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Pex7p and Pex20p of *Neurospora crassa* Function Together in PTS2-dependent Protein Import into Peroxisomes

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Recruiting matrix proteins with a peroxisomal targeting signal type 2 (PTS2) to the peroxisomal membrane requires species-specific factors. In *Saccharomyces cerevisiae*, the PTS2 receptor Pex7p acts in concert with the redundant Pex18p/Pex21p, whereas in *Yarrowia lipolytica*, Pex20p might unite the function of both *S. cerevisiae* peroxins. Herein, the genome of the filamentous fungus *Neurospora crassa* was analyzed for peroxin-encoding genes. We identified a set of 18 peroxins that resembles that of *Y. lipolytica* rather than that of *S. cerevisiae*. Interestingly, proteins homologous to both *S. cerevisiae* Pex7p and *Y. lipolytica* Pex20p exist in *N. crassa*. We report on the isolation of these PTS2-specific peroxins and demonstrate that *Nc*Pex20p can substitute for *S. cerevisiae* Pex18p/Pex21p, but not for *Sc*Pex7p. Like Pex18p, *Nc*Pex20p did not bind PTS2 protein or the docking proteins in the absence of *Sc*Pex7p. Rather, *Nc*Pex20p was required before docking to form an import-competent complex of cargo-loaded PTS2 receptors. *Nc*Pex7p did not functionally replace yeast Pex7p, probably because the *N. crassa* PTS2 receptor failed to associate with Pex18p/Pex21p. However, once *Nc*Pex7p and *Nc*Pex20p had been coexpressed, it proved possible to replace yeast Pex7p. Pex20p and Pex18p/Pex21p are therefore true orthologues, both of which are in need of Pex7p for PTS2 protein import.

INTRODUCTION

Peroxisome biogenesis has been studied in various model organisms ranging from yeast to human (for recent reviews, see Holroyd and Erdmann, 2001; Purdue and Lazarow, 2001; Sacksteder and Gould, 2000; Subramani *et al.*, 2000). This has led to the identification of a number of *PEX* genes that are specifically implicated in that process. Many of the corresponding gene products, the so-called peroxins, are involved in peroxisomal matrix protein import, which can take place via two pathways, the first of which is dependent on the type 1 peroxisomal targeting signal (PTS1), and the second on the type 2 (PTS2). The cognate receptors for the PTS1 and the PTS2 signal are Pex5p and Pex7p, respectively, both of which bind their cargo proteins in the cytosol. On docking of the cargo-loaded receptors to the peroxisomal membrane, the two pathways seem to merge, because the peroxins of the docking complex, Pex14p and Pex13p, are mandatory for both routes.

Although several peroxins have been identified in all organisms analyzed, some of them seem to be species specific. One example is the class of proteins required to direct PTS2 cargo proteins to the peroxisomal membrane. In mammals, the PTS2 signal-recognition factor Pex7p acts in concert with Pex5pL, the long isoform of the PTS1 receptor (Braverman *et al.*, 1998; Otera *et al.*, 2000), whereas *Saccharomyces cerevisiae* uses Pex7p and the redundant Pex18p and Pex21p for that purpose (Purdue *et al.*, 1998). In the case of *Yarrowia lipolytica*, which generally seems to carry a number of unique peroxins, Pex20p is essential for PTS2-dependent protein import (Titorenko *et al.*, 1998), whereas a Pex7p orthologue has not been found in that organism.

On the other hand, evidence for a more conserved function of these divergent factors has recently emerged. The insertion within Pex5pL that is encoded by an additional exon and necessary for the Pex5pL interaction with Pex7p shows similarity to a region within Pex18p and Pex21p that is also involved in contacting Pex7p (Dodt *et al.*, 2001; Otera *et al.*, 2002). Even Pex20p contains such a Pex7p-binding

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Table 1. *S. cerevisiae* strains used

S. cerevisiae strain	Description	Source or reference
UTL-7A	*MATα leu2-3, 112 ura3-52 trp1*	(Erdmann *et al.*, 1989)
HF7c	*MAT*a *ura3-52 his3-200lys2-801 ade2-101 trp1-901 ssleu2-3/112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17-mers)$_3$-CYC1-LacZ*	(Feilotter *et al.*, 1994)
UTL-7A *pex7*Δ	*pex7*Δ*::LEU2*	(Marzioch *et al.*, 1994)
UTL-7A *pex14*Δ	*pex14*Δ*::LEU2*	(Albertini *et al.*, 1997)
HF7c *pex7*Δ	*pex7*Δ*::LEU2*	(Stein *et al.*, 2002)
yKat36	UTL-7A*pex18*Δ*::kanMX4 pex21*Δ*::loxP*	(Stein *et al.*, 2002)
yKat110	UTL-7A*pex14*Δ*::LEU2 pex18*Δ*::kanMX4 pex21*Δ*::loxP*	(Stein *et al.*, 2002)
yKat111	UTL-7A *pex18*Δ*::kanMX4 pex21*Δ*::loxP* [pHPR131]	(Stein *et al.*, 2002)
yHPR251	UTL-7A [pHPR131]	(Stein *et al.*, 2002)
yHPR252	UTL-7A *pex7*Δ*::LEU2* [pHPR131]	(Stein *et al.*, 2002)
yAS3	UTL-7A *pex7*Δ *PEX18-TAP TRP1*	(Stein *et al.*, 2002)
yAS4	UTL-7A *pex21*Δ *PEX18-TAP TRP1*	(Stein *et al.*, 2002)

domain and somewhat unexpectedly, has been shown to partially complement an *S. cerevisiae pex18* Δ*pex21*Δ mutant (Einwächter *et al.*, 2001). However, Pex20p might have acquired functions in addition to those of Pex18p/Pex21p, because only *Yl*Pex20p 1) directly binds endogenous thiolase (Titorenko *et al.*, 1998), 2) interacts with the *S. cerevisiae* docking proteins Pex13p and Pex14p in the absence of Pex7p (Einwächter *et al.*, 2001; Stein *et al.*, 2002), and 3) directly binds intraperoxisomal *Yl*Pex8p (Smith and Rachubinski, 2001). Pex20p might therefore compensate a possible absence of Pex7p in *Y. lipolytica*, although the lack of a completed *Y. lipolytica* genome sequence still leaves room for a Pex7p in that species.

The filamentous fungus *Neursospora crassa* has a long history as a model organism for addressing both genetic and biochemical questions. Although *S. cerevisiae* has proved to be a more tractable organism for many purposes, the release of the complete genome sequence of *N. crassa* might lay the foundation for a revival in *Neurospora* research, including research on peroxisome biogenesis. *N. crassa* harbors at least two compartments of the microbody family: 1) glyoxysomes, which house the fatty acid β-oxidation enzymes and two key enzymes of the glyoxylate cycle (Kionka and Kunau, 1985); and 2) the Woronin body, whose main function is to reseal membrane lesions and whose predominant matrix enzyme, Hex1p, contains a PTS1 (Jedd and Chua, 2000; Tenney *et al.*, 2000). The peroxisomal marker enzyme catalase does not colocalize with either organelle and might thus be present in yet another microbody subclass (Thieringer and Kunau, 1991).

Herein, we report the identification of *N. crassa* peroxins and the isolation of both Pex7p and Pex20p from this fungus. We expressed the two proteins in *S. cerevisiae* and analyzed their ability to complement the yeast PTS2-specific counterparts. We provide evidence that Pex20p, just like Pex18p/Pex21p, only functions in a complex with Pex7p and discuss these results in terms of a mechanism for the early steps in PTS2 protein import that is conserved among species.

