Peroxisomal Membrane Proteins Insert into the Endoplasmic Reticulum

Adabella van der Zand, Ineke Braakman,* and Henk F. Tabak*

Cellular Protein Chemistry, Faculty of Science, Utrecht University, NL-3584 CH Utrecht, The Netherlands

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We show that a comprehensive set of 16 peroxisomal membrane proteins (PMPs) encompassing all types of membrane topologies first target to the endoplasmic reticulum (ER) in Saccharomyces cerevisiae. These PMPs insert into the ER membrane via the protein import complexes Sec61p and Get3p (for tail-anchored proteins). This trafficking pathway is representative for multiplying wild-type cells in which the peroxisome population needs to be maintained, as well as for mutant cells lacking peroxisomes in which new peroxisomes form after complementation with the wild-type version of the mutant gene. PMPs leave the ER in a Pex3p-Pex19p–dependent manner to end up in metabolically active peroxisomes. These results further extend the new concept that peroxisomes derive their basic framework (membrane and membrane proteins) from the ER and imply a new functional role for Pex3p and Pex19p.

INTRODUCTION

Peroxisomes belong to the basic repertoire of organelles in eukaryotic cells. They were first isolated by the group of De Duve, and initial studies focused on the enzymes they contained to understand their contribution to cellular metabolism (De Duve and Baudhuin, 1966). The enzyme content can vary depending on species, explaining why initial description led to different names: peroxisomes, glyoxysomes, and glycosomes. Now, it is clear that these organelles all belong to the same microbody family based on conserved features. For example, the ability to degrade fatty acids as well as the presence of conserved peroxisomal targeting signal (PTS) 1 and PTS2 on enzymes is widespread (Gould et al., 1987; Swinkels et al., 1992; Gabaldon et al., 2006; Schluter et al., 2007). Enzymes are imported from the cytosol into peroxisomes by a protein import machinery located in the peroxisomal membrane (importor) (Rachubinski et al., 1984). Particularly, this last feature formed the basis for the proposal that peroxisomes are autonomous organelles that multiply by growth and division (Lazarow and Fujiuki, 1985; Subramani, 1998).

New tools for investigating this concept became available when genetic screens were used to isolate mutants with disturbances in peroxisome function. It led to the discovery of Pex proteins with a function in peroxisome formation and maintenance (Erdmann et al., 1989; Tsukamoto et al., 1990; Elgersma et al., 1993; Wanders, 1999; Gould and Valle, 2000). Remarkable representatives were Pex3p and Pex19p, because cells harboring mutant versions of these genes have lost the complete peroxisome population. However, upon reintroduction of a wild-type PEX3 or PEX19 gene in such mutants, the peroxisome population is restored even after many generations of growth without peroxisomes (Höfled et al., 1991). The question arises: where do these new peroxisomes come from?

Already in the early days of peroxisome research there were hints that the endoplasmic reticulum (ER) might be involved. Electron microscopic observations indicated that peroxisomes are often found in close association with the ER (Novikoff and Novikoff, 1972), and pulse-chase experiments using germinating castor beans showed that peroxisomal proteins passed through the ER before they appeared in glyoxysomes (specialized peroxisomes) (Gonzalez and Beevers, 1976; Gonzalez, 1986). The Δpex3/Δpex19 mutants sparked a new interest in the relationship between ER and peroxisomes, and evidence for an important contribution of the ER to peroxisome formation was mounting.

Pulse-chase analysis in Yarrowia lipolytica showed that Pex2p and Pex16p appeared first in the ER, were glycosylated and then accumulated in peroxisomes (Titorenko and Rachubinski, 1998). The ER concept gained further momentum with the discovery, by immunoelectron microscopy in mouse dendritic cells, of Pex13p being present in specialized parts of the ER and in lamellar structures (peroxisomal precompartments) (Geuze et al., 2003). In three-dimensional reconstructions, these lamellae were shown to be in continuity with peroxisomes (Tabak et al., 2003). The nature and distribution of proteins over these various compartments suggested a developmental pathway starting in the ER and leading to mature peroxisomes. This was further elaborated in Saccharomyces cerevisiae whereby the formation of peroxisomes was followed using real-time fluorescence microscopy (Hoepfner et al., 2005). Newly synthesized yellow fluorescent protein (YFP)-tagged Pex3p appeared first in the ER and subsequently via preperoxisomal structures in peroxisomes. A similar trafficking route for Pex3p was also reported by others in yeast (Kragt et al., 2005; Tam et al., 2005; Haan et al., 2006; Motley and Hettema, 2007), plants (Karnik and Trelease, 2007), and mammalian cells (Kim et al., 2006).

