Endoplasmic Reticulum-directed Pex3p Routes to Peroxisomes and Restores Peroxisome Formation in a Saccharomyces cerevisiae pex3Δ Strain*

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Recent studies on the sorting of peroxisomal membrane proteins challenge the long-standing model in which peroxisomes are considered to be autonomous organelles that multiply by growth and division. Here, we present data lending support to the idea that the endoplasmic reticulum (ER) is involved in sorting of the peroxisomal membrane protein Pex3p, a protein required early in peroxisome biogenesis. First, we show that the introduction of an artificial glycosylation site into the N terminus of Pex3p leads to partial mitochondrial localization to the ER and the cytosol and cannot complement the processed protein localization to peroxisomes. In contrast, a Pex3p construct in which cleavage of the signal peptide is blocked by a mutation localizes to the ER and the cytosol and cannot complement pex3Δ cells. Together, these results strongly suggest that ER-targeted Pex3p indeed routes via the ER to peroxisomes, and we hypothesize that this pathway is also used by endogenous Pex3p.

Peroxisomes are eukaryotic organelles surrounded by a single membrane. Depending on organism and cell type they can have different functions, two of which are well conserved, i.e. hydrogen peroxide decomposition and fatty acid β-oxidation. Peroxisome biogenesis is carried out by so-called peroxins encoded by PEX genes, 32 of which have been identified so far (1, 2). Peroxosomal matrix proteins are synthesized on free polyribosomes and posttranslationally imported into peroxisomes. Targeting of peroxisomal matrix proteins from the cytosol to peroxisomes is mediated by two types of peroxisomal targeting signals, PTS1 and PTS2, that are recognized by the corresponding mobile import receptors Pex5p and Pex7p, respectively (for review, see Ref. 3).

Although trafficking of peroxisomal membrane proteins (PMPs) is less well understood, two observations indicate that distinct pathways exist for targeting of membrane and matrix proteins. First, in most pex mutants PMPs are correctly targeted to peroxisomal remnants (membrane structures lacking peroxisomal matrix proteins), whereas matrix proteins are found in the cytosol. The only pex mutants in which peroxisomal remnants are absent and PMPs are mislocalized and degraded are pex3 and pex19 in yeast and pex3, pex16, and pex19 in mammals (4–6). This indicates that Pex3D, Pex16p, and Pex19p play important roles in peroxisomal membrane biogenesis in these organisms. Second, PMPs do not contain a PTS1 or PTS2 but, rather, possess a distinct membrane peroxisomal targeting signal consisting of a transmembrane domain in conjunction with a cluster of basic residues that frequently also contains a number of hydrophobic residues (7–11).

Recent studies of Gould and co-workers (9, 12) explain why Pex19p is essential for peroxisomal membrane biogenesis. These authors show that Pex19p functions as a chaperone and import receptor for PMPs, stabilizing newly synthesized PMPs and specifically interacting with the hydrophobic domains of the membrane peroxisomal targeting signals in these proteins. The fact that Pex19p is a predominantly cytosolic protein, with only a small percentage (<5%) localized to peroxisomes, suggests that it is a shuttling receptor, similar to Pex5p and Pex7p, binding its PMP cargo in the cytosol followed by transport to the peroxisome (13–17). Current evidence suggests that the docking of Pex19p at the membrane is mediated by the PMP Pex3p (18). Interestingly, peroxisomal targeting of Pex3p is not dependent on Pex19p, which has led to the proposal that two distinct membrane peroxisomal targeting signal pathways exist, i.e. Pex19p-dependent (class I) and Pex19p-independent (class II) (12).

The classical model for peroxisome biogenesis formulated in 1985 by Lazarow and Fujiki (19) states that peroxisomes are autonomous organelles that multiply by growth and division of pre-existing ones. However, if de novo synthesis of organelles is excluded, the model cannot explain the formation of peroxisomes that follows the reintroduction of the corresponding wild-type gene into mutant cells which completely lack peroxisomal remnants (e.g. pex3Δ, pex16Δ, pex19Δ) (5, 6, 15, 20–27). It has been envisaged, therefore, that the ER plays a role in peroxisome biogenesis, triggering the formation of new peroxisomes, which would imply that at least some PMPs target to peroxisomes via the ER. Indeed, targeting of PMPs to the ER has been described. First, Pex2p and Pex16p of Yarrowia lipolytica are N-glycosylated, a process specific for the ER (28). Importantly, N-glycosylated Pex2p and Pex16p localized to peroxisomes, indicating that in Y. lipolytica these two PMPs route via the ER to peroxisomes. However, in other organisms there is no evidence for N-glycosylation of these or other peroxins. Second, in mouse dendritic cells Pex13p is predominantly located in a specialized ER subdomain that is connected with a peroxisomal reticulum, from which peroxisomes appear to originate (29). Third, Hansenula polymorpha Pex3p has been suggested to route to the ER in view of the fact that a fusion of the N-terminal 16 amino acids of Pex3p efficiently targets a marker protein to the ER (21). Fourth, in Saccharomyces cerevisiae, the integral PMP Pex15p is present in the ER when it is overexpressed or when 30 amino acids are deleted from the C terminus (30).

