Inactivation of the endoplasmic reticulum protein translocation factor, Sec61p, or its homolog, Ssh1p, does not affect peroxisome biogenesis

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Peroxisomes are single membrane-bound organelles present in virtually all eukaryotes. These organelles participate in several important metabolic processes, and defects in peroxisome function and biogenesis are a significant contributor to human disease. Several models propose that peroxisomes arise from the endoplasmic reticulum (ER) in a process that involves the translocation of ''group I'' peroxisomal membrane proteins into the ER, the exit of these group I peroxisomal membrane proteins from the ER by vesicle budding, and the formation of nascent peroxisomes from vesicles containing the group I peroxisomal membrane proteins. A central prediction of these models is that the formation of nascent peroxisomes requires protein translocation into the ER. Sec61p is an essential component of the ER translocon, and we show here that loss of Sec61p activity has no effect on peroxisome biogenesis. In addition, loss of the *SEC61*-related gene, *SSH1*, also has no effect on peroxisome biogenesis. Although some proteins may enter the ER independently of Sec61p or Ssh1p, none are known, leading us to propose that peroxisome biogenesis may not require protein import into the ER, and by extension, transfer of proteins from the ER to the peroxisome.

Peroxisomes are single membrane-bound organelles present in virtually all eukaryotes (1). Peroxisomes lack nucleic acids and import all of their proteins and virtually all of their membrane lipids. Peroxisomal matrix enzymes are synthesized on cytosolic polyribosomes and are imported posttranslationally into the peroxisome (1, 2); integral peroxisomal membrane proteins apparently also use this biogenic route (3–5). These and other observations have supported the hypothesis, originally proposed by Lazarow and Fujiki (1), that peroxisomes arise by growth and division of preexisting peroxisomes.

In humans, disorders of peroxisome biogenesis cause the lethal inherited disorders Zellweger syndrome and rhizomelic chondrodysplasia punctata, and their clinical variants (6). The *PEX* genes that are mutated in these patients are evolutionarily conserved, and yeast mutants (*pex*) lacking homologs of these genes are also defective in peroxisome biogenesis (7). Most patients with Zellweger syndrome and yeast *pex* mutants contain numerous peroxisomes and import peroxisomal membrane proteins (PMPs), but a few lack detectable peroxisome membranes (8, 9). Inactivating mutations in the human or yeast *PEX3* gene cause precisely this phenotype, indicating that Pex3p plays an essential role in the formation of the peroxisome membrane (9, 10). In these mutants, all known PMPs are either rapidly degraded without insertion into cellular membranes, or are mislocalized to the mitochondria. Although it is formally possible that these cells may contain undetectable preperoxisome membranes, the aberrant fates of so many PMPs in these cells indicate that even if such structures do exist, they are fundamentally distinct from true peroxisomes.

By introducing the normal copy of the affected gene into mutants that lack detectable peroxisomes, several investigators have found that peroxisomes can, in fact, be synthesized in the absence of preexisting peroxisomes (9–12). The origin of these peroxisomes is currently a matter of debate (6–8, 13–18). The endoplasmic reticulum (ER) is an attractive candidate for the origin of these membranes because the ER is the direct or indirect progenitor of so many other organelles. Accordingly, several models of peroxisome biogenesis have proposed that peroxisomes arise by vesicle budding from the ER and that a subset of PMPs, termed the "group I" PMPs, are translocated first into the ER and mediate vesicle transport between the ER and the peroxisome (13–18). Here we tested the hypothesis that peroxisome biogenesis requires protein import into the ER. Working with the yeast *Saccharomyces cerevisiae*, we find that peroxisome biogenesis is unaffected in a *sec61* mutant that is defective in co- and posttranslational import of both luminal and membrane proteins into the ER, as well as in cells that lack the *SEC61* homolog, *SSH1*.