This involvement of the ER could also explain the Δpex3/Δpex19 paradox mentioned above: the lost population of peroxisomes can be restocked through the contribution of the ER. But how extensive is this contribution? Here, we show that 16 peroxisomal membrane proteins (PMPs) dis-
playing all types of membrane topology entering the ER via the Sec63p translocon or the Get3 protein, attain their correct topology, and travel to peroxisomes. Thus, the basic framework for a functional peroxisome, membrane with PMPs, is delivered by the ER, making peroxisomes an intrinsic member of the endomembrane family.

MATERIALS AND METHODS
DNA Manipulations, Cloning Procedures, and Strain Constructions
Yeast strains used in this study are listed in Supplemental Table S1. Polymerase chain reaction (PCR)-based methods were used to construct gene deletion cassettes, cyan fluorescent protein (CFP)/YFP-fusion- or GAL1-fusion-cassette for transformations (Janke et al., 2004). Oligonucleotides (oligos) are listed in Supplemental Table S2. DNA of Escherichia coli plasmids pFA6-kanMX6, pFA6-HisMX6, pFA6-bLacZ (Wach et al., 1994), pFA6-NetMX (Goldstein and McCusker, 1999), pD13 and pD6 (Yeast Genetic Stock Center, University of Washington, Seattle, WA), and pFA6-KanMX6-PGAL1-CFP and a pFA6-His3MX6-PGAL1-GFP (Longtine et al., 1998) variant with the green fluorescent protein (GFP) exchanged for YFP served as template for preparative PCR reactions. Genomic integration of the corresponding construct was verified by analysis of PCR (Wach et al., 1994). Plasmid pSW17 containing CFP-PTS1 was constructed as described previously (Hoepfner et al., 2001). The GAL1-PEX3 integration plasmid (pAZU2) was constructed by excising the YFP open reading frame from pDIhs1 (Hoepfner et al., 2005) with XmaI and HindIII. The GAL1-PEX3-YFP integration plasmid (pTH2) was constructed as follows: YFP was amplified by PCR from pDH5 using oligonucleotides introducing flanking HindIII and XhoI sites. The fragment was cloned into the corresponding sites of pNBS27 (liang and Ferro-Novick, 1994), resulting in pTH1. PEX5 was amplified by PCR from the genomic DNA from strain FY1679 (Winston et al., 1995) using oligos that introduced HindIII sites. The fragment was cloned into the corresponding site of pTH1. The GAL1-PEX3-YFP integration plasmid (pYEW200) was constructed as follows: the GAL1 promoter was amplified by PCR from pA6a-kanMX6-PGAL1 using oligonucleotides introducing flanking EcoRI and SacI sites (Hoepfner et al., 2005). The fragment was cloned into the corresponding sites of pYiplac128 (Gietz and Sugino, 1988). An NH-tagged PEX3-YFP fragment from p20.19 (Erlanger et al., 1999) was subcloned into the corresponding sites of pNBS27. The resulting construct was cloned into the corresponding sites of pNBS28 (liang and Ferro-Novick, 1994), resulting in pTH5. PEX1 was amplified by PCR from the genomic DNA from strain FY1679 (Winston et al., 1995) using oligos that introduced BamH1 and HindIII sites. The fragment was cloned into the corresponding sites of pTH5.

Cellular Fractionation
AZY155 cells were grown in YPD at 30°C. Cells were harvested into spheroplasts as described previously (Franzusoff et al., 1991). To digest the cell wall, yeast lytic enzyme (derived from Anthrobotus lacteus, 82,800 U/g; ICN) was used at a final concentration of 1.5 U/1.0 OD600 cell units. All subsequent steps were performed in a cold room. Spheroplasts were harvested, resuspended in lysis buffer (20 mM HEPES, pH 7.4, 50 mM KAc, 100 mM sorbitol, 1 mM EDTA, 1 mM diethiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and homogenized by vortexing in presence of 0.5-mm glass beads (Biospec Products, Bartlesville, OK). Cell debris was sedimented at 10,000 × g for 10 min, and the remaining postnuclear supernatant was centrifuged at 200,000 × g for 20 min. The resulting membrane pellet was washed twice and resuspended in membrane storage buffer (MSB) (20 mM HEPES, pH 7.4, 50 mM KAc, 250 mM sorbitol, 1 mM DTT, and 1 mM PMSF). Five OD590 units of membranes were treated with 1 M NaCl in MSB for 30 min at 4°C or 125 mM Na2CO3, pH 11.5, for 30 min at 4°C. Membrane pellets were resolubilized by ultracentrifugation at 132,000 × g for 10 min in a TL120.1 rotor (Beckman Coulter, Fullerton, CA) and resuspended in equal volumes of MSB. Alternatively, resuspended membrane pellets were treated with protease. Typically, 200-μl reactions containing 5 OD590 units of membranes (in MSB without PMSF) were incubated with fresh 100 μg/ml proteinase K for 20 min at 30°C in presence or absence of 1% Triton X-100. Samples were cooled on ice for 5 min and 10 mM PMSF was added. Fractions were precipitated using methanol and chloroform (4:1). Equivalent amounts were analyzed on 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and analyzed by Western blot and enhanced chemiluminescence (ECL).

