Pex18p and Pex21p are Structurally Related Yeast Peroxisomal Proteins

Peroxisome biogenesis proceeds via a complex, branched pathway, in which a cellular machinery consisting of more than 20 proteins (peroxins) effects the recognition, targeting, and import of proteins containing peroxisomal targeting sequences (PTSs)\(^1\) (reviewed in Refs. 1–5). Multiple classes of PTS exist, of which one of the best characterized is the PTS2 family of peroxisomal biogenesis proteins. These proteins contain a C-terminal tripeptide sequence (6–9) utilized by thiolase and several other peroxisomal proteins, which are imported into peroxisomes via interaction with Pex7p, the PTS2 receptor (10–15). The importance of this pathway is highlighted by the observation that it is evolutionarily conserved between yeast and humans and by the fact that loss of the PTS2 branch of peroxisomal biogenesis through PEX7 mutation causes the lethal disorder rhizomelic chondrodysplasia punctata in humans (12–14), and inviability on oleic acid as carbon source in yeast.

In this paper, we report that the functioning of Pex18p and Pex21p is accompanied by their rapid proteolytic turnover and that this pathway of peroxin degradation is obligatorily connected to ongoing peroxisome assembly. This represents, to the best of our knowledge, the first instance of an organelle biogenesis factor that is constitutively degraded during peroxisome biogenesis.

**Experimental Procedures**

*Antibody Production and Purification—Polyclonal rabbit antisera against *S. cerevisiae* Pex7p and Pex18p were raised at Covance Research Products, Inc. (Denver, PA) using standard procedures. Antigens were made available to investigators through the yeast research community in accordance with 18 U.S.C. Section 1734 in accordance with 18 U.S.C. Section 1734.

\(^{1}\) The abbreviations used are: PTS, peroxisomal targeting sequence; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; Ub, ubiquitin; E2, ubiquitin carrier protein.
RNA and Protein Methods—Preparation of total cellular RNA by the hot acidic phenol method and Northern blotting were as described (38). 32P-Labeled probes were prepared by random priming from the coding regions of yeast PEX18 and actin genes, the latter of which were a kind gift of Drs. Igor Karpichev and Gillian Small (Mount Sinai School of Medicine, New York). Autoradiographs of the blots were visualized by autoradiography or with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Preparation by glass bead homogenization of total cellular protein extracts for immunoblotting was as described previously (16). Preparation by glass bead homogenization of total cellular protein extracts for immunoprecipitation with anti-Pex18p (Figs. 2B and 4B) was by this strategy, except that prior to blotting with anti-ubiquitin, the nitrocellulose was washed and collected as usual. All subsequent steps were performed at room temperature. Pellets were resuspended in 0.5 ml of 40% trichloroacetic acid and centrifuged at 14,000 rpm. The supernatant was discarded, and the pellets were briefly frozen in liquid nitrogen and then thawed and resuspended in 0.5 ml of 20% trichloroacetic acid. Glutaraldehyde was added, and the mixture was incubated on ice for 5 min prior to protein extraction. Deproteinization of Pex18p-ubiquitin conjugates with anti-FLAG, a modified extraction procedure was employed in order to minimize the risk of proteolytic degradation of these conjugates. Cells were grown in 25 ml of YPEO and washed and collected as usual. All subsequent steps were performed at room temperature. Pellets were resuspended in 0.5 ml of 40% trichloroacetic acid and centrifuged at 14,000 rpm. The supernatant was discarded, and the pellets were briefly frozen in liquid nitrogen and then thawed and resuspended in 0.5 ml of 20% trichloroacetic acid. Glutaraldehyde was added, and the mixture was incubated on ice for 5 min prior to protein extraction. For FLAG tagging, the complete coding sequence of the Pex18p Abundance Varies Markedly between Wild Type and pex Mutant Strains—A polyclonal antiserum raised against bacterially expressed Pex18p (see “Experimental Procedures”) was used to monitor Pex18p expression levels in wild-type and various pex mutant yeast strains. Pex18p could be detected in extracts of oleate-induced wild-type yeast as a doublet with an estimated size of about 36 kDa (see Fig. 1), compared with the predicted size, based on the sequence of the PEX18 gene, of ~32 kDa (16). The reason for this apparent size difference is unclear, but it was also observed for bacterially expressed Pex18p (which likewise runs as a doublet), indicating that its mobility in SDS-PAGE may be an intrinsic feature of the protein rather than due to covalent modifications. Treatment of extracts with iodoacetamide, phosphatases, or hydroxylamine did not alter the Pex18p gel migration pattern (data not shown). Both bands of the doublet were absent in extracts from W303Δpex18 yeast, confirming that they correspond to Pex18p (Fig. 1A).

Remarkably, the abundance of Pex18p was greatly increased in strains with generalized defects in the import of peroxisomal matrix proteins (W303Δpex1, W303Δpex4, W303Δpex13, and W303Δpex14) (Figs. 1 and 3B). On the other hand, Pex18p (16).
abundance was close to normal in W303Δpex7 and W303Δpex5, which display partial defects in this process.

