University of Groningen

A conserved alpha helical domain at the N-terminus of Pex14p is required for PTS1 and PTS2 protein import in Hansenula polymorpha

de Vries, Bart; Kiel, Jan A. K. W.; Scheek, Ruud; Veenhuis, Marten; van der Klei, Ida J.

Published in:
FEBS Letters

DOI:
[10.1016/j.febslet.2007.11.012](https://doi.org/10.1016/j.febslet.2007.11.012)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

de Vries, B., Kiel, J. A. K. W., Scheek, R., Veenhuis, M., & van der Klei, I. J. (2007). A conserved alpha helical domain at the N-terminus of Pex14p is required for PTS1 and PTS2 protein import in Hansenula polymorpha. *FEBS Letters*, 581(29), 5627-5634. <https://doi.org/10.1016/j.febslet.2007.11.012>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

A conserved alpha helical domain at the N-terminus of Pex14p is required for PTS1 and PTS2 protein import in *Hansenula polymorpha*

Bart de Vries^a, Jan A.K.W. Kiel^a, Ruud Scheek^b, Marten Veenhuis^a, Ida J. van der Klei^{a,*}

^a Molecular Cell Biology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

^b Molecular Dynamics, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, 9747 AG Groningen, The Netherlands

Received 20 August 2007; revised 5 October 2007; accepted 5 November 2007

Available online 20 November 2007

Edited by Ulrike Kutay

Abstract We have analyzed the highly conserved N-terminus of *Hansenula polymorpha* Pex14p for its function in peroxisomal matrix protein import. The region comprising aa 10–54 of HpPex14p is predicted to contain three α -helices. Its α -helical structure was confirmed by CD analysis of a synthetic peptide, corresponding to residues 8–58. Deletion of aa 1–21 of HpPex14p, but not of aa 1–9, completely abolished PTS1 and PTS2 matrix protein import. An extensive mutational analysis of the first α -helix (aa 10–21) demonstrated that its secondary structure, as well as residues Phe20 and Leu21, are essential for PTS1 and PTS2 matrix protein import. © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Peroxisome; Pex14p; Biogenesis; Protein import

1. Introduction

Peroxisomal matrix protein import requires the function of various peroxins. These include soluble receptor proteins (Pex5p and Pex7p) and two protein complexes in the peroxisome membrane, namely the docking complex (Pex13p, Pex14p, Pex17p) and the RING finger complex (Pex2p, Pex10p, Pex12p). These two complexes are thought to form the peroxisomal importomer [1].

Pex14p, together with Pex13p, is regarded to be a key component of the receptor docking site. Pex14p has been identified and characterized in a variety of organisms, including lower and higher eukaryotes [2]. Pex5p is the receptor for the Peroxisomal Targeting Signal 1 (PTS1), which is a C-terminal tripeptide that is present in most peroxisomal matrix proteins. Pex7p specifically recognizes the PTS2, a degenerated nonapeptide present at the N-terminus of few matrix proteins. Pex5p and Pex7p shuttle in and out of peroxisomes, but are predominantly cytosolic in stationary cells [3]. A third component of the docking site is Pex17p, a membrane-bound peroxin that interacts with Pex14p, but not with Pex13p or the receptor proteins. Pex17p can only be identified in yeast species [4] and its function is still unresolved.

Pex14p contains three conserved structural elements [2]. (i) Within the first 80 N-terminal residues, a conserved core region of approximately 50 amino acids exists. In *Saccharomyces cerevisiae* this region is involved in Pex5p binding. In mammalian Pex14p, also Pex13p and Pex19p interact with this N-terminal domain. The association of Pex19p with the N-terminus of HsPex14p has been suggested to play a role in modulating the association of Pex5p with the docking site [5,6]. (ii) A central coiled-coil region in Pex14p may play a role in dimerization. Between the extreme N-terminus and the coiled coil domain a hydrophobic region is located, which might represent a possible membrane spanning domain. (iii) The C-terminal half of Pex14p lacks conserved domains and its function is still highly speculative. In *S. cerevisiae*, a second Pex5p binding domain together with a Pex7p binding region, have been identified in this C-terminal region [2]. This was however not confirmed in other organisms.