Buoyant Density Centrifugation
Yeast cells were grown in YPD, and 1000 OD600 units of AZY193 cells were harvested, spheroplasted, and postnuclear supernatants were derived after glass bead homogenization (see above). Postnuclear supernatants were layered on top of a 55% sucrose cushion made up in MSB. Gradients were centrifuged at 237,000 × g for 2 h at 4°C (SW41Ti rotor; Beckman Coulter). The recovery of membrane material was confirmed by Western blot with antibodies against Pex13p and Sec63p. The membrane fraction was collected and overlaid with a nonlinear (18–54%) sucrose step-gradient (12 ml) made up in MSB and spun to equilibrium for 16 h at 237,000 × g (SW41Ti rotor; Beckman Coulter). Twenty-two fractions (540 μl) were collected starting from the top of the gradient using a micropipette. Fractions were treated with 1 M NaCl and chloroform (4:1). Equivalent amounts were analyzed on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by Western blot and ECL.

Antibodies and Immunoblot Analysis
Mouse polyclonal anti-GFP antibody 1814460 was purchased from Roche Diagnostics. Antibodies against Hsp90 (Roepert et al., 1994), Pex13p (Egersma et al., 1996), Pex14p (Böttger et al., 2000), and Kar2p (Hettema et al., 1998) have been described previously. Anti sera against Sec61p, Sec62p, Sec63p, Nyv1p, and Sso1p were kindly donated by Dr. C. J. Stirling (University of Manchester, Manchester, United Kingdom) and antisera of Pex12p and Pex15p were kindly donated by Dr. Ralf Erdmann (University of Bochum, Bochum, Germany). Immunoreactive complexes were visualized using anti-rabbit, anti-mouse, or anti-sheep immunoglobulin G-coupled horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) in combination with the ECL system from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). The PVDF membranes were exposed to Kodak MR BioMax films (PerkinElmer Life and Analytical Sciences, Boston, MA) and recorded under ultraviolet light image processing using Photoshop (Adobe Systems, Mountain View, CA).

Fluorescence Pulse-Chase Assay
Strains for fluorescence pulse-chase analysis were grown overnight in 10 ml of YP-2% raffinose medium to early log phase at 30°C. The cells were spun down and resuspended in an equal volume of YP-2% galactose for 5-30 min and then washed in phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) and taken up in 20 ml of YP-2% glucose. For microscopy, 500-1000 μl of cells was washed with PBS, and 1 μl was spread on a slide overlaid with a coverslip and used immediately for microscopy.

Microscopy Setup and Image Acquisition
Live cells were taken using an Axiosvert 200M fluorescent microscope (Carl Zeiss, Jena, Germany) fitted with a Plan-Neofluar 100×/1.3 numerical aperture Ph3 × 0.17 oil immersion objective lens (Carl Zeiss), a Xenon XBO 75W/2 illuminator, and a CoolSNAP HQ monochrome camera (Photometrics, Tucson, AZ). We acquired one phase contrast image and three z-planes spaced by 0.8 μm. In each z-plane we acquired one YFP and one cyan fluorescent protein (CFP) image with up to 5-s exposure times and 100% fluorescence transmission without binning. Separately, the YFP and CFP images of the stack were averaged into a single projection with a maximum intensity algorithm (stack arithmetic: maximum command of MetaMorph, version 5; Molecular Devices, Sunnyvale, CA). The YFP/C FP planes were scaled and converted to 8-bit images. The drastic difference in YFP signal intensity during the galactose induction time courses made it necessary to scale individual time points of a time course differently (as apparent by the intensity differences of the background). The phase-contrast, YFP, and CFP images were overlaid with default color balance settings assigning false-color look-up tables using the Overlay Image command of MetaMorph from the main taskbar. Blue color was applied to the phase-contrast picture, green for the YFP channel, and red for the CFP channel. Images were cropped and assembled into figures using Photoshop CS4 (Adobe Systems). To reduce background intensities, we used the Levels command, and to enhance brightness we applied the Curves command in Photoshop to the whole image by using the RGB channel only. The original images we acquired at a resolution of 72 dpi (1392 × 1046 pixels) and were resized to 300 dpi using Photoshop by reducing the physical size of the original image.