Methods

Plasmids, Strains, and Culture. The *LEU2*-based plasmid p*sec61-11* was a generous gift from R. Schekman (University of California, Berkeley). The plasmid p*PGK1*-GFP-PTS1 contains the GFP-PTS1 ORF (19) downstream of the *S. cerevisiae PGK1* promoter in the *URA3*, cen/ars shuttle vector pRS316 (20). The plasmid p*GAL1-PEX3* contains the *S. cerevisiae PEX3* gene downstream of the *GAL1* promoter in the *TRP1*, cen/ars shuttle vector pRS314 (20). All strains were based on BY4733 (*MAT***a**, *his3*Δ*200*, *leu2*Δ*0*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*; ref. 21) and were grown in standard yeast media (22). SY24 [*MAT***a**, *his3*Δ*200*, *leu2*Δ*0*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*, *pex11*Δ::*kanMX*, *pex3*Δ::*MET15*, *pPGK-GFP-PTS1 (URA3)*], SY25 [*MAT***a**, *his3*Δ*200*, *leu2*Δ*0*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*, *pex11*Δ::*kanMX*, *pex3*Δ::*MET15*, *pPGK-GFP-PTS1 (URA3)*, *pGAL1-PEX3 (TRP1)*], SY36 [*MAT***a**, *his3*Δ*200*, *leu2*Δ*0*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*, *pex11*Δ::*kanMX*, *pex3*Δ::*MET15*, *sec61*Δ::*HIS3*, *pPGK-GFP-PTS1 (URA3)*, *pGAL1-PEX3 (TRP1)*, *psec61–11 (LEU2)*], and SY37 [*MAT***a**, *his3*Δ*200*, *leu2*Δ*0*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*, *pex11*Δ::*kanMX*, *pex3*Δ::*MET15*, *ssh1*Δ::*HIS3*, *pPGK-GFP-PTS1 (URA3)*, *pGAL1-PEX3 (TRP1)*] were generated by PCR-mediated gene disruption and standard transformation techniques (21). Yeast were grown in either minimal (S) medium [1.7 g/liter yeast nitrogen base without amino acids, uracil, or adenine (Difco)/5 g/liter $(NH_4)_2SO_4$] containing either 2% glucose (SD) or 2% galactose (Sgal), supplemented with amino acids, uracil and adenine, as required by strain auxotrophies and plasmid selections (22).

Pulse Labeling and Immunoprecipitations. Cells were labeled with [^{35}S]methionine for 10 min and lysed, and the lysates were

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Abbreviations: ER, endoplasmic reticulum; PMPs, peroxisomal membrane proteins; CPY, carboxypeptidase Y; GFP, green fluorescent protein; PTS1, peroxisomal targeting signal type 1.

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processed for immunoprecipitation as described (23). Immunoprecipitations were performed with anti-carboxypeptidase Y (CPY) antibodies kindly provided by C. Sterling (University of Manchester, U.K.). The immunoprecipitates were separated by SDS/PAGE, and labeled proteins were detected by fluorography.

Microscopy. For confocal fluorescence microscopy and phase-contrast microscopy, cells were affixed to poly-L-lysine-coated coverglasses and observed with use of a Noran confocal microscope (Noran Instruments, Middleton, WI). For immunoelectron microscopy, cells were harvested, converted to spheroplasts, fixed, dehydrated, and embedded in Unicryl essentially as described (24, 25). Ultrathin sections were cut, blocked in 4% BSA in Tris-buffered saline (20 mM Tris·HCl, pH 7.4/150 mM NaCl/0.1% BSA), and incubated with either rabbit anti-green fluorescent protein (GFP) antibodies (Abcam Ltd., Cambridge, U.K.) alone or with both mouse anti-GFP antibodies (Santa Cruz Biotechnology) and rabbit anti-yeast thiolase antibodies (23). After extensive washes in Tris-buffered saline, the samples were incubated with 18-nm colloidal gold particles bound to donkey anti-rabbit antibodies or both 18-nm colloidal gold particles bound to donkey anti-rabbit antibodies and 6-nm colloidal gold particles bound to donkey anti-mouse antibodies (Jackson ImmunoResearch). The samples were again washed in Tris-buffered saline and then contrasted with uranyl acetate and lead citrate (25). Images were obtained by using a Philips CM120 electron microscope (FEI Company, Hillsboro, OR).

Results

Before testing the role of ER protein translocation in peroxisome biogenesis we first established a system for detecting and regulating peroxisome synthesis in yeast cells that lack detectable peroxisomes. The *S. cerevisiae* strain BY4733 (21) was modified so that it lacks the *PEX3* gene and constitutively expresses GFP-PTS1 (19), a fusion between GFP and the type I peroxisomal targeting signal [PTS1 (26)], creating the strain SY24. GFP-PTS1 serves as a fluorescent marker of functional peroxisomes, is imported into the peroxisome matrix of wild-type strains, and accumulates in the cytoplasm of *pex3* mutants (19, 27). The plasmid *pGAL1-PEX3* expresses *PEX3* from the glucose-repressible, galactose-inducible *GAL1* promoter and was introduced into SY24 cells, generating the strain SY25.