Several mutagenesis studies and analyses of protein–protein interactions have been carried out to determine the role of the conserved core region in the N-terminus of Pex14p of various organisms [5–9]. However, translation of all data into a general model for the function of the Pex14p N-terminus in matrix protein import is still difficult. This is among others due to the fact that many mutations have only been analyzed for their effects on specific protein–protein interactions, but not for their functional role in vivo. Moreover, conflicting data from in vitro binding studies have been presented (e.g. the role of residues F35 and L36 in HsPex14p in binding of HsPex5p [5,6]). Studies in mammals have the additional disadvantage that mutations in the N-terminal region of Pex14p may cause mislocalization of the protein to mitochondria, which severely hampers the interpretation of in vivo data [5,6].

We analyzed the N-terminus of *Hansenula polymorpha* Pex14p (HpPex14p) and focussed on its functional role in PTS1 and PTS2 protein import in vivo as well as on the structural properties of this domain. *H. polymorpha* PEX14 has been shown to be involved in both PTS1 and PTS2 protein import [10]. Previous studies have revealed that in *H. polymorpha* the N-terminus of Pex14p is not essential for the proper localization of the protein to the peroxisomal membrane [11].

We now show that the conserved N-terminal domain is largely alpha helical. In addition we demonstrate that in the first short alpha helix (12 residues long) only two residues are important for PTS1 and PTS2 protein import.

*Corresponding author. Fax: +31 50 3638280.

E-mail address: I.J.van.der.Klei@rug.nl (I.J. van der Klei).

2. Materials and methods

2.1. Micro-organisms and growth conditions

The *H. polymorpha* strains used in this study are listed in Table 1. Cells were grown at 37 °C on mineral medium (MM, [13]) or yeast nitrogen base (YNB) using as carbon source 0.5% glucose, 0.5% methanol, 0.5% glycerol or 0.05% glycerol + 0.5% methanol. 0.25% ammonium sulfate or 0.25% methylamine were used as nitrogen sources. For cloning purposes, *Escherichia coli* DH5 α (Gibco-Brl, Gaithersburg, MD) was used.

To test growth of *H. polymorpha* cells on methanol, yeast colonies from fresh YNB-glucose plates were resuspended in sterile milli-Q water and diluted to OD₆₆₀ = 5.0. 10-fold, 100-fold and 1000-fold dilutions were prepared and 4 μ l of each dilution was spotted onto YNB-methanol plates and incubated for 3 days at 37 °C.

2.2. Miscellaneous DNA techniques

The plasmids and oligonucleotide primers used in this study are listed in Tables 2 and 3, respectively. All DNA manipulations were carried out according to standard techniques. *H. polymorpha* cells were transformed by electroporation [14]. DNA modifying enzymes were used as recommended by the supplier (Roche, Almere, and The Netherlands). *Pwo* polymerase was used for preparative polymerase chain reactions (PCR). For DNA sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, USA) was used. Sequence alignments were made using ClustalX software [15] in combination with Genedoc (available at <http://www.psc.edu/biomed/genedoc>). The JPRED program (see <http://www.compbio.dundee.ac.uk/~www-jpred/submit.html>) was used to analyze the alignment shown in Fig. 1 for the presence of α -helices [16].

2.3. Construction of strains

Detailed information on the construction of the *pex14* strain and strains that contain GFP.SKL or the first 50 amino acids of bakers yeast thiolase fused to GFP (ThioN₅₀-GFP) is available on request.

Point mutations in *PEX14* were made by two-step-PCR. First, a DNA fragment was obtained by PCR with a mutagenesis primer and primer PEX14-deltaC108 using plasmid pBSK-PER10 as template. The resulting PCR product was isolated and used as primer in combination with the M13/pUC reverse sequencing primer in a second PCR again using pBSK-PER10 as template. The resulting PCR product was digested with *Bam*HI and *Nco*I and cloned in *Bam*HI + *Nco*I-digested pHIPX10-PEX14. The presence of the mutation was confirmed by sequencing.

2.4. Biochemical methods

Crude cell extracts of TCA precipitated cells were prepared as described [19]. SDS-PAGE and Western blotting were performed by established procedures. Blots were probed with polyclonal antibodies against HpPex14p [10].

2.5. Microscopy

Fluorescence microscopy studies were performed as described [19]. Intact cells were prepared for electron microscopy and immunocytochemistry as described previously [20].