N-[3-Triethylammoniumpropyl]-4-[p-diethylaminophenyl]-hexatrienyl]pyridinium Dibromide (FM4-64) Staining
FM4-64 staining of live yeast cells was performed as described previously (Vida and Emr, 1995).

RESULTS
Trafficking of PMPs in Wild-Type Cells
A group of ~22 PMPs are known to have a function in peroxisome formation and maintenance (Marelli et al., 2004). We studied the biogenesis of Pex2p, Pex8p, Pex11p, Pex11p, Pex13p, Pex14p, Pex15p, and Ant1p in wild-type cells by using fluorescence microscopy to investigate whether they
followed the same trafficking route as Pex3p: inserting first in the ER before ending up in peroxisomes. Pex2p, Pex10p, Pex11p, and Pex13p are polytopic PMPs with two membrane spans and N and C termini facing the cytosol (Okumoto et al., 1998; Girzalsky et al., 1999; Harano et al., 1999; Anton et al., 2000), whereas Ant1p (PMP47), which encodes an ATP/ADP translocator, has six membrane spans (McCaman et al., 1994). Pex14p is a type I integral membrane protein (Brocard et al., 1997) and Pex15p a C-terminally anchored protein (Elgersma et al., 1997). Finally, Pex8p, which associates peripherally with the luminal face of the peroxisomal membrane (Agne et al., 2003).

PMPs were C-terminally tagged with YFP, put under control of the GAL1 promoter and integrated into the yeast genome. This allowed us to produce a limited wave of PMP-YFP synthesis comparable with endogenous expression levels. Before induction, cells were grown in raffinose, a medium in which the promoter is on stand-by. PMP synthesis was started by growth in galactose and stopped by repression of the promoter by adding glucose. Using this protocol, we showed before that the amount of Pex3p-YFP that is synthesized corresponds well with the endogenous level and that overproduction is prevented (Hoepfner et al., 2005). Here, we show by Western blot analysis that the amount of Pex13p-YFP produced after 15-min galactose induction was less than the level of endogenous Pex13p present (Figure 1A, lanes 4–6). We present the trafficking route of Pex13p as an example using fluorescence pulse-chase experiments. Before induction, no YFP-tagged Pex13p (Figure 1, B and C) was detectable, yet the multipunctate CFP-PTS1 signal indicated the presence of functional peroxisomes (Figure 1C). After induction, Pex13p-YFP first appeared in ER-localized foci that did not colocalize with peroxisomes (Figure 1, B and C). Only later in time, after 300 min, the fluorescent signal of Pex13p-YFP started to coincide with that of peroxisomes (Figure 1C). We found the same route from ER to peroxisomes for other PMPs (Supplemental Figure S1, A–C). Surprisingly, also Pex8p, which needs to translocate across the membrane to reach the lumen, targeted to the ER first (Supplemental Figure S1C). These results demonstrate that in wild-type cells harboring functional CFP-PTS1–containing peroxisomes, the ER is the transit compartment in the trafficking of PMPs to peroxisomes.
To confirm the fluorescence microscopy results, we studied Membrane Topology of PMPs in the ER function and membrane topology. Other PMPs (Supplemental Figure S2, A–D), irrespective of and Pex14p-YFP marked puncta colocalized with CFP-PTS1 import starts at 120 min with this is that in the corresponding strain expressing (Figure 2, 90–120 min) followed by multiple dots, which intensity, Pex14p-YFP appeared at distinct puncta on the ER 60 min). Later, when the fluorescence signal increased in YFP started to colocalize with the perinuclear ER (Figure 2, induction (Figure 2, 60 and 90 min), the additional Pex14p-YFP of endogenously expressed Pex14p-YFP colocalized with the ER promoter. As a result, these strains have no peroxisomes when grown on raffinose. To induce peroxisome formation in these cells and study the behavior of PMPs, cells were exposed to galactose for 30 min to transiently express Pex3p, followed by glucose to repress the promoter again (Hoepfner et al., 2005). We performed the fluorescence pulse-chase assay on the following proteins: Pex1p, Pex2p, Pex3p, Pex4p, Pex6p, Pex8p, Pex10p, Pex11p, Pex12p, Pex13p, Pex14p, Pex15p, Pex19p, Pex25p, Pex27p, and Ant1p. We show results representative for this group.