MATERIALS AND METHODS

Strains, Media, and General Methods

The *Escherichia coli* strain DH5α was used for all plasmid amplifications and isolations. The *S. cerevisiae* strains used in this study are listed in Table 1. Standard and oleic acid-containing media were prepared as described previously (Stein *et al.*, 2002). Induction of *CUP1* promoter-dependent *myc*-Pex7p was achieved by adding 25 mg/l $CuSO_4$ to the medium. Standard recombinant DNA techniques were performed essentially as described previously (Sambrook *et al.*, 1989).

Plasmids and Oligonucleotides

Table 2 provides a list of plasmids and oligonucleotides used in this study. *NcPEX7* (GenBank accession no. AY141206) and *NcPEX20* (GenBank accession no. AY141207) were amplified from a *N. crassa* cDNA library by polymerase chain reaction with primer pair RE579/580 and RE575/576, respectively. The two products were subcloned into *Eco*RV-cut pBluescript SK$^+$ (Stratagene, La Jolla, CA), resulting in pMAR28 (*PEX7*) and pMAR14 (*PEX20*). To express *Nc*Pex7p in *S. cerevisiae*, the *NcPEX7* cDNA fragment was excised from pMAR28 with *Eco*RI/*Cla*I and cloned into the appropriately cut yeast expression vector pRS-FOXP-TERM (pMAR39). *Nc*Pex20p was similarly cloned by ligating an *Eco*RI/*Xho*I *PEX20* cDNA fragment from pMAR14 into pRS-FOXP-TERM (pMAR22). The *NcPEX20* expression construct was also subcloned into the *TRP1*-based expression vector YCplac22 as a *Bam*HI/*Kpn*I fragment (pAS5). The *PEX18* open reading frame, including 292 base pairs of its promoter and 329 base pairs of its 3′ region, was amplified from *S. cerevisiae* genomic DNA by using primer pair RE221/222 and cloned into *Eco*RV-cut pBluescript SK$^+$ (pAS1). This fragment was excised as an *Xba*I/*Hin*dIII fragment and cloned into YCplac33 (pAS3) and YCplac22 (pAS4).

Yeast Two-Hybrid Assays

To analyze the interactions of *Nc*Pex20p and *Nc*Pex7p with *S. cerevisiae* peroxins in the yeast two-hybrid system, the *NcPEX20* cDNA fragment was cloned as a *Bgl*II/*Not*I fragment from pMAR14 into a similarly digested pPC86 (pMAR26). The *NcPEX7* cDNA fragment was excised from pMAR28 with *Sal*I/BglII and cloned into pPC97 (pMAR30). All constructs containing *S. cerevisiae* peroxins or truncations thereof have been described previously (Stein *et al.*, 2002). Two-hybrid plasmids were cotransformed into the HF7c wild-type or the otherwise isogenic *pex7*Δ strain and selected on SD plates without tryptophane and leucine. His-auxotrophy of transformed strains was determined by growth on selective plates lacking leucine, tryptophane, and histidine but containing 1 or 5 mM 3-aminotriazole.

Antibodies and Western Blotting

The antibodies used have either been described previously, namely, anti-Fox3p (Erdmann and Kunau, 1994), anti-Cta1p (Gurvitz *et al.*,

Table 2. Plasmids and oligonucleotides used

Plasmid or oligonucleotide	Description	Source or reference
Plasmid		
YCplac22	*TRP1*-marked centromeric plasmid	(Gietz and Sugino, 1988)
YCplac33	*URA3*-marked centromeric plasmid	(Gietz and Sugino, 1988)
pRS-FOXP-TERM	*URA3*-based yeast expression vector	C. Clayton, Heidelberg
pJR233	*MLS1*pr-*GFP-SKL*	(Lametschwandtner *et al.*, 1998)
pIH217	LEU2-based *MLS1*pr-*GFP-SKL*	I. Heiland, Berlin
pHPR131	*ADH2pr-PTS2-DsRed*	(Stein *et al.*, 2002)
YEpmycPex7	*CUP1pr-myc-PEX7*	(Rehling *et al.*, 1996)
pPC97-PEX7		(Albertini *et al.*, 1997)
pPC97-PEX13		(Albertini *et al.*, 1997)
pPC97-PEX14		(Albertini *et al.*, 1997)
pPC97-FOX3		(Rehling *et al.*, 1996)
pKat31	pPC97-PEX13 (1–151)	(Stein *et al.*, 2002)
pMar14	pSK-*NcPEX20*	This study
pMar22	pRS-FOXP-TERM-*NcPEX20*	This study
pMar26	pPC86-*NcPEX20*	This study
pMar28	pSK-*NcPEX7*	This study
pMar30	pPC97-*NcPEX7*	This study
pMar39	pRS-FOXP-TERM-*NcPEX7*	This study
pAS1	pSK-*PEX18pr-PEX18*	This study
pAS3	YCplac33-*PEX18pr-PEX18*	This study
pAS4	YCplac22-*PEX18pr-PEX18*	This study
pAS5	YCplac22-*NcPEX20*	This study
Oligonucleotide (5′–3′)		
RE 221	GTATAATCAGGTATGTAAGGG	
RE 222	CGACAACTAAGTTCCAGAAAG	
RE 575	ATAGATCTACGAATTCATGTCTGACAGCATGTGC	
RE 576	ATGCGGCCGCCTCGAGTTAAGCGGAAGAAGCA	
RE 579	ATGTCGACCGAATTCATGGCGTCCATGCTGGAATTT	
RE 580	ATAGATCTATCGATCCTCTTTCAAACAACCTGCTTC	

1997), and anti-Pex7p (Stein *et al.*, 2002), or purchased in the case of anti-protein A (Sigma-Aldrich, St. Louis, MO) and monoclonal anti-9E10 *c-myc* (Babco, Richmond, CA) antibodies. Immunoblotting was performed according to standard protocols. Horseradish peroxidase-coupled anti-rabbit or anti-mouse IgGs, in combination with the enhanced chemiluminscence system (Amersham Biosciences, Freiburg, Germany), were used to detect immunoreactive complexes.

Fluorescence Microscopy

Live cells were analyzed for DsRed and green fluorescent protein (GFP) fluorescence by using an Axioplan 2 microscope equipped with a Plan-Neofluar 1003/1.30 Ph3 oil objective and a 100-W mercury lamp and filter sets (Carl Zeiss, Jena, Germany). Images were recorded with a SPOT-cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI) and processed with Lite MetaMorph imaging software (Universal Imaging, West Chester, PA). Immunofluorescence microscopy was carried out as described previously (Erdmann, 1994) by using anti-Fox3p or anti-Cta1p antibodies followed by an incubation with Cy3-conjugated donkey anti-rabbit IgGs (Jackson Immunoresearch Laboratories, West Grove, PA) for detection.

Coimmunoprecipitation and Subcellular Fractionation

Myc-Pex7p and Pex18p-TAP were immunoprecipitated from soluble protein extracts essentially as described previously (Stein *et al.*, 2002). Strains were grown and induced in appropriate selective media to maintain extrachromosomal plasmids. Cells were broken open in solution A (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.2% Triton X-100) that contained the Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) with a French press (Pex18p-TAP) or glass beads (*myc*-Pex7p). The cleared homogenates were incubated for 1 h at 4°C with IgG-Sepharose (Amersham Biosciences) in the case of Pex18p-TAP or with magnetic beads (Dynal, Hamburg, Germany) covered with monoclonal anti-*myc* antibodies (*myc*-Pex7p). After a repeated washing step with solution A, bound *myc*-Pex7p was released by cooking the beads in SDS sample buffer, whereas bound Pex18p was released from the beads by adding 5 U of tobacco etch virus (TEV) protease (Invitrogen, Karlsruhe, Germany) in solution A, adjusted to 10 mM dithiothreitol and 1 mM EDTA. All eluates were analyzed for the presence of Pex7p and Fox3p. Yeast lysates were prepared and fractionated by differential centrifugation as described previously (Erdmann *et al.*, 1989).