SY24 and SY25 cells were grown in minimal glucose (SD) or minimal galactose (Sgal) media, and the distribution of GFP-PTS1 was used to assess the presence or absence of functional peroxisomes. GFP-PTS1 was cytoplasmically localized in 100% of SY24 cells, regardless of whether they were grown on glucose or galactose. When grown in minimal glucose medium almost all SY25 cells showed a cytosolic distribution of GFP-PTS1 (Fig. 1*a*), although we could detect some punctate GFP-PTS1 in ≈10% of these cells, presumably because of low and variable transcription from the *GAL1* promoter even during growth on glucose (28). In contrast, when SY25 cells were transferred to galactose medium, which induces *PEX3* gene expression, GFP-PTS1 was imported into peroxisomes (Fig. 1*b*). Time-course experiments established that SY25 cells required ≈20 h incubation in Sgal medium before significant rescue was detected (Fig. 1*c*). This period is similar to the amount of time required for rescue of *PEX3*-deficient human cells after introduction of a functional *PEX3* cDNA expression vector (10). The lack of complete rescue may reflect several factors, including the inhibitory effects of *PEX3* overexpression (29, 30) and plasmid loss.

Our ability to control peroxisome biogenesis in SY25 cells by a simple switch of growth medium suggested that we could use this system to assess the role of ER protein translocation in peroxisome biogenesis. *SEC61* is required for protein translocation across the ER membrane (31–33), and numerous conditional alleles of *S. cerevisiae SEC61* have been characterized in regard to their protein translocation defect (34–37). Of the 11 conditional *sec61* alleles examined by Pilon *et al.* (37), the cold-sensitive *sec61-11* mutant displays the earliest and most severe block in protein translocation. In *sec61* deletion strains that carry the *sec61-11* allele on a plasmid, protein translocation into the ER is undetectable at the restrictive temperature of 17°C for both cotranslationally inserted luminal (Kar2p) and membrane (dipeptidyl-aminopeptidase B) proteins and posttranslationally inserted luminal (α-factor) and membrane (CPY) proteins (37). We therefore modified SY25 by the simultaneous disruption of the *SEC61* gene and the addition of the *sec61-11* expression vector, p*sec61-11*, generating the strain SY36. Like the original *sec61-11* strain, SY36 cells grow well at 30°C but are unable to grow at 17°C (Fig. 2*a*), indicating that they too are defective in protein translocation into the ER. To test the ER translocation defect of SY36 cells more directly, we examined the biogenesis of CPY, which enters the ER before its transport to