Pex14p is shown here as an example. Before expression of PEX3 from the GAL1 promoter, weak punctate fluorescence of endogenously expressed Pex14p-YFP colocalized with the ER marker Sec63p-CFP (Figure 2, 0 min). Sixty minutes after induction (Figure 2, 60 and 90 min), the additional Pex14p-YFP started to colocalize with the perinuclear ER (Figure 2, 60 min). Later, when the fluorescence signal increased in intensity, Pex14p-YFP appeared at distinct puncta on the ER (Figure 2, 90–120 min) followed by multiple dots, which became independent of the ER (Figure 2, 300 min). Consistent with this is that in the corresponding strain expressing the peroxisomal marker CFP-PTS1 import starts at 120 min and Pex14p-YFP marked puncta colocalized with CFP-PTS1 (Figure 2, bottom). This description is representative for all other PMPs (Supplemental Figure S2, A–D), irrespective of function and membrane topology.

Membrane Topology of PMPs in the ER

To confirm the fluorescence microscopy results, we studied the membrane topology of PMPs using biochemical techniques. PMPs were expressed from their endogenous promoters in PEX19-deleted cells (the Δpex19 phenotype is identical to the Δpex3 phenotype: both lack peroxisomes; Hettema et al., 2000). In both mutant cells, PMP-YFP proteins accumulate either in the perinuclear ER or in an ER-localized dot (specialized ER) (Supplemental Figure S3). Homogenates of Δpex19 cells were enriched in an organellar fraction, which was analyzed by buoyant density centrifugation in nonlinear sucrose gradients. Immunoblot analysis of the fractions demonstrated that all PMPs equilibrated at the same density (1.192 g/cm³) coinciding with the first ER peak, indicated by the ER markers Kar2p and Sec63p (Figure 3A). The vacuolar membrane protein Nyp1p, the plasma membrane protein Sso1p and mitochondrial Hsp60p were found to peak at lighter densities. This demonstrates that PMPs localize to the ER, confirming our fluorescence microscopic data in Figures 1 and 2.

We next studied the topology of three different PMPs that form part of the peroxisomal importomer complex: Pex8p, Pex13p, and Pex14p when they are present in the ER (in Δpex19 cells). All three proteins fractionated completely into a crude organelar pellet (Figure 3B, lane P), also upon disruption of protein–protein interactions by treating the membrane fractions with 1 M NaCl (Figure 3B, lane NP). However, after treatment at alkaline pH, which converts vesicles into membrane sheets, Pex8p-YFP, like Kar2p, fractionated into the supernatant fraction consistent with it being a luminal protein (Figure 3B, lane CS). Pex13p and Pex14p, as integral membrane proteins, colo nated together with Sec63p-CFP in the pellet fraction (Figure 3B, lane CP). The crude organellar pellet fraction was also treated with proteinase K. Pex8p-YFP and Kar2p remained fully protected against protease and only degraded after addition of detergent (Figure 3B, lanes PK and PK + Triton X-100 [TX100]). The cytosolically faced SH3 epitope of Pex13p was degraded upon proteinase K treatment, as well as Pex14p. The experiment was controlled using Kar2p as luminal and

![Figure 2. Pex14p trafficking during peroxisome biogenesis in Δpex3 cells.](image-url)

**Figure 2.** Pex14p trafficking during peroxisome biogenesis in Δpex3 cells. Fluorescence pulse-chase experiment in peroxisome-deficient cells. Peroxisome formation is started by short exposure to galactose (30 min) to induce synthesis of Pex3p. Before expression of PEX3, most cells displayed no Pex14p-YFP (AZY191). However, a small but significant population of Pex14p-YFP-marked cells showed punctate staining that colocalized with the ER marker Sec63p-CFP (red). Sixty minutes after limited expression of PEX3, all cells expressing PEX14-YFP showed a weak signal that colocalized with the perinuclear ER (red). At a later stage, Pex14p-YFP started to concentrate into dots frequently localized on or at the periphery of the ER (90 min). At 120 min, Pex14p-YFP localized exclusively to foci mostly no longer overlapping with the Sec63p-CFP signal. At 300 min, the peroxisomal population in the cell has been restored and up to five individual Pex14p-YFP dots per cell were visible. The experiment was also performed in cells expressing CFP-PTS1 to label peroxisomes (AZY192). At the start of the experiment, the exclusively cytosolic CFP-PTS1 demonstrated the absence of importcompetent peroxisomes (0 min). Despite detectable Pex14p-YFP signal 60–90 min after induction, CFP-PTS1 was uniformly cytosolic, indicating that no import competent peroxisomes had yet been formed. The formation of ER-independent dot-like Pex14p-YFP structures at 120–180 min was accompanied by onset of CFP-PTS1 import, discernible by accumulation of CFP-PTS signal into dots (120 min). At 300 min, the cytosolic background of CFP-PTS1 dropped below detection level and the protein was exclusively peroxisomal, indicating that mature, import-competent peroxisomes have been formed. Bar, 2 μm.
Sec63p-CFP as membrane marker of the ER (Sec63p-CFP is protease sensitive because we monitored the behavior of the CFP tail, which extends into the cytosol). Together, these experiments illustrate that PMPs in \( \text{H9004} \) \( \text{pex19} \) cells are present in the ER and attain their properly folded state and polarity in the membrane. An implication of these findings is that Pex3p and Pex19p are not essential for the recognition and membrane insertion of newly synthesized PMPs into the peroxisomal membrane but are necessary for exit from the ER.

