Hansenula polymorpha Pex20p is an oligomer that binds the peroxisomal targeting signal 2 (PTS2)

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Summary

We have cloned and characterized the *Hansenula polymorpha PEX20* gene. The Hp*PEX20* gene encodes a protein of 309 amino acids (HpPex20p) with a calculated molecular mass of ~35 kDa. In cells of an Hp*PEX20* disruption strain, PTS2 proteins were mislocalized to the cytosol, whereas PTS1 matrix protein import proceeded normally. Also, the PTS2 proteins amine oxidase and thiolase were normally assembled and active in these cells, suggesting HpPex20p is not involved in oligomerization/activation of these proteins. Localization studies revealed that HpPex20p is predominantly associated with peroxisomes. Using fluorescence correlation spectroscopy we determined the native molecular mass of purified HpPex20p and binding of a synthetic peptide containing a PTS2 sequence. The data revealed that purified HpPex20p forms oligomers, which specifically bind PTS2-containing peptides.

Key words: Yeast, PTS2 protein import, Peroxisomes, FCS, *Hansenula polymorpha*

Introduction

Peroxisomes are membrane-bound organelles, present in virtually all eukaryotic cells. Peroxisomal matrix proteins are synthesized on free polysomes and subsequently transported to the target organelle. The routing of most matrix proteins depends on one of the two conserved peroxisomal targeting signals, designated PTS1 and PTS2, which are recognized by their specific soluble receptor protein, Pex5p or Pex7p. A current model describing the import of peroxisomal matrix proteins predicts that these receptor molecules may enter the peroxisomal lumen together with their cargo and subsequently return to the cytosol for another round of import (Kunau, 2001; Dammai and Subramani, 2001; Purdue and Lazarow, 2001a; Nair et al., 2004). In line with this model are data from in vitro binding experiments, which revealed that Pex8p, a matrix-localized peroxin, physically interacts with Pex5p resulting in Pex5p-PTS1 cargo dissociation (Rehling et al., 2000; Wang et al., 2003).

It is now generally accepted that targeting of PTS2 proteins requires auxiliary factors in addition to Pex7p. These include the long isoform of Pex5p (Pex5pL) in mammalian cells (Braverman et al., 1998; Otera et al., 1998), Pex18p and Pex21p in *Saccharomyces cerevisiae* (Purdue et al., 1998), and Pex20p in *Yarrowia lipolytica* and *Neurospora crassa* (Titorenko et al., 1998; Sichting et al., 2003). Although these proteins display only a weak sequence homology, several studies demonstrated that they might fulfil a generalized function in PTS2 protein import. This assumption is based on the observation that synthesis of NcPex20p or YlPex20p in an *S. cerevisiae pex18 pex21* double knockout strain could partially complement the PTS2 protein import defect (Einwächter et al., 2001; Sichting et al., 2003). Also, the 37 amino acid insertion within Pex5pL, which is required for the interaction with Pex7p (Otera et al., 2000), shows similarity to a region in ScPex18p, YlPex20p, NcPex20p and ScPex21p, which is involved in Pex7p interaction (Einwächter et al., 2001; Dodt et al., 2001; Sichting et al., 2003).

Recently, Schäfer et al. (Schäfer et al., 2004) demonstrated that in *S. cerevisiae* a fusion protein consisting of ScPex18p (lacking the Pex7p-binding site) and the C-terminal PTS1 binding domain of ScPex5p, was able to partially complement the PTS1 protein import defect in a *PEX5* deletion strain (Schäfer et al., 2004). Based on these data the authors suggested a model in which ScPex18p is predicted to be required for protein translocation into the peroxisome and ScPex7p for recognition of the PTS2 signal.

A *Y. lipolytica* Pex7p has not been identified yet, suggesting that YlPex20p may fulfil the functions of Pex7p as well. In this organism YlPex20p functions in the oligomerization of thiolase in the cytosol (Titorenko et al., 1998). YlPex20p is also involved in later events of PTS2 protein import, and may enter the organelle lumen, as suggested by the observed interaction with matrix-localized YlPex8p (Smith and Rachubinski, 2001).

In this study we analyzed the PTS2 protein pathway in the methylotrophic yeast *H. polymorpha*. The genome of this yeast contains both a *PEX7* and a *PEX20* gene (our unpublished results). Here, we report the cloning of Hp*PEX20* and characteristics of the corresponding protein, HpPex20p. Our data show that HpPex20p is essential for the import of PTS2 matrix proteins into peroxisomes. Furthermore, we demonstrate that purified HpPex20p forms oligomers that have affinity for PTS2-containing synthetic peptides.

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Table 1. Strains used in this study

Strains	Relevant properties	Reference
NCYC495	Wild type, *ura3 leu1.1*	Gleeson and Sudbery, 1988
Hp*pex20*	Hp*PEX20* disruption strain, *leu1.1*	This study
NCYC495::P_{AMO}*Thio*$_{N50}$-*GFP*::P*aox*DsRed-SKL	NCYC495 with one-copy integration of plasmid pHIPX5-Thio$_{N50}$-GFP and pHIPZ4-DsRed-SKL	This study
Hp*pex20*::P_{AMO}*Thio*$_{N50}$-*GFP*::P*aox*DsRed-SKL	Hp*pex20* with one-copy integration of plasmid pHIPX5-Thio$_{N50}$-GFP and pHIPZ4-DsRed-SKL	This study
Hp*pex20*::P_{PEX20}*PEX20-GFP*::P_{AMO}DsRed-SKL	NCYC495 with one-copy integration of plasmid pHIPZ-PEX20-GFP, and one-copy integration of plasmid pHIPX6-DsRed-T1-SKL	This study

Materials and Methods

Organisms and growth

The *Hansenula polymorpha* strains used in this study are listed in Table 1. Yeast cells were grown in batch cultures at 37°C on 1% yeast extract, 1% peptone and 1% glucose (YPD), selective minimal media containing 0.67% Yeast Nitrogen Base without amino acids (DIFCO) or in minimal medium (Van Dijken et al., 1976) using glucose (0.5%), methanol (0.5%) or glycerol (0.5%) as carbon source and methylamine (0.25%) or ammonium sulphate (0.25%) as nitrogen sources. When required, uracil or amino acids were added to a final concentration of 30 μg ml^{-1}. For growth on agar plates the media were supplemented with 1.5% agar. To test the effect of proteasome inhibition on HpPex20p levels MG132 was added to exponentially growing cultures as described previously (Baerends et al., 2000).

Escherichia coli strains were grown on LB medium (Sambrook et al., 1989). When required, ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) was supplemented to the media.