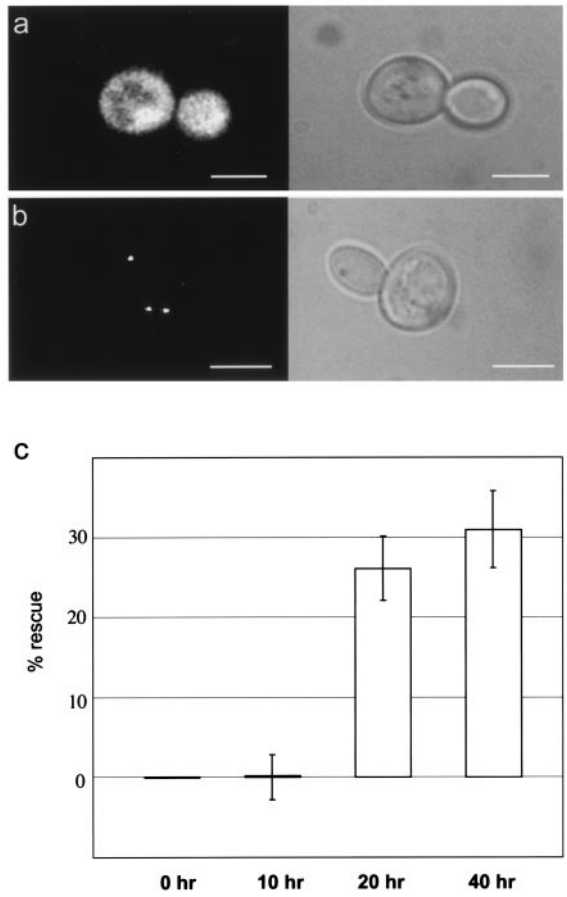

Fig. 1. Galactose control of peroxisome biogenesis in SY25 cells. Phase-contrast and confocal fluorescence microscopy were used to determine the subcellular distribution of GFP-PTS1 in SY25 cells grown at 17°C in (*a*) glucose or (*b*) galactose for 24 h. The left side of each panel shows the fluorescence from GFP-PTS1, whereas the right side of each panel shows a phase-contrast image of the same cells. (Scale bar, 5 μm.) (*c*) The time course and extent of peroxisome biogenesis mediated by p*GAL1-PEX3* after a shift from glucose to galactose. The percentage of cells in which GFP-PTS1 was imported into peroxisomes at each time point was determined by counting a minimum of 500 cells from each time point in four independent trials. The peak of each bar represents the average percent rescue of these four trials, and the error bars represent the standard deviation. The percent rescue at each time point was calculated from the actual percentage of cells displaying punctate GFP-PTS1 less the percentage of cells displaying punctate GFP-PTS1 at time zero in each individual trial.

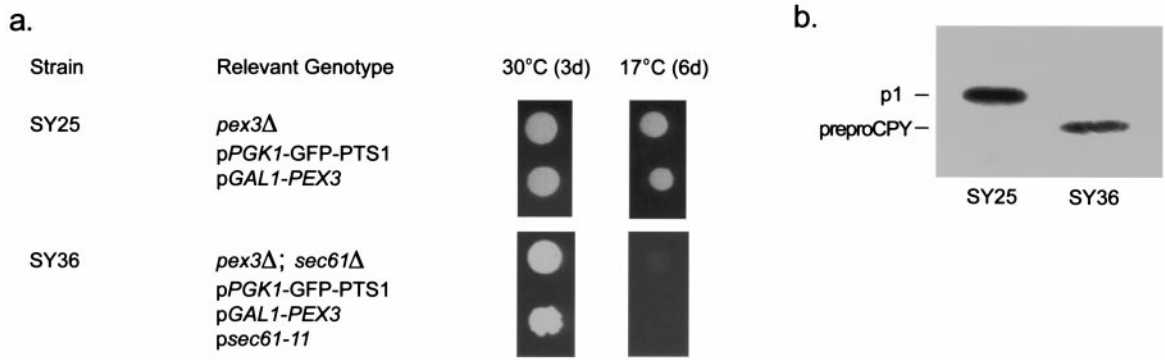

Fig. 2. SY36 cells are defective in protein translocation into the ER. (*a*) SY36 cells carry the *sec61-11* allele and display cold-sensitive growth. SY25 and SY36 cells were grown in minimal glucose medium at 30°C to an A_{600} of 1.0. From these cultures, 10 μl (upper spot) and 1 μl (lower spot) were placed on minimal glucose agar plates and incubated at either 30°C for 3 days or at 17°C for 6 days. (*b*) SY36 cells are unable to import preproCPY into the ER. SY25 and SY36 cells were grown in minimal glucose medium, shifted to 17°C for 15 min, then labeled with [^{35}S]methionine in minimal galactose medium for 10 min at 17°C, and finally lysed. CPY was immunoprecipitated from both lysates, the immunoprecipitates were separated by SDS/PAGE, and the size of CPY was determined by fluorography. The position of preproCPY and partially processed (p1) CPY are indicated.

the vacuole (37, 38). In normal cells, CPY is both proteolytically processed and *N*-glycosylated during and/or shortly after its posttranslational translocation into the ER, and this processed form of CPY (p1) migrates with a M_r of 67 kDa (38). In contrast, strains that are defective in protein translocation into the ER synthesize preproCPY, which migrates at a M_r of 60 kDa, but they fail to translocate preproCPY into the ER and are therefore unable to process CPY to the larger p1 form (38). At the restrictive temperature of 17°C, SY36 cells synthesize preproCPY but cannot mature it to the p1 form (Fig. 2*b*), confirming that SY36 cells are defective in protein translocation into the ER. SY25 cells rapidly converted preproCPY to the p1 form.

