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# 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\*

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**Pex3p is a peroxisomal membrane protein that is essential for peroxisome biogenesis. Here, we show that a conserved stretch of positively charged amino acids (Arg<sup>11</sup>-X-Lys-Lys-Lys<sup>15</sup>) in the N terminus of *Hansenula polymorpha* Pex3p is involved in incorporation of the protein into its target membrane. Despite the strong conservation, this sequence shows a high degree of redundancy. Substitution of either Arg<sup>11</sup>, Lys<sup>13</sup>, Lys<sup>14</sup>, or Lys<sup>15</sup> with uncharged or negatively charged amino acids did not interfere with Pex3p location and function. However, a mutant Pex3p, carrying negatively charged amino acids at position 13 and 15 (K13E/K15E), caused moderate but significant defects in peroxisome assembly and matrix protein import. Additional changes in the N terminus of Pex3p, e.g. replacing three or four of the positively charged amino acids with negatively charged ones, led to a typical *pex3* phenotype, i.e. accumulation of peroxisomal matrix proteins in the cytosol and absence of peroxisomal remnants. Also, in these cases, the mutant Pex3p levels were reduced. Remarkably, mutant Pex3p proteins were mislocalized to mitochondria or the cytosol, depending on the nature of the mutation. Furthermore, in case of reduced amounts of Pex3p, the levels of other peroxisomal membrane proteins, e.g. Pex10p and Pex14p, were also diminished, suggesting that Pex3p maybe involved in the recruitment or stabilization of these proteins (in the membrane).**

respect to the mechanisms of matrix protein import (3–6). On the other hand, little still is known of the biogenesis of the peroxisomal membrane and the function of the individual peroxisomal membrane proteins (7, 8). Sequences involved in sorting of peroxisomal membrane proteins (termed mPTS)<sup>1</sup> have been described for only a few proteins, namely *Candida boidinii* PMP47 (9), *Saccharomyces cerevisiae* Pex15p (10), and Pex3p proteins of several organisms (11–14). Pex3p is essential for peroxisome biogenesis and is proposed to play a role in the early steps of the biogenesis (and maintenance) of the peroxisomal membrane (12, 13). Most Pex3p orthologues contain in their N termini a hydrophobic domain that may span the membrane. A stretch of positively charged amino acids, which is highly conserved among various Pex3p proteins, precedes this hydrophobic domain (Fig. 1). Together with uncharged conserved flanking residues, this sequence was postulated to play a role in sorting of Pex3p (13, 14). In order to analyze the possible role of the positively charged amino acids in Pex3p sorting, we performed a detailed mutational analysis of this sequence in *Hansenula polymorpha* Pex3p. For this purpose, various mutant alleles of *PEX3* have been constructed that encode mutant Pex3p proteins containing one or more amino acid substitutions. All mutant *PEX3* alleles were expressed in *H. polymorpha pex3* deletion strains and analyzed for their ability to restore peroxisome biogenesis and function in the host cells. The subcellular location of the mutant Pex3p proteins was also determined. The results of these studies are included in this paper.

## EXPERIMENTAL PROCEDURES

### Microorganisms and Growth Conditions

The *H. polymorpha* strains used in this study are listed in Table I. *H. polymorpha* cells were grown at 37 °C in batch cultures on mineral medium (15) supplemented with 0.5% carbon source (i.e. glucose (w/v) or methanol (v/v)) and 0.25% nitrogen source (i.e. ammonium sulfate or methylamine (w/v)). During proteasome inhibition experiments, the proteasome inhibitor MG-132 (Calbiochem, Omnilabo International b.v., The Netherlands) was added to the cultures to final concentrations of 20–100 μM. For growth on agar plates, the media were supplemented with 1.5% granulated agar. *Escherichia coli* DH5α (*supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) (16) was used for plasmid amplification and grown on LB medium supplemented with the appropriate antibiotics.

### DNA Procedures

Standard recombinant DNA (16) and genetic manipulations of *H. polymorpha* (18, 19) were carried out as detailed before. Site-directed

Peroxisomes are ubiquitous subcellular organelles that play an essential role in intermediary metabolism in eukaryotes. The organelles are characterized by the presence of enzymes that produce and degrade H<sub>2</sub>O<sub>2</sub> (1). In yeasts, peroxisomes are generally involved in the primary metabolism of unusual carbon and/or nitrogen sources, which are used for growth (2). The isolation and analysis of peroxisome-deficient yeast mutants has led to a rapid progress in our understanding of the principles of peroxisome biogenesis and function, in particular with

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<sup>1</sup> The abbreviations used are: AMO, amine oxidase; AO, alcohol oxidase; GFP, green fluorescent protein; eGFP, enhanced GFP; kb, kilobase pair(s); mPTS, membrane peroxisomal targeting signal; PMP, peroxisomal membrane protein; P<sub>AMO</sub>, amine oxidase promoter; P<sub>AOX</sub>, alcohol oxidase promoter; WT, wild type; Pex3p<sub>N50</sub>, N-terminal 50 amino acids of Pex3p; Pex3p<sub>N224</sub>, N-terminal 224 amino acids of Pex3p.

	hydrophobic domain	
Hp-Pex3p	MFQYCRDLVSRKKKLLFGTGVIAVSYAVSS---FVSNKLAEL	40
Pp-Pex3p	MLEYTAGLIRRRKRFLLISSGIIIGVGYIVTK----TINNKIQEF	40
Sc-Pex3p	MAPNQRSRSLLQRRRCVLLISLTGIAALFTTGSVVVFVKKRWLYKQ	46
Kl-Pex3p	MTGN-RSLVQRERKRFVSVSVLFAFLFATCAITVYFVKRWLYKQ	43
Hs-Pex3p	MLRSVWNFLKRRKKKCFILGTVLGGVYILGK---YQKKIREI	40
Ca-pPex3p	MAIFSSLAGFFNRKRRFFITSVAVTVSYLLINE---FVVKTFRRY	43
Dm-pPex3p	MLSLRQDFLSRRRCRFIVTGVVGGTIFAAR---YAQRFFVEF	40
Ce-pPex3p	MLASAWEFARNRKRCFLTAGGVLVGSAIAYVQS---SSRPKTFLEK	41

FIG. 1. Alignment of the amino termini of Pex3 proteins from the yeasts *H. polymorpha* (Hp-Pex3p), *Pichia pastoris* (Pp-Pex3p), *S. cerevisiae* (Sc-Pex3p), *Kluyveromyces lactis* (Kl-Pex3p; GenBank™ accession number AF012338), human (Hs-Pex3p), and their putative homologues in *Candida albicans* (Ca-pPex3p; EST 384128F09.s1.seq of the *C. albicans* Sequencing Project), *Drosophila melanogaster* (Dm-pPex3p; EST alignment dot number 132 of the Berkeley *Drosophila* Genome Project, Berkeley, CA) and *Caenorhabditis elegans* (Ce-pPex3p; gene C15H9.8, GenBank™ accession number U56965). Conserved positively charged amino acids (black background) precede the proposed transmembrane domain (gray background) with the consensus sequence RX(K/R)XK.

mutagenesis of *PEX3* was performed by using the polymerase chain reaction with *Pwo* polymerase according to the instructions of the supplier (Roche Molecular Biochemicals). The oligonucleotides (Eurogentec, Seraing, Belgium) and plasmids used in this study are listed in Tables II and III, respectively. Endonuclease restriction enzymes and biochemicals were obtained from Roche Molecular Biochemicals and used as directed by the manufacturer. For amino acid sequence analysis, the PC-GENE™ program (Release 6.70, IntelliGenetics, Mountain View, CA) was used. The TBLASTN algorithm (21) was used to search the data bases at the National Center for Biotechnology Information (Bethesda, MD).

#### Construction of a pex3 Deletion Strain

Construction of a strain in which the complete open reading frame of the *PEX3* gene was deleted was performed as follows: first, a 0.5-kb *SacI*-*Bam*HI fragment of pHIPX6-HpPEX3, containing the *PEX3* promoter, was cloned in pBluescript II SK+ digested with *SacI*-*Bam*HI (resulting in pRBG1). The plasmid pRBG1 was cut with *Bam*HI-*Sal*I and a 2.3-kb *Bam*HI-*Sal*I fragment containing the *H. polymorpha* *URA3* gene (22) was inserted (resulting in pRBG2). Subsequently a 0.1-kb 3'-untranslated region of the *PEX3* gene was amplified by polymerase chain reaction using the primers *-50 reverse* and *pex3utr3* (and pBS-HpPEX3-2.7 kb) as a template, cut with *Bgl*II+*Sal*I, and inserted into pRBG2 digested with *Bgl*II+*Sal*I, resulting in plasmid pRBG3. Finally, a 2.3-kb linear DNA fragment, containing the *URA3* gene flanked by *PEX3* upstream and downstream sequences, was isolated from pRBG3 using *Sal*I and partial *Sac*I digestion. This DNA fragment was used to transform *H. polymorpha* NCYC495 (*leu1.1 ura3*) cells; transformants were selected for uracil prototrophy and inability to grow on methanol (Mut<sup>-</sup>). One Mut<sup>-</sup> strain was selected that harbored the expected chromosomal alterations, as determined by Southern blot analysis.