Molecular techniques

Standard recombinant DNA techniques were carried out essentially as described by Sambrook et al. (Sambrook et al., 1989). PCR was performed using Pwo Polymerase according to the instructions of the supplier (Roche Diagnostics, Almere, The Netherlands). Transformation of *H. polymorpha* cells and site-specific integrations of single or multiple copies of plasmid DNA was performed as described (Faber et al., 1992; Faber et al., 1994). Correct integration in the *H. polymorpha* genome was analyzed by Southern blotting using the ECL direct nucleic acid labeling and detection system according to the instructions of the supplier (Amersham Corp., Arlington Heights, IL).

Isolation and characterization of the *H. polymorpha* PEX20 gene

The *H. polymorpha PEX20* gene was identified by RALF mutagenesis (van Dijk et al., 2001). DNA sequencing was performed at Baseclear (Leiden, The Netherlands) using a Licor automated DNA sequencer and dye primer chemistry (LiCor, Lincoln, NB). After sequencing of both strands, the BLASTN algorithm (Altschul et al., 2001) was used to search the GenBank database for DNA and protein sequences, showing similarity to this gene and its translation product.

HpPEX20 disruption

An *HpPEX20* disruption strain was constructed as follows: the *H. polymorpha URA3* gene was isolated as a *Bgl*II, *Pst*I fragment and ligated between the two flanking regions of the *HpPEX20* gene. These regions were obtained by PCR using chromosomal DNA of a wild type (WT) strain as a template and primers Pex20del-1 (AAACT-GCAGGTGGTGACTGCTTGTGGAG) and Pex20-5 (CGGTCT-CAACATGCTTGTTC), resulting in a product containing the sequence upstream the start codon, digested with *Pst*I. Furthermore, the primers Pex20del-2 (GAAGATCTAGCCTCCGGCGATATATCG) and Pex20del-3 (AGAGAGAGGCGGCCGCAACAGTGGAACG-GCATGTGC) were used, resulting in a product containing the last 163 base pairs of the *HpPEX20* gene together with the sequence downstream the stopcodon, digested with *Bgl*II. This fragment was used to transform *H. polymorpha* NCYC495.

Plasmid constructions

To analyze the localization of PTS2 proteins in living cells, a DNA fragment encoding the first 50 amino acids of *S. cerevisiae* thiolase, including its PTS2 targeting signal, was fused to the gene encoding enhanced green fluorescent protein (eGFP; CLONTECH). To this purpose the first 150 base pairs of the thiolase gene were amplified using primers KN31 (CCCACTAGTGGATCCATGTCTCAAAGAC-TACAAAG) and KN32 (GGGAGATCTAAAACCTTTACCGATG-GC). The PCR product was then fused in frame to the 5′ end of the *eGFP* gene, and subsequently ligated behind the amine oxidase promoter region, resulting in plasmid pHIPX5-Thio$_{N50}$-GFP. For stable integration of the expression cassette into the *H. polymorpha* genome, the plasmid was linearized with *Aoc*I in the promoter region and transformed into *H. polymorpha*.

To determine import of PTS1 proteins, plasmid pHIPZ4-DsRed-T1.SKL (Monastyrska et al., 2005) was integrated in the genome of the same strain. The plasmid was linearised with *SphI* prior to transformation.

For the purification of HpPex20p, a gene encoding HpPex20-His$_8$ was amplified using the primers Pex20His-start (ACCAC-CATGGGCTTCAGCAACGCCTTTG) and Pex20His-stop (CG-CAAGCTTTCAGTGATGGTGATGGTGATGGTGATGCTGCCAC-ATGCTGGGTCTCA). This resulted in a 969 base pair (bp) product containing an in frame fusion between the Hp*PEX20* gene and a *HIS*$_8$-tag. Subsequently, the PCR product was digested with *Nco*I and *Hin*dIII, and ligated into *Nco*I-*Hin*dIII digested pQE60 (Qiagen, Leusden, The Netherlands). The resulting plasmid pQE60-Pex20-His8 was then introduced into *E. coli*.

To analyze the localization of HpPex20p in intact cells, plasmid pHIPZ-PEX20-GFP was constructed. To this purpose the Hp*PEX20* gene was amplified using the primers 20GFP-start (CCCAAGCT-TCAACAACTCGAGTCTGAGTAC) and 20GFP-stop (GGAAGA-TCTCTGCCACATGCTGGGTCTCA), resulting in a product lacking the stop codon of the Hp*PEX20* gene. This PCR product was then digested with *Hin*dIII and *Bgl*II, and ligated into the *Hin*dIII-*Bgl*II digested pANL31 (Leao-Helder et al., 2003), resulting in plasmid pHIPZ-PEX20-GFP, containing an in frame fusion of the whole Hp*PEX20* gene fused to the *eGFP* gene. Subsequently, linearization of this plasmid was performed with *Apa*I in the *PEX20* open reading frame to enable integration in the *H. polymorpha* genome.

To identify peroxisomes, the gene encoding DsRed-T1-SKL was expressed under control of the amine oxidase promoter. For this plasmid pHIPX6-DsRed-T1-SKL, the 743 bp *Sma*I-*Bam*HI fragment from pHIPZ4-DsRed-T1.SKL (Monastyrska et al., 2005) was ligated into *Sma*I-*Bam*HI digested pHIPX5 (Kiel et al., 1995). The resulting plasmid was linearized with *Bsi*WI in the P_{AMO} region to enable integration in the *H. polymorpha* genome.

Biochemical methods

Crude extracts of *H. polymorpha* cells were prepared as described before (Baerends et al., 2000). Cell fractionation studies were performed as detailed previously (van der Klei et al., 1998), except that 1 mM NaF and 1 mM PMSF was added to all buffers.

Protein concentrations were determined using the Bio-Rad protein assay system (Biorad GmbH, Munich, Germany) using bovine serum albumine as a standard. SDS-PAGE (Laemmli, 1970) and native gel electrophoresis (Musgrove et al., 1987) were carried out as described. Western blotting was performed as detailed before (Kyhse-Andersen, 1984). Blots were probed using specific antibodies against various *H. polymorpha* proteins using the BM Chemiluminiscence Western Blotting kit (Boehringer Mannheim BV, Almere, The Netherlands).

To analyze the native molecular mass of peroxisomal enzymes, intact cells were harvested by centrifugation, resuspended in buffer A [50 mM potassium phosphate buffer pH7.5, containing 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete™ (Roche, Almere, the Netherlands)] and disrupted using glass beads. Subsequently, the cell lysates were clarified by centrifugation at 4°C for 5 minutes at 21,000 ***g***. The resulting lysate contains soluble cellular proteins (i.e. including cytosolic and peroxisomal matrix proteins). Proteins in the lysate were separated using an ÄKTA™ FPLC system (Amersham Biosciences, The Netherlands, Roosendaal). Gel filtration was performed at 4°C using a Superose 6 column connected to a Superose 12 column, using buffer A as a running buffer at a flow rate of 0.1 ml per minute. The eluate was collected in fractions of 1 ml.