To determine whether ER protein translocation was essential for peroxisome biogenesis, SY25 and SY36 cells were grown at 30°C in minimal glucose medium and then shifted to 17°C for 15 min, which is sufficient to block protein entry into the ER. The two strains were then transferred to galactose medium (equilibrated to 17°C) to induce *PEX3* expression. Cells were removed immediately before the shift to galactose medium ($t = 0$) and after additional 20- and 40-h incubations at 17°C in galactose medium. At each time point, cells were removed from the culture and fixed, and the distribution of GFP-PTS1 was determined by fluorescence microscopy. Incubation in galactose medium induced the formation of functional peroxisomes in both strains, as deduced from the punctate distribution of GFP-PTS1 after incubation in galactose medium (Fig. 3 *a–d*). Immunoelectron microscopic analysis of SY36 cells with anti-GFP antibodies confirmed that the punctate distribution of GFP-PTS1 reflected its import into membrane-bound organelles with the typical appearance of peroxisomes (Fig. 3*e*). Double-label immunoelectron microscopy experiments with anti-GFP and antiperoxisomal thiolase antibodies confirmed the identity of these structures as peroxisomes (Fig. 3*f*). Quantitation of the percentage of each cell population importing GFP-PTS1 into peroxisomes revealed that peroxisome biogenesis occurred with the same kinetics and to the same extent in SY25 and SY36 cells (Fig. 3*g*). Taken together, these results indicate that the inactivation of Sec61p-mediated protein import into the ER does not affect peroxisome biogenesis.

Although there is no evidence that any protein enters the ER independently of Sec61p, *S. cerevisiae* does express a Sec61p-related protein, Ssh1p (39). Sec61p associates with Sss1p and Sbh1p to form the heterotrimeric Sec61p complex, which is required for both co- and posttranslational protein import into the ER (40). Similarly, Ssh1p forms a heterotrimeric protein complex in the ER membrane with Ssh1p and Sbh2p, an Sbh1p-related protein, raising the possibility that it may represent a second protein translocon in the ER membrane (39). Therefore, we examined peroxisome biogenesis in cells lacking Ssh1p. SY25 cells were modified by the deletion of the *SSH1* gene, which is not essential, generating the strain SY37. For consistency, we examined the restoration of functional peroxisomes in SY37 cells at 17°C. Cells were once again analyzed at each time point after the shift to minimal galactose medium by fluorescence microscopy. Restoration of functional peroxisomes occurred ≈20 h after induction of *PEX3* expression, and the kinetics and percentage of rescued peroxisomes was not affected by the loss of Ssh1p (Fig. 3*h*).

Discussion

The hypothesis that peroxisomes arise by vesicular transport from the ER predicts that some PMPs transit through the ER en route to the peroxisome (13–18). These as-yet-unknown group I PMPs would presumably enter the ER by the Sec61p complex, which is required for all known protein import into the ER (31–33, 41, 42). Our data revealed that peroxisome biogenesis occurs at the same rate and to the same extent in wild-type cells as it does in a *sec61–11* strain maintained at the restrictive temperature, in which protein entry into the ER seems to be blocked. We also showed that peroxisome biogenesis was unaffected by loss of Ssh1p, a Sec61p-related protein. Our observation that peroxisome biogenesis occurs independently of Sec61p and Ssh1p, together with the fact that peroxisome biogenesis is neither blocked nor inhibited by loss of COPI- or COPII-mediated vesicle transport processes (10, 11), seriously undermines several tenets of the hypothesis that peroxisomes arise from the ER by vesicle budding (13–17) and supports the hypothesis that PMPs do not transit through the ER but are instead imported directly from the cytoplasm (1).

The nature of the experimental design and the fact that we do not solve the question of how Pex3p mediates the formation of detectable peroxisomes means that there are several caveats to this study. For example, our data cannot exclude the possibility that some small amount of Pex3p enters the ER even after the inactivation of Sec61p or Ssh1p, either through these or some other as-yet-unknown protein translocon. Another possibility is that Pex3p is not a group I PMP and that it is other PMPs that enter the ER and mediate the formation of nascent peroxisomes. This caveat, however, is only valid if the loss of Pex3p leads to the accumulation of some other PMPs in the ER or ER-derived, preperoxisomal vesicles, and that Pex3p expression induced peroxisome formation without itself entering the ER. This