Database Analysis

Homologues to known peroxins were identified by a TBLASTN search within the database of the Center for Genome Research at the Whitehead Institute (WICGR) by using the default search algorithm. Genes that were also annotated at Munich Information Center for Protein Sequences were compared for identity with the corresponding WICGR open reading frame. The query was carried out with the *S. cerevisiae* peroxins, except *PEX8*, *PEX9*, *PEX20*, *PEX23*, and *PEX24* (*Y. lipolytica*) and *PEX2* and *PEX16* (*Homo sapiens*).

RESULTS

Predicted Peroxins in N. crassa

The release of the genomic sequence of *N. crassa* allowed us to search for the presence of potential orthologous peroxins

Table 3. *In silico* identification of peroxins in *N. crassa*

Neurospora protein name[a]	Peroxin (organism)[b]	Description	*S.c.*[c]	*Y.l.*[c]	*H.s.*[c]	*Neurospora* contig[a]	Score[d]	Length[d]	Identity[d] (%)
NCU08118.1	PEX1 (*S. cerevisiae*)	Member of the AAA-protein family	+	+	+	3.487 (scaffold 46)	e-109	1139	30
NCU02070.1	PEX2 (*H. sapiens*)	Zinc-binding integral peroxisomal membrane protein	+	+	+	3.91 (scaffold 5)	2e-23	270	29
NCU06175.1	PEX3 (*S. cerevisiae*)	Integral peroxisomal membrane protein	+	−	+	3.359 (scaffold 26)	1e-14	251	27
NCU02636.1	PEX4 (*S. cerevisiae*)	Member of ubiquitin-conjugating protein family	+	−	−	3.137 (scaffold 7)	3e-18	110	42
NCU02960.1	PEX5 (*S. cerevisiae*)	69-kDa protein containing tetratricopeptide repeat (TPR), PTS1-receptor	+	+	+	3.153 (scaffold 8)	3e-81	494	38
NCU08373.1	PEX6 (*S. cerevisiae*)	Member of the AAA-protein family	+	+	+	3.501 (scaffold 50)	e-134	829	34
NCU07662.1 (AY141206)	PEX7 (*S. cerevisiae*)	40 kDa-protein containing WD40-repeats, PTS2-receptor	+	−	+	3.458 (scaffold 41)	1e-59	131	48
NCU00032.1	PEX8 (*Y. lipolytica*)	Integral peroxisomal membrane protein	+	+	−	3.1 (scaffold 1)	7e-30	590	24
—	PEX9 (*Y. lipolytica*)	Integral peroxisomal membrane protein	−	+	−	—	—	—	—
NCU03277.1	PEX10 (*S. cerevisiae*)	Zinc-binding integral peroxisomal membrane protein	+	+	+	3.171 (scaffold 9)	3e-35	420	26
NCU04802.1	PEX11 (*S. cerevisiae*)	Peroxisomal membrane protein, proliferation	+	−	+	3.263 (scaffold 16)	1e-15	147	37
NCU05245.1	PEX12 (*S. cerevisiae*)	Zinc-binding integral peroxisomal membrane protein	+	−	+	3.295 (scaffold 19)	2e-17	440	23
NCU02618.1	PEX13 (*S. cerevisiae*)	Peroxisomal membrane protein, contains SH3 domain	+	−	+	3.135 (scaffold 7)	3e-60	332	44
NCU03901.1	PEX14 (*S. cerevisiae*)	Component of peroxisomal import machinery	+	+	+	3.212 (scaffold 12)	3e-11	127	28
—	PEX15 (*S. cerevisiae*)	Peroxisomal membrane protein	+	−	−	—	—	—	—
NCU01850.1	PEX16 (*H. sapiens*)	Peroxisomal membrane protein	−	+	+	3.82 (scaffold 5)	5e-11	302	24
—	PEX17 (*S. cerevisiae*)	Peroxisomal membrane protein	+	−	−	—	—	—	—
—	PEX18 (*S. cerevisiae*)	Involved in PTS2-protein import	+	−	−	—	—	—	—
NCU04301.1	PEX19 (*S. cerevisiae*)	40 kDa farnesylated protein associated with peroxisomes	+	+	+	3.222 (scaffold 13)	2e-11	177	25
NCU04062.1 (AY141207)	PEX20 (*Y. lipolytica*)	Involved in PTS2-protein import	−	+	−	3.214 (scaffold 12)	8e-12	337	24
—	PEX21 (*S. cerevisiae*)	Involved in PTS2-protein import	+	−	−	—	—	—	—
—	PEX22 (*S. cerevisiae*)	Peroxisomal membrane protein	+	−	−	—	—	—	—
NCU05564.1	PEX23 (*Y. lipolytica*)	Peroxisomal membrane protein	−	+	−	3.312 (scaffold 21)	2e-58	283	33
NCU06637.1	PEX24 (*Y. lipolytica*)	Peroxisomal membrane protein	−	+	−	3.384 (scaffold 30)	2e-25	394	22
—	PEX25 (*S. cerevisiae*)	Peroxisomal membrane protein, proliferation	+	−	−	—	—	—	—

[a] Annotated *N. crassa* protein names and contig numbers are according to Whitehead Institute/MIT Centre for Genome Research (http://www-genome.wi.mit.edu/annotation/fungi/neurospora).

[b] Protein sequence of peroxin used as bait in BLAST search at the Whitehead Institute/MIT Center for Genome Research.

[c] Peroxin present (+) or not identified (−) in indicated organism.

[d] Score from BLAST search.

in this organism by using the database of the Center for Genome Research at the Whitehead Institute, Cambridge, MA (Neurospora Sequencing Project. Whitehead Institute/MIT Center for Genome Research, assembly version 3; www-genome.wi.mit.edu/annotation/fungi/neurospora). The results from this search are summarized in Table 3. Proteins with significant similarity to 18 of the 25 currently known peroxins were identified. Interestingly, the search revealed that the *N. crassa* genome contains homologues to all known human peroxins. On the other hand, peroxins specific to *S. cerevisiae* and/or *Pichia pastoris*, i.e., Pex15p, Pex17p, Pex18p, Pex21p, Pex22p (Purdue and Lazarow,

2001), and Pex25p (Smith *et al.*, 2002), are likely to be absent from *N. crassa* (Table 3). The set of *N. crassa* peroxins seems to resemble fairly closely that of *Y. lipolytica*, because it also includes Pex20p, Pex23p, and Pex24p, peroxins that had previously been found only in *Y. lipolytica*. A BLAST search with Pex9p, the remaining *Y. lipolytica*-specific peroxin did not yield hits with significant homologies and was therefore classified as being absent from *N. crassa*. From the peroxins that are specifically involved in the PTS2 branch of peroxisomal matrix protein import, *N. crassa* lacks Pex18p and Pex21p homologues, but, as outlined previously (Einwächter *et al.*, 2001), it does contain stretches with strong similarity to both *Sc*Pex7p and *Yl*Pex20p (see below). So far, these two peroxins have not been identified together in any other organism.