2.6. Circular dichroism analysis

A synthetic peptide corresponding to amino acids 8–58 of *H. polymorpha* Pex14p: C-TSRAELVSSAVEFLLDQSIADSLAKKVE-FLESKGLTQQEIEEALQKARTG-CO-HN2 was obtained from Pepscan Systems (Lelystad, The Netherlands). The peptide was dissolved to a concentration of 40 μ M in 10 mM potassium phosphate buffer, pH 6.5. Circular dichroism spectra were recorded from 190 to 250 nm on an Aviv 62A DS CD spectrometer, using a 1 mm quartz cuvette. The sample compartment was flushed with nitrogen gas and the temperature was kept constant at 25 °C, unless indicated otherwise. Six scans were averaged, using a bandwidth of 1 nm, a stepsize of 1 nm and 3 s averaging per point. The spectra were corrected using a reference solution without the protein.

Table 1
Strains used in this study

Strains	Genotype and characteristics	References
WT	NCYC495 <i>leu1.1</i>	[12]
WT	NCYC495 <i>leu1.1. ura3</i>	[12]
<i>pex14::pHIPX10-PEX14-ΔC58</i>	<i>pex14</i> with plasmid containing <i>PEX14</i> lacking the final 58 aa	[11]
<i>pex14</i>	<i>pex14::HpURA3 leu1.1</i>	This study
<i>pex14::P_{AOX}GFP.SKL</i>	<i>pex14</i> with single-copy integration of pANL29 at P _{AOX} locus <i>leu1.1</i> , <i>zeo</i> ^R	This study
<i>pex14::P_{AMO}PTS2.GFP</i>	<i>pex14</i> with single-copy integration of pHIPX5-ThioN ₅₀ -GFP at P _{AMO} locus, <i>leu1.1</i> , <i>zeo</i> ^R	This study

Table 2
Plasmids used in this study

Plasmid name	Characteristics	Reference
pBSK-PER10	pBluescript SK ⁺ containing the <i>PEX14</i> gene	[10]
pANL29	Plasmid containing P _{AOX} driven GFP.SKL, <i>Zeo</i> ^R	[17]
pHIPX5-ThioN ₅₀ -GFP	Plasmid containing P _{AMO} driven PTS2.GFP, Kan ^R , ScLeu2	[18]
pHIPX5-ThioN ₅₀ -GFP	Plasmid containing P _{AMO} driven PTS2.GFP, Amp ^R , <i>Zeo</i> ^R	This study
pHIPX10-PEX14	<i>E. coli/H. polymorpha</i> shuttle plasmid containing P _{PEX14} driven <i>PEX14</i> gene. Kan ^R , ScLeu2	[11]
pHIPX10-PEX14-R10E	pHIPX10-PEX14, with <i>PEX14</i> Arg10Glu mutation	This study
pHIPX10-PEX14-E12L	pHIPX10-PEX14, with <i>PEX14</i> Glu12Leu mutation	This study
pHIPX10-PEX14-L13D	pHIPX10-PEX14, with <i>PEX14</i> Leu13Asp mutation	This study
pHIPX10-PEX14-V14E	pHIPX10-PEX14, with <i>PEX14</i> Val14Glu mutation	This study
pHIPX10-PEX14-S15V	pHIPX10-PEX14, with <i>PEX14</i> Ser15Val mutation	This study
pHIPX10-PEX14-S16V	pHIPX10-PEX14, with <i>PEX14</i> Ser16Val mutation	This study
pHIPX10-PEX14-A17P	pHIPX10-PEX14, with <i>PEX14</i> Ala17Pro mutation	This study
pHIPX10-PEX14-V18E	pHIPX10-PEX14, with <i>PEX14</i> Val18Glu mutation	This study
pHIPX10-PEX14-E19L	pHIPX10-PEX14, with <i>PEX14</i> Glu19Leu mutation	This study
pHIPX10-PEX14-F20S	pHIPX10-PEX14, with <i>PEX14</i> Phe20Ser mutation	This study
pHIPX10-PEX14-L21D	pHIPX10-PEX14, with <i>PEX14</i> Leu21Asp mutation	This study
pHIPX10-PEX14-ΔN9	pHIPX10-PEX14, with <i>PEX14</i> lacking the first 9 aa	This study
pHIPX10-PEX14-ΔN21	pHIPX10-PEX14, with <i>PEX14</i> lacking the first 9 aa	This study
pHIPX10-PEX14-ΔC58	pHIPX10-PEX14, with <i>PEX14</i> lacking the final 58 aa	[11]