#### Construction of Plasmids

**Mutant PEX3 Genes**—Site-directed mutations were introduced by polymerase chain reaction using pBS-HpPEX3-2.7 kb as template and the primers *pex3.xx* and *pex3-1B* (see Table II). The amplified mutant *PEX3* fragments were subcloned in pRBG21. All mutations were confirmed by sequencing. Finally, mutant *PEX3* genes were cloned behind the *PEX3* promoter in plasmid pHIPX6. The resulting plasmids (designated pRBGx in Table III) were used to transform  $\Delta$ pex3 cells (for designations, see Table I).

**PEX3-Green Fluorescent Protein Hybrids**—A hybrid gene encoding the amino-terminal 224 amino acids of Pex3p and the enhanced green fluorescent protein (Pex3p<sub>N224</sub>-eGFP) was constructed as follows. By polymerase chain reaction, using the primers *eGFP-dATG/eGFP-SalI* and plasmid pEGFP-C1 as template, the gene encoding *eGFP* was obtained. The amplified fragment was cut with *Sma*I-*Sal*I and ligated into a *Nco*I (Klenow-filled in)-*Sal*I-digested pBS-*PEX3*. The hybrid *PEX3-eGFP* gene was subsequently cloned in *Bam*HI-*Sma*I-digested pHIPX5 as a *Bam*HI-*Sal*I (Klenow-filled in) fragment behind the amine oxidase promoter (*P*<sub>AMO</sub>), resulting in pFEM67. The mutations generated in *PEX3* of pRBG44 (K13E/K15E) and pRBG48 (R11E/K13E/K14D/K15E) were introduced in the hybrid gene by exchanging the *Bam*HI-*Nhe*I fragment from pFEM67 with *Bam*HI-*Nhe*I inserts of pRBG44 and pRBG48, resulting in pFEM113 and pFEM117, respec-

tively. The resulting plasmids were integrated in the genomic amine oxidase locus of NCYC495 (*leu1.1*) cells as described before (23). Correct integration in the *P*<sub>AMO</sub> locus was determined by Southern blot analysis (data not shown). Integrants with single and multiple copies of the expression cassette in the genome were selected for further analysis (for designations of the strains, see Table I).

A hybrid gene encoding the amino-terminal 50 amino acids of Pex3p and eGFP (Pex3p<sub>N50</sub>-eGFP) was constructed. By polymerase chain reaction, using the primers *Universalpex3-FT2* and plasmid pBS-*PEX3* as template, part of the *PEX3* gene containing a *Nco*I site was obtained and subcloned in pBS-*PEX3*, resulting in pBS-*PEX3*\*. The previously amplified GFP fragment was cut with *Sma*I-*Sal*I and ligated into a *Nco*I (Klenow-filled in)-*Sal*I-digested pBS-*PEX3*\*. The hybrid *PEX3-eGFP* gene was subsequently cloned in *Bam*HI-*Sal*I-digested pHIPX4-HpPEX3 as a *Bam*HI-*Sal*I fragment behind the alcohol oxidase promoter (*P*<sub>AOX</sub>), resulting in pFEM75. This plasmid was integrated in the genomic alcohol oxidase locus of NCYC495 (*leu1.1*) cells as described before (23). Correct integration in the *P*<sub>AOX</sub> locus was determined by Southern blot analysis (data not shown). Integrants with a single copy of the expression cassette in the genome were selected for further analysis (see Table I).

#### Biochemical Methods

Total cell extracts were prepared as follows: an aliquot of a culture, corresponding to 3.0 *A*<sub>660</sub> units (volume × cell density), was centrifuged. After resuspension of the cell pellet in 300  $\mu$ l of 50 mM potassium phosphate buffer, pH 7.5, 100  $\mu$ l of 50% trichloroacetic acid was added and incubated for 30 min at  $-80^{\circ}\text{C}$  to allow protein precipitation. Trichloroacetic acid-treated cells were pelleted by centrifugation (10 min at 16,000 × *g*, room temperature), washed twice with ice-cold 80% acetone, and air-dried. After dissolving the pellets in 80  $\mu$ l of 1% SDS/0.1 N sodium hydroxide, 20  $\mu$ l of 5× SDS sample buffer was added (24). Samples were boiled for 5 min. For SDS-polyacrylamide gel electrophoresis, 10  $\mu$ l of each sample was used per lane.

Subcellular fractionation of *H. polymorpha* cells was performed as described (25). Enzyme activities of AO (26) and cytochrome *c* oxidase (27) were assayed by established procedures. Protein concentrations were determined using the Bio-Rad protein assay kit and bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis (24) and Western blotting (28) were carried out as described. Proteins on Western blots were detected using the chromogenic or chemiluminescent Western blotting kit (Roche Molecular Biochemicals) after decoration with polyclonal antibodies against various *H. polymorpha* proteins. Polyclonal antibodies against GFP were a gift from Dr. W.-H. Kunau (Ruhr University, Bochum, Germany).

#### Fluorescence and Electron Microscopy

GFP fluorescence in living cells was analyzed using the fluorescein isothiocyanate channel of an Axioskop H fluorescence microscope (Zeiss Netherlands b.v., Weesp, The Netherlands) equipped with a Plan-neofluar 100/1.3 (oil) objective and a Princeton Instruments CCD camera (RTE/CCD-1300 Y; Princeton Instruments b.v., Groenekan, The Netherlands).

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described (29). Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells, using polyclonal antibodies against various *H. polymorpha* peroxisomal proteins or GFP and gold-conjugated goat anti-rabbit antibodies (29).

#### RESULTS

**Multiple Amino Acid Substitutions in the Stretch of Positively Charged Amino Acids of Pex3p Affect Peroxisome Biogenesis**—Previously, we demonstrated that the N-terminal 37 amino acids of *H. polymorpha* Pex3p are sufficient to target *H. polymorpha* catalase, lacking its peroxisomal targeting signal 1, to the peroxisomal membrane (12). To exclude the possibility that internal sequences of peroxisomal catalase were involved in sorting of this hybrid protein, we sought to confirm these studies with the heterologous reporter protein eGFP. A hybrid gene was constructed encoding the first 50 amino acids of HpPex3p and eGFP; this gene was expressed in *H. polymorpha* wild type (WT) cells. Analysis of these cells by fluorescence microscopy (Fig. 2) and immunocytochemistry (not shown) for the location of eGFP demonstrated that the fusion protein was



TABLE I  
*H. polymorpha* strains used in this study

Strain	Genotype	Ref.
NCYC495	<i>leu1.1</i> derivative	17
NCYC495	<i>leu1.1, ura3</i> derivative	17
RBG1 ( $\Delta$ <i>pex3</i> )	<i>pex3::HpURA3, leu1.1</i>	This study
PEX3(P <sub>AOX</sub> )	RBG1 + pHIPX4-HpPEX3	This study
PEX3(P <sub>PEX3</sub> )	RBG1 + pHIPX6-HpPEX3	This study
PEX3(QXKKK)	RBG1 + pRBG27	This study
PEX3(RXQKK)	RBG1 + pRBG28	This study
PEX3(RXKQK)	RBG1 + pRBG29	This study
PEX3(RXEKK)	RBG1 + pRBG30	This study
PEX3(RXKKQ)	RBG1 + pRBG31	This study
PEX3(RXNQK)	RBG1 + pRBG43	This study
PEX3(RXEKE)	RBG1 + pRBG44	This study
PEX3(QXEKE)	RBG1 + pRBG45	This study
PEX3(EXEKE)	RBG1 + pRBG46	This study
PEX3(QXNQN)	RBG1 + pRBG47	This study
PEX3(EXEDE)	RBG1 + pRBG48	This study
PEX3(EXKKK)	RBG1 + pRBG58	This study
PEX3(RXKEK)	RBG1 + pRBG59	This study
HF74	NCYC495::pFEM75, single copy	This study
PEX3 <sub>N224</sub> (RXKKK)/GFP	NCYC495::pFEM67, single or multicopy	This study
PEX3 <sub>N224</sub> (RXEKE)/GFP	NCYC495::pFEM113, single or multicopy	This study
PEX3 <sub>N224</sub> (EXEDE)/GFP	NCYC495::pFEM117, single or multicopy	This study