In common with many peroxisomal proteins and peroxins, Pex18p expression was oleate-inducible, as shown by the apparent absence or near absence of immunoreactive protein in strains grown in YPD or YPE (Fig. 1B).

The Variation among Strains in Pex18p Abundance Is Not Primarily Due to Variations in PEX18 mRNA Levels or Pex18p Synthesis—To investigate the mechanism of the variation in Pex18p levels, RNA was extracted from oleate-induced strains and analyzed by Northern blotting. PEX18 mRNA levels were similar to the wild-type level in several pex mutants, including W303Δpex5, W303Δpex7, W303Δpex13, and W303Δpex14 (shown for W303Δpex14 in Fig. 2A), suggesting that the variation in Pex18p levels is not primarily due to differences in abundance of PEX18 message. In agreement with the immunoblot data shown in Fig. 1B, PEX18 mRNA levels were much lower in the absence of oleate. The rate of Pex18p synthesis was tested directly by pulse-labeling oleate-grown cells with [35S]methionine and then analyzing incorporation into Pex18p by immunoprecipitation with anti-Pex18p, followed by SDS-PAGE and fluorography (Fig. 2B). Little difference was observed between wild-type and W303Δpex14, suggesting that Pex18p is made at similar rates in these strains.

Pex18p is Rapidly Turned Over in Wild-type Cells but Not in Certain pex Mutants—Since neither the levels of PEX18 mRNA nor the rates of Pex18p synthesis vary substantially between strains with grossly different levels of Pex18p, we next addressed the question of Pex18p turnover. Cells were grown and induced as usual, and then growth was continued in the presence of cycloheximide. As shown in Fig. 3A, Pex18p rapidly disappeared from wild-type cells treated with cycloheximide, being barely detectable within 20 min. In contrast, the high level of Pex18p in W303Δpex14 cells was maintained after 20 min in cycloheximide (Fig. 3A) and showed no discernible decline even 5 hr after administration of cycloheximide (not shown). The turnover of Pex18p in W303Δpex7 cells, which maintain Pex18p at a level similar to that seen in wild-type cells, is similar to the turnover rate in wild-type cells (Fig. 3A).

Analysis of additional pex mutants revealed a consistent pattern. Mutants that have a generalized defect in peroxisome biogenesis accumulated high levels of Pex18p, which turned over slowly, if at all, whereas W303Δpex7 and W303Δpex5, which are defective only for PTS2 and PTS1 targeting, respectively, behaved similarly to wild type (Fig. 3B). Thus, in mutants W303Δpex13 and W303Δpex14, where Pex18p is unable to fulfill its role of PTS2 protein delivery to peroxisomes, Pex18p is stabilized. Notably in W303Δpex4, which lacks the peroxisomal member of the ubiquitin-conjugating enzyme family, Pex18p is likewise stabilized. Stabilization also occurs in W303Δpex1. In W303Δpex5, where PTS2 packaging is functional, Pex18p is turned over as in wild-type cells. Pex18p is known to interact with Pex7p (16). The instability of Pex18p in W303Δpex7 suggests that Pex18p may be stabilized by its interaction with Pex7p.

Pex21p Also Turns Over Rapidly—Pex21p, whose function can partially compensate for an absence of Pex18p (16), also turns over rapidly in wild-type yeast (data not shown). In cells defective in peroxisome biogenesis (W303Δpex13 or W303Δpex14), Pex21p is stabilized little, if at all, unless Pex18p is missing, in which case there is a striking stabilization and accumulation of Pex21p. As for Pex18p, this stabilization requires Pex7p to be present.

Pex7p Forms a Complex with Pex18p and Is Required for Its Stabilization—In order to investigate further the possible involvement of Pex7p in Pex18p stabilization, we disrupted the PEX7 gene from several pex mutants and assessed the consequences for Pex18p accumulation and turnover. As shown in Fig. 4A, the absence of Pex7p completely abolishes Pex18p stabilization in the W303Δpex13 background. W303Δpex13Δpex7 appeared identical to W303Δpex7 with respect to the abundance of Pex18p and its turnover. Similar Pex18p instability was observed in W303Δpex14Δpex7 (not shown). These data support the idea that Pex18p becomes stabilized in pex mutants through persistence of its interaction with Pex7p.
To test directly whether the elevated Pex18p is associated with Pex7p, anti-Pex18p immunoprecipitates were immunoblotted with anti-Pex7p antisera (Fig. 4B). Pex7p was co-immunoprecipitated with Pex18p from wild-type cells; the amount of immunoprecipitated Pex7p was considerably greater with W303/H9004pex14 cells. In neither case was immunoprecipitation of Pex7p complete, suggesting that the cellular Pex7p is not saturated with Pex18p, even when the latter is accumulated to the levels seen in W303/H9004pex14.