Despite increasing evidence that some PMPs may reach the peroxisome via the ER, it remains unclear whether the ER plays an essential,
general role in PMP routing. Arguments against such a role are divers (for an in depth discussion, see Ref. 3). For example, inhibitors of COPI and COPII that block vesicular transport in the early secretory pathway do not have an effect on PMP targeting (31, 32). Also, mutants of the ER translocation factors Sec61p or its homologue Ssh1p do not inhibit peroxisome biogenesis (33). In addition, in many studies that reported targeting of PMPs to the ER, overexpressed or truncated PMPs were used, which may have caused mislocalization (21, 30, 34–36).

In an attempt to shed further light on the possible involvement of the ER in PMP sorting, we studied the targeting of Pex3p in S. cerevisiae, making use of manipulated versions of the protein. In one series of experiments, we introduced a recognition site for ER-specific, N-linked core glycosylation and show that Pex3p is indeed modified. We also fused the signal peptide of the ER protein invertase to Pex3p and demonstrate that the construct is efficiently processed. Importantly, the processed Pex3p reaches the peroxisome and can rescue the pex phenotype of a pex3Δ strain, whereas a construct in which cleavage of the signal peptide is blocked fails to do so. Together, these studies provide strong evidence for an ER-to-peroxisome pathway of Pex3p.

**EXPERIMENTAL PROCEDURES**

Yeast Strains and Culture Conditions—Yeast strains used in this study were S. cerevisiae wild-type BJ1991 (Mata, leu2, trp1, ura3-251, prb1-1122, pep4-3, gal2), wild-type UTL-7A (Mata, ura3-52, trp1, leu2-3/112), pex3ΔBJ1991 (Mata, pex3::kanMX4, leu2, trpl, ura3-251, prb1-1122, pep4-3, gal2), and pex3ΔUTL-7A (Mata, pex3::LEU2, ura3-52, trpl, leu2-3/112).

Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO) (Difco), 2% glucose, and amino acids (20–30 μg/ml) as needed. The liquid media used for culturing of the cells for total protein isolation and (immuno)fluorescence microscopy contained 0.5% potassium phosphate buffer, pH 6.0, 0.3% yeast extract, 0.5% peptone, 0.1% (v/v) oleate, and 0.2% (v/v) Tween 40. Before shifting to this medium cells were grown on 0.67% YNB-WO containing 0.3% glucose for at least 24 h. For galactose induction experiments cells were precultured on 0.3% glucose medium (see above) and subsequently shifted to liquid media containing 2% galactose, 0.5% potassium phosphate buffer, pH 6.0, 0.3% yeast extract, 0.5% peptone, 0.1% (v/v) oleate, and 0.2% (v/v) Tween 40. Precultured cells were either harvested and resuspended or 1:10-diluted in this galactose media. Oleate plates contained 0.67% YNB-WO, 0.1% oleate (v/v), 0.5% (v/v) Tween 40, 2% agar, and amino acids as needed.

Cloning Procedures—Standard techniques for DNA manipulations were used (37). pAsk18 was generated by cloning the Narl-BamHI fragment of pEII130 containing the catalase promoter and the NH tag (MQDLPGNDNSTAGGS, corresponding to the N terminus of the mature hemagglutinin protein) in front of the BamHI-Sphl-cloned open reading frame of PEX3 in YCplac33 (38, 39). All the GAL1/10 promoter-controlled expression constructs are based on the EcoRI-Sacl GAL1/10 promoter of pA20 (40), which was cloned Narl-Sacl in front of PEX3 in pAsk18, generating pAsk19. The SP-Pex3p expression plasmid was constructed by cloning the SacI-HindIII SP-GFP-HDEL fragment of pEW109 (41), of which the HindIII end was made blunt by the Klenow polymerase, into the SacI-Smal sites of pAsk30, an YCplac22-derived plasmid that contained an EcoRI-Sacl GAL1/10 promoter fragment. This resulted in plasmid pAsk37. By PCR with primers PEX3Eag-F and PEX3Myc-R, PEX3-Myc was constructed (see TABLE ONE for primers used in this study). The PCR product was cloned EagI-SphI into pAsk37, generating pAsk52. Because the Myc tag was not used in this study for antibody recognition, SP-Pex3-Myc is referred to as SP-Pex3p.