 TABLE II  
 Oligonucleotides used in this study

Primer	Sequence
-50 reverse	5'-GCT CGT ATG TTG TGT GG-3'
Universal	5'-GTA AAA ACG ACG GCC AGT-3'
pex3utr3	5'-AGA AGA TCT TGA TGA TGA TTG GCA GC-3'
eGFP-dATG	5'-AGA GGA TCC CCG GGG TGA GCA AGG GCG AG-3'
eGFP-Sall	5'-TCG CGT CGA CTT TAC TTG TAC AGC-3'
pex3-1B	5'-GTC GTC GAC GAT ATC TAA TCA GTA TAC ATG C-3'
pex3-FT2	5'-G TTC CAT GGC AAA ATT CTC CTC C-3'
pex3.27 (R11Q)	5'-GT AGA GAT CTT GTG TCG CAG CAC AAG AAA AAG C-3'
pex3.28 (K13Q)	5'-GT AGA GAT CTT GTG TCG AGG CAC CAG AAA AAG CTT C-3'
pex3.29 (K14Q/K15Q)	5'-GT AGA GAT CTT GTG TCG AGG CAC AAG CAA CAG CTT CTT TTT GGC-3'
pex3.30 (K13E)	5'-GT AGA GAT CTT GTG TCG AGG CAC GAG AAA AAG CTT C-3'
pex3.31 (K15Q)	5'-GT AGA GAT CTT GTG TCG AGG CAC AAG AAA CAG CTT CTT TTT GGC-3'
pex3.43 (K13N/K14Q)	5'-GT AGA GAT CTT GTG TCG AGG CAC AAT CAA AAG CTT CTT TTT GGC-3'
pex3.44 (K13E/K15E)	5'-GT AGA GAT CTT GTG TCG AGG CAC GAG AAA GAG CTT CTT TTT GGC-3'
pex3.45 (R11Q/K13E/K15E)	5'-GT AGA GAT CTT GTG TCG CAG CAC AAG AAA CAG CTT CTT TTT GGC-3'
pex3.46 (R11E/K13E/K15E)	5'-GT AGA GAT CTT GTG TCG GAG CAC GAG AAA GAG CTT CTT TTT GGC-3'
pex3.47 (R11Q/K13N/K14Q/K15N)	5'-GT AGA GAT CTT GTG TCG CAG CAC AAC CAG AAC CTT CTT TTT GGC-3'
pex3.48 (R11E/K13E/K14D/K15E)	5'-GT AGA GAT CTT GTG TCG GAG CAC GAG AAC GAG CTT CTT TTT GGC-3'
pex3.58 (R11E)	5'-GT AGA GAT CTT GTG TCG GAG CAC AAG AAA AAG C-3'
pex3.59 (K14E)	5'-GT AGA GAT CTT GTG TCG AGG CAC AAG GAG AAG CTT CTT TTT GGC-3'

located at the peroxisomal membrane.

In an initial set of experiments, aimed at analyzing the significance of the conserved positively charged amino acids at positions 11, 13, 14, and 15 on Pex3p sorting, we substituted them all by uncharged residues (strain PEX3(QXNQN), Table IV). In order to determine the fate of the mutant Pex3p, the protein was synthesized in  $\Delta$ *pex3* cells and analyzed for its ability to restore peroxisome biogenesis and growth of the transformants on methanol. As shown before, cells of the  $\Delta$ *pex3* strain are unable to grow on media containing methanol because they lack functional peroxisomes (12). Clearly, the growth of PEX3(QXNQN) cells on methanol was retarded (Table IV). Electron microscopical analysis revealed that, compared with WT controls, in methanol-induced PEX3(QXNQN) cells, peroxisome biogenesis was severely affected (Fig. 3). The majority of the cells lacked peroxisomes but contained cytosolic AO crystalloids and/or aggregates, indicative of a *pex* phenotype (Fig. 3D). Infrequently, cells were observed that contained one large or a few smaller organelles (Fig. 3C), a phenomenon that is characteristic of reduced amounts of functional Pex3p (30).

*Single or Double Amino Acid Substitutions Do Not Affect Pex3p Incorporation*—The above experiment suggests that the

conserved positively charged amino acids at positions 11–15 are important for proper Pex3p function in peroxisome biogenesis. In order to obtain more insight in the significance of the individual positively charged amino acids in this region, we investigated the effect of single or double amino acid substitutions. To this end, one or two positively charged amino acid(s) at different positions in the N-terminal sequence were substituted by uncharged residues; the resulting mutant Pex3 proteins are summarized in Table IV. The mutant PEX3 alleles were expressed in  $\Delta$ *pex3* cells and analyzed for their ability to restore growth on methanol and peroxisome biogenesis in the host cells. The results revealed that single amino acid substitutions in Pex3p did not affect functional complementation, *i.e.* growth of the transformants on methanol (strains PEX3(QXKKK), PEX3(RXQKK) and PEX3(RXKKQ); Table IV). Also, mutant Pex3p proteins in which two amino acids had been replaced by uncharged residues were still able to fully rescue the methanol-growth defect of  $\Delta$ *pex3* cells (strains PEX3(RXNQK) and PEX3(RXKQK); see Table IV). Electron microscopy revealed that in all these strains peroxisomes were normally present; immunocytochemically, Pex3p was confined to the peroxisomal membrane (data not shown).

Because substitution of one or two positively charged amino

TABLE III  
Plasmids used in this study

Plasmid	Characteristics	Ref.
pBluescript II SK+		Stratagene, La Jolla, CA
pEGFP-C1		Clontech, Palo Alto, CA
pBS-HpPEX3-(2.7 kb)	pBluescript II KS+ with 2.7-kb <i>SaI</i> I PEX3 fragment	12
pHIPX5	<i>H. polymorpha</i> expression plasmid with <i>AMO</i> promoter	20
pHIPX6	<i>H. polymorpha</i> expression plasmid with PEX3 promoter	20
pHIPX4-HpPEX3	pHIPX4 containing the <i>H. polymorpha</i> PEX3 gene	20
pHIPX6-HpPEX3	pHIPX6 containing the <i>H. polymorpha</i> PEX3 gene	20
pBS-PEX3	pBS KS+ with 1.5-kb <i>Bam</i> HI- <i>SaI</i> I PEX3 gene	This study
pRBG3	pBS SK+ with <i>HpURA3</i> gene flanked by 5' and 3' UTRs PEX3 <sup>a</sup>	This study
pRBG21	pBS-PEX3 with 599-bp <i>Nhe</i> I- <i>Stu</i> I deletion in PEX3 gene	This study
pRBG27	pHIPX6 with mutated PEX3 (R11Q)	This study
pRBG28	pHIPX6 with mutated PEX3 (K13Q)	This study
pRBG29	pHIPX6 with mutated PEX3 (K14Q/K15Q)	This study
pRBG30	pHIPX6 with mutated PEX3 (K13E)	This study
pRBG31	pHIPX6 with mutated PEX3 (K15Q)	This study
pRBG43	pHIPX6 with mutated PEX3 (K13N/K14Q)	This study
pRBG44	pHIPX6 with mutated PEX3 (K13E/K15E)	This study
pRBG45	pHIPX6 with mutated PEX3 (R11Q/K13E/K15E)	This study
pRBG46	pHIPX6 with mutated PEX3 (R11E/K13E/K15E)	This study
pRBG47	pHIPX6 with mutated PEX3 (R11Q/K13N/K14Q/K15N)	This study
pRBG48	pHIPX6 with mutated PEX3 (R11E/K13E/K14D/K15E)	This study
pRBG58	pHIPX6 with mutated PEX3 (R11E)	This study
pRBG59	pHIPX6 with mutated PEX3 (K14E)	This study
pFEM75	pHIPX4 with PEX3 <sub>N50</sub> -eGFP hybrid gene	This study
pFEM67	pHIPX5 with PEX3 <sub>N224</sub> -eGFP hybrid gene	This study
pFEM113	pFEM66 with mutated PEX3 (K13E/K15E)	This study
pFEM117	pFEM66 with mutated PEX3 (R11E/K13E/K14D/K15E)	This study

<sup>a</sup> UTR, untranslated region; bp, base pair(s).