Table 3
Primers used in this study

Primer name	Primer sequence
PEX14-P1	5' GAGCATCGCAGAAATCGACATCTG 3'
PEX14-P2-U1	5' GCGTTTGCTGAACATCAGTTGAAGCTCGGTGAGGAAGAAAAAGAGACAAC 3'
PEX14-T1-U2	5' GAAGAAGCGACGCCGATCCAGTTGATCCTAAACCATCCTTCTACCGTACC 3'
PEX14-T2	5' CAACAGACCAAGACACGCTCCAC 3'
PEX14-R10E	5' GCAACGACACGCGAAGCAGAGCTCGTATCC AGCGCA 3'
PEX14-E12L	5' GACCAGCCGTGCACTGCTGGTATCCAGC 3'
PEX14-L13D	5' AGCCGTGCAGAGGACGCTCTCCAGCGCAGTT 3'
PEX14-V14E	5' CGTGACAGAGCTGGAGTCCAGCGCAGTT 3'
PEX14-S15V	5' GCAGAGCTGGTAGTCAGCGCAGTTGAG 3'
PEX14-S16V	5' GAGCTGGTATCCGTCGAGTTGAGTTTC 3'
PEX14-A17P	5' CTGGTATCCAGCCGTTGAGTTTCTTTTG 3'
PEX14-V18E	5' GTATCCAGCGCAGAGGAGTTTCTTTTG 3'
PEX14-E19L	5' TCCAGCGCAGTTCTGTTTCTTTTGGAC 3'
PEX14-F20S	5' CAGCGCAGTTGAAAGCTTGTGGACCGAGC 3'
PEX14-L21D	5' GCAGTTGAGTTGACTTGGACCGAGC 3'
PEX14-ΔN9	5' CACAGGATCCATGCG TGCAGAGCTGGTATCCAG 3'
PEX14-ΔN21	5' CACAGGATCCATGTTGGACCGAGCATTGCGGAC 3'
PEX14-delta-C108	5' AAGCTTTTAAGCCACGGCGATGCCAGAGG 3'
M13/pUC reverse	5' CAGGAAACAGCTATGAC 3'

Mutant codons are underlined.

Fig. 1. Sequence alignment of the N-termini of various Pex14 proteins, using the Clustal_X and GeneDoc programs. Gaps were introduced to maximize the similarity. Abbreviations of species and accession numbers are, Hp: *Hansenula polymorpha* (GenBank Accession No: AAB40596); Pp: *Pichia pastoris* (GenBank Accession No: AAG28574); An: *Aspergillus nidulans* (GenBank Accession No: EAA61046); Nc: *Neurospora crassa* (GenBank Accession No: EAA28304); Sc: *Saccharomyces cerevisiae* (GenBank Accession No: AAS56829); *Schizosaccharomyces pombe* (GenBank Accession No: CAA18656); Ce: *Caenorhabditis elegans* (GenBank Accession No: CAB03225); Hs: *Homo sapiens* (GenBank Accession No: CAI19198); Dm: *Drosophila melanogaster* (GenBank Accession No: NP_649253). Residues that are similar in all proteins are shaded black. Similar residues in at least seven of the proteins are shaded dark grey while those that are similar in at least five of the proteins are shaded light grey. The putative alpha helices identified, using the JPRED program, are indicated below the alignment. Amino acid substitutions introduced in the first predicted alpha helix are indicated above the alignment.

3. Results

3.1. Secondary structure of the N-terminal domain of *H. polymorpha* Pex14p

To determine conserved residues in the extreme N-terminus of HpPex14p, we aligned the first 58 residues with the corresponding regions in Pex14p from various other organisms. The alignment (Fig. 1) indicates that especially residues 10–55 of HpPex14p are highly conserved. This region of 46 residues starts with RxxL/M in all species analyzed. Another highly conserved feature is the presence of two FL/I dipeptides. As evident from Fig. 1, additional highly conserved residues occur as well. Using the JPRED program [16], three short alpha helices (12, 9 and 10 residues long, respectively) are predicted (Fig. 1). Between helices 1 and 2 one or two prolines are generally present that are absent between the helices 2 and 3.

To determine whether the N-terminus of HpPex14p indeed is largely alpha helical, we analyzed a synthetic peptide corresponding to residues 8–58 of HpPex14p by circular dichroism (Fig. 2). The maximum at 190 nm and the two minima at 208 and 222 nm are indicators of a predominantly α -helical secondary structure. The spectrum closely resembles that of the N-terminal domain (residues 1–51) of the lac repressor [21], which contains three α -helices. A temperature scan revealed that at 90 °C more than 50% of the 220 nm circular dichroism was still present, indicating a remarkably stable α -helical fold.