The SP-FLAG-Pex3p fusion protein (consisting of the signal peptide of invertase (SLIC2), the FLAG epitope, and Pex3p) was generated using gene assembly by overlap extensions. Primers SP-F and FLAGSP-R were used to amplify the DNA encoding the SP sequence. The second pair of primers (FLAGPEX3-F and PEX3-R) was used to amplify the PEX3 open reading frame lacking the ATG. Templates for the amplification of both plasmids were generated as described above. The two sets of primers were used in two separate amplification reactions to amplify the overlapping DNA fragments, which were gel-purified using the QiAEX II gel extraction kit (Qiagen). The overlapping fragments were mixed and used in a single amplification cycle without primers to create double-stranded SP-FLAG-Pex3 DNA. This DNA was amplified in a third reaction by primers SP-F and PEX3-R and cloned SacI-Sphl into the EcoRI-Sacl-cloned GAL1/10 promoter into a YCplac22-based plasmid (39), resulting in pAsk56. SP*-FLAG-Pex3p is a point mutant of SP-FLAG-Pex3p in which the original alanine at position 1 relative to the signal peptide cleavage site is substituted for a tyrosine. SP*-FLAG-Pex3p was assembled in a similar way as SP-FLAG-Pex3 with the use of primers SP-F, FLAGSP*-Y-R, FLAGPEX3-F, and PEX3-R. Met-FLAG-Pex3 was generated by PCR on pAsk54 with primers FLAG-F1 and PEX3-R. Both SP*-FLAG-Pex3 and Met-FLAG-Pex3 were cloned like SP-FLAG-Pex3 downstream of the EcoRI-Sacl-cloned GAL1/10 promoter, resulting in pAsk54 and pAsk68, respectively.

The GAL1/10 promoter in pAsk54, pAsk56, and pAsk68 was replaced by the 207-bp EcoRI-Sacl PEX3 promoter fragment generated by PCR with primers PEX3-F-207 and PEX3-R-1 from genomic DNA, resulting in pAsk77, pAsk78, and pAsk79, respectively. All constructs were verified by sequencing.

**TABLE ONE**

| Name          | Sequence                        |
|---------------|---------------------------------|
| PEX3-F-207    | CCGGAATTTCAGCTAAGATGTGTATTAAACAG |
| PEX3-R-1      | GCCGAGCTCCCTTCTTTAGTGTTGCTTCC |
| PEX3Eag-F     | AATTATCGGCCGCTTCAAAATCAAGATCAGTC |
| PEX3-R        | ATACGTCATGCTAAGGCTTGAGGAAAGAGAG |
| SP-F          | CGACTCATCACAAACAAAC              |
| FLAGSP-R      | CTTGTCTATCTGCTCCTTTGATGTCGCTTACAAAC |
| FLAGPEX3-F    | GACTACAAGGAGCAGATGCAAGGGATCCGCCCCAAATCAAGTACG |
| FLAGSPY-R     | CTTGTCTATCTGCTCCTTTGATGCTATAGATATTGTCGCTGCAAAC |
| FLAG-F1       | GCCGAGCTCATGACATGACTAAGGAGCG |
| PEX3Myc-R     | ATACGTCATGCTATACATTTCTTCAGAAATCATATTTTTGTTCAGAACCAGAA |

CCCTGCAGAGGCCTTGAAGGAAAGAACGAGC
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Endoglycosidase H (Endo H) Digestion—Endo H digestion was carried out essentially as described by Coligan et al. (42). Two A<sub>opt</sub> equivalents of cells were harvested by centrifugation and resuspended in 10% trichloroacetic acid. Protein extracts were made as described (see "Miscellaneous"). The protein sample was dissolved in 50 μl of 1% 2-mercaptoethanol, 0.5% SDS and heat-denatured for 5 min at 100 °C. To the denatured protein sample 10 μl of 0.5 M sodium citrate, pH 5.5, 38 μl of deionized water, 1 μl of 100 mU phenylmethylsulfonyl fluoride, and 1 μl (5 units/μl) of Endo H (Roche Applied Science) was added. For the control digestion the same reagents were added, but the enzyme was substituted by water. Samples were incubated overnight at 30–35 °C. To inactivate Endo H, samples were heated for 5 min at 100 °C. Samples were analyzed by SDS-PAGE and immunoblotting.

Antibodies—Antibodies used were rabbit polyclonal anti-Pex3p (a kind gift from Prof. Dr. R. Erdmann, rabbit polyclonal anti-NH (a kind gift from Dr. P. van der Sluijs), mouse monoclonal anti-FLAG M1 (Sigma-Aldrich), mouse monoclonal anti-FLAG M2 (Sigma-Aldrich), rabbit polyclonal anti-3-ketoacyl-CoA thiolase, rabbit polyclonal anti-Kar2p, fluorescein isothiocyanate-conjugated goat-anti-rabbit, and TRITC-conjugated goat anti-mouse (Jackson Immuno Research Laboratories, West Grove PA).

Immunofluorescence and Immunoelectron Microscopy—Immunofluorescence microscopy was performed as previously described (43) with the following modifications. Blocking solution contained 1% bovine serum albumin, 0.05% Tween 20, and 1× Tris-buffered saline. Samples were incubated for 1 h with secondary antibody. Mowiol (Hoechst) was used as mounting solution. Cells were examined using a Zeiss Axiophot 2 microscope, and images were captured by a Coolpix HQ CCD camera (Roper Scientific) and processed in Adobe Photoshop. For immunocytochemistry oleate-induced cells were fixed and prepared as described (6). Immunolabeling was performed on ultrathin sections using antibodies against 3-ketoacyl-CoA thiolase and gold-conjugated protein A.