Polyclonal antibodies against HpPex20p were generated in rabbit, using a C-terminal His_8-tagged version of HpPex20p. Growth and purification of HpPex20-His8 in *E. coli* was performed according to the instructions of the manufacturer (Qiagen, Leusden, The Netherlands).

Peptides and proteins

Peptides PTS2-FITC [peptide MERLRQIASQATAASAAPARPAH labeled to fluorescein 5-isothiocyanate (FITC) at the C-terminus] and peptide nonPTS2-FITC (peptide MEDDRQIASDETAASA-APARPAH labeled to FITC at the C-terminus) were purchased from Isogen (Maarssen, The Netherlands). Peptide FITC-PTS1 (peptide ASSASKL labeled with FITC at the N-terminus) was purchased from Eurosequence (Groningen, The Netherlands). The concentrations of the peptides were determined spectrophotometrically using the molar extinction coefficient of FITC (ε_{450}=7.7×10^4 M cm^{-1}).

For HpPex20p purification, *E. coli* transformants containing the plasmid pQE60-Pex20-His8 were grown as detailed in the QIA*expressionist*™. All subsequent steps were performed at 4°C. Cells were harvested by centrifugation and resuspended in 50 mM phosphate buffer, pH 7.4, containing 300 mM NaCl, 1% Tween 20, 10% glycerol, 0.2 mM β-mercaptoethanol, 1 mM sodium azide, 5 mM sodium fluoride, 1 mM phenylmethyl sulfonyl fluoride (PMSF), complete™ (Roche, Almere, the Netherlands) (buffer A) and subsequently disrupted using a French Press. Cell debris was removed by centrifugation (10,000 ***g***, 20 minutes). Supernatants were incubated for 1 hour with Ni-NTA resin (Qiagen, Hilden, Germany; 500 mg protein per ml resin) followed by extensive washing with buffer B (50 mM phosphate buffer, pH 7.4, containing 10 mM NaCl and 40 mM imidazole), and subsequent elution with buffer B containing 250 mM imidazole. HpPex20-His8 containing fractions, determined by western blotting using anti-HpPex20p antiserum, were further purified by anion exchange chromatography (MonoQ, Amersham Pharmacia, Uppsala, Sweden) using a linear gradient of 0.1 to 1 M NaCl in 20 mM Tris-HCl buffer (pH 8.5). When required, HpPex20-His8 containing fractions were labeled with Alexa Fluor 488, using the protein labeling kit of Molecular Probes (Leiden, the Netherlands). This procedure was followed by another anion exchange chromatography step as detailed above. The concentration of HpPex20p protein concentrations was calculated from the absorption at 280 nm, using a molar extinction coefficient of 2.3×10^4 M cm^{-1}.

Fluorescence correlation spectroscopy (FCS)

All measurements were carried out essentially as described before (Otzen et al., 2004). Autocorrelation traces were acquired during 10 seconds at room temperature and repeated 20 times. Furthermore, autocorrelation curves were globally analyzed using the FCS data processor 1.3 software (the Scientific Software Technologies Center of Belarusian State University, Belarus) as detailed before (Beechem et al., 1991; Wang et al., 2003).

Microscopy

Fluorescence microscopy was performed as described before (Baerends et al., 2000). Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as detailed previously (Waterham et al., 1994). Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against *H. polymorpha* alcohol oxidase protein and gold conjugated goat-anti-rabbit antibodies (Waterham et al., 1994).

Results

The *Hansenula polymorpha PEX20* gene

In an earlier search for new *H. polymorpha* genes involved in peroxisome biogenesis (van Dijk et al., 2001), a DNA fragment showing sequence homology to *PEX20* genes was identified. Sequencing of the complete putative Hp*PEX20* gene revealed an open reading frame (ORF) encoding a protein of 309 amino acids with a calculated molecular mass of ~35 kDa. The nucleotide sequence of Hp*PEX20* was deposited at GenBank (accession number AY788916). Database searches revealed that the protein encoded by Hp*PEX20* showed highest homology to *Yarrowia lipolytica* Pex20p (23% identity). Also, HpPex20p contained two WxxxF motifs (residues 87-91 and 118-122) involved in binding to the docking site proteins Pex13p and Pex14p (Schliebs et al., 1999; Otera et al., 2002) and the predicted conserved Pex7p binding site (residues 232 to 261; Dodt et al., 2001). Both characteristics are typical for auxiliary proteins that function in PTS2 protein import (Einwächter et al., 2001).

To analyze the function of HpPex20p, an Hp*PEX20* deletion strain (Hp*pex20*) was constructed by replacing most of the gene by the *URA3* gene. Correct integration of the disruption cassette was confirmed by Southern blot analysis (data not shown). Growth experiments revealed that Hp*pex20* cells grew like wild-type cells (WT) on all carbon (glucose, methanol and glycerol) and nitrogen sources analyzed (ammonium sulphate, methylamine and ethylamine).

To study the localization of PTS2 proteins in Hp*pex20*, cells were grown on glucose/methylamine to induce synthesis of the peroxisomal PTS2 protein amine oxidase (AMO). In WT cells these growth conditions generally result in the presence of one, and occasionally few, small peroxisomes per cell. Differential centrifugation of homogenized protoplasts of Hp*pex20* cells revealed that AMO protein was predominantly present in the 30,000 ***g*** supernatant (S3), indicative for a cytosolic location (Fig. 1A). Similar experiments on WT controls revealed that in these cells AMO protein was predominantly present in the 30,000 ***g*** organellar pellet (P3) in conjunction with some

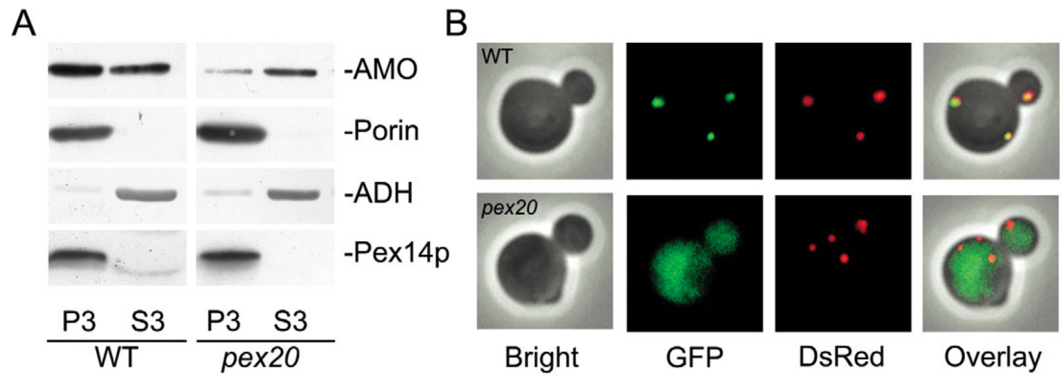

Fig. 1. PTS2 protein mislocalization in Hp*pex20* cells. (A) Post nuclear supernatants prepared from glucose/methylamine-grown wild type (WT) and Hp*pex20* cells were subjected to differential centrifugation. P3-30,000 ***g*** pellet, S3-30,000 ***g*** supernatant. Western blots were probed with antibodies against several *H. polymorpha* proteins: AMO (PTS2 matrix protein); porin (mitochondria); ADH-alcohol dehydrogenase (cytosol) and Pex14p (peroxisomal membrane). Equal portions of each fraction were loaded per lane. (B) Fluorescence microscopy of WT and Hp*pex20* cells expressing a fusion protein consisting of the first 50 amino acids of *S. cerevisiae* thiolase and GFP and DsRed containing the PTS1 sequence –SKL. In WT cells, green and red fluorescence is present in spots, indicative of peroxisomes. In *pex20* cells, green fluorescence is observed in the cytosol, whereas red fluorescence is localized in spots.