Pex18p Is Ubiquitinated—To investigate the mechanism of the rapid turnover of Pex18p in wild-type cells, we first analyzed Pex18p turnover in strains defective in various aspects of degradation of cellular proteins. W303Δpep4, which lacks the vacuolar proteinase A, had wild-type levels of Pex18p abundance and turnover (Fig. 5). On the other hand, Y0238, which lacks two functionally related ubiquitin-conjugating enzymes, Ubc4p and Ubc5p, showed accumulation of high levels of Pex18p and impaired Pex18p turnover. A congenic wild type strain (Y0002; Fig. 5) and various other ubc mutants, including a strain lacking Ubc6p and Ubc7p (Y0241; not shown), demonstrated normal Pex18p levels and rates of turnover. Finally, we analyzed strain MHY623, in which ubiquitin homeostasis is severely impaired due to a lack of the deubiquitinating enzyme, Doa4p (36). Cells lacking Doa4p have decreased ubiquitin levels and display strongly reduced turnover of several proteins degraded via ubiquitination. As shown in Fig. 5, Pex18p turnover is severely deficient in Δdoa4 yeast (but normal in a congenic wild-type strain).

Higher molecular weight forms of Pex18p (which would be consistent with the existence of ubiquitinated degradation intermediates) could not be detected in immunoprecipitations and immunoblots with anti-Pex18p serum (not shown). However, when FLAG-tagged Pex18p was immunoprecipitated directly from total cellular protein extracts (prepared by glass bead homogenization in the presence of trichloroacetic acid), two higher molecular weight species in addition to FLAG-Pex18p were discernible (Fig. 6A, left panel). The stronger of these bands had an estimated mass about 8 kDa greater than FLAG-Pex18p, consistent with the presence of a single 76-amino acid ubiquitin moiety (or related peptide), and the fainter band was a further 8 kDa larger. Curiously, the larger, but not the smaller of these putative ubiquitin conjugates could be detected with anti-ubiquitin antiserum (Fig. 6A, right panel). Co-expression of ubiquitin tagged with the hemagglutinin epitope (HA-Ub, 85 amino acids) reduced the intensities of both of these bands and generated two slightly larger new bands (Fig. 6B, arrowheads), indicating the incorporation of one or two HA-Ub moieties into FLAG-Pex18p. Taken together, these results indicate that Pex18p is ubiquitinated.
peroxisomal member of the E2 family of ubiquitin-conjugating enzymes, Ubc4p and Ubc5p, and is deficient in strains lacking the ubiquitin-conjugating enzyme Doa4p, and the congenic wild-type strain, MHY501 (bottom panel).

**DISCUSSION**

In this paper, we report that Pex18p, a peroxin previously shown to be essential for peroxisome biogenesis via the PTS2 pathway, turns over rapidly during normal peroxisome biogenesis in wild-type cells. This is, to the best of our knowledge, the first report of an organelle biogenesis factor that is constitutively degraded in the course of its normal function. In pex mutants in which peroxisomal matrix protein import is completely abolished, Pex18p undergoes a remarkable increase in stability and abundance. Stabilization of Pex18p is entirely dependent upon the presence of Pex7p, the PTS2 receptor, with which it forms a complex both in vivo and in vitro. Pex18p turnover is impaired in cells lacking the ubiquitin-conjugating enzymes Ubc4p and Ubc5p and is deficient in Δadp4 cells, which display a generalized defect in ubiquitin homeostasis. Finally, mono- and diubiquitinated forms of Pex18p can be detected in wild-type cells during unperturbed peroxisome biogenesis.

The finding that Pex18p is rapidly turned over in wild-type cells growing on oleic acid was unexpected. Such turnover has not been described for mitochondrial or chloroplast biogenesis, nor for other organelles, so far as we are aware. Various peroxins, including Pex5p and several peroxisomal membrane proteins, have been shown to display altered abundance in certain pex mutants (40–43), but rapid turnover of any peroxin during normal peroxisome biogenesis has not been reported. The observed rate of Pex18p degradation is as fast as that of some transcriptional regulators, for example the a2 homeodomain protein encoded by the MATα locus (44).

Pex18p is stabilized in pex mutants with diverse defects. These include W303Δpex14 and W303Δpex14, which lack peroxisomal membrane proteins implicated in Pex7p docking at the peroxisomal surface, and mutants lacking either Pex4p, a peroxisomal member of the E2 family of ubiquitin-conjugating enzymes (35), or Pex1p, a peroxin whose function is uncertain. The common property of these mutants is a defect in the assembly of all peroxisomal matrix proteins. In contrast, in cells lacking Pex7p, but with all other peroxins intact, Pex18p abundance, and turnover are indistinguishable from wild type (Fig. 4A). Double mutant analysis demonstrated a requirement for PEX7 for the stabilization of Pex18p. Consistent with this, Pex7p and Pex18p form a complex in cells as determined by co-immunoprecipitation with anti-Pex18p. The abundance of Pex7p does not change when PEX18, PEX13, PEX14, PEX1, or PEX4 is knocked out (data not shown). A small amount of Pex7p is associated with Pex18p (lane 4) or, as a control, untagged Pex18