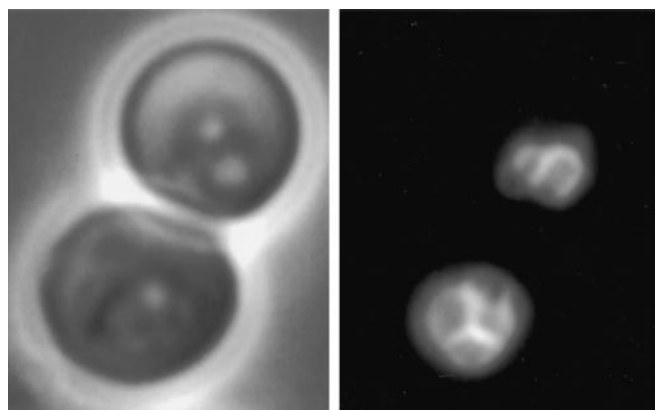


FIG. 2. Subcellular location of the Pex3p<sub>N50</sub>-eGFP hybrid protein in *H. polymorpha* HF74 cells. Cells were grown for 16 h on methanol-containing media and examined by fluorescence microscopy. The cells (left panel; phase-contrast light microscopy) have large, clustered peroxisomes that contain the Pex3p<sub>N50</sub>-eGFP hybrid protein (right panel; fluorescence microscopy).

acids by uncharged residues did not affect the sorting of Pex3p, we determined the effect of substituting each of these amino acids by a negatively charged residue (strains PEX3(EXKKK), PEX3(RXEKK) and PEX3(RXKEK); see Table IV). Again, all mutant Pex3p proteins were fully functional, because growth on methanol (Table IV) and peroxisome proliferation in these transformants was indistinguishable from WT controls (Fig. 3B). Immunocytochemical experiments confirmed that in these cells the mutant Pex3p proteins were normally associated with peroxisomes; also, AO protein, a major component of the peroxisomal matrix, was confined to peroxisomes, a prerequisite for *H. polymorpha* cells to grow on methanol (data not shown) (31). Analysis of the growth of the transformants in methanol-containing media at different temperatures (*i.e.* 25, 30, 37, and 43 °C) also showed no differences in growth characteristics compared with WT cells, thus ruling out a possible temperature-sensitive phenotype of one of the mutant alleles (data not shown).

Taken together, these results demonstrate that none of the individual positively charged amino acids in the conserved N-terminal sequence of *H. polymorpha* Pex3p are essential for Pex3p sorting and function. We subsequently checked the effect of more drastic alterations. Initially, we constructed a mutant in which two positively charged amino acids of Pex3p were replaced by glutamate-residues PEX3(RXEKE). This mutant did not display an apparent growth defect on methanol (Table IV). By electron microscopy, it was shown that methanol-grown PEX3(RXEKE) cells contained virtually normal peroxisomes. However, after immunocytochemical experiments using  $\alpha$ -AO or  $\alpha$ -catalase antibodies, a minor but significant labeling was found on the cytosol and the nucleus, suggesting a low AO and catalase import defect (Fig. 4, A and B).

Subsequently, an additional mutation was introduced (R11Q). Cells containing this mutant Pex3p, PEX3(QXEKE) (see Table IV), were hampered in growth on methanol. Electron microscopical analysis of these cells showed that normal peroxisome biogenesis was severely affected. The morphology of methanol-induced PEX3(QXEKE) cells was similar to PEX3(QXNQN) cells described before in that the cells harbored only one or—infrequently—two very large peroxisomes, which contained AO protein as judged from immunocytochemistry (Fig. 4C), or displayed cytosolic AO crystalloids, indicative of a severe peroxisomal matrix protein import defect (Fig. 4D). Finally, two mutants were constructed in which three or four conserved positively charged amino acids were substituted by negatively charged residues (PEX3(EXEKE) and PEX3(EXEDE); Table IV). Both strains behaved like the  $\Delta$ pex3 host strain, as they were unable to grow on methanol. Methanol-induced cells of these strains lacked normal peroxisomes. Also, peroxisomal membrane ghosts were undetectable; AO protein had predominantly accumulated in cytosolic aggregates (Fig. 5, A and B).

In summary, these data demonstrate that mutant Pex3p proteins, in which more than two positively charged amino acids in the conserved amino acid region 11–15 have been replaced by negatively charged ones, led to aberrant peroxisome assembly as well as to peroxisomal matrix protein import

TABLE IV

Characteristics of *H. polymorpha* WT,  $\Delta$ pex3 cells, and  $\Delta$ pex3 cells synthesizing mutant Pex3 proteins, incubated in media containing methanol

Strain	Charges <sup>a</sup>	A <sub>660</sub> <sup>b</sup>	Peroxisomal import defect <sup>c</sup>	Pex3p level <sup>d</sup>	Pex3p location <sup>e</sup>
$\Delta$ pex3	–	0.3	+	ND	ND
WT	+.+++ (+4)	3.2	–	+	p.m.
PEX3(QXKKK)	0.+++ (+3)	2.9	–	++	p.m.
PEX3(RXQKK)	+.0++ (+3)	3.1	–	++	p.m.
PEX3(RXKKQ)	+.++0 (+3)	2.8	–	++	p.m.
PEX3(EXKKK)	–.+++ (+2)	3.3	–	++	p.m.
PEX3(RXEKK)	+.+++ (+2)	2.8	–	++	p.m.
PEX3(RXKEK)	+.+-+ (+2)	2.7	–	++	p.m.
PEX3(RXNQK)	+.00+ (+2)	2.8	–	++	p.m.
PEX3(RXKQQ)	+.+00 (+2)	2.6	–	++	p.m.
PEX3(RXEKE)	+.--+ (0)	2.6	+	++	p.m.
PEX3(QXEKE)	0.-+- (-1)	1.4	+	+	ND
PEX3(QXNQN)	0.000 (0)	0.6	+	±	ND
PEX3(EXEKE)	–.+-+ (-2)	0.3	+	±	ND
PEX3(EXEDE)	–.--- (-4)	0.3	+	ND	ND

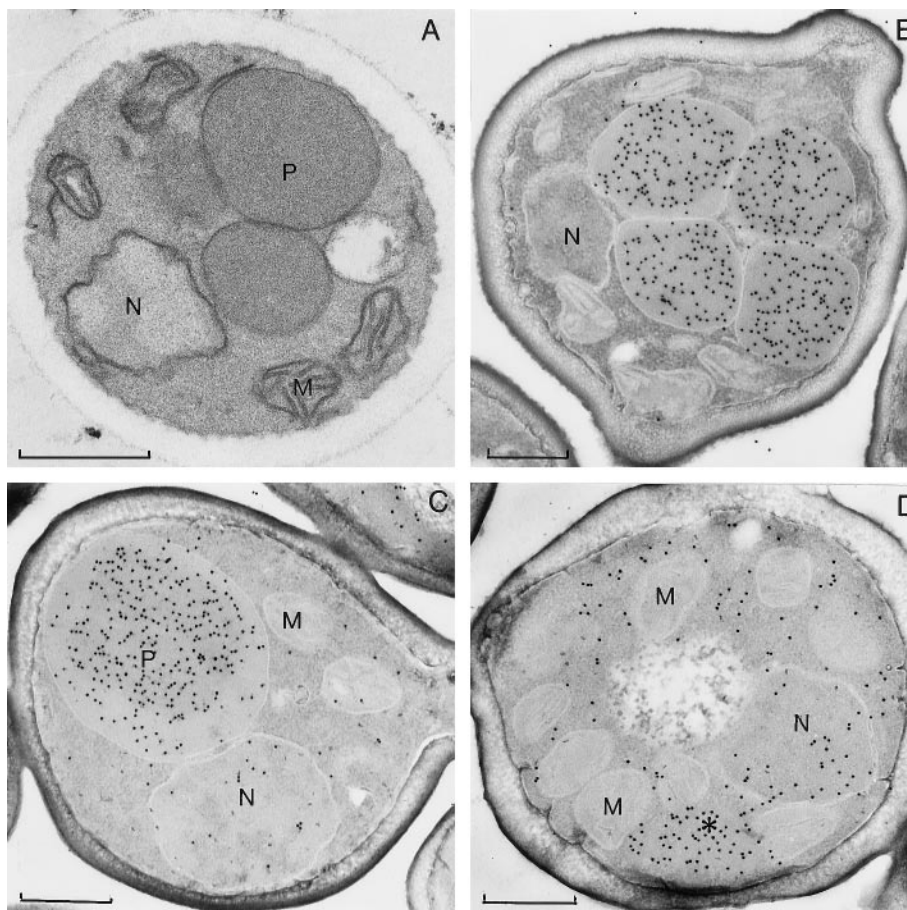
<sup>a</sup> Charged amino acids present in the RX(K/R)XK sequence; value in parentheses is the overall charge.<sup>b</sup> Cells were cultivated three times in mineral medium containing glucose and subsequently inoculated in methanol-containing media at an absorbance at 660 nm of 0.1. Growth after 18 h of incubation at 37 °C is indicated as A<sub>660</sub>.<sup>c</sup> Immunocytochemical analysis of the subcellular location of peroxisomal matrix proteins (e.g. AO and CAT). –, all matrix proteins peroxisomal; +, peroxisomal import defect.<sup>d</sup> Pex3p levels in (mutant) *H. polymorpha* cells compared to those in WT cells. ++, enhanced level; +, similar to WT; ±, decreased level; ND, not detectable.<sup>e</sup> Immunocytochemical localization of (mutant) Pex3p. p.m., peroxisomal membrane.