Isolation of PEX7 and PEX20 from N. crassa cDNA

The homology search with Pex7p yielded two adjacent, nonoverlapping regions within the *N. crassa* genome, covering most of *Sc*Pex7p. The proper contig (3.458) was therefore analyzed for the presence of a potential start and a stop codon, both of which were found in proximity to the ends of the homologous stretches. The 5′ and 3′ ends found by this process were incorporated into the primer sequence designed to amplify the *PEX7* open reading frame from *N. crassa* cDNA. The *PEX20* gene was amplified according to the predictions quoted at the Munich Information Center for Protein Sequences (www.mips.gsf.de). Sequencing of the polymerase chain reaction products thus obtained for *PEX7* and *PEX20* revealed that they were identical to the respective genomic loci in the database with the exception of the presence of a single intron in each reading frame, ranging from base pairs 400–496 in *PEX7* and from base pairs 121–197 in *PEX20*. An alignment of the deduced amino acid sequence of *Nc*Pex7p with Pex7p of both *S. cerevisiae* and *P. pastoris* is shown in Figure 1A. *Nc*Pex7p showed significant similarity to the two yeast proteins throughout its sequence, with 36.3% identical and 28.7% similar residues to the *S. cerevisiae* protein and 36.4% identical and 31.7% similar residues to Pex7p of *P. pastoris*. Likewise, the sequence of Pex20p was compared with that of *Yl*Pex20p. *Nc*Pex20p is less conserved, with a portion of 14.7% identical and 35.1% similar residues to *Yl*Pex20p; however, two short stretches of ~30 amino acids in length are highly similar. Of these two, the one closer to the C terminus represents the region that is also conserved in the PTS2 auxiliary factors Pex5pL and Pex18p/Pex21p and that is required to interact with Pex7p (Figure 1B, box). The significance of the conserved N-terminal homologous region in Pex20p remains to be determined, but it might represent a second protein–protein interaction domain.

Expression of NcPEX20 in S. cerevisiae Complements a pex18Δpex21Δ Mutation

The recent demonstration of a yeast *pex18Δpex21Δ* mutant being partially complemented by *Yl*Pex20p allowed bridging of the function of these two proteins (Einwächter *et al.*, 2001). We therefore analyzed whether *Nc*Pex20p would be similarly proficient in substituting Pex18p/Pex21p. For that purpose, *NcPEX20* was cloned behind the oleic acid-inducible *FOX3* promoter and expressed in a *pex18Δpex21Δ* mutant. In addition, fluorescent marker proteins for both the PTS1 and the PTS2 pathway, whose fluorescence patterns reflect the ability of a strain to import PTS1 and PTS2 substrates respectively, were coexpressed in this strain. After 2 d of incubation on oleic acid plates, cells were inspected under the fluorescence microscope.

In the *pex18Δpex21Δ* mutant, the PTS2 marker protein PTS2-DsRed showed a diffuse fluorescence pattern, whereas the PTS1 protein GFP-SKL exhibited a punctate staining pattern, which was in line with the specific PTS2 protein import defect of the analyzed mutant (Figure 2). Expression of *Nc*Pex20p in the *pex18Δpex21Δ* mutant caused PTS2-DsRed to be localized in punctate structures that colocalized with GFP-SKL. Similar results were obtained when *Sc*Pex18p was expressed in this mutant strain. This result indicated that *Nc*Pex20p was able to restore the import of a synthetic PTS2-containing substrate in the *pex18Δpex21Δ* mutant.

To now test whether *Nc*Pex20p is also able to physiologically replace Pex18p/Pex21p, these strains were spotted onto plates containing oleic acid as sole carbon source. As expected, it proved possible to rescue the growth phenotype of the *pex18Δpex21Δ* mutant on fatty acids by the expression of Pex18p in that strain (Figure 3). Importantly, also *Nc*Pex20p restored the mutant's ability to grow on oleic acid, thereby demonstrating the conserved function of these peroxins.

Interactions of NcPEX20 Resemble Those of PEX18

To nail down the function of *Nc*Pex20p in PTS2-dependent import, the protein's ability to interact with the known partners of Pex18p was addressed in a yeast two-hybrid assay (Stein *et al.*, 2002). *Nc*Pex20p did indeed interact with Fox3p, Pex7p, and $Pex13p_{1–151}$ (Figure 4A, left). Moreover, the previously observed dependency of the Pex18p–Fox3p and Pex18p–$Pex13p_{1–151}$ interactions on Pex7p was also found to apply to the *Nc*Pex20p interactions (Figure 4A, right). In addition, *Nc*Pex20p interacted strongly with Pex14p in the wild-type strain, but only very weakly in the absence of Pex7p. The inability of *Nc*Pex20p to interact with Fox3p or the docking proteins Pex13p and Pex14p in the absence of Pex7p already implies that Pex7p is essential for *Nc*Pex20p to function in *S. cerevisiae*. In fact, growth of a *pex7Δ* mutant on oleic acid medium did not resume upon introduction of *Nc*Pex20p, and the PTS2-DsRed marker protein remained cytosolic in that strain (see below). These data favor a close link between Pex18p and *Nc*Pex20p.

We reported previously that Pex18p is instrumental in the formation of an import-competent complex containing the PTS2 receptor Pex7p and its cargo protein Fox3p (Stein *et al.*, 2002). The accumulation of this complex in a *pex14Δ* mutant vanishes when Pex18p and Pex21p are concomitantly absent. Introduction of a plasmid-borne copy of *PEX18* into a *pex14Δpex18Δpex21Δ* triple mutant caused Fox3p to be present in significant amounts in the immunoprecipitate of *myc*-Pex7p, indicating the reappearance of the accumulated complex as in a *pex14Δ* single mutant (Figure 4B). The same observation was made when a strain that had expressed *Nc*Pex20p in this triple mutant was used for analysis. Thus, like Pex18p, *Nc*Pex20p also possesses PTS2 complex-forming potential, which is required before docking because this complex is generated in a *pex14Δ* mutant.