Miscellaneous—Protein extracts were prepared by breaking the cells with glass beads and acid precipitated as described (44). SDS-PAGE and immunoblotting were performed as described (45). Antibody binding was detected using either goat-anti-rabbit IgG conjugated by horseradish-peroxidase in combination with ECL reagents from Amersham Biosciences. The SignalP server was used to predict the presence and location of signal peptide cleavage sites (46).

RESULTS

NH-tagged Pex3p Is N-Glycosylated and Localizes to the ER—Pex3p is an integral PMP with a predicted type I topology (N<sub>lumen</sub>-C<sub>cytosol</sub>). In an attempt to find evidence for routing of Pex3p through the ER, we epitope-tagged full-length Pex3p at its N terminus with a synthetic NH tag (MQDLPGNDFSTTGGGS), which corresponds to the N-linked core glycosylation site (NH-Pex3p, see Fig. 1 and Ref. (38)). NH-Pex3p cells expressing NH-Pex3p from the strong catalase promoter were first analyzed for growth on oleate as the sole carbon source and for peroxisomal import of PTS1-tagged green fluorescent protein (GFP-SKL). These experiments showed that NH-Pex3p can rescue the pex phenotype of pex3Δ cells and, thus, is a functional protein (results not shown). To analyze the glycosylation status of the NH-Pex3p protein in oleate-induced pex3Δ cells, crude cellular extracts were subjected to immunoblot analysis using NH and Pex3p antibodies. As controls, total cell lysates of oleate-induced untransformed wild-type and pex3Δ cells were also analyzed. Both Pex3p and NH antibodies detected two protein bands of about 50 and 52 kDa, respectively, in the pex3Δ strain expressing NH-Pex3p (Fig. 2A, lane 3). In contrast, the Pex3p antibody recognized only one protein band of 48 kDa in untransformed wild-type cells, corresponding to endogenous Pex3p (lane 1). None of the three bands were present in extracts from pex3Δ cells (lane 2). Taken together, the results imply that about 50% of NH-tagged Pex3p is modified, whereas endogenous Pex3p is not. To identify the modification as glycosylation, extracts of pex3Δ cells expressing NH-Pex3p were incubated with endoglycosidase H (Endo H, Fig. 2B). As a result of Endo H treatment, the slower migrating NH-Pex3p band disappeared, demonstrating that NH-Pex3p is indeed modified by N-linked core glycosylation. The difference in apparent molecular mass of about 2 kDa between Endo H-treated and untreated samples suggested that Pex3p receives a single core oligosaccharide chain (42). Because N-linked core glycosylation is an ER-specific modification, this result implies that a substantial portion of NH-Pex3p has been in the ER.
It is well documented that overexpression of PMPs may lead to mistargeting to subcellular compartments other than peroxisomes (6, 30, 47). To address this problem, we cloned NH-PEx3 downstream of the inducible galactose promoter (P\textsubscript{GAL1/10}), which is repressed by glucose and induced in galactose-containing media. Anti-NH immunoblotting of total lysates from pex3\textDelta cells expressing P\textsubscript{GAL1/10}·NH-PEx3 showed that expression of the protein was indeed repressed in media containing 0.3% glucose (Fig. 3, lane 1) and that there was a gradual increase in expression upon shifting the cells to rich oleate media containing 2% galactose (lanes 2–5). Importantly, at low expression levels both glycosylated and unglycosylated NH-PEx3 were detected, whereas the ratio of the two bands remained the same at higher expression levels. This implies that NH-PEx3 is not located in the ER as a result of overexpression but, rather, as part of a normal routing process. However, Nycodenz gradient analysis revealed that N-glycosylated NH-PEx3 largely co-migrated with Dpm1p (an ER membrane marker) but not with the peroxisomal membrane marker Ant1p, whereas most of the unmodified NH-PEx3 was detected in peroxisomes (data not shown). This seems to suggest that the N-glycosylation of NH-PEx3 turns the protein into a permanent resident of the ER, unable to leave this compartment and reach its destination (see “Discussion”).

The Signal Peptide of Invertase Targets FLAG-tagged Pex3p to the ER—The results described above suggest that N-glycosylation cannot be used as a tool to study all aspects of Pex3p trafficking. Therefore, we took a different approach fusing the signal peptide (SP) of invertase (SUC2) to the N terminus of Pex3p (SP-Pex3p, Fig. 1) to direct it deliberately to the ER. Fig. 4 shows that SP-Pex3p, when expressed from the galactose promoter, restores the capacity to grow on oleate of pex3\textDelta cells at growth rates comparable with that of wild-type cells. This indicates that when Pex3p is sent through the ER on purpose, it can trigger the formation of new, functional peroxisomes in pex3\textDelta cells.