FIG. 3. Ultrathin sections of methanol-incubated cells of *H. polymorpha* WT and PEX3(QXNQN). WT cells typically contain 2–4 peroxisomes (A), in which matrix proteins are efficiently imported, as indicated by immunocytochemical experiments using  $\alpha$ -AO antibodies, which show that the labeling is confined to peroxisomes (B). In contrast, PEX3(QXNQN) cells a matrix protein import defect indicated by cytosolic and nuclear labeling (C and D). In addition, cells of this strain are heterogeneous as a subset of cells contain a few, enlarged peroxisomes (C;  $\alpha$ -AO labeling), whereas others lacked peroxisomes and contained cytosolic aggregates (\*) containing alcohol oxidase protein (D;  $\alpha$ -AO labeling). The electron micrographs are taken from KMnO<sub>4</sub>-fixed (A) or glutaraldehyde-fixed (B–D) cells. N, nucleus; M, mitochondrion; P, peroxisome; \*, alcohol oxidase. The bars represent 0.5  $\mu$ m aggregate.

defects.

**Subcellular Localization of Mutant Pex3 Proteins**—In order to address the question whether the peroxisomal abnormalities observed in some of the transformants was indeed due to a defect in mutant Pex3p sorting, we investigated the subcellular location of the Pex3p(QXEKE) protein, as a representative of the defective strains. Postnuclear supernatants, prepared from methanol-induced Pex3p(QXEKE)-producing cells, were subjected to sucrose density gradient centrifugation. Analysis of

the various fractions obtained from the gradient revealed that a minor peak of AO activity was present at 53% sucrose (Fig. 6A, fraction 7), a density at which peroxisomes of methanol-grown WT cells are normally located (1.24 g·cm<sup>-3</sup>) (32). The bulk of the AO activity remained at the top of the gradient (Fig. 6A, fractions 20–26), indicating that most of the AO protein was not associated with peroxisomes. The dual distribution of AO in the gradient was confirmed after Western blotting (Fig. 6B). The membrane-bound peroxins Pex3p, Pex10p, and



FIG. 4. Ultrathin sections of methanol-incubated cells of *H. polymorpha* PEX3(RXEKE) and PEX3(QXEKE). PEX3(RXEKE) cells are characterized by a morphology comparable to WT cells, but minor portions of matrix proteins, alcohol oxidase, and catalase were mislocated in the cytosol and nucleus, as indicated by labeling with  $\alpha$ -AO (A) or  $\alpha$ -catalase antibodies (B). In contrast, PEX3(QXEKE) cells display a heterogeneous phenotype; a subset of cells contain a few, enlarged peroxisomes containing most of the matrix proteins (C;  $\alpha$ -AO labeling) or lacked peroxisomes and contained a large cytosolic crystalloid (\*) containing alcohol oxidase protein (D;  $\alpha$ -AO labeling). The electron micrographs are taken from glutaraldehyde-fixed cells. N, nucleus; M, mitochondrion; P, peroxisome; \*, alcohol oxidase crystalloid. The bars represent 0.5  $\mu$ m.

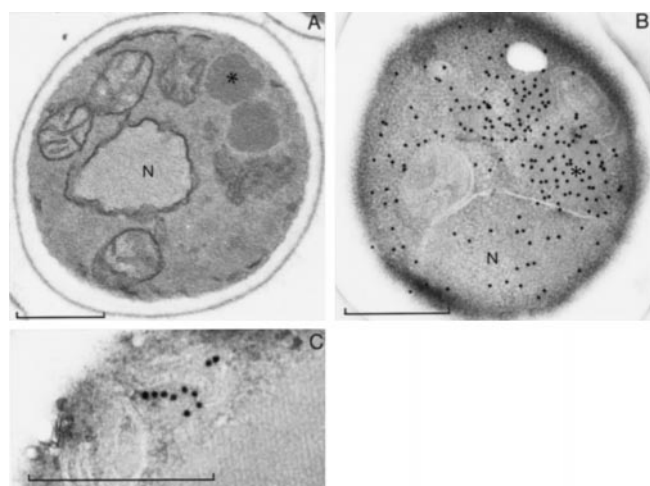
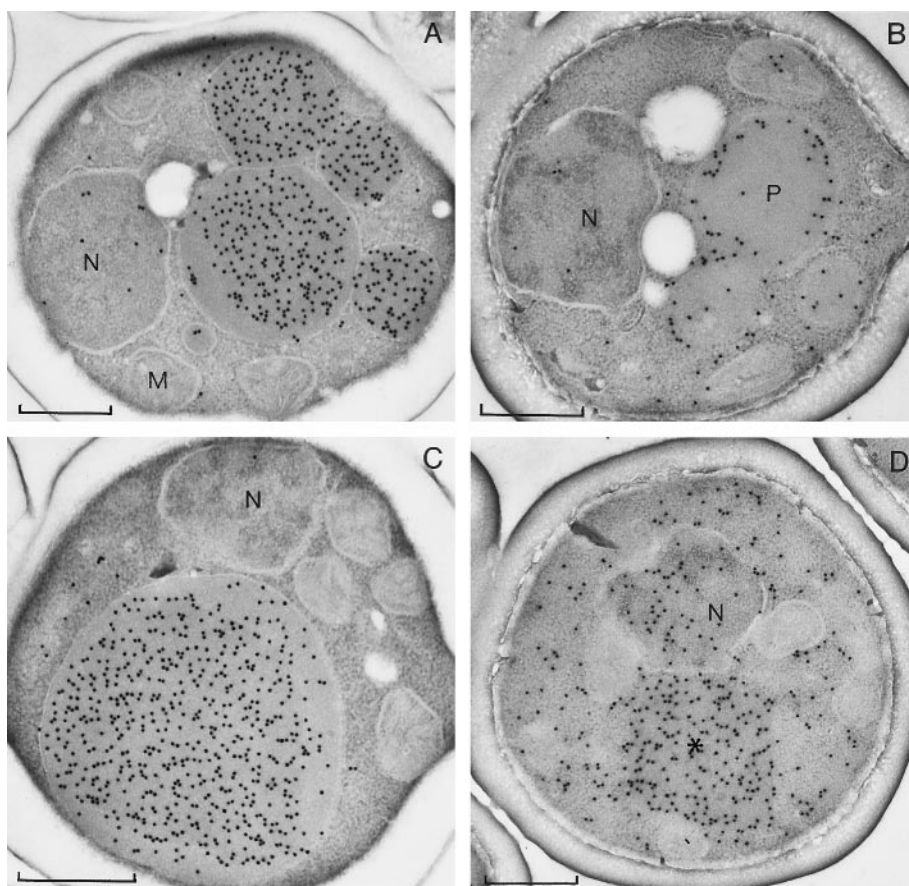


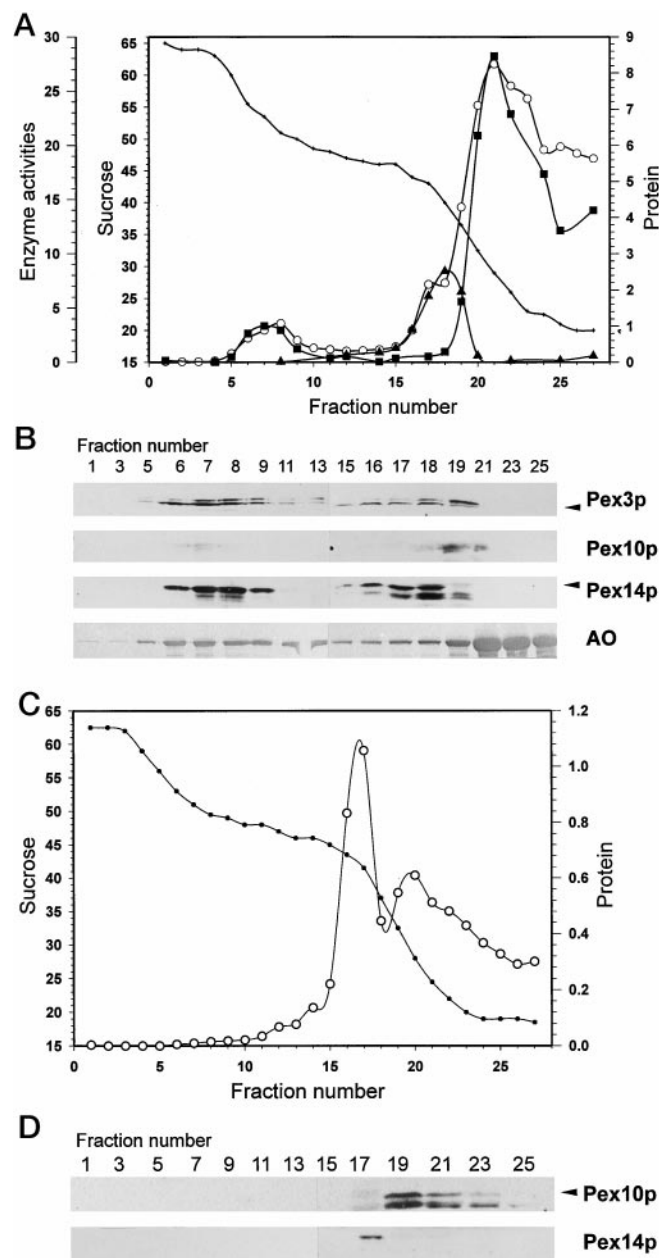
FIG. 5. Ultrathin sections of methanol-incubated cells of *H. polymorpha* PEX3(EXEKE) and PEX3(EXEDE). PEX3(EXEKE) and PEX3(EXEDE) cells displayed similar peroxisome-deficient phenotypes, in that they lacked peroxisomes, but instead, cytosolic aggregates (\*) were observed (A) containing the peroxisomal matrix proteins, as indicated by labeling with  $\alpha$ -AO antibodies (B) (shown for PEX3(EXEKE) cells). C, immunocytochemical experiments showing that Pex14p is located to mitochondria in  $\Delta$ pex3 cells, which lack recognizable peroxisomal membrane remnants. The electron micrographs are taken from  $\text{KMnO}_4$ -fixed (A) or glutaraldehyde-fixed (B and C) cells. N, nucleus; \*, alcohol oxidase aggregate. The bars represent 0.5  $\mu$ m.