soluble AMO protein (S3; Fig. 1A). The soluble fraction of AMO is most probably due to leakage of this protein as a result of the fractionation procedure (Salomons et al., 2000). Additionally, mitochondrial porin and cytosolic alcohol dehydrogenase, used as controls, were in their expected fractions, indicating that appropriate organelle separation in these experiments had occurred (Fig. 1A). Like porin, the peroxisomal membrane protein Pex14p sedimented to the organellar pellet (P3), indicating that peroxisomes were properly pelleted. These data suggest that HpPex20p is important for import of the PTS2 protein AMO into peroxisomes.

To analyze whether HpPex20p is essential for import of PTS2 proteins in general, a strain was constructed that produced a fusion protein consisting of the first 50 amino acids of the *Saccharomyces cerevisiae* thiolase (including the PTS2 signal) and eGFP (thiolase$_{N1\text{-}50}$.eGFP), under control of the AMO promoter (P_{AMO}). This construct was integrated into the genome of both the WT and the Hp*pex20* strains. As a control, we also introduced a gene encoding DsRed containing a

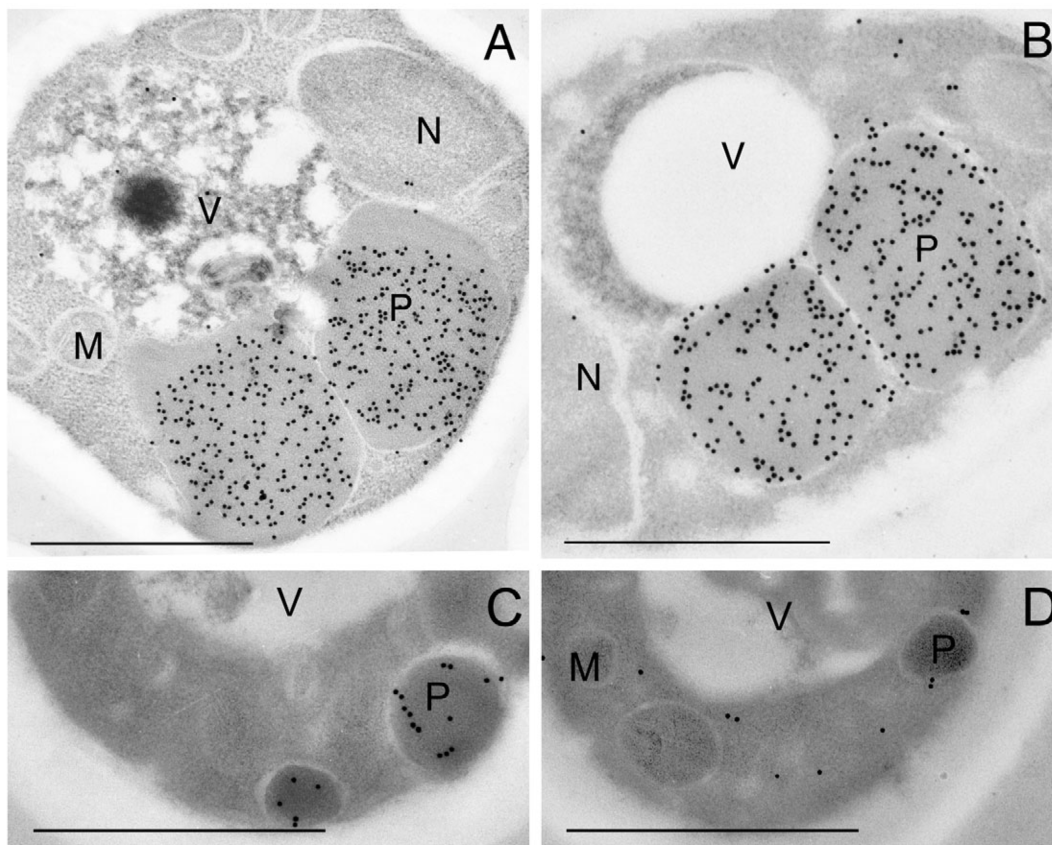

Fig. 2. Normal PTS1 protein localization in Hp*pex20* cells. (A,B) Ultrathin sections of WT and *pex20* cells were used for immunolabeling experiments using antibodies against the PTS1 protein alcohol oxidase (A,B) or the PTS2 protein amine oxidase (C,D). Both in WT (A) and in *pex20* cells (B) AO labeling is found at the peroxisomal profiles. AMO is normally localized to peroxisomes in WT cells (2C), but mislocalized to the cytosol in *pex20* cells (D). Bars, 0.5 µm. M, mitochondrion; N, nucleus, P, peroxisome; V, vacuole.

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PTS1 (DsRed-SKL) into the Hp*pex20* strain. Fluorescence microscopy of glucose/methylamine-grown WT cells revealed a punctuate pattern of the thiolase$_{N1-50}$.eGFP fluorescence, indicative for peroxisomal localization of the fusion protein. DsRed-SKL co-localized to the same spots in WT cells. In Hp*pex20* cells however, GFP fluorescence was dispersed throughout the cell, indicating that thiolase$_{N1-50}$.eGFP was mislocalized to the cytosol. In these cells DsRed fluorescence showed a punctuate pattern like in WT cells, indicating that sorting of DsRed-SKL is not affected in Hp*pex20* cells (Fig. 1B).

Electron microscopy was performed to study the import of the peroxisomal matrix proteins alcohol oxidase (AO) and AMO in Hp*pex20* cells. To this purpose WT and Hp*pex20* cells were grown in a glucose-limited chemostat using choline as sole nitrogen source. These conditions result in massive peroxisome proliferation and induction of both PTS1 and PTS2 matrix proteins (Zwart et al., 1983). Immunocytochemistry revealed that in Hp*pex20* cells the PTS1-protein AO was localized in peroxisomes as in WT cells (Fig. 2A,B). However, the PTS2-protein AMO was mislocalized to the cytosol in Hp*pex20* cells, but normally localized to peroxisomes in WT controls (Fig. 2C,D). These data suggest that HpPex20p is specifically required for PTS2 protein import, but not for import of peroxisomal matrix proteins that contain a PTS1.