**Insertion of PMPs via the ER-specific Translocons**

We next studied whether the Sec61p complex supported the entry of PMPs into the ER, because the majority of proteins entering the ER do so via the essential Sec61p complex. We therefore gradually depleted cells of their capacity to import proteins into the ER. Wild-type cells were used to replace both endogenous promoters of \( \text{SEC62} \) and \( \text{SEC63} \) with the repressible \( \text{MET3} \) promoter (Young et al., 2001) and chromosomally tagged \( \text{PEX8-YFP} \) (AZY250) or \( \text{PEX13-YFP} \) (AZY289) were used to test the contribution of the Sec61p translocon to PMP insertion. Unrepressed (0 h) cells expressing \( \text{SEC62} \) and \( \text{SEC63} \) contained multiple Pex8p-YFP or Pex13p-YFP puncta representing peroxisomes. Six to 8 h after addition of 2 mM methionine (Met), both Pex8p- and Pex13p-YFP started to accumulate diffusely in the cytosol, and the number of YFP fluorescent puncta decreased compared with untreated cells. Bar, 2 \( \mu \)m.

This results in depletion of the essential Sec proteins and decreases the protein import capacity via both co- and post-translational translocation into the ER (Young et al., 2001). For fluorescence microscopy, cells were grown in the presence or absence of methionine, and images were acquired at different time points. At 0 min, both Pex8p-YFP and Pex13p-YFP were present in several fluorescent foci, demonstrating...
their specific localization to peroxisomes (Figure 4A). Six to 8 h after addition of methionine, part of the YFP fluorescence became diffuse, indicating that both Pex8p-YFP and Pex13p-YFP were accumulating in the cytosol (Figure 4A). During this experiment, we also monitored the amount of essential Sec proteins by using Western blotting. Cells were grown in the presence or absence of methionine, and total cell extracts were prepared. Seven hours after shutoff, the levels of Sec62p and Sec63p became significantly reduced but not completely depleted, whereas the levels of Sec61p remained unaffected (Figure 4B). We confirmed these results using biochemical fractionation combined with Western blotting. To avoid the problem of the possibility of leakage from peroxisomes contaminating the supernatant fraction, we performed the cellular fractionation experiments in cells that lack peroxisomes, and we blotted for various PMPs. As a positive control for ER translocation capacity we used Kar2p, which uses both co- and posttranslational translocation routes (Ng et al., 1996). Like the fluorescence microscopy results, the translocation defect emerged 7 h after shutoff, as indicated by the appearance of the slower migrating precursor form of Kar2p in addition to the mature luminal form (Figure 4C). (Kar2p is not glycosylated and the lower mobility of the precursor form represents the presence of an uncleaved signal sequence.) The majority of Kar2p was processed (Figure 4C), indicating that despite the decrease in import the ER remained fully stocked with resident proteins (Figure 4B), thus minimizing the risk of indirect effects on PMP import into the ER.

Pex8p-YFP, Pex13p, and Pex14p were expressed from their corresponding endogenous promoters and codelivered in the pellet fraction in cells without methionine repression (Figure 4C, lane P, 0 h). Yet, 7 h after Sec62p and Sec63p depletion, PMPs showed a translocation defect reflected in a significant pool of untranslocated, soluble protein (Figure 4C, lane S, 7 h). To verify the specificity of the Sec62p/Sec63p depletion, we studied the behavior of the tail-anchored protein Nyv1p, a vacuolar vesicle-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE). Tail-anchored proteins translocate independently of the Sec61p translocon into the ER (Steel et al., 2002). Indeed, Nyv1p was stably integrated under all conditions tested, and there was no evidence of a soluble pool of Nyv1p (Figure 4C). In addition, this control indicates that the ER remains functional during the depletion period. Together, the Western blot data and microscopy results shown for several topologically distinct PMPs suggest the involvement of the general protein import machinery (the Sec61p complex) for PMP import into the ER.