Pex14p also showed a dual location. A portion of these proteins co-localized with AO protein in fractions 6–9 and most probably represents the intact peroxisomes that were infrequently observed in these cells. However, a significant portion of Pex3p and Pex14p are present in fractions 16–19 (at a density of 1.18  $\text{g cm}^{-3}$ ) and co-sedimented with mitochondria, as indicated by

cytochrome *c* oxidase activity, whereas Pex10p was predominantly found in fractions 19–21 (density, 1.15–1.16  $\text{g cm}^{-3}$ ). These results suggest that Pex3p and Pex14p are associated with mitochondria, whereas Pex10p may be attached to unidentified subcellular structures.

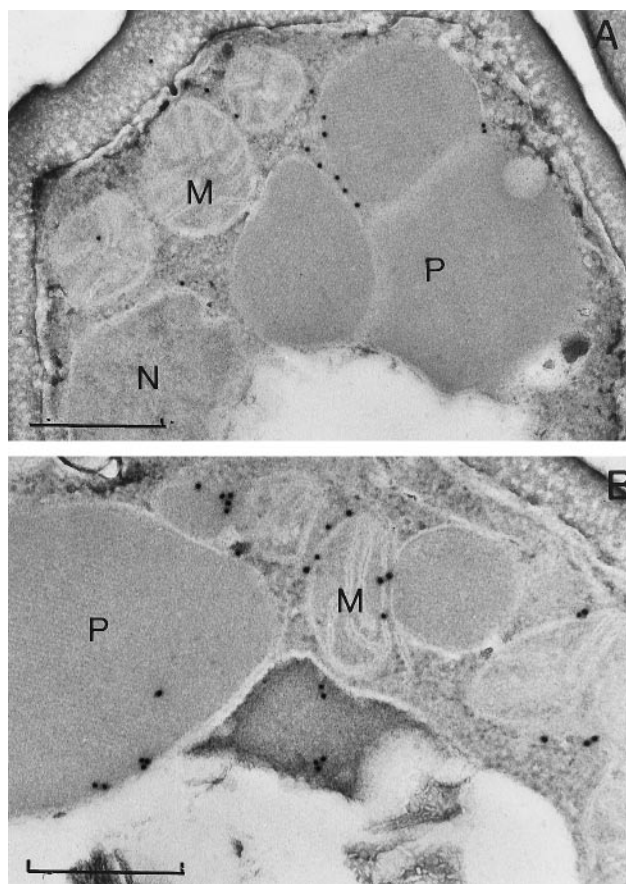
Control experiments performed on homogenates of methanol-induced  $\Delta$ pex3 cells showed a similar distribution of Pex10p and Pex14p, suggesting that in the absence of Pex3p, these peroxins are associated with structures similar to those in PEX3(QXEKE) cells (Fig. 6, C and D). In contrast, in control experiments using WT cells, Pex3p, Pex10p, and Pex14p invariably co-fractionated with peroxisomes (data not shown; see Refs. 32–34). Immunocytochemically, the location of Pex14p on mitochondria of PEX3(QXEKE) and  $\Delta$ pex3 cells was confirmed (Fig. 5C). Immunocytochemical experiments to localize Pex10p were inconclusive (data not shown); similarly, the subcellular location of mutant Pex3p in PEX3(QXEKE) cells could not be established, most likely due to the reduced levels of the protein which hampered an accurate localization (see below).

**Incorporation of Pex3p<sub>N224</sub>-eGFP Is Affected by Mutations in the Conserved Region of Pex3p**—In a further approach to analyze the location of mutant Pex3p, chimeric proteins were constructed by fusing the first 224 amino acids of these proteins to an eGFP. This approach was chosen because 1) these N-terminal parts of Pex3p are known to efficiently sort reporter proteins to peroxisomes (11–14), and 2) expressing the chimeric genes in a WT background allowed us to study the sorting of mutant Pex3p in cells that already contain peroxisomes (sorting is thus independent of whether the protein is functional or not). To avoid peroxisome assembly defects as a result of Pex3p overproduction (30), the chimeric genes were expressed under control of the substrate-inducible  $P_{\text{AMO}}$ . Two mutant Pex3p proteins were selected for this purpose, giving rise to either a slight (Pex3p(RXEKE)) or a severe peroxisome assembly defect



**FIG. 6. Subcellular fractionation of methanol-induced  $\Delta pex3$  and PEX3(QXEKE) cells.** *A*, distribution pattern of AO activity (■; units·ml<sup>-1</sup>), cytochrome C oxidase activity (▲; 10<sup>-1</sup> units·ml<sup>-1</sup>), protein concentration (○; mg·ml<sup>-1</sup>), and sucrose concentration (+; % (w/w)) after sucrose density centrifugation of postnuclear supernatant from homogenized protoplasts of PEX3(QXEKE) cells. *B*, Western blots of the various fractions obtained (see *A*) using antibodies against *H. polymorpha* Pex3p (arrow), Pex10p, Pex14p (arrow) and AO. Equal portions of each fraction were loaded per lane. Peroxisomal proteins were found in two peaks, namely in fractions 6–9 and fractions 16–19. *C*, distribution pattern of protein concentration (○; mg·ml<sup>-1</sup>) and sucrose concentration (●; % (w/w)) after sucrose density centrifugation of postnuclear supernatant from homogenized protoplasts of methanol-induced  $\Delta pex3$  cells. *D*, Western blots of the various fractions obtained (see *C*) using antibodies against *H. polymorpha* Pex10p and Pex14p.

(Pex3p(EXEDE)), using WT Pex3p<sub>N224</sub> as control (Table I). Cells of the two transformants were grown in methanol/methylamine-containing media (the amine substrate is used to induce the P<sub>AMO</sub>) and subsequently analyzed immunocytochemically for the subcellular location of the Pex3p-eGFP fusion proteins. As expected, in the control cells producing Pex3p<sub>N224</sub>(RXKKK)-eGFP, an  $\alpha$ -GFP-dependent labeling was found exclusively at the peroxisomal membrane (Fig. 7A; see also Ref. 12). The morphological phenotype of



**FIG. 7. Immunocytochemical localization of hybrid protein Pex3p<sub>N224</sub>-eGFP in *H. polymorpha* WT cells grown for 18 h on methanol/methylamine-containing media.** *A*, Pex3p-eGFP hybrid protein was predominantly located at the peroxisomal membrane (labeling with  $\alpha$ -GFP antibodies) of PEX3<sub>N224</sub>(RXKKK)GFP cells. In contrast, Pex3p-eGFP hybrid protein synthesized in PEX3<sub>N224</sub>(RXEKE)GFP cells was observed on both mitochondrial profiles and on the peroxisomal membrane (*B*). The electron micrographs are taken from glutaraldehyde-fixed cells. *N*, nucleus; *M*, mitochondrion; *P*, peroxisome. The bars represent 0.5  $\mu$ m.

these cells was indistinguishable from WT cells, and all AO protein was incorporated into peroxisomes (data not shown). In Pex3p<sub>N224</sub>(RXEKE)-eGFP producing cells, however, the eGFP protein had a dual location. Using  $\alpha$ -GFP antibodies, specific labeling was observed both on mitochondrial profiles and on the peroxisomal membrane (Fig. 7B). This suggests that the mutations introduced in Pex3p (K13E/K15E) indeed resulted in a significant mislocalization of the fusion protein to mitochondria. The labeling densities in Pex3p<sub>N224</sub>(EXEDE)-eGFP producing cells were too low to obtain conclusive results. In order to determine whether these low labeling densities reflected decreased levels of the fusion protein or had to be attributed to intrinsic features of the hybrid protein, Western blot analyses were performed. These experiments revealed that in crude extracts of methanol/methylamine-grown PEX3<sub>N224</sub>(RXKKK)GFP and PEX3<sub>N224</sub>(RXEKE)GFP cells, the fusion protein was readily detectable, using  $\alpha$ -GFP antibodies (Fig. 8). However, in Pex3p<sub>N224</sub>(EXEDE)-eGFP-producing cells, Pex3p<sub>N224</sub>-eGFP was hardly detectable, indicating that the protein level was strongly reduced (Fig. 8). The amounts of peroxisomal amine oxidase and Hsp70, used as markers for cytosolic proteins, remained approximately constant in these strains (Fig. 8).