Oligomerisation of AMO and thiolase is not dependent of the function of HpPex20p

In *Y. lipolytica*, Pex20p is required for the oligomerisation and subsequent sorting of thiolase protein to peroxisomes (Titorenko et al., 1998). To analyze whether PTS2 protein oligomerisation in *H. polymorpha* is also dependent on Pex20p, the native molecular mass of AMO and thiolase was analyzed in Hp*pex20* cells, relative to those identified in WT controls. Total cell extracts (containing cytosolic and peroxisomal proteins) were analyzed by gel filtration chromatography and western blotting. The data indicate that, based on the calculated molecular mass of both proteins (47 kDa for thiolase and 78 kDa for AMO respectively), AMO and thiolase are probably present as dimers in Hp*pex20* and WT cells (Fig. 3). Similarly, the PTS1 proteins AO and catalase (CAT), used as controls, were normally assembled in *pex20* cells. Enzyme activity measurements revealed that AMO is enzymatically active in *pex20* cells (data not shown). Hence, our results reveal that oligomerisation of the PTS2 proteins AMO and thiolase is not dependent of the presence of HpPex20p.

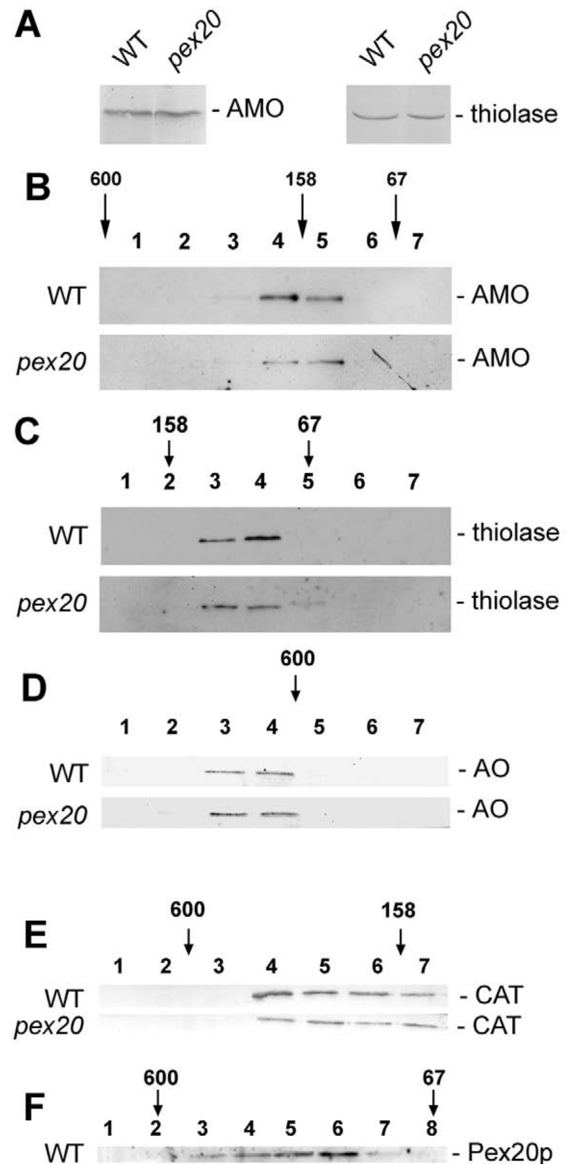

Fig. 3. HpPex20p is not required for oligomerisation of AMO and thiolase. (A) Western blot analysis of crude cell extracts revealed that the levels AMO and thiolase were similar in WT and Hp*pex20* cells. Equal amounts of protein were loaded per lane. (B-F) The oligomeric state of AMO (B), thiolase (C), AO (D), catalase (CAT; E) and HpPex20p (F) were determined by gel filtration analysis. Western blotting of FPLC elution fractions of WT and *pex20* cell free extracts revealed similar native sizes for AMO (~150 kDa; B), thiolase (~100 kDa; C), AO (~600 kDa; D) and catalase (~240 kDa). In crude extracts of WT *H. polymorpha* cells HpPex20p was estimated to be ~180 kDa (F). WT and Hp*pex20* cells were grown to the late exponential growth phase on glucose/methylamine for the detection of AMO, CAT, AO and HpPex20p (Fig. 3A,B,D-F) or shortly induced on oleic acid/ammonium sulphate to induce thiolase (A,C).

HpPex20p levels and localization

Western blot analysis of crude extracts, prepared from TCA-precipitated intact methanol/methylamine-grown *H. polymorpha* WT cells, probed with anti-HpPex20p antibodies, visualized a protein band with an apparent molecular mass of approximately 35 kDa (Fig. 4A). This size was similar to the calculated molecular mass based on the amino acid sequence of HpPex20p. Since this protein band was absent in crude extracts prepared of similarly grown Hp*pex20* cells, we concluded that the HpPex20p antiserum specifically recognized HpPex20p (Fig. 4A). Using these antibodies, we analyzed HpPex20p levels in cells grown at specific growth conditions. To this purpose WT cells were grown at peroxisome repressing (glucose) or inducing (methanol) cultivation media. These experiments demonstrated that HpPex20p levels were significantly enhanced in glucose-

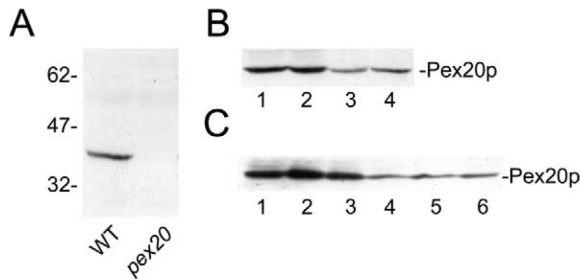

Fig. 4. Levels of HpPex20p. (A) WT and Hp*pex20* cells were grown on glucose/methylamine medium. Western blots, probed with α-HpPex20p antibodies, revealed that in lysates of TCA-precipitated WT cells a cross-reacting protein band with an apparent molecular mass of 35 kDa was present. This band was absent in a similar blot prepared from Hp*pex20* cells. (B) Crude cell extracts were prepared from TCA-precipitated WT cells grown on glucose (lanes 1,2) or methanol (lanes 3,4) as carbon source in the presence of ammonium sulphate (lanes 1,3) or methylamine (lanes 2,4) as nitrogen source. Western blots, probed with α-HpPex20p antibodies, revealed that HpPex20p levels were not significantly increased when cells were grown on media containing methylamine, relative to ammonium sulphate. However, HpPex20p levels appeared to be dependent on the carbon source and higher in glucose grown cells (lanes 1,2) compared with methanol-grown cells (lanes 3,4). (C) Western blot analysis of crude cell extracts prepared from TCA precipitated glucose- (lanes 1-3) and methanol grown (lanes 4-6) *H. polymorpha* WT cells. Cells were collected prior to (lanes 1,4) or after growth for 60 minutes in the presence (lanes 2,5) or absence (lanes 3,6) of proteasome inhibitor MG-132. Blots were probed with anti-HpPex20p antibodies. Equal amounts of protein were loaded per lane. The addition of MG-132 resulted in an increase in HpPex20p levels during growth of cells on glucose (lane 2), but not on methanol (lane 5).