3.2. The first N-terminal alpha helix in HpPex14p is important for PTS1 protein import

We previously showed that two N-terminally truncated HpPex14p variants, namely Δ N31 and Δ N64, could not restore the PTS1 protein import defect of *H. polymorpha* pex14

Fig. 2. Circular dichroism spectrum of a synthetic peptide comprised of amino acids 8–58 of HpPex14p is shown. The maximum at 190 nm and the two minima at 208 and 222 nm point towards a predominantly α -helical structure.

null mutant cells [11]. To analyze whether shorter N-terminal deletions also affected PTS1 protein import, we constructed two additional truncated genes encoding HpPex14p variants lacking either the first 9 N-terminal non-conserved residues (Δ N9) or lacking the first 21 residues (Δ N21, which includes the first predicted alpha helix). *pex14* cells, expressing these truncated *PEX14* genes were analyzed for growth on methanol as carbon and energy source, which requires the proper peroxisomal localization of the PTS1 proteins alcohol oxidase, dihydroxyacetone synthase and catalase [10]. Cultivation experiments revealed that Δ N9, but not Δ N21 cells, were still capable to grow on methanol (Fig. 3A). Fluorescence microscopy analysis of strains producing GFP.SKL confirmed that PTS1 protein import was prohibited in Δ N21, but not in Δ N9 cells (Fig. 3C). The PTS1 protein import defect in Δ N21 cells was not related to highly reduced steady state HpPex14p levels as shown by Western blot analysis using α -HpPex14p antibodies (Fig. 3B).

To establish the importance of the first alpha helix in HpPex14p, we introduced a proline in this region (substitution of Ala17 into Pro, PEX14^{A17P}). Western blotting showed that the mutant protein was normally produced (Fig. 3A), but unable to restore the PTS1 import defect (Fig. 3C) and the methanol growth defect of *pex14* cells (Fig. 3A). Electron microscopy confirmed that only very small peroxisomal structures were observed in these cells (Fig. 3D) when compared to peroxisomes in WT *H. polymorpha* cells [19], but similar as observed in *pex14* cells (Fig. 3D).

3.3. Mutational analysis of the first alpha helix of HpPex14p

To analyze whether predominantly the structure or also specific conserved residues were important for the function of HpPex14p in PTS1 protein import, we performed an extensive mutational analysis of all residues present in the first predicted alpha helix (residues 10–21). Amino acid substitutions were chosen such that the changes do not disturb the alpha helical structure of this domain (Fig. 1), while the characteristics of

the side chains are changed dramatically. As shown in Fig. 4A, only mutations Phe20Ser and Leu21Asp completely inhibited methanol growth, indicative for a severe PTS1 protein import defect. This import defect was also evident from fluorescence microscopy. In most mutants GFP.SKL is localized to distinct spots (Fig. 4C; shown for mutants S15V and E19L), indicating that the protein is properly sorted to peroxisomes. However, in strains producing Pex14p^{F20S} or Pex14p^{L21D}, GFP fluorescence was observed throughout the cytosol (Fig. 4C). Western blotting revealed that the import defect was not due to the absence of mutant Pex14p proteins (Fig. 4B). Comparable peroxisome structures as in Pex14p^{A17P} were observed in Pex14p^{F20S} and Pex14p^{L21D} by electron microscopy (Fig. 4D).

3.4. PTS2 protein import

We also analyzed the role of the N-terminal domain of Pex14p in PTS2 protein import. An expression cassette for the production of GFP containing a PTS2 (PTS2.GFP[18]) was introduced into the various constructed mutant strains. As shown in Fig. 5 deletion of the first 9 N-terminal residues of Pex14p did not affect import of PTS2.GFP into peroxisomes. However, like import of GFP.SKL, import of PTS2.GFP was abolished upon deletion of the first 21 N-terminal residues. Remarkably, the point mutations defective in PTS1 import (A17P, F20S or L21D) also displayed a defect in PTS2.GFP import (Fig. 5).

We previously showed that the last C-terminal 58 residues of HpPex14p are redundant for PTS1 protein import [11]. Immunocytochemical staining using antibodies against the PTS2 protein amine oxidase (AMO) revealed that also PTS2 protein import is normal in a strain producing this C-terminal truncated version of Pex14p (PEX14^{ΔC58} cells; Fig. 6).