**Specific Mutations in the N Terminus of Pex3p Affect the Protein Level**—The clear-cut reduction of Pex3p<sub>N224</sub>(EXEDE)-eGFP protein led us to analyze the levels of the various other Pex3p mutants, using WT cells as a control. Western blot analysis showed that the amounts of WT Pex3p, when pro-



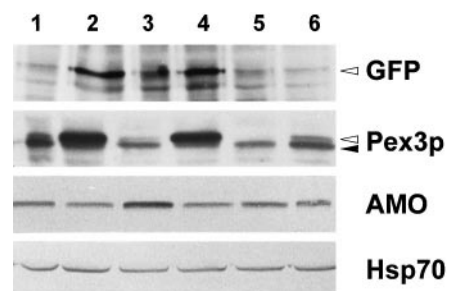


FIG. 8. Western blot analysis of crude extracts prepared from methanol/methylamine-grown PEX3<sub>N224</sub>(RXKKK)/GFP (lanes 1 and 2), PEX3<sub>N224</sub>(RXEKE)/GFP (lanes 3 and 4), and PEX3<sub>N224</sub>(EXEDE)/GFP (lanes 5 and 6) cells producing the fusion protein Pex3p-eGFP (lanes 1, 3, and 5, single copy expression; lanes 2, 4, and 6, multicopy expression). The blots were decorated with antibodies against GFP (upper open arrowhead), Pex3p (lower open arrowhead), Pex3p-eGFP; closed arrowhead, endogenous Pex3p), AMO, and cytosolic Hsp70. The latter two represent controls for P<sub>AMO</sub> expression and gel loading. Equal amounts of crude extracts were loaded per lane.

duced in  $\Delta pex3$  cells from autonomously replicating plasmids (PEX3(P<sub>PEX3</sub>)), are slightly enhanced compared with WT cells (Fig. 9A). Similarly, slightly enhanced Pex3p levels were found in cells of the strains PEX3(RXKEK), PEX3(EXKKK), PEX3(RXEKK), and PEX3(RXEKE), which showed normal growth in methanol-containing media (compare Table IV). In cells of strain PEX3(QXEKE) a comparable level of Pex3p was observed as found in WT cells. However, in PEX3(EXEKE), PEX3(QXNQN), and PEX3(EXEDE) cells, the Pex3p levels were significantly decreased. Only after overexposure of the detecting films could a very weak signal of Pex3p in blots prepared from crude extracts of PEX3(EXEKE) and PEX3(QXNQN) cells be observed; in extracts prepared from PEX3(EXEDE) cells, Pex3p remained invariably undetectable (data not shown). The levels of catalase, used a marker for matrix proteins, were comparable in the various mutants (Fig. 9A). Remarkably, in those cases in which mutant Pex3p was barely or not detectable, the levels of the cytosolic Hsp70 were enhanced (Fig. 9B). Taken together, these data clearly demonstrate that replacement of three or four positively charged amino acids in the N-terminal region strongly affects the mutant Pex3p level.

**The Protein Levels of Pex10p and Pex14p Are Related to the Presence of Functional Pex3p**—To address the question of whether the altered Pex3p levels in specific mutants also affect the amounts of other peroxins, Western blot analyses were performed. As shown in Fig. 9B, in methanol-induced cells of strains PEX3(RXKEK), PEX3(EXKKK), PEX3(RXEKK), and PEX3(RXEKE) the amounts of Pex3p are comparable to control WT levels; the levels of Pex10p and Pex14p were also unaltered. Conversely, the decrease in Pex3p levels detected in extracts of PEX3(QXEKE), PEX3(EXEKE), PEX3(QXNQN), and PEX3(EXEDE) cells was associated with lowered amounts of Pex10p and Pex14p, suggesting that the ultimate levels of these proteins are influenced by Pex3p. A similar correlation was found for two other membrane-bound peroxins, namely *H. polymorpha* Pex1p and Pex6p (data not shown) (35). Again, the levels of the major matrix proteins, as exemplified by AO, and also the peroxisomal targeting signal 1 receptor Pex5p were not significantly affected in transformants that displayed decreased levels of Pex3p (shown for PEX3(EXEDE) cells in Fig. 9C). Also, the levels of Pex10p and Pex14p were not significantly altered in cells in which Pex3p was overproduced from the alcohol oxidase promoter (Fig. 9).

**Mutations in the N Terminus of Pex3p<sub>N224</sub>-eGFP affect its incorporation (and stability)**—In the above experiments, we dem-

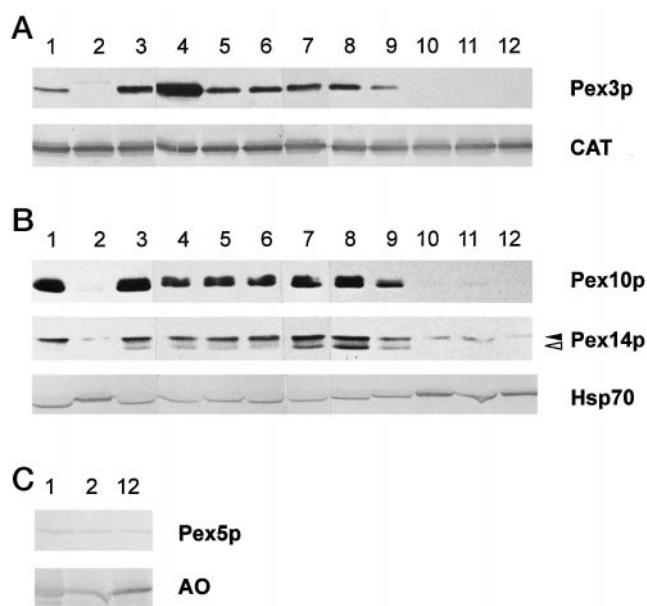


FIG. 9. Western blot analysis of crude extracts prepared from methanol-induced WT,  $\Delta pex3$ , and  $\Delta pex3$  cells expressing WT and mutant *pex3* alleles. Lanes contain crude extracts from WT (lane 1),  $\Delta pex3$  (lane 2), PEX3(P<sub>PEX3</sub>) (lane 3), PEX3(P<sub>AO</sub>) (lane 4), PEX3(RXKEK) (lane 5), PEX3(EXKKK) (lane 6), PEX3(RXEKK) (lane 7), PEX3(RXEKE) (lane 8), PEX3(QXEKE) (lane 9), PEX3(EXEKE) (lane 10), PEX3(QXNQN) (lane 11), and PEX3(EXEDE) (lane 12). Western blots were decorated with antibodies against the following proteins: A, Pex3p and catalase; B, Pex10p, Pex14p (closed arrowhead, phosphorylated protein; open arrowhead, nonphosphorylated protein) (36), and cytosolic Hsp70; and C, Pex5p and alcohol oxidase.

onstrated that specific alterations in the amino acid sequence of Pex3p resulted in decreased levels of the mutant protein (Fig. 9A). Also the hybrid protein, consisting of the N terminus of Pex3p with mutations (EXEDE) and eGFP was found at reduced levels, even when produced in a multicopy transformant (Fig. 8). Typically, no degradation products containing eGFP were observed in Western blots prepared from crude extracts of methanol-induced cells of this strain. Because full-length eGFP is rather resistant against *H. polymorpha* vacuolar proteases,<sup>2</sup> our data indicate that Pex3p<sub>N224</sub>(EXEDE)-eGFP may be degraded by the proteasome. In order to investigate this possibility, we analyzed whether inhibition of proteasome activity in *H. polymorpha* cells by the specific proteasome-inhibitor MG-132 affected the Pex3p<sub>N224</sub>(EXEDE)-eGFP levels. First, the effect of MG-132 on the growth characteristics of *H. polymorpha* was determined. PEX3<sub>N224</sub>(RXKKK)/GFP cells were pregrown for 4 h on media containing methanol/methylamine, and subsequently, MG-132 was added at final concentrations varying from 20 to 100  $\mu$ M. During further incubation, growth of the cultures grown in the presence of MG-132 was retarded (varying from ~5 to 20%) compared with untreated controls. These results suggest that MG-132 is indeed taken up by intact *H. polymorpha* cells. Subsequent experiments showed that administration of MG-132 to cultures of PEX3<sub>N224</sub>(EXEDE)/GFP cells led to a significant increase in the amounts of Pex3p<sub>N224</sub>-eGFP in these cells, whereas the levels of endogenous amine oxidase and Pex14p were hardly affected (Fig. 10A). These results suggest that the decrease of Pex3p<sub>N224</sub>(EXEDE)-eGFP protein is indeed due to specific degradation by the proteasome. Subsequently, the subcellular location of the protein was analyzed by cell fractionation. To this end, Pex3p<sub>N224</sub>(EXEDE)-eGFP-producing cells were grown in media containing methanol/methylamine for 8 h and subsequently

<sup>2</sup> K. N. Faber and R. J. S. Baerends, unpublished results.