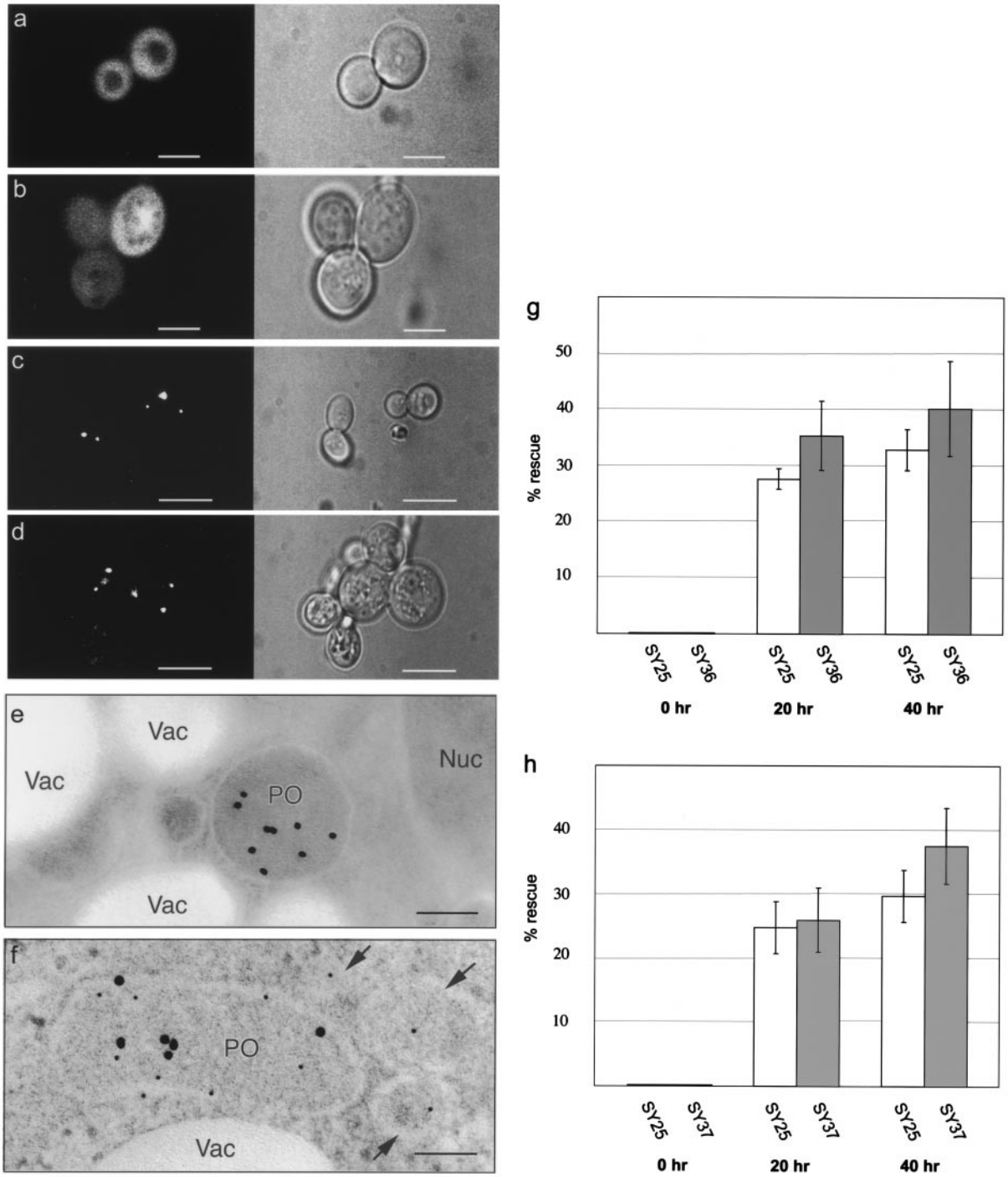

Fig. 3. Peroxisome biogenesis is unaffected by the loss of *SEC61*. (*a–d*) Confocal fluorescence (left side of each panel) and phase-contrast (right side of each panel) images of (*a* and *c*) SY25 and (*b* and *d*) SY36 cells grown at 17°C in minimal glucose medium (*a* and *b*) or after 40-h growth at 17°C in minimal galactose medium (*c* and *d*). (Scale bars in *a* and *b*, 5 μm; scale bars in *c* and *d*, 10 μm.) (*e* and *f*) Immunoelectron microscopic analysis of ultrathin sections from SY36 cells that had been incubated for 40 h at 17°C in minimal galactose medium. (*e*) Single antibody labeling with rabbit antibodies to GFP and 18-nm colloidal gold particles bound to donkey anti-rabbit antibodies. PO, peroxisome; Vac, vacuole; Nuc, nucleus. (Scale bar, 200 nm.) (*f*) Double-antibody labeling with mouse antibodies to GFP and rabbit antibodies to yeast peroxisomal thiolase, followed by 6-nm colloidal gold particles bound to anti-mouse antibodies and 18-nm colloidal gold particles bound to anti-rabbit antibodies. Arrows point to the membranes of three extremely small peroxisomes that label for GFP-PTS1 and are located next to the larger peroxisome that labeled for both GFP and thiolase. PO, peroxisome; Vac, vacuole. (Scale bar, 100 nm.) (*g*) Quantitative analysis of peroxisome biogenesis in SY25 and SY36 cells at 17°C. The percentage of cells in which GFP-PTS1 was imported into peroxisomes at each time point was determined by counting a minimum of 500 cells from each time point in four independent trials. The peak of each bar represents the average percent rescue of these four trials, and the error bars represent the standard deviation. The percent rescue at each time point was calculated from the actual percentage of cells displaying punctate GFP-PTS1 less the percentage of cells displaying punctate GFP-PTS1 at time zero in each individual trial. (*h*) Peroxisome biogenesis is also independent of *SSH1*. Quantitative analysis of peroxisome biogenesis in SY25 and SY37 cells at 17°C. The percentage of cells in which GFP-PTS1 was imported into peroxisomes at each time point was determined by counting a minimum of 500 cells from each time point in three independent trials. The peak of each bar represents the average percent rescue of these three trials, and the error bars represent the standard deviation.