To firmly establish that SP·Pex3p is indeed routed to peroxisomes via the ER, we cloned the FLAG epitope (sequence DYKDDDDK) between the SP and the N terminus of Pex3p (SP-FLAG-Pex3p, Fig. 1). The FLAG tag is recognized by two antibodies with different specificity, i.e. anti-FLAG M1 (M1) and anti-FLAG M2 (M2). The M2 antibody recognizes all FLAG-tag-containing proteins, whereas the M1 antibody recognizes the FLAG tag only when it is present at the extreme N terminus of a FLAG-Pex3p fusion protein from which the signal peptide has been removed by the signal peptidase in the ER. This cleavage exposes the N-terminal Asp residue of the FLAG tag, which is essential for recognition by the M1 antibody (48). This antibody can, therefore, be used to unambiguously determine whether a given FLAG-Pex3p variant has been inside the ER, irrespective of its final destination in the cell. As a control, we introduced an Ala-to-Tyr change at position -1 relative to the cleavage site of SP-FLAG-Pex3p (SP*-FLAG-Pex3p, Fig. 1), a mutation predicted to block cleavage of the signal peptide (prediction program SignalP: www.cbs.dtu.dk/services/SignalP (46)). Accordingly, SP*-FLAG-Pex3p will be recognized by the M2 antibody but not by the M1 antibody. Finally, we also constructed an N-terminal FLAG-tagged Pex3p without a signal peptide as a control on the effect of the FLAG tag on Pex3p function (Met-FLAG-Pex3p, see Fig. 1 and below).

Total lysates of pex3\textDelta strains expressing SP-FLAG-Pex3p or SP*-FLAG-Pex3p from the endogenous PEX3 promoter or the strong GAL1/10 promoter were analyzed by immunoblotting using the M1 and M2 antibodies (Fig. 5A). The protein product generated with the SP*-FLAG-Pex3p construct migrated in a single band in the polyacrylamide gel and was only recognized by the M2 antibody (Fig. 5, lanes 2 and 3), demonstrating that SP*-FLAG-Pex3p indeed has an uncleavable signal peptide. The protein produced from the SP-FLAG-Pex3p construct also migrated in a single band but was recognized by both antibodies (Fig. 5, lanes 4 and 5), indicating that the signal peptide of SP-FLAG-Pex3p is properly cleaved. Importantly, we observed a small but reproducible difference in molecular mass of about 2 kDa between the bands derived from the two constructs, supporting the notion that the signal peptide of SP-FLAG-Pex3p is removed (cf. Fig. 5, lanes 2 and 3 to lanes 4 and 5 of the M2 blot). Signal peptide cleavage appeared to be efficient as shown by the apparent absence of the slower migrating, uncleaved form of the protein in the lanes probed with the M2 antibody. The use of the GAL1/10 promoter resulted in a substantial increase in the production of only the faster migrating, cleaved form of the protein (cf. Fig. 5, lanes 4 and 5), suggesting that efficient cleavage of the signal peptide of SP-FLAG-Pex3p is independent of expression level. Finally, the M1 antibody failed to detect Met-FLAG-Pex3p (Fig. 5B), confirming that the M1 antibody recognizes the FLAG tag only when it is present at the N terminus of the product generated by the signal peptidase in the ER. Taken together, the results of Fig. 5 suggest that SP-FLAG-Pex3p is efficiently processed by the ER signal peptidase and, therefore, that it is routed to the ER.

ER-directed FLAG-Pex3p Can Restore Peroxisome Formation in a pex3\textDelta Deletion Strain—Next, we asked whether PEX3 promoter-controlled SP-FLAG-Pex3p is able to restore peroxisome formation in a pex3\textDelta strain. We first analyzed the capacity of SP-FLAG-PEX3-transformed pex3\textDelta cells to grow on media with oleate as the sole carbon source (Fig. 6A). Indeed, in contrast to the untransformed strain, the transformed cells were perfectly capable of growth on oleic acid, indicating that they contain functional peroxisomes. Growth of the SP-FLAG-Pex3p-expressing cells was slightly reduced in comparison to wild-type cells but comparable with Met-FLAG-Pex3p-expressing cells, indicative of a small inhibitory effect of the FLAG tag on Pex3p functioning. Importantly, the construct with the uncleavable SP (SP*-FLAG-Pex3p) was unable to complement the growth deficiency of the pex3\textDelta
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FIGURE 5. The signal peptide of SP-FLAG-Pex3p is correctly processed. A, the signal peptide of SP-FLAG-Pex3p is efficiently cleaved both at low and high expression levels. Trichloroacetic acid lysates of oleate-induced pex3Δ cells expressing SP-FLAG-Pex3p or SP-FLAG-Pex3p under control of the PEX3 or the GAL1/10 promoter (pEX3, and Ppex3, respectively) were analyzed using M1 and M2 antibodies. A negative control for SP*/SP/FLAG-Pex3p expression was a sample of pex3Δ cells. B, expression of Met-FLAG-Pex3p. Trichloroacetic acid lysates of oleate-induced pex3Δ cells expressing Met-FLAG-Pex3p under control of the PEX3 promoter were analyzed using M1 and M2 antibodies. Similar results were obtained with the GAL1/10 promoter-controlled constructs (data not shown).