4. Discussion

We have performed a structural and mutational analysis of a highly conserved 46 amino acid region at the N-terminus of *H. polymorpha* Pex14p. Circular dichroism analysis of this region indicated that it has an alpha helical structure, which is in agreement with the secondary structure prediction based on the JPRED program. The latter program predicted three short alpha helices of 9–12 residues.

Deletion of the extreme 9 N-terminal residues from HpPex14p, which precede the conserved region, had no effect on PTS1 matrix protein import. However, when in addition the first predicted alpha helix in the conserved region was deleted (Δ N21), a block in PTS1 import was observed. The same phenotype was obtained upon introduction of a helix-breaking proline residue in the middle of the first predicted alpha helix (A17P), suggesting that the secondary structure indeed is important for the function of this domain.

A detailed mutational analysis of all 10 conserved residues in the first predicted alpha helix revealed that solely the two residues of the conserved PheLeu dipeptide (Phe20 or Leu21) were essential for PTS1 protein import. Hence, these two residues are essential for the proper functioning of HpPex14p in PTS1 protein import in vivo. Data obtained with Pex5p from several species indicated that this protein interacts with the conserved N-terminal domain of Pex14p [2]. WxxxY/F motifs

Fig. 3. (A) Growth of WT, *pex14*, *PEX14^{ΔN9}*, *PEX14^{ΔN21}* and *PEX14^{A17P}* cells on methanol. Spots were placed on YNB-methanol plates. (B) Western blot of same strains decorated with α-HpPex14p antibodies. Equal amounts of protein were loaded per lane. (C) Fluorescence microscopy of GFP.SKL localization in WT, *pex14*, *PEX14^{ΔN9}*, *PEX14^{ΔN21}* and *PEX14^{A17P}* cells, induced on methanol. (D) Electron micrographs of *pex14* and *PEX14^{A17P}* cells, grown on glycerol/methanol. Peroxisomes remnants are shown. Key: P, peroxisome; N, nucleus; M, mitochondria; V, vacuole. The bar represents 0.5 μm.

in human, plant and trypanosomal Pex5p were shown to be important for this interaction. In *S. cerevisiae* Pex5p however not this pentapeptide motif, but instead an inverted FxxxW motif is important for association with Pex14p [22]. All these pentapeptide motifs are parts of amphipathic helices in which the most critical aromatic residues are positioned at one side of the helix providing a hydrophobic anchor for Pex14p. Also HpPex5p has two WxxxY/F motifs as well as a conserved

FxxxW motif. This might suggest that the FL dipeptide in the N-terminus of HpPex14p could provide a binding site for an aromatic residue of one of the pentapeptides present in HpPex5p. However, mutations in the first FL dipeptide of HsPex14p gave varying results with respect to Pex5p binding in vitro [5,6]. An HsPex14p variant containing the mutation F35S did not abolish interaction with Pex5p, whereas Pex5p binding was enhanced for mutant L36R [5]. On the other hand

Fig. 4. (A) Spot assay of point mutants *PEX14*^{R10E}, *PEX14*^{E12L}, *PEX14*^{L13D}, *PEX14*^{V14E}, *PEX14*^{S15V}, *PEX14*^{S16V}, *PEX14*^{V18E}, *PEX14*^{E19L}, *PEX14*^{F20S}, *PEX14*^{L21D} on YNB-methanol plates. WT *H. polymorpha* was used as a control. (B) Western blot of the same strains decorated with α -HpPex14p antibodies. Equal amounts of protein were loaded per lane. (C) Fluorescence microscopy of GFP::SKL produced in the strains indicated above. (D) Electron micrographs to show peroxisomal structures in *PEX14*^{F20S} and *PEX14*^{L21D} cells. Cells were grown on methanol or glycerol/methanol. Key: P, peroxisome; N, nucleus; M, mitochondria; V, vacuole. The bar represents 0.5 μ m.

the double mutant F35A L36A showed reduced Pex5p binding. What the effect of these mutations is on the function of HsPex14p in vivo is difficult to study, because these mutant HsPex14 proteins mislocalize to mitochondria [5,6].