caveat should be viewed in light of the fact that all PMPs examined to date in *pex3* mutants are either (*i*) rapidly degraded without ever being inserted into a membranous vesicle or (*ii*) mislocalized to the mitochondrion, not the ER. Another caveat is that Pex3p might be a multifunctional protein with one population of Pex3p targeted into the ER and responsible for generating preperoxisomal membranes, whereas a second population of Pex3p is targeted directly to peroxisomes and mediates the maturation of nascent peroxisomes, perhaps participating in PMP import. If this possibility is true, it could be that slight *PEX3* expression from the *GAL1* promoter during growth on glucose allows the formation of preperoxisomes before the block in ER protein import, and that Pex3p import into these structures after the ER translocation block allows them to mature into functional peroxisomes.

In addition to considering the evidence for and against our proposal that peroxisome biogenesis may not require protein import into the ER, we should also examine the role that ER-to-peroxisome protein transport might perform. Detailed studies have clearly established that peroxisomal matrix and membrane proteins can be imported into peroxisomes directly, without trafficking through the ER. Thus, there is no conceptual need for an ER-to-peroxisome vesicle transport system for delivering proteins to peroxisomes. In fact, the only reasonable explanation for why some peroxisomal proteins might enter the ER and traffic to the peroxisome by vesicle budding and fusion is to deliver phospholipids from their site of synthesis in the ER to the peroxisome so that peroxisome membranes can grow. The precedent for this role is that vesicle transport is used to deliver phospholipids synthesized in the ER to virtually all secretory and endocytic organelles, including the Golgi apparatus, plasma membrane, endosome, and lysosome. However, these vesicular transport events also serve to deliver nearly all of the proteins to these organelles, and it may be that vesicle traffic evolved to solve a protein biogenesis problem rather than a phospholipid transport problem. The absence of any evidence for vesicle transport between the ER and the peroxisome, mitochondrion, and chloroplast might reflect the existence of other mechanisms for transporting phospholipids between organelles, such as enzyme-mediated phospholipid transport (43). The same considerations are relevant to the formation of new peroxisomes during rescue of *pex3* mutants. If we assume that *pex3* mutants are devoid of all peroxisome-related membranes, it could be that Pex3p expression mediates the formation of peroxisomes *de novo* by extracting phospholipids from the ER membrane and nucleating the formation of phospholipid micelles and vesicles that will later mature to recognizable peroxisomes. Alternatively, it may be that *pex3* mutants do contain a preperoxisomal membrane, which is competent for phospholipid uptake from the ER but unable to import most PMPs, and that Pex3p expression mediates their conversion to mature peroxisomes, perhaps by allowing PMP import.

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