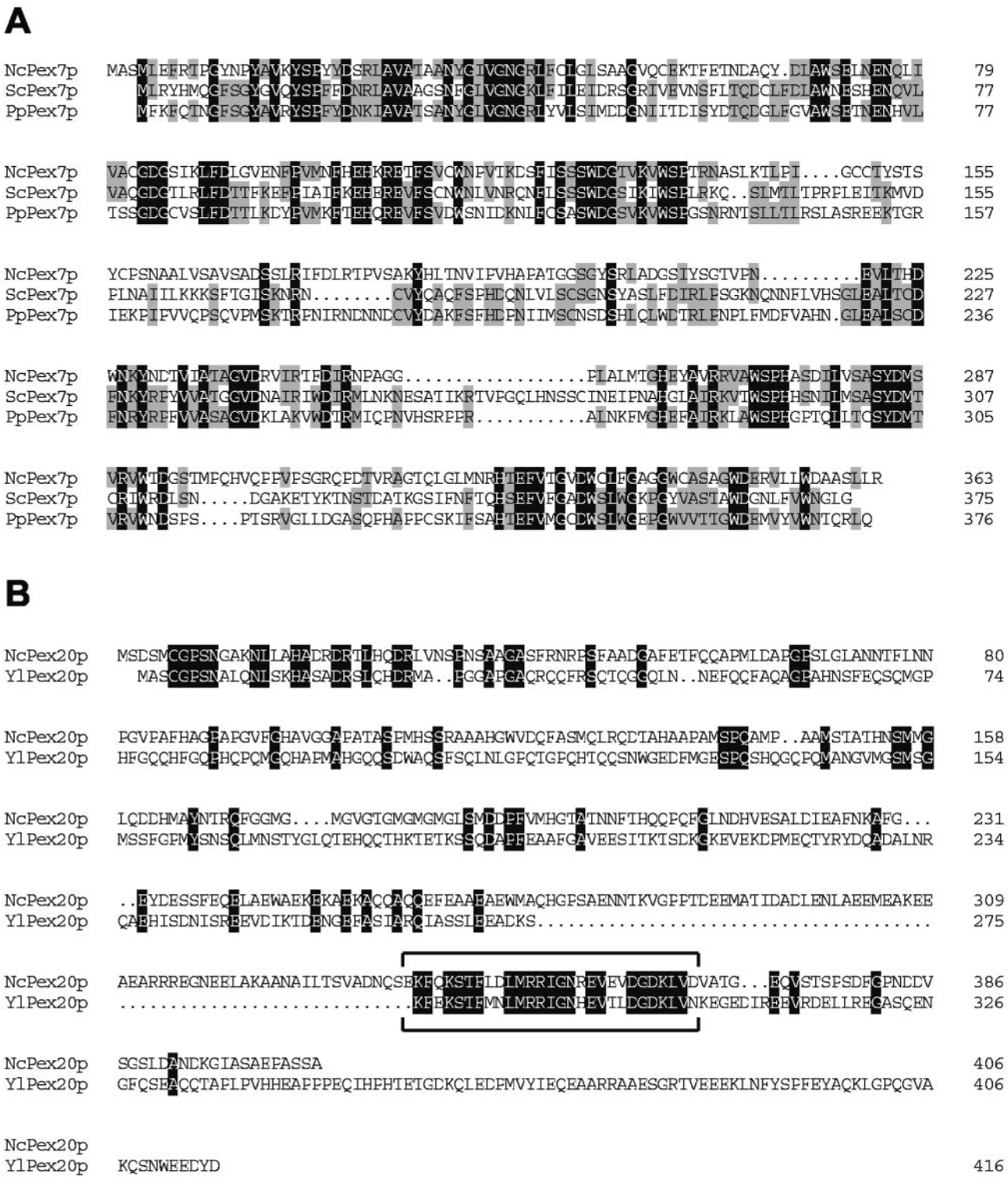

Figure 1. Sequence alignment of Pex7p and Pex20p. (A) Amino acid sequences of Pex7p from *N. crassa*, *S. cerevisiae*, and *P. pastoris* were aligned with DNAMAN (Lynnon BioSoft, Quebec, Canada). Identical residues in all three sequences are shaded in black. Residues that are either similar in all three sequences or identical in two out of three sequences are shaded in gray. Similarity rule: D = E, R = K, Q = N, S = T, I = L = V = A. (B) Amino acid sequences of Pex20p from *N. crassa* and *Y. lipolytica* were similarly aligned, but only identical residues (shaded in black) are shown. The box marks the region within Pex20p that is conserved in Pex18p and Pex5pL.

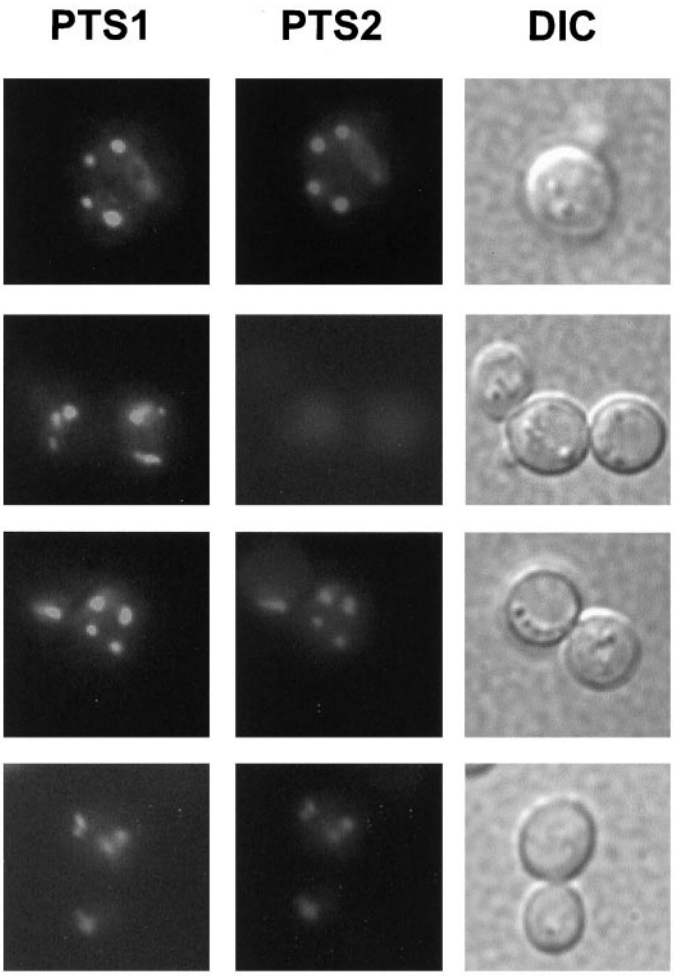

Figure 2. NcPex20p restores the PTS2 import deficiency of a *pex18*Δ*pex21*Δ mutant in *S. cerevisiae*. The *pex18*Δ*pex21*Δ strains expressing *Sc*Pex18p (*pex18*Δ*pex21*Δ [*PEX18*]) or *Nc*Pex20p (*pex18*Δ*pex21*Δ [*NcPEX20*]) together with PTS2-DsRed and GFP-PTS1 were analyzed for the fluorescence pattern of the PTS marker proteins. The wild-type and the untransformed mutant strain (*pex18*Δ*pex21*Δ) served as controls. Before inspection, cells were kept on oleic acid-containing plates for 2 d. A punctate pattern indicates that protein import is enabled. Structural integrity of the cells is documented by differential interference contrast (DIC) microscopy.

NcPex7p Fails to Functionally Replace ScPex7p

The putative *N. crassa PEX7* gene was cloned behind the *FOX3* promoter and expressed in a *pex7*Δ mutant strain. The ability of *NcPEX7* to complement the respective *S. cerevisiae* gene was again assessed by determining the cellular distribution of the PTS2-DsRed marker protein and the growth rate on oleic acid plates. The transformed strain did not lead to growth on oleic acid (see below). The PTS2 marker protein was dispersed in cells grown on oleic acid or ethanol, albeit on ethanol, punctate structures were discernible in rare cases (our unpublished data). We did not further investigate the identity of these structures because it became obvious that *Nc*Pex7p barely substituted the yeast protein. Rather, we inquired why *Nc*Pex7p was inefficient in functioning as a PTS2 receptor in yeast. Due to a fortuitous cross-reactivity of our anti-*Sc*Pex7p antibody, expression of *Nc*Pex7p could be analyzed immunologically. The protein was stably expressed at high levels under oleic acid-induction conditions, in agreement with *NcPEX7* being under the control of the *FOX3* promoter (Figure 5, bottom, lane 3). The failure of *Nc*Pex7p to functionally replace *Sc*Pex7p could therefore be due to the lack of interaction with a *S. cerevisiae* protein that is involved in PTS2 import.