In yeast, mutations or deletions at the N-terminus of Pex14p generally do not affect the localization of the protein. In *S. cerevisiae* deletion of the N-terminal 58 residues did not affect proper sorting [23]. Similarly the N-terminal deletions Δ N31 and Δ N64 in *H. polymorpha* Pex14p did not affect the localization of HpPex14p [11]. Also, fluorescence microscopy analysis of WT *H. polymorpha* cells producing HpPex14p^{F20S}-GFP revealed that this protein is correctly

sorting to peroxisomes (our unpublished data). This indicates that the conserved N-terminal domain of HpPex14p is indeed directly involved in matrix protein import, most likely in the transient association of Pex5p to the docking site during PTS1 protein import.

S. cerevisiae Pex14p contains a second Pex5p binding site in the C-terminal half of the protein, which overlaps with the sole Pex7p binding region [23,24]. These Pex5p and Pex7p binding domains have not been identified in the C-terminal region of Pex14p of other species. In HpPex14p this second Pex5p binding site and Pex7p binding site are most likely also absent or redundant, because C-terminal deletions (up to 58 residues)

Fig. 5. Fluorescence microscopy, showing the location of PTS2.GFP in WT, *pex14*, PEX14^{ΔN9}, PEX14^{ΔN21}, PEX14^{A17P}, PEX14^{F20S} and PEX14^{L21D} cells. Cells were grown on glucose/methylamine.

Fig. 6. Immunocytochemical localization of AMO in cells producing C-terminally truncated form of HpPex14p (PEX14^{ΔC58}). Cells were grown on methanol/methylamine to induce peroxisome proliferation and the synthesis of AMO. The labelling is present on peroxisomal profiles. Key: P, peroxisome; M, mitochondria. The bar represents 0.5 μm.

in HpPex14p had no effect on PTS1 [11] or PTS2 (Fig. 6, this study) matrix protein import.

Our current data indicate that the conserved N-terminal region in HpPex14p is important for both PTS1 and PTS2 protein import and that the same mutations that affect PTS1 import also block import of PTS2 proteins. This lends support to the view that HpPex7p indirectly associates to the N-terminus of HpPex14p via HpPex20p. The N-terminal half of Pex5p and Pex20p (or Pex18p and Pex21p in *S. cerevisiae*) have sequence and functional similarities [22,25]. Moreover, HpPex20p contains two WxxxY/F motifs [18]. Hence, it is possible that both Pex5p and Pex20p functionally associate to the same domain in Pex14p. As the PTS2 receptor Pex7p interacts with a conserved Pex7p binding box in Pex20p, Pex7p might then indirectly associate to Pex14p via Pex20p [3]. Alternatively, HpPex7p may also directly bind to the Pex14p N-terminus.

Acknowledgements: We thank Dr. Xiaoqin Wang for assistance in obtaining the CD-spectrum and R.S. Booij for skillful assistance in electron microscopy. BdV and JAKWK are supported by ALW/NWO.

References

- [1] Platta, H.W. and Erdmann, R. (2007) The peroxisomal protein import machinery. FEBS Lett. 581, 2811–2819.
- [2] Azevedo, J.E. and Schliebs, W. (2006) Pex14p, more than just a docking protein. Biochim. Biophys. Acta 1763, 1574–1584.
- [3] Erdmann, R. and Schliebs, W. (2005) Peroxisomal matrix protein import: the transient pore model. Nat. Rev. Mol. Cell Biol. 6, 738–742.
- [4] Kiel, J.A., Veenhuis, M. and van der Klei, I.J. (2006) PEX genes in fungal genomes: common, rare or redundant. Traffic 7, 1291–1303.
- [5] Franssen, M., Vastiau, I., Brees, C., Brys, V., Mannaerts, G.P. and van Veldhoven, P.P. (2004) Potential role for Pex19p in assembly of PTS-receptor docking complexes. J. Biol. Chem. 279, 12615–12624.
- [6] Itoh, R. and Fujiki, Y. (2006) Functional domains and dynamic assembly of the peroxin Pex14p, the entry site of matrix proteins. J. Biol. Chem. 281, 10196–10205.
- [7] Nito, K., Hayashi, M. and Nishimura, M. (2002) Direct interaction and determination of binding domains among peroxisomal import factors in *Arabidopsis thaliana*. Plant Cell Biol. 43, 355–366.
- [8] Choe, J., Moyersoen, J., Roach, C., Carter, T.L., Fan, E., Michels, P.A. and Hol, W.G. (2003) Analysis of the sequence motifs responsible for the interactions of peroxins 14 and 5, which are involved in glycosome biogenesis in *Trypanosoma brucei*. Biochemistry 42, 10915–10922.
- [9] Kerssen, D., Hambruch, E., Klaas, W., Platta, H.W., de Kruijff, B., Erdmann, R., Kunau, W.H. and Schliebs, W. (2006) Membrane association of the cycling peroxisome import receptor Pex5p. J. Biol. Chem. 281, 27003–27015.
- [10] Komori, M., Rasmussen, S.W., Kiel, J.A., Baerends, R.J., Cregg, J.M., van der Klei, I.J. and Veenhuis, M. (1997) The *Hansenula polymorpha* PEX14 gene encodes a novel peroxisomal membrane protein essential for peroxisome biogenesis. EMBO J. 16, 44–53.
- [11] Bellu, A.R., Komori, M., Kiel, J.A., van der Klei, I.J. and Veenhuis, M. (2001) Peroxisome biogenesis and selective degradation converge at Pex14p. J. Biol. Chem. 276, 44570–44574.
- [12] Gleeson, M.A.G. and Sudbery, P.E. (1998) Genetic analysis in the methylotrophic yeast *Hansenula polymorpha*. Yeast 4, 293–303.
- [13] Van Dijken, J.P., Otto, R. and Harder, W. (1976) Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. Arch. Microbiol. 111, 137–144.
- [14] Faber, K.N., Haima, P., Harder, W., Veenhuis, M. and AB, G. (1994) Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*. Curr. Genet. 25, 305–310.
- [15] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