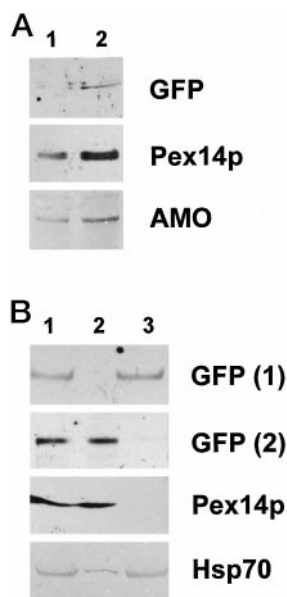


FIG. 10. A, Western blot analysis of crude extracts prepared from methanol/methylamine-induced PEX3<sub>N224</sub>(EXEDE)GFP cells incubated in the absence (lane 1) or presence (lane 2) of the proteasome inhibitor MG-132 (after an incubated period of 4 h). The Western blots were decorated with antibodies against GFP, Pex14p, and AMO showing the specific increase of Pex3p<sub>N224</sub>(EXEDE)-eGFP in cells grown in the presence of MG-132. Equal amounts of crude extracts were loaded per lane. B, homogenates, obtained from methanol/methylamine-induced PEX3<sub>N224</sub>(EXEDE)GFP (*GFP(1)*), Pex14p, and Hsp70) and PEX3<sub>N224</sub>(RXKKK)GFP cells (*GFP(2)*) and incubated in the presence of MG-132 (after an incubated period of 4 h), were subjected to differential centrifugation. Western blots were prepared from the postnuclear supernatant (lane 1), organellar pellet (30,000 × g; lane 2) and 30,000 × g supernatant (lane 3) and decorated with antibodies against GFP, Pex14p, and the cytosolic Hsp70. Pex3p<sub>N224</sub>(EXEDE)-eGFP (*GFP(1)*) behaves like the cytosolic Hsp70; Pex3p<sub>N224</sub>(RXKKK)-eGFP (*GFP(2)*) is organelle-bound, like Pex14p.

treated with 50 μM MG-132. After an additional incubation period of 4 h, the cells were harvested, converted to protoplasts, and gently homogenized. The resulting homogenate was subsequently subjected to differential centrifugation. Analysis of the various fractions obtained after centrifugation revealed that Pex3p<sub>N224</sub>(EXEDE)-eGFP was soluble, whereas control WT Pex3p<sub>N224</sub>(RXKKK)-eGFP was pelletable under these conditions (Fig. 10B). These results indicate that Pex3p<sub>N224</sub>(EXEDE)-eGFP, synthesized in WT cells, is mislocalized to the cytosol, where it most probably is rapidly degraded by the proteasome. Taken together, these data suggest that such mutations in the N terminus of Pex3p affect the incorporation of the protein into its target membrane.

#### DISCUSSION

In this paper, we provide evidence that a stretch of positively charged amino acids in the N terminus of *H. polymorpha* Pex3p (R<sub>11</sub>XKKK<sub>15</sub>) is involved in incorporation of the protein into its target membrane. This sequence was selected because it is highly conserved within the first 16 amino acids of various Pex3p proteins, which was shown to contain sorting information because it was able to direct a reporter protein to the peroxisomal membrane (Fig. 1) (12). The mutational analysis of amino acids 11–15 of HpPex3p revealed that none of the individual positively charged amino acids are crucial for sorting of the protein. This redundancy is remarkable in view of the strong conservation of the sequence. However, replacement of two positively charged amino acids by negatively charged residues had a distinct effect, as was exemplified by the mislocalization of a minor portion of a mutant Pex3p<sub>N224</sub>(RXEKE)-

eGFP hybrid protein to mitochondria. Remarkably, full-length Pex3p(RXEKE) could restore growth on methanol and peroxisome assembly in Δ*pex3* cells and gave rise to only a minor matrix protein import defect. The most likely explanation for this is that in these cells, the protein is slightly overproduced because it is synthesized from a plasmid-borne expression system. As shown before (30), the levels of WT Pex3p may modulate to a certain extent without affecting peroxisome biogenesis, and apparently, sufficient Pex3p(RXEKE) is correctly localized to account for the observed functional complementation. In line with this reasoning, these findings imply that the mutations most likely do not affect the function of the protein. It should furthermore be emphasized that the mitochondrial location of a portion of the protein is related to the mutations: in cells overproducing WT Pex3p, the protein was never localized to mitochondria (30).

Additional mutations, e.g. by replacing three conserved positively charged amino acids by glutamate residues, resulted in mutant alleles unable to complement Δ*pex3* cells. Unexpectedly, in Δ*pex3* cells expressing these alleles, the level of mutant Pex3p was strongly reduced compared with WT controls, suggesting that these proteins are unstable and/or rapidly degraded. Active degradation was in particular suggested by the reduced levels of the GFP fusion protein (Pex3p<sub>N224</sub>(EXEDE)-eGFP). Indeed, inhibition of the proteasome activity by MG-132 led to an increase in the level of Pex3p<sub>N224</sub>(EXEDE)-eGFP, which was localized in the cytosol.

Taken together, these data indicate that the positively charged amino acids at positions 11–15 in the N terminus of HpPex3p are involved in recruiting the protein to the peroxisomal membrane. Mutagenesis of this stretch may lead to Pex3p incorporation defects and thereby, depending on the nature of the mutations, cause insufficient Pex3p to be made available to allow normal peroxisome assembly. Mutant Pex3p proteins may be mislocalized, again depending on the nature of the mutations, to various compartments, where they may be stabilized (e.g. in mitochondria) or subject to degradation (e.g. in the cytosol). Although we demonstrated that the stretch of positively charged amino acids at the N terminus of HpPex3p is involved in incorporation of the protein into the peroxisomal membrane, our results do not discriminate between a possible role as a true sorting sequence (i.e. one that is recognized by a receptor) and a situation in which it may be necessary to keep the protein in association with the peroxisomal membrane.

An important question is whether the N-terminal sequence described above contains the only information for incorporation of Pex3p into the peroxisomal membrane. As shown before (12), Pex3p may reach its target membrane via the endoplasmic reticulum. This pathway is also suggested for other proteins (10, 13, 14), although definite proof is still lacking. In line with this still hypothetical possibility, HpPex3p may contain two sorting sequences, namely one that guides the protein to the endoplasmic reticulum and a second one that mediates the subsequent routing to the peroxisomal membrane. The first peroxisomal sorting signal identified thus far (termed mPTS) was described by Goodman and co-workers (9), who defined the sorting information in *C. bovidinii* PMP47. Remarkably, the conserved stretch of positively charged amino acids in the N termini of various Pex3p proteins resembles the PMP47 mPTS sequence, which was therefore proposed to be the core of the mPTS (9, 13, 14). In addition, it was proposed that Pex3p proteins contain the true PMP47 mPTS motif (KKX<sub>3</sub>TX<sub>2</sub>D) (9). In *H. polymorpha* Pex3p, this motif is located at positions 56–64. However, in HpPex3p, this sequence is not essential for sorting because fusion proteins consisting of first 37 or 50 N-terminal amino acids (excluding the PMP47 motif) and a



reporter protein (*i.e.* catalase or eGFP, respectively) are invariably correctly transported to the peroxisomal membrane (Ref. 12 and this study). Therefore, further studies are required to unravel the sorting information of Pex3p. These studies may also elucidate whether the endoplasmic reticulum-based pathway really exists and, if so, which of the two was affected by the mutagenesis of the stretch of positively charged amino acids.

A second important finding of our studies includes the fact that missorting of Pex3p also affected the levels of Pex10p and Pex14p. One possible explanation is that Pex3p plays a direct role in the incorporation/insertion of these proteins into the peroxisomal membrane. A second possibility relates to the previous finding that Pex3p may be part of a functional protein complex of a rather fixed stoichiometry, localized at the peroxisomal membrane (30, 37). Lowered levels of Pex3p may influence the normal formation of these putative protein complexes and, as a consequence, prevent uptake/binding of other constituents and thus lead to mislocalization of these proteins. In line with this reasoning is the finding of Huhse *et al.* (38) that the amount of *S. cerevisiae* Pex17p, a component of the matrix protein import machinery shown to interact with Pex14p, was drastically reduced in  $\Delta pex14$  cells. The option that the levels of proteins that are assumed to function in a complex are correlated is certainly not unique to peroxisomes and has been described before for proteins involved in the mitochondrial (39, 40) and the endoplasmic reticulum import machinery (41). Attempts to purify the putative protein complexes from the peroxisomal membrane of methanol-grown *H. polymorpha* are currently under way.

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