NcPex7p Does Not Interact with Pex18p

A fusion of *Nc*Pex7p with the Gal4p BD turned out to be autoactive in the yeast two-hybrid assay (our unpublished data). As a consequence, this method was not considered further for analyzing *Nc*Pex7p interactions, given that *Sc*Pex7p was previously found to interact with Fox3p as a Gal4p BD- but not as a Gal4p AD-fusion protein (Rehling *et al.*, 1996). Instead, *Nc*Pex7p was tested biochemically for interaction with Pex18p by means of coimmunoprecipitation. For that matter, a functional TAP-tagged version of Pex18p was precipitated from the control strain *pex21*Δ as well as from *pex7*Δ strains overexpressing *Nc*Pex7p and *myc-Sc*Pex7p, respectively (Figure 5). The precipitate obtained from the *pex21*Δ strain did also contain *Sc*Pex7p (Figure 5, top, lane 1), demonstrating the interaction between endogenous *Sc*Pex7p and Pex18p. Likewise, it proved possible to coimmunoprecipitate *myc-Sc*Pex7p with Pex18p, as shown by the presence of the slightly slower migrating band representing *myc-Sc*Pex7p in the precipitate of this strain. In contrast, *Nc*Pex7p was not found in the respective Pex18p precipitate. In addition, only background levels of the PTS2 cargo protein Fox3p were detected in this precipitate, whereas a significant amount of Fox3p was found in the

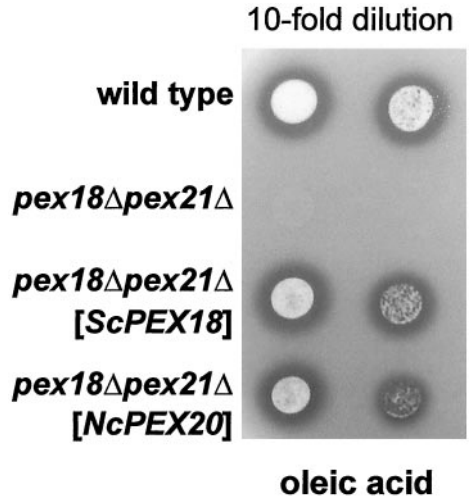

Figure 3. Expression of *Nc*Pex20p restores growth of *pex18*Δ*pex21*Δ cells on oleic acid. Tenfold dilutions of the wild-type, the untransformed *pex18*Δ*pex21*Δ and the *pex18*Δ*pex21*Δ strain expressing either *Sc*Pex18p or *Nc*Pex20p were spotted onto an oleic acid plate and incubated for 5 d at 30°C. Halos surrounding the spots indicate utilization of the fatty acid from the medium.

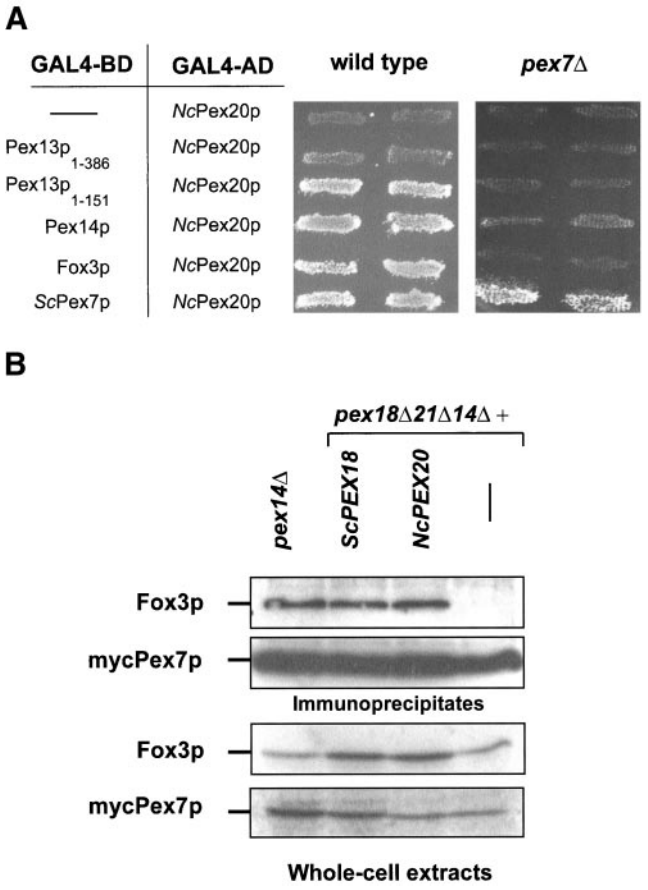

Figure 4. Function of NcPex20p resembles that of Pex18p. (A) Dependence of the *Nc*Pex20p interactions on *Sc*Pex7p. The indicated peroxins were tested in a two-hybrid assay for interaction with *Nc*Pex20p in the HF7c wild-type strain (left) and the otherwise isogenic *pex7*Δ strain (right). Interactions are indicated by the histidine prototrophy of two independent transformants at each case. (B) Accumulation of a Pex7p–Fox3p complex through substitution of Pex18p/Pex21p with *Nc*Pex20p in the docking mutant *pex14*Δ. The indicated strains were analyzed for the presence of Fox3p in the immunoprecipitate (20% of total) of *myc*-Pex7p by immunoblotting (top). The bottom panel shows 0.5% of the used total cell lysates.

precipitates containing *Sc*Pex7p (Figure 5, top), in agreement with a Pex7p-mediated interaction between Pex18p and Fox3p (Stein *et al.*, 2002). Taken together, these data indicate that *Nc*Pex7p does not interact with *Sc*Pex18p, which could explain the failure of the *N. crassa* PTS2 receptor to substitute for the *S. cerevisiae* counterpart.

*Coexpression of NcPex7p and NcPex20p Rescues the PTS2 Import Defect of an S. cerevisiae pex7*Δ *Mutant*

Our observation of *Nc*Pex20p being the orthologue of *Sc*Pex18p/Pex21p led us to suspect that an interaction between *Nc*Pex20p and *Nc*Pex7p should be relevant to PTS2-dependent import in *N. crassa*. Pursuing this train of thought further, by concomitantly expressing both peroxins in a *S. cerevisiae pex7*Δ strain, the requirement of *Nc*Pex7p to interact with Pex18p might be circumvented. Thus, a *pex7*Δ strain was transformed with expression plasmids carrying *NcPEX7*, *NcPEX20*, or both genes and the resulting strains were subjected to growth analysis on oleic acid plates. Compared with the untransformed *pex7*Δ strain, neither the expression of *Nc*Pex20p nor that of *Nc*Pex7p had any discernible effect. Interestingly, coexpression of *Nc*Pex7p and *Nc*Pex20p in *pex7*Δ did lead to a significant increase in cell mass and halos around the colonies were visible (Figure 6A). The concomitant presence of *Nc*Pex7p and *Nc*Pex20p did not result in higher levels of *Nc*Pex7p (Figure 6B), suggesting that *Nc*Pex20p actively converted *Nc*Pex7p into a functional PTS2 receptor that was able to translocate significant amounts of Fox3p into the peroxisomal lumen. We therefore separated postnuclear lysates of the same strains into a supernatant and an organellar pellet fraction that was enriched in peroxisomes and mitochondria. In all strains, the PTS1 protein Cta1p was predominately found in the pellet fraction, which was in line with the PTS2-specific defect of the *pex7*Δ mutation (Figure 7). The PTS2 protein thiolase was also preferentially present in the pellet fraction in the wild-type strain, but in the absence of *Sc*Pex7p, Fox3p was found in the supernatant fraction. This cytosolic distribution was not altered upon expression of *Nc*Pex7p or *Nc*Pex20p in the *pex7*Δ mutant. In contrast, coexpression of *Nc*Pex7p and *Nc*Pex20p resulted in the appearance of some thiolase in the pellet fraction (Figure 7).