- [16] Cuff, J.A. and Barton, G.J. (2000) Application of multiple sequence alignment profiles to improve protein secondary structure prediction. *Proteins* 40, 502–511.
- [17] Leão-Helder, A.N., Krikken, A.M., van der Klei, I.J., Kiel, J.A. and Veenhuis, M. (2003) Transcriptional down-regulation of peroxisome numbers affects selective peroxisome degradation in *Hansenula polymorpha*. *J. Biol. Chem.* 278, 40749–40756.
- [18] Otzen, M., Wang, D., Lunenborg, M.G. and van der Klei, I.J. (2005) *Hansenula polymorpha* Pex20p is an oligomer that binds the peroxisomal targeting signal 2 (PTS2). *J. Cell Sci.* 118, 3409–3418.
- [19] Baerends, R.J., Faber, K.N., Kram, A.M., Kiel, J.A., van der Klei, I.J. and Veenhuis, M. (2000) A stretch of positively charged amino acids at the N terminus of *Hansenula polymorpha* Pex3p is involved in incorporation of the protein into the peroxisomal membrane. *J. Biol. Chem.* 275, 9986–9995.
- [20] Waterham, H.R., Titorenko, V.I., Haima, P., Cregg, J.M., Harder, W. and Veenhuis, M. (1994) The *Hansenula polymorpha* *PER1* gene is essential for peroxisome biogenesis and encodes a peroxisomal matrix protein with both carboxy- and amino-terminal targeting signals. *J. Cell Biol.* 127, 737–749.
- [21] Schnarr, M. and Maurizot, J.C. (1982) Secondary structure of the lac repressor headpiece. Possibilities and limitations of a joint infrared and circular dichroism study. *Eur. J. Biochem.* 128, 515–520.
- [22] Schäfer, A., Kerssen, D., Veenhuis, M., Kunau, W.H. and Schliebs, W. (2004) Functional similarity between the peroxisomal PTS2 receptor binding protein Pex18p and the N-terminal half of the PTS1 receptor Pex5p. *Mol. Cell Biol.* 24, 8895–8906.
- [23] Niederhoff, K., Meindl-Beinker, N.M., Kerssen, D., Perband, U., Schäfer, A., Schliebs, W. and Kunau, W.H. (2005) Yeast Pex14p possesses two functionally distinct Pex5p and one Pex7p binding sites. *J. Biol. Chem.* 280, 35571–35578.
- [24] Williams, C., van den Berg, M. and Distel, B. (2005) *Saccharomyces cerevisiae* Pex14p contains two independent Pex5p binding sites, which are both essential for PTS1 protein import. *FEBS Lett.* 579, 3416–3420.
- [25] Dodt, G., Warren, D., Becker, E., Rehling, P. and Gould, S.J. (2001) Domain mapping of human *PEX5* reveals functional and structural similarities to *Saccharomyces cerevisiae* Pex18p and Pex21p. *J. Biol. Chem.* 276, 41769–41781.