Recently, a dedicated protein translocation machinery has been identified that is specific for delivering and inserting tail-anchored proteins into the ER membrane. This novel ATP-dependent pathway requires the contribution of the cytosolic protein Asna-1/TRC40 in mammalian cells (Stefanovic and Hedge, 2007) or Get3p in yeast (Schuldiner et al., 2008). Because peroxisomal Pex15p is a tail-anchored protein (Elgersma et al., 1997), which was shown to interact with Get3p via its transmembrane domain (Schuldiner et al., 2008), we tested the effect of deleting GET3 on ER targeting of Pex15p in a fluorescent pulse chase. Wild-type and Δget3 cells were used in which the chromosomal copy of PEX15 was replaced for YFP-PEX15 (15 min galactose treatment), YFP fluorescence (green) colocalized with CFP-PTS1-labeled peroxisomes (red) in wild-type cells. In Δget3 cells, CFP-PTS1 was predominantly cytosolic before induction. CFP-PTS1 import was rescued by limited expression of YFP-PEX15. However, the YFP-Pex15p signal itself remained largely undetectable and failed to reach peroxisomes in the Δget3 cells. Bar, 2 μm.
block imposed by deleting GET3 is apparently not tight enough to prevent insertion of YFP-Pex15p and prevent maintenance of some functional peroxisomes. Nonetheless, these results demonstrate that deletion of GET3 has a profound effect on the kinetics of peroxisomal delivery of Pex15p and formation of peroxisomes, indicating that Pex15p, too, is taken up by an ER-specific protein import machinery. Our data suggest that when import of Pex15p into the ER becomes rate limiting (as in Δget3 cells), it cannot bypass the ER and insert into peroxisomes directly, because peroxisomally localized YFP-Pex15p never recovered fully in Δget3 cells. Probably, the pool of untranslocated YFP-Pex15p was quickly degraded in the cytosol.

To confirm that the phenotype of the GET3 deletion is specific to ER-targeted tail-anchored proteins only, we performed fluorescence pulse-chase analyses in Δget3 cells of similar genetic design as described above, with the vacuolar tail-anchored protein Nyv1p and with the non-tail-anchored (polytopic) PMP Pex13p. YFP-Nyv1p accumulated in the cytosol in Δget3 cells compared with the wild-type control (Supplemental Figure S4A, 120 min). Insufficient Nyv1p targeting to the vacuole leads to a vacuolar fusion defect, which can be visualized by allowing cells to take up the styryl dye FM4-64. Wild-type cells indeed showed fragmented vacuoles before expression of YFP-NYV1 from the GAL1 promoter (Supplemental Figure S4A, 0 min). After 30 min of galactose induced synthesis of YFP-Nyv1p, wild-type cells recovered and showed large fused vacuoles, whereas in the GET3-deleted strain vacuoles remained smaller and more fragmented (Supplemental Figure S4A, 120 min). In contrast, the nontail-anchored protein Pex13p-YFP appeared in CFP-PTS1-labeled peroxisomes in Δget3 cells (Supplemental Figure S4B, 120 min), identical to its localization in wild-type cells. The only difference we observed was a difference in the number of peroxisomes between wild-type and Δget3 cells, probably due to defective insertion of Pex15p because deletion of PEX15 stalls peroxisome development.

In accordance with the data published in Schuldiner et al. (2008), our results support the notion that Get3p contributes to insertion of tail-anchored proteins of different organelar destination into the ER and that Pex15p makes use of this pathway.

DISCUSSION

Import of PMPs into the ER

A contribution of the ER to peroxisome biogenesis was demonstrated recently, as proof of principle, by way of trafficking of the peroxisomal membrane protein Pex3p in various yeast (Hoepfner et al., 2005; Kräg et al., 2005; Tam et al., 2005; Haan et al., 2006) and Pex16p in mammalian and plant cells (Kim et al., 2006; Karnik and Trelfa, 2007). The question remained how extensive this involvement of the ER is and how this new concept impacts on current ideas about peroxisome formation and maintenance.