These strains were also analyzed for the location of native Fox3p by means of indirect immunofluorescence. Spheroplasts from oleic acid-induced cells were labeled with antibodies directed against the PTS1 protein Cta1p (Figure 8, left). The punctate staining pattern typical for peroxisomes was observed in all samples, including the untransformed *pex7*Δ strain, thereby demonstrating the occurrence of intact

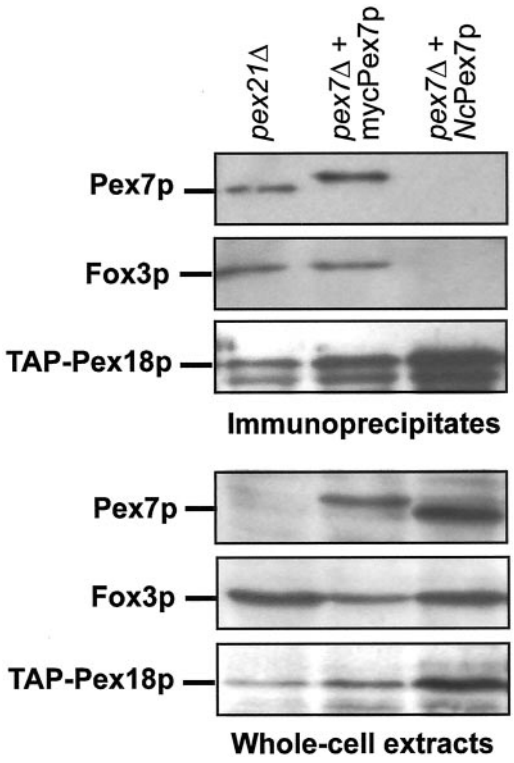

Figure 5. NcPex7p does not interact with *Sc*Pex18p. Pex18p-TAP was immunoprecipitated from whole-cell lysates of the strains *pex21*Δ *PEX18-TAP* as well as *pex7*Δ *PEX18-TAP* expressing *myc*-Pex7p or *Nc*Pex7p. Precipitates were analyzed for the presence of Pex7p (top, row 1), Fox3p (top, row 2), and Pex18p-TAP (top, row 3) by immunoblotting. The bottom panel shows 0.2% of the total cell lysates.

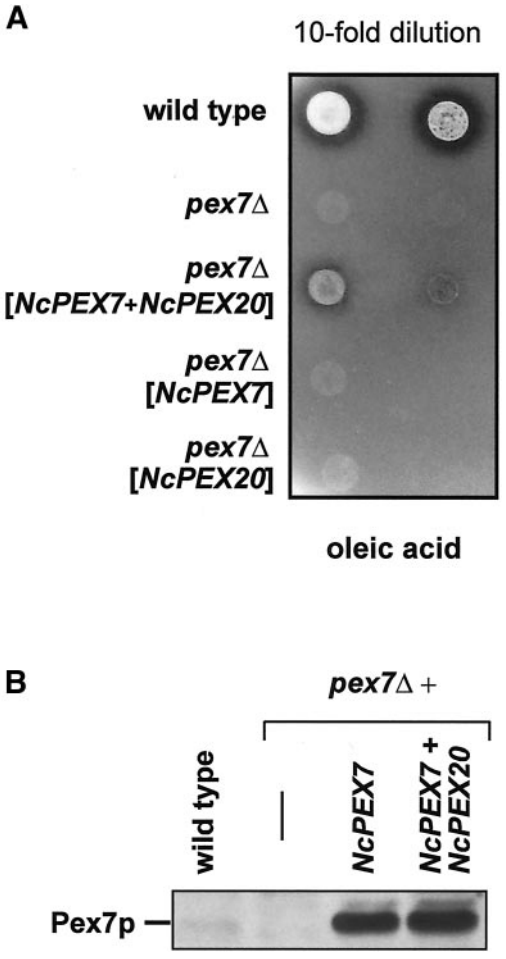

Figure 6. NcPex20p converts *Nc*Pex7p into a functional PTS2 receptor in *S. cerevisiae*. (A) Growth of a *pex7*Δ strain on oleic acid resumes in the concomitant presence of *Nc*Pex7p and *Nc*Pex20p. Tenfold dilutions of the wild-type, the untransformed *pex7*Δ as well as the *pex7*Δ strain transformed with either *NcPEX7*, *NcPEX20*, or *NcPEX7* plus *NcPEX20* were spotted onto oleic acid plates and incubated for 5 d at 30°C. Halo formation was observed in the strain coexpressing both *N. crassa* peroxins. (B) Expression of *Nc*Pex7p does not change in the presence of *Nc*Pex20p. Whole-cell extracts of the indicated strains that had been induced by oleic acid were analyzed for the expression level of *Nc*Pex7p using Pex7p-specific antibodies. The amount of *Nc*Pex7p exceeded that of native *Sc*Pex7p (lane 1) but was comparable in the absence or presence of *Nc*Pex20p (lanes 3 and 4).

peroxisomal structures. Decoration with anti-Fox3p antibodies caused a diffuse staining in the *pex7*Δ strain, which reflects a nonperoxisomal location of Fox3p (Figure 8, right). The diffuse fluorescence pattern was maintained upon expression of *Nc*Pex20p or *Nc*Pex7p in that strain; however, the presence of both *N. crassa* proteins caused Fox3p to reappear in punctate structures. This result indicated that Fox3p relocated into peroxisomes only when *Nc*Pex20p and *Nc*Pex7p were present at the same time. Thus, the two *N. crassa* proteins are functionally linked and act together in PTS2-dependent matrix protein import.

DISCUSSION

In essence, peroxisome biogenesis is evolutionary conserved. However, the degree of deviance seemed more pronounced for the PTS2 branch of the peroxisomal matrix protein import. In this report, we have shown that the filamentous fungus *N. crassa* possesses two PTS2-specific peroxins; the PTS2-signal recognition factor Pex7p that is common to many organisms, as well as Pex20p, which had hitherto been identified only in *Y. lipolytica*. These findings unequivocally dispose Pex20p into the group of PTS2-specific peroxins that are auxiliary to Pex7p, which includes Pex18p/Pex21p and human Pex5pL. Our observation that *Nc*Pex20p, when expressed in *S. cerevisiae*, substituted for Pex18p/Pex21p but not for Pex7p, supports this classification. The dependency of *Nc*Pex20p on Pex7p was corroborated in a two-hybrid assay because *Nc*Pex20p interacted with *Sc*Fox3p and the docking proteins Pex13p and Pex14p, but only in the presence of Pex7p.

In analogy to the function of *Nc*Pex20p, *Yl*Pex20p has been shown to interact with *Sc*Fox3p via *Sc*Pex7p. This led Einwächter *et al*. (2001) to propose the existence of a hitherto undetected Pex7p in *Y. lipolytica*. Our data on the interplay

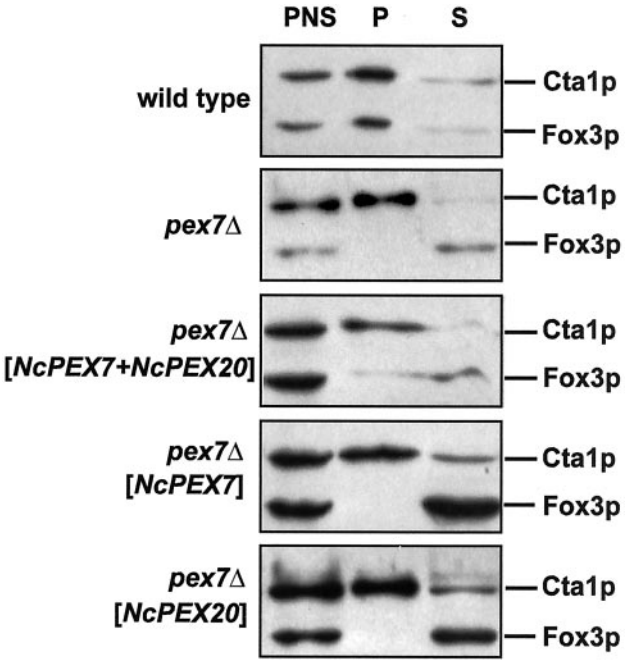

Figure 7. Redistribution of the PTS2 protein Fox3p into the organellar fraction in a *pex7*Δ strain in the concurrent presence of *Nc*Pex7p and *Nc*Pex20p. Postnuclear supernatants (PNS) of the indicated oleic acid-induced strains were subjected to differential centrifugation at 20,000 × *g* for 20 min. Equal portions of the resulting organellar pellet (P) and supernatant (S) fractions were analyzed for the distribution of Fox3p. The PTS1 protein Cta1p was monitored as control for the intactness of the isolated peroxisomes.