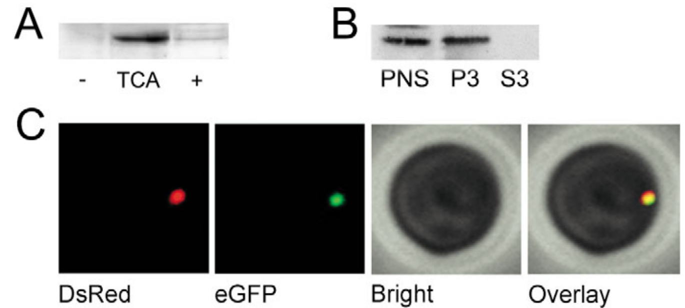

Fig. 5. Subcellular localization of HpPex20p. (A) Glucose/choline grown WT cells were disrupted using glass beads in buffer lacking (–) or containing (+) a protease inhibitor cocktail and incubated for 10 minutes at room temperature. Crude extracts prepared from cells that were immediately TCA precipitated upon harvesting were used as a control (TCA). HpPex20p levels were analysed by western blotting using anti-Pex20p antibodies. Equal amounts of protein were loaded per lane. These experiments revealed that HpPex20p is partially protected by the addition of the protease inhibitor cocktail (+). However, still approximately 90% is degraded relative to cells that were immediately TCA precipitated. (B) From glucose/choline-grown WT cells a post nuclear supernatant (PNS) was prepared in the presence of the protease inhibitor cocktail and subsequently centrifuged at 30,000 ***g***. Western blot analysis revealed that HpPex20p is present in the organellar pellet. P3-30.000g pellet, S3-30,000 ***g*** supernatant. (C) Fluorescence microscopy of WT cells expressing HpPex20.eGFP and DsRed-SKL. This revealed that the green fluorescence was present in a spot at the same localization as the DsRed-SKL fluorescence.

grown cells relative to methanol-grown cells (Fig. 4B). At conditions that induce the synthesis of the PTS2 protein AMO (methylamine as sole nitrogen source) HpPex20p levels are not significantly elevated when compared with growth conditions that fully repress the synthesis of AMO (ammonium sulphate) (Fig. 4B). The reduced levels of HpPex20p in methanol-grown cells are not due to massive degradation of the protein by the Ub-proteasome pathway as previously reported for *S. cerevisiae* Pex18p (Purdue and Lazarow, 2001b). No increase in HpPex20p levels was observed upon addition of the proteasome inhibitor MG-132 to methanol cultures of WT cells (Fig. 4C). In glucose cultures a slight increase in HpPex20p levels was observed upon growth in the presence of MG-132 (Fig. 4C).

Using anti-HpPex20p antibodies, HpPex20p was not detectable after sucrose density centrifugation of homogenized protoplasts prepared from glucose-limited chemostat cells. To address the possible instability or susceptibility of HpPex20p to proteolytic degradation, we analyzed the stability of the protein in crude extracts prepared via glass bead disruption, in the presence or absence of a protease inhibitor cocktail (PMSF, NaF and Complete^{TM}), relative to the HpPex20p levels in crude extracts of TCA precipitated cells. The data revealed that approximately 10% of the protein can be recovered in the presence of protease inhibitors (Fig. 5A). Upon adding this protease inhibitor cocktail to all buffers during cell fractionation, a portion of HpPex20p was detectable in the post nuclear supernatant, which was predominantly present in the 30,000 ***g*** organellar pellet (Fig. 5B), containing peroxisomes.

The subcellular localization of HpPex20p was also studied by fluorescence microscopy. To this purpose, a strain was constructed in which the authentic Hp*PEX20* gene was replaced by a Hp*PEX20e.GFP* hybrid gene. In this strain (*pex20*::P_{PEX20}*Pex20-GFP*) a gene encoding peroxisome targeted DsRed (DsRed-SKL) under control of the AMO promoter (*pex20*::P_{PEX20}*Pex20.eGFP*::P_{AMO}*DsRedSKL*) was introduced as well, to enable visualization of peroxisomes. Fluorescence microscopy of glucose/methylamine-grown cells of this strain revealed that GFP fluorescence is generally present at a single spot in the cell. Cytosolic GFP fluorescence was below the limit of detection. The GFP fluorescent spot co-localized with DsRed fluorescence, suggesting HpPex20p is predominantly present at peroxisomes (Fig. 5C).

Purified HpPex20p forms oligomers

Fluorescence correlation spectroscopy (FCS) is a very sensitive technique, which enables the study of dynamic processes using fluorescently marked molecules at equilibrium. Using this technique the mobility of fluorescent molecules can be determined, so that their size can be estimated (Hink et al., 2002; Bacia and Schwille, 2003).

First, we used FCS to estimate the native molecular mass of purified HpPex20p in vitro. To facilitate purification of HpPex20p, a C-terminal His_8-tagged version of HpPex20p was overproduced in *E. coli*. The His_8-tagged HpPex20p was

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purified to approximately 95% homogeneity by Ni-NTA affinity chromatography followed by anion exchange chromatography (data not shown). The purified protein was labeled with the fluorescent dye Alexa Fluor 488, which allows us to monitor HpPex20p by FCS. In Fig. 6 three autocorrelation curves (of 20) are shown together with fitted curves and residuals. The autocorrelation curves were fitted globally to a diffusion model including triplet kinetics (Wang et al., 2003). The autocorrelation curve of Alexa Fluor 488-labeled HpPex20p fitted best with a two-component fit from which two diffusion times were deduced, one corresponding to the free Alexa Fluor 488 (diffusion time 34 microseconds) and the other one to Alexa Fluor 488 bound to HpPex20p (average diffusion time 223 microseconds). The molecular mass of HpPex20p, estimated from this diffusion time was 198 kDa, presuming that the protein had a globular conformation. Because the molecular mass of monomeric HpPex20p is 35 kDa, based on its deduced amino acid composition, we concluded that the purified HpPex20p was not present as a monomer, but formed an oligomeric structure (probably a hexamer) under the conditions tested. This finding was confirmed by gel filtration analysis of HpPex20p present in crude extracts of *H. polymorpha* WT cells (Fig. 3F). Using a standard curve based on gel filtration analysis of proteins with a known native molecular mass, the molecular mass of HpPex20p in *H. polymorpha* extracts was estimated to be approximately 180 kDa.