As an additional check on peroxisome formation in (SP-)FLAG-PEX3-transformed strains, we introduced the reporter protein GFP-PTS1 and analyzed its location with fluorescence microscopy (Fig. 6A). Pex3Δ cells expressing SP-FLAG-Pex3p or Met-FLAG-Pex3p from the endogenous PEX3 promoter exhibited a predominantly punctated pattern, typical for cells with functional peroxisomes that import GFP-SKL (49). In contrast, a diffuse cytosolic fluorescence was observed in pex3Δ cells transformed with the non-cleavable SP*-FLAG-Pex3p fusion protein (also expressed from the endogenous PEX3 promoter), a pattern very similar to that observed in pex3Δ cells transformed with an empty vector and typical for cells without functional peroxisomes. Also in this experiment, a (small) inhibitory effect of the FLAG tag on Pex3p functioning is noticeable. Although in wild-type cells peroxisomes were evenly distributed, peroxisomes in SP-FLAG-Pex3p and Met-FLAG-Pex3p-expressing cells appeared to cluster, often forming one large fluorescent structure. To investigate whether these structures represent bona fide peroxisomes, we performed immunoelectron microscopy with antibodies against the peroxisomal matrix enzyme thiolase. In cells transformed with SP-FLAG-Pex3p (Fig. 6, G and H) or Met-FLAG-Pex3p (Fig. 6, D and E), two types of thiolase-containing peroxisomal profiles were observed; the first (Fig. 6, D and G) was very similar to wild-type peroxisomes (cf. Fig. 6C), and the second (Fig. 6, E and H) indeed consisted of clustered organelles, highly reminiscent of the large fluorescent structures seen in panel B. We found clusters of 3–5 organelles that were occasionally positioned at or close to the nuclear envelope (Fig. 6H) or appeared to be associated with strands of membranes (Fig. 6E). Nevertheless, the density of thiolase-labeling in clustered peroxisomes appears to be quite similar to that in wild-type organelles, suggesting that they contain a functional PTS2 import system. SP*-FLAG-Pex3p cells failed to form peroxisomal structures and mislocalized thiolase to the cytosol (Fig. 6F). Together, the experiments of Figs. 6, A–H, demonstrate that despite a small inhibitory effect of the FLAG tag, ER-targeted SP-FLAG-Pex3p is able to mediate formation of functional peroxisomes in pex3Δ cells, provided the fusion protein is equipped with a cleavable signal peptide.

Processed SP-FLAG-Pex3p Is Localized Both in the ER and Peroxisomes—To further substantiate the notion that SP-FLAG-Pex3p routes from the ER to peroxisomes, we analyzed the subcellular localization of the fusion protein by double indirect immunofluorescence in pex3Δ cells. Because the PEX3 promoter-controlled construct is expressed at relatively low levels, we used pex3Δ cells expressing SP-FLAG-Pex3p from the stronger GAL1/10 promoter. The latter construct is fully functional, since it is efficiently processed and comple-

ments the pex3Δ strain (Fig. 5 and data not shown). In this experiment we applied M1 antibodies that specifically detect processed SP-FLAG-Pex3p in combination with antibodies raised against the peroxisomal matrix protein thiolase or the ER luminal protein Kar2p. In some cells processed SP-FLAG-Pex3p appeared to exclusively co-localize with either Kar2p or thiolase (Fig. 7B, top row of panels). In other cells the overlap between processed SP-FLAG-Pex3p and the marker protein was only partial (Fig. 7B, bottom row of panels). These results can best be explained by assuming that processed SP-FLAG-Pex3p has a dual localization and is present in both the ER and the peroxisomes, apparent differences in fluorescence between cells being generated by a varying amount of SP-FLAG-Pex3p in the two compartments in different cells. We speculate that these differences may be related to differences in expression levels of the construct; in cells with a relatively low expression level the protein appears to localize mainly to the ER, whereas high expression levels are required to get a predominant peroxisomal location. Next we analyzed SP*-FLAG-Pex3p-expressing pex3Δ cells using Kar2p and thiolase antibodies in combination with the M2 antibody. These cells lack peroxisomes (see Fig. 6, B and F), resulting in a cytoplasmic location of thiolase. Co-localization of SP*-FLAG-Pex3p with Kar2p was observed in the majority of the cells, indicating that it is present in the ER (Fig. 7C). In addition to ER staining, a weak diffuse fluorescence was seen that colocalized with thiolase, implying that SP*-FLAG-Pex3p is also present in the cytoplasm. These data again support the notion that SP-FLAG-Pex3p is targeted from the ER to peroxisomes depending on the presence of a cleavable pre-sequence.