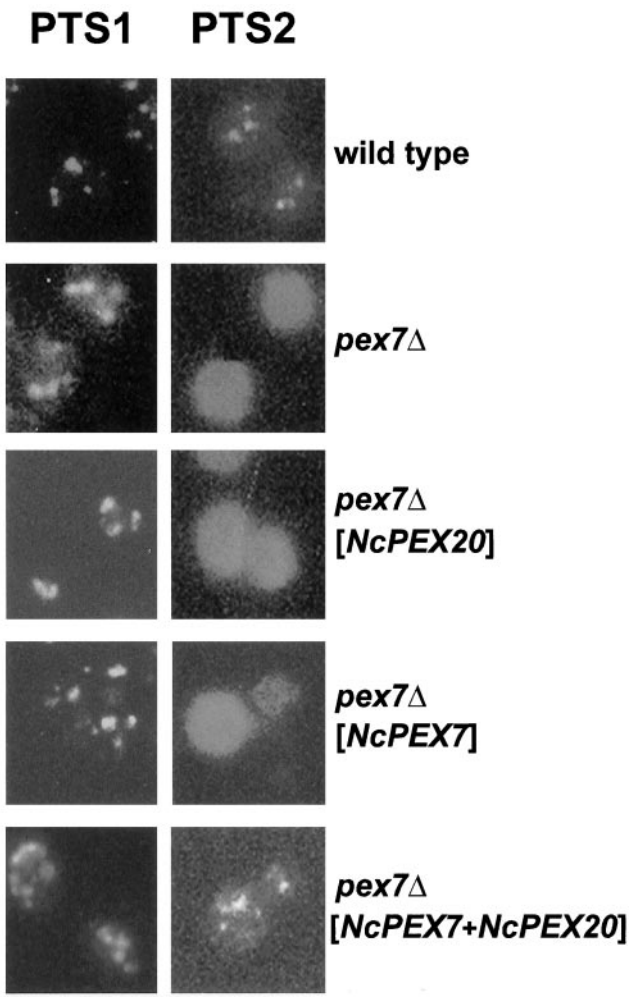

Figure 8. Fox3p relocates into peroxisomes in a *pex7*Δ mutant upon coexpression of *Nc*Pex20p and *Nc*Pex7p. The indicated strains were induced on oleic acid-containing medium for 12 h and subjected to immunofluorescence analysis. Right (PTS2), cells that had been decorated with antibodies against Fox3p and subsequently with CY3-coupled anti-rabbit antibodies. Left (PTS1), same strains but treated with anti-Cta1p antibodies. Only coexpression of *Nc*Pex7p and *Nc*Pex20p (pex7Δ [*NcPEX7+NcPEX20*]) restored the punctate fluorescence pattern of thiolase in a *pex7*Δ strain.

of *N. crassa* Pex7p and Pex20p clearly support this notion. On the other hand, the seemingly cross-functional *Yl*Pex20p and *Nc*Pex20p may still possess properties that are unique to each protein in its native environment. Particularly if *Y. lipolytica* were indeed found to lack Pex7p, the additional interactions of *Yl*Pex20p such as that with *Yl*Fox3p might be required to recruit and target thiolase to the docking complex, as proposed previously (Titorenko *et al.*, 1998). It will be possible to give a definite answer by the time the genomic sequence of *Y. lipolytica* is released. In this respect, it should be noted that the genome of the nematode *Caenorahabditis elegans* does indeed lack an apparent orthologue of the PTS2 receptor Pex7p (Gurvitz *et al.*, 2000; Motley *et al.*, 2000). But in this case, *C. elegans* orthologues of PTS2-containing enzymes have gained a PTS1, suggesting that the PTS2 branch is entirely absent from the nematode (Motley *et al.*, 2000), which is clearly not the case in *Y. lipolytica*.

The heterologous test system used in our study allowed us to demonstrate that the PTS2 receptor of *N. crassa* is not capable of importing PTS2 cargo proteins in the absence of Pex20p. This conclusion is based on the fact that on the one hand, *Nc*Pex7p failed to interact with Pex18p, and on the other it was not self-sufficient in doing the job. However, upon coexpression of *Nc*Pex20p and *Nc*Pex7p in *pex7*Δ cells, the import of Fox3p resumed. In these cells, thiolase was found in punctate structures and significant amounts were present in an organellar pellet. Strikingly, this strain was able to grow slowly on oleic acid plates, whereas strains harboring only *Nc*Pex7p or *Nc*Pex20p failed to do so at all. Since *Nc*Pex20p did not interact with Pex13p and Pex14p in the absence of *Sc*Pex7p in a two-hybrid assay, *Nc*Pex7p is the likely interaction partner of these *S. cerevisiae* peroxins upon docking and the same holds true for PTS2 signal recognition. Thus, by taking into account the multitude of interactions *Nc*Pex7p has to make with *S. cerevisiae* proteins, it is not surprising that the complementation observed was only partial.

The precise role of Pex20p in PTS2 import is not yet completely clear, but it is similar to that of Pex18p. *Nc*Pex20p was required for the formation of an import-competent complex containing thiolase and Pex7p. This complex accumulates in a *pex14*Δ mutant but not in a wild-type strain,

suggesting that it represents an intermediate of the import process of PTS2 proteins (Stein *et al*., 2002). Because neither Pex18p nor Pex20p was able to interact directly with *Sc*Fox3p, these peroxins could promote the oligomerization or aggregation of cargo-loaded receptor complexes, which would be a prerequisite for the import of Fox3p. Similar ideas as to the role of Pex18p/Pex21p were recently put forward in a hypothesis that postulates the existence of such an oligomeric complex, a so-called preimplex (Gould and Collins, 2002). In mammals, Pex5pL would represent this oligomerization factor that might additionally undertake docking (Otera *et al*., 2000; Dodt *et al*., 2001). In the higher plant *Arabidopsis thaliana*, the PTS2 pathway depends on Pex5p just as in mammals; however, a single Pex5p isoform seems to be required for the import of both PTS1 and PTS2 proteins (Nito *et al*., 2002).

One final point that warrants discussion is our revelation of remarkably similar peroxin contents of *N. crassa* and *Y. lipolytica*. This indicates that the interplay of peroxins in *Y. lipolytica* is probably conserved to a larger degree than appreciated so far. In fact, our genome-wide search for peroxins also revealed a link between these two fungi and *H. sapiens* (Table 3). For instance, all three organisms but not *S. cerevisiae* contain Pex16p, a peroxin that is crucial for the early steps in peroxisome biogenesis in humans (South and Gould, 1999). On the other hand, *N. crassa* and *H. sapiens* probably lack several yeast-specific peroxins such as Pex15p and Pex17p (Table 3).

N. crassa only grows as a mycelium but *Y. lipolytica* can also undergo a transition to a mycelial form, which has been shown to require peroxisomes (Titorenko *et al*., 1997). *N. crassa* might therefore be the organism of choice to test whether intact peroxisomes are indeed mandatory for mycelial growth. Another challenging discovery stemming solely from work carried out with *Y. lipolytica* is the fusion of peroxisomal subpopulations in the course of peroxisome maturation (Titorenko and Rachubinski, 2001). Using *N. crassa* as a novel model organism to study peroxisome biogenesis might now allow an independent assessment of the more general validity of this and other hypotheses. Demonstrating herein the concerted action of *Nc*Pex7p and *Nc*Pex20p in PTS2-dependent protein import has shown that this could be indeed a promising approach.

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