A synthetic peptide containing a PTS2 sequence interacts with oligomeric HpPex20p

FCS was also used to analyze whether HpPex20p has affinity for the PTS2 peroxisomal targeting signal. To this purpose, a peptide was synthesized that contained the PTS2 signal of *H. polymorpha* AMO (MERLRQIASQATAASAAPARPAH). This peptide was labeled with fluorescein 5-isothiocyanate (FITC) at the C-terminus (PTS2-FITC), which allowed us to monitor the mobility of the peptide by FCS. Autocorrelation curves of the labeled peptide (PTS2-FITC) were collected in the absence and presence of (unlabeled) HpPex20p. In the absence of HpPex20p a single diffusion time of 49 microseconds, corresponding to free PTS2-FITC peptide, was observed. However, upon addition of HpPex20p the curve shifted to longer diffusion times, indicating binding of the peptide to a larger molecule. The autocorrelation curves now fitted best with a two-component fit, which generated two diffusion times corresponding to free peptide and peptide bound to a larger structure. The additional diffusion time corresponded to a molecular mass of 178 kDa, which is similar to that observed for purified HpPex20p. These data therefore suggest that PTS2-FITC had bound to oligomeric HpPex20p. Based on the fraction of PTS2-FITC peptide that was bound to HpPex20p (in an experiment using 50 nM peptide and 50 nM HpPex20p), a K_d of ~400 nM was estimated. This suggests that the binding is relatively weak [e.g. compared with a K_d of 18 nM for the HpPex5p-PTS1 peptide interaction (Wang et al., 2003)].

To analyze whether the observed interaction was related to the presence of the PTS2 signal in the synthetic peptide, we performed control experiments using FITC, a FITC containing peptide in which the PTS2 consensus sequence was destroyed (non-PTS2-FITC; MEDDRQIASDETAASAAPARPAH) (Gietl et al., 1994) and a peptide containing a PTS1 signal coupled to FITC (FITC-PTS1) (Wang et al., 2003). As indicated in Table 2, FITC has no affinity for HpPex20p. Interaction between HpPex20p and the non-PTS2 peptide or PTS1 peptide was also not detectable. Finally, we tested whether the PTS2-FITC peptide had affinity for the control protein lysozyme and we found that no association could be observed. These findings clearly indicate that the PTS2-FITC-HpPex20p interaction is highly specific and dependent on the PTS2 consensus sequence in the synthetic peptide.

To analyze whether the binding between PTS2 peptide and HpPex20p is reversible, excess unlabeled PTS2 peptide (10-fold excess) was added to the solution containing one-

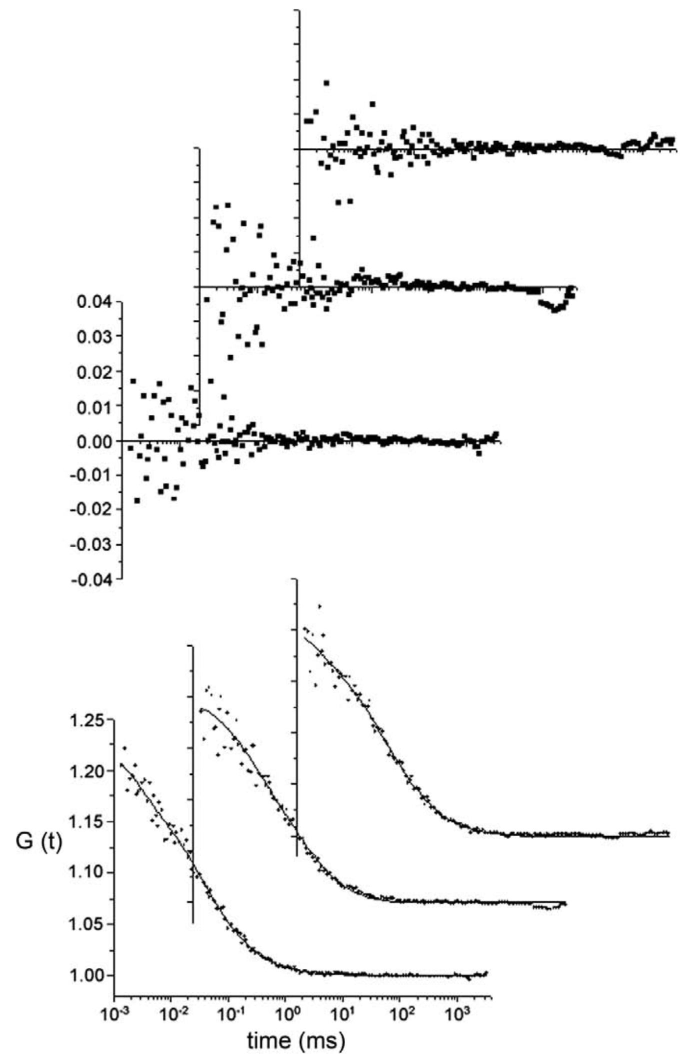

Fig. 6. Examples of experimental autocorrelation curve (dots), fitted (solid line), and residuals (upper inset) of HpPex20p. HpPex20p was labeled with Alexa Fluor 488. In all experiments the concentration of HpPex20p was 50 nM. After global analysis of 20 experimental curves, a diffusion time of 223 microseconds was obtained (confidence limit: 218-277 microseconds). Based on these data the calculated molecular mass of the fluorescent protein complex is estimated to amount 198 kDa (confidence limit: 185-380 microseconds) assuming that the complex is globular in shape.

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Table 2. Diffusion times of FITC, FITC-PTS1, PTS2-FITC and nonPTS2-FITC in the presence or absence of HpPex20p

	HpPex20p	Diffusion time (μs) $\tau1$*	$\tau2$*	Fraction of 2nd component (%)	Calculated M_r of 2nd component (kDa)
FITC	–	29	–	0	–
FITC	+	29	–	0	–
FITC-PTS1	–	40	–	0	–
FITC-PTS1	+	40	–	0	–
PTS2-FITC	–	49	–	0	–
PTS2-FITC[†,‡]	+	49	223	10	178
Non-PTS2-FITC	–	47	–	0	–
Non-PTS2-FITC	+	47	–	0	–
PTS2-FITC	+ Lysozyme	49	–	0	–

The peptides were present at a concentration of 50 nM, FITC was used at a concentration of 5 nM. Both Pex20p and the control protein lysozyme were added to a final concentration of 500 nM. The measurements were performed at room temperature.

*Data were calculated with fixing the diffusion time of free Alexa Fluor 488 at 34 microseconds (μs).

[†]$\tau1$ and $\tau2$: diffusion times of the first and second component.

[‡]Data were fitted with the two-component model.

component fit, corresponding to free PTS2-FITC. These data suggest that most of the initially bound and labeled PTS2 peptide was replaced by the unlabeled PTS2 peptide.