DISCUSSION

The conserved peroxin Pex3p is required early in peroxisome membrane biogenesis, and in all organisms so far investigated deletion of PEX3 results in cells without peroxisomal structures (20–22, 25, 50). Here, we study the routing of this essential peroxin and present new evidence suggesting that Pex3p targets first to the ER before reaching the peroxisome.

In a first series of experiments (Figs. 2 and 3), we introduced an artificial N-glycosylation site into the N terminus of Pex3p and showed that this construct receives a single core N-linked oligosaccharide chain. This indicates that Pex3p is inserted into the ER membrane with its N terminus exposed to the ER lumen, supporting the model proposed by Höfeld (20) that Pex3p is a type I membrane protein (Nlumen,Ncytosol). N-Linked glycosylation of Pex3p also occurred at low expression levels, which suggests that the ER localization of the protein is not caused by overexpression. It is very unlikely that the added NH tag contains ER targeting information, since (i) it does not appear to contain a potential signal sequence when analyzed with the SP prediction program SignalP (46), and (ii) it does not induce ER targeting and/or N-glycosylation in fusions with other peroxins and peroxisomal matrix proteins (30). Thus, the data imply that Pex3p by itself contains topogenic information for sorting to the ER, in agreement with the observation that the 16 N-terminal amino acids of Pex3p can sort a reporter protein to the ER (21). Nevertheless, the results do not provide conclusive evidence for ER-to-peroxisome routing of Pex3p, in view of the fact that although about 50% of Pex3p is glycosylated, peroxisomes seem to harbor only the non-glycosylated form (data not shown). One way to explain this would be to assume that (artificially) N-glycosylated Pex3p is retained in the ER and that Pex3p can only reach the peroxisomes when it is not modified. However, since unglycosylated Pex3p is almost exclusively found in peroxisomes (data not shown), it cannot be ruled out that it never sees the ER and is directly sorted from the cytosol to peroxisomes.

3 A. Kräg, T. Voorn-Brouwer, M. van den Berg, and B. Distel, unpublished observations.
To obtain more information on Pex3p routing, we fused an ER signal peptide to its N terminus to send it deliberately to the ER and followed the pathway taken by the fusion protein. The introduction of a FLAG tag immediately after the SP allowed us to monitor whether the construct had indeed passed through the ER en route to its final location. These experiments demonstrate (i) that SP-Pex3p and SP-FLAG-Pex3p are functional proteins that can restore peroxisome formation in pex³/H9004 cells (Figs. 4 and 6, A, B, G, and H), (ii) that SP-FLAG-Pex3p is efficiently processed by the signal peptidase complex of the ER, irrespective of expression levels (Fig. 5), and (iii) that cleaved SP-FLAG-Pex3p is localized both in the ER and peroxisomes (Fig. 7B). Together, these results strongly suggest that Pex3p equipped with an ER signal peptide indeed routes via the ER and show that deliberate ER routing of this protein still results in the formation of functional peroxisomes in a pex³ strain. Functional complementation of the pex³ strain is strictly dependent on the presence of a cleavable signal peptide, as shown by the inability to induce peroxisome formation of the SP*-FLAG-Pex3p containing a mutation in the signal peptide recognition site (Fig. 6, A, B, and F).

Why the non-cleaved SP*-FLAG-Pex3p is unable to complement pex³ cells is unclear. The fusion protein is targeted to the ER as it, partially, colocalizes with the ER marker Kar2p (Fig. 7C), but it is not correctly processed (Fig. 5A). It is possible that the uncleaved, hydrophobic, signal peptide anchors SP*-FLAG-Pex3p in the ER membrane, preventing further sorting out of this compartment. Similar observations have been reported for human coagulation factor X, harboring a mutant signal peptide that resists cleavage by the signal peptidase (51). Our immunofluorescence suggests, however, that SP*-FLAG-Pex3p is also partially located in the cytosol (Fig. 7C). The cytosolic localization of SP*-FLAG-Pex3p could be the consequence of inefficient ER targeting or, more likely, may be the result of retrotranslocation of misfolded/ not properly processed Pex3p that had first been inserted into the ER. Irrespective of what causes SP*-FLAG-Pex3p to be non-functional,

![FIGURE 6. SP-FLAG-Pex3p can restore functional peroxisomes in pex³ cells. A, growth characteristics on oleate of pex³ cells expressing FLAG-Pex3p, SP-FLAG-Pex3p, or SP*-FLAG-Pex3p under control of the PEX3 promoter. 10-Fold serial dilutions were spotted onto plates containing oleate as sole carbon source. Untransformed wild type (WT) and pex³ cells served as positive and negative controls for growth, respectively. B, wild-type cells, pex³ cells, and pex³ cells expressing Met-FLAG-Pex3p, SP-FLAG-Pex3p, or SP*-FLAG-Pex3p under control of the PEX3 promoter were (co)transformed with a plasmid expressing GFP-SKL. Subcellular distribution of GFP-SKL in oleate-induced cells was visualized by fluorescence microscopy. Bar, 5 μm. C–H, electron microscopy analysis of wild type-cells (C) and pex³ cells expressing either Met-FLAG-Pex3p (D and E), SP*-FLAG-Pex3p (F), or SP-FLAG-Pex3p (G and H). Cryo-sections of cells grown on oleate were labeled with anti-thiolase antibodies, and immunogold particles were conjugated to protein A. P, peroxisome; M, mitochondrion; N, nucleus; arrowhead, membrane elements. Bar, 0.5 μm.](image-url)
these results further support the notion that only the processed, ER-targeted Pex3p is responsible for restoring peroxisome formation in pex3Δ cells. Even if there would be a small amount of non-cleaved SP-FLAG-Pex3p in the cell, unnoticed in our experimental set-up, such unprocessed SP-FLAG-Pex3p would most likely not be functional.