We have studied the biogenesis of a representative set of 16 peroxisomal membrane proteins differing in function and membrane topology. We showed by fluorescence pulse-chase analysis that all these proteins first target to the ER before arriving in peroxisomes. We used two genetically different contexts: wild-type cells, where peroxisomes are already present and a Δpex3 or a Δpex19 mutant in which the full complement of peroxisomes needs to be restored. Biochemical fractionation combined with equilibrium density gradient analysis confirmed that PMPs locate first to the ER. Differential carbonate extraction and protease protection assays demonstrated that the proteins translocate properly into the ER and attain the same topology as described when they are present in the peroxisomal membrane. Extrapolating from this representative set of proteins, we conclude that peroxisomal membrane proteins in S. cerevisiae as a rule enter the ER. They subsequently leave the ER in preperoxisomal structures at distinct exit sites. The precompartments are uncoupled from the ER and become competent to import PTS1/PTS2-containing enzymes, forming a new metabolically active peroxisome. Subsequent fission events supported by the dynamins Vps1p and/or Dnm1p keep the number of peroxisomes constant in growing cells (Hoepfner et al., 2001; Kuravi et al., 2006; Motley and Hettema, 2007; Motley et al., 2008). Because peroxisomal fission cannot go on indefinitely, peroxisome numbers can only be maintained through constant supply of ER-derived preperoxisomal structures.

This view is not commonly shared, and authors state that the ER-mediated pathway only comes into play when peroxisomes need to be reformed in mutant cells (Motley and Hettema, 2007). We consider the notion that two distinct mechanisms exist for the formation of peroxisomes, one mechanism in wild-type cells and the other mechanism in mutant cells, unlikely for the following reasons. First, we have experimentally shown here that one and the same principle for peroxisome formation is followed in wild-type and mutant cells. Second, why would single pex mutants be found if a separate backup mechanism would exist. Third, this so-called backup system will be called into action rarely and only when a mutation in a particular PEX gene occurs that is subsequently restored or compensated for in some other way, again a very rare event. When a biological process is almost never called into operation, it is nearly impossible to keep it functional. The most plausible hypothesis therefore is that one and the same process of peroxisome formation operates in wild-type as well as in mutant cells.

Involvement of ER Protein Import Complexes

The topological variety of PMPs requires the cooperation of different ER-specific translocons. We showed that the general-purpose ER import complex, the Sec61p translocon, is involved. Based on this proof of principle, we consider it likely that other PMPs follow the same route. Application of the SignalP 3.0 algorithm (Bendtsen et al., 2004) did not reveal probable signal peptide or signal anchor sequences, nor did we observe telltale marks of protein processing. Thus, the signal(s) that target PMPs to the ER remain to be found.

An exception is the group of tail-anchored proteins, such as Pex15p, which are not inserted via the Sec61p complex. We found that deletion of the tail-anchor protein-specific chaperone encoding GET3 caused a severe delay in the ER insertion of Pex15p. Surprisingly, the knockout mutant of GET3 remained viable (Schuldiner et al., 2008). This explains why some peroxisomes are still present in the Δget3 mutant.

Implications

Our findings that protein import complexes of the ER take care of uptake of PMPs into this compartment disagree with the view on insertion of PMPs into membranes. A current model implicates Pex3p and Pex19p in the direct insertion of peroxisomal proteins from the cytosol into the peroxisomal membrane (Fang et al., 2004; Jones et al., 2004; Fujiki et al., 2006; Matsuozono and Fujiki, 2006). It proposes that Pex19p acts as a chaperone (Jones et al., 2004) for newly synthesized PMPs, binding them at a consensus amino acid sequence (mPTS) (Rottensteiner et al., 2004) for delivery to the peroxisomal membrane. Interaction between Pex19p and Pex3p
then would lead to the insertion of PMPs into peroxisomal membranes (Fang et al., 2004). Our data argue instead for a role of Pex3p and Pex19p in ER budding of the peroxisomal precompartment from the ER, whereby the mPTS may provide a signature for the ER to sort peroxisome-specific cargo from ER resident proteins, to enable specific exit from the ER at distinct sites.

Here, we illustrate that the ER forms an obligate requirement to sustain peroxisomal maintenance in multiplying cells. It is therefore legitimate to view peroxisomes as a specialized subcompartment of the endoplasmic reticulum system.

Our results confirm new ideas on the evolutionary past of these organelles. Phylogenetic analysis of the peroxisomal proteome showed that the common core of the microbody family consists of Pex proteins and proteins involved in fatty acid metabolism (Girzalsky et al., 2006; Schlüter et al., 2007).

Moreover, some of the oldest Pex proteins are homologous to proteins of the endoplasmic reticulum associated degradation pathway. It all fits well into a concept in which peroxisomes developed from the protoendomembrane system in an early pre-eukaryote (Tabak et al., 2006; Cavalier-Smith, 2009).

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