Discussion

In this paper we report the cloning of *H. polymorpha PEX20* and the characterization of the corresponding peroxin HpPex20p. Our data indicate that HpPex20p is essential for import of PTS2 proteins into peroxisomes. *H. polymorpha* also contains a gene encoding the PTS2 receptor, *PEX7* (our unpublished results). Also, in other organisms additional proteins have been shown to be required for PTS2 protein import besides the PTS2 receptor Pex7p. However, depending on the organism different additional proteins have been identified. These include the long isoform of the mammalian PTS1 receptor, Pex5pL, *S. cerevisiae* Pex18p and Pex21p and *Y. lipolytica* and *N. crassa* Pex20p (Braverman et al., 1998; Otera et al., 1998; Purdue et al., 1998; Titorenko et al., 1998; Sichting et al., 2003). A general feature of the auxiliary proteins, including HpPex20p, is the presence of a conserved domain that is involved in Pex7p binding and one or more WxxxF motifs (Dodt et al., 2001; Einwächter et al., 2001).

A general function of HpPex20p in the PTS2 import pathway in *H. polymorpha* was indicated by the finding that the protein is also necessary for efficient import of a heterologous fusion protein consisting of the first 50 amino acids of the *S. cerevisiae* thiolase (that carry the PTS2) and GFP. HsPex5pL and ScPex18p have also been described to have a general function in PTS2 matrix protein import (Braverman et al., 1998; Purdue et al., 1998).

In the yeast *Y. lipolytica*, in which no Pex7p has been described yet, YlPex20p is required for oligomerization and subsequent import of the PTS2 protein thiolase into peroxisomes (Titorenko et al., 1998). We observed that HpPex20p is not required for the oligomerization of the PTS2 proteins thiolase or amine oxidase in *H. polymorpha*. Whether NcPex20p is involved in protein assembly has not been studied so far. Similarly, it is not yet known whether the other auxiliary proteins (Pex5L, Pex18p, Pex21p) are involved in oligomerization of PTS2 proteins.

For *S. cerevisiae* Pex18p, Purdue and Lazarow reported that this peroxin is rapidly degraded by the proteasome (Purdue and Lazarow, 2001b). We observed a slight increase in HpPex20p levels upon addition of a proteasome inhibitor to *H. polymorpha* cells during growth on glucose. During growth of cells on methanol this effect was not observed. Compared with the HpPex20p levels in glucose-grown cells, HpPex20p levels were significantly reduced in methanol-grown cells, conditions that strongly induce peroxisome proliferation. All *H. polymorpha* peroxins studied so far are either constitutively expressed or induced during growth of cells on methanol. HpPex20p is the first *H. polymorpha* peroxin whose levels are reduced during growth of cells on methanol. The reasons behind this phenomenon are not known, but may be related to the fact that the three major peroxisomal enzymes during methanol growth all are imported via the PTS1 pathway.

Biochemistry and fluorescence microscopy revealed that bulk of HpPex20p co-localizes with peroxisomes. However, a partial cytosolic localization of this peroxin can not be excluded. Studies in other organisms reveal that the Pex7p auxiliary proteins are involved in early stages of PTS2 protein import and most likely form a cytosolic complex, together with Pex7p and the PTS2 cargo protein, prior to the actual protein translocation process. Data obtained in *Y. lipolytica* indicate that YlPex20p can interact with the matrix-localized peroxin YlPex8p, suggesting that YlPex20p may accompany the PTS2-cargo into peroxisomes (Smith and Rachubinski, 2001). A similar dual localization has been proposed for the PTS1 receptor Pex5p, which also functionally interacts with Pex8p (Rehling et al., 2000; Wang et al., 2003). Interestingly, recent data from Schäfer et al. (Schäfer et al., 2004) also reveal functional similarity between ScPex18p and the N-terminal half of ScPex5p. Based on these findings it was proposed that Pex7p and the C-terminal domain of Pex5p may be required for cargo recognition, whereas the N-terminal domain of Pex5p and full length Pex18p may fulfill functions in the actual protein import process (Schäfer et al., 2004). As in the N-terminal domain of Pex5p, WxxxF motifs that are implicated in binding to the docking proteins Pex13p and Pex14p are also found in ScPex18p, ScPex21, YlPex20p, NcPex20p and HpPex20p (Schliebs et al., 1999; Otera et al., 2002). Moreover, these proteins most likely all interact with Pex8p upon exposure to the peroxisomal matrix (Rehling et al., 2001; Smith and Rachubinski, 2001; Wang et al., 2003).

Our FCS data revealed that HpPex20p forms oligomers. Moreover, we showed that PTS2 containing synthetic peptides specifically associated with oligomeric HpPex20p. Titorenko et al. (Titorenko et al., 1998) previously showed that YlPex20p directly interacts with the PTS2 protein thiolase (Titorenko et al., 1998). This interaction was still observed when the PTS2 of thiolase was removed. This result does not exclude the possibility that YlPex20p can bind the PTS2 of thiolase, as multiple interaction domains may be present in this protein. For example, this was recently shown for *H. polymorpha* Pex5p, whose C-terminal domain recognizes the PTS1 of peroxisomal alcohol oxidase. However, upon removal of the PTS1, alcohol oxidase still interacted with HpPex5p, a process

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that involved the N-terminal domain of HpPex5p (Gunkel et al., 2004).

Except for the thiolase-YlPex20p interaction, no direct binding has been reported for other auxiliary proteins with the PTS2 or PTS2 proteins. Two hybrid studies and co-immunoprecipitation experiments reveal an interaction between ScPex18p and thiolase, however this interaction was dependent on the presence of Pex7p (Purdue et al., 1998; Stein et al., 2002). However, relative to the two-hybrid technique, FCS is much more sensitive and capable of detecting weak transient interactions. Moreover, authentic proteins are used in the FCS technique.

The finding that PTS2 peptides specifically bind to oligomeric HpPex20p may fit in the proposed pre-implex model for PTS1-peroxisomal matrix protein import (Gould and Collins, 2002). This model was based on the observation that Pex5p is a tetramer, in which each subunit is capable of binding one PTS1 matrix protein. Because peroxisomal matrix proteins are predominantly imported as oligomers, large complexes containing oligomeric Pex5p molecules and different oligomeric matrix proteins may be formed before import. For the import of the PTS2 matrix protein, a similar large protein complex might be formed. The oligomeric state of HpPex20p may be important for pre-implex formation in PTS2 protein import. As each HpPex20p has a Pex7p binding site, very large protein complexes can potentially be formed.

We speculate that the capacity of HpPex20p to bind PTS2 peptides may be important in an early stage of PTS2 import. After initial binding of a newly synthesized PTS2 protein to HpPex20p, HpPex20p may associate to Pex7p to travel to the docking site. Consistent with this are the relatively high levels that are observed in association with peroxisomes. Alternatively, PTS2-binding to HpPex20p may function as a kind of rescue mechanism next to the major PTS2 receptor Pex7p. We are currently studying these possibilities and the putative pre-implex formation in PTS2 import by FCS, thereby also including the role of HpPex7p.

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