What do these experiments tell us about the routing of endogenous Pex3p? As discussed above, NH-Pex3p is N-glycosylated, indicating that Pex3p contains topogenic information that directs the protein to the ER. However, unlike proteins that are destined for the secretory pathway, Pex3p does not possess a classical signal sequence, and an alternative ER targeting signal must be used. South et al. (33) showed that inactivation of the ER translocation factors Sec61p or its homologue Ssh1p does not affect Pex3p-mediated peroxisome biogenesis. This indeed suggests that Pex3p insertion into the ER may not require a canonical signal peptide and occurs independently of the classic Sec61p/Ssh1p translocation machinery. Sec61p/Ssh1p-independent insertion into the ER membrane is not without precedent, since it has also been found for tail-anchored proteins (52, 53), which are characterized by a single transmembrane domain close to the short luminal C terminus and a large cytosolic N-terminal domain. Like Pex3p, they lack an N-terminal signal sequence and also appear to be inserted posttranslationally into the ER membrane. Given the resemblance of certain features of (the ER import of) tail-anchored proteins and Pex3p, it could be envisaged that they follow similar, Sec61p/Ssh1p-independent import routes.

Our observation that the processed SP-FLAG-Pex3p is found in peroxisomes indicates the presence of a functional ER-to-peroxisome pathway in yeast. The near wild-type growth rates on oleate and the normal import of PTS1- and PTS2-containing proteins of SP-FLAG-Pex3p-transformed pex3Δ cells indicate that ER-targeted Pex3p can efficiently restore the formation of functional peroxisomes. Assuming that Pex3p activity in these cells is the rate-limiting step in peroxisome formation, these results suggest that the ER-to-peroxisome pathway represents the route normally followed by endogenous Pex3p. This suggestion is supported by very recent results obtained from time-lapse studies in which endogenous levels of GFP-labeled Pex3p localized to the ER before redistributing into peroxisomes (54).

How Pex3p travels from the ER to the peroxisomes is currently unclear, however. It is unlikely to be mediated by COP-coated vesicles because inhibition of COPI- or COPII-mediated vesicular transport does not affect peroxisome biogenesis (31, 32). In support of this, there
is recent evidence in mouse dendritic cells for the existence of a route from the ER to peroxisomes that does not rely on the components needed for vesicle formation in the secretory pathway (29, 55). In these cells the peroxisomal membrane protein Pex3p seems to be present in specialized subdomains of the ER, the so-called lamellar extensions, which were essentially free of COP coats and ribosomes and contained only very small amounts of luminal ER markers. These lamellae appear to be continuous with a peroxisomal reticulum from which mature peroxisomes arise. Also, in plants there is evidence for the existence of a specialized subdomain of the ER through which some peroxisomal membrane proteins travel before reaching the peroxisome (36). Together, these data imply that ER-to-peroxisome maturation pathways, in which specialized ER structures participate, may indeed exist.

The specific role, biogenesis, and protein composition of these structures remains to be elucidated, but it could be hypothesized that Pex3p may be one of the key players in this process. Loss of Pex3p results in cells without peroxisomes and peroxisomal remnants, but peroxisomes are reformed after re-introduction of Pex3p expression. Because de novo synthesis of peroxisomes cannot occur, these observations could best be explained by Pex3p-mediated priming of an endomembrane, such as the ER, that could serve as a starting point for the development of new peroxisomes. Another protein involved in this process could be Pex16p, because cells lacking this peroxin also have a peroxisome-deficient phenotype (5, 56). Other data also suggest that Pex3p may indeed prime the formation of new peroxisomes at the ER. Faber et al. (35) showed that overexpression of a truncated, non-functional Pex3p in pex2Δ cells resulted in the production of (presumably) ER-derives vesicles and that these vesicles could develop in functional peroxisomes upon expression of full-length Pex3p. In addition, it was shown in Y. lipolytica that the formation of new peroxisomes in temperature-sensitive pex3 mutants occurred in close association with the ER (50). Finally, the mechanism of Pex3p trafficking to peroxisomes is essentially different from that used by most other proteins; Pex3p is the only protein so far identified that targets to peroxisomes independent of Pex19p (12) or any other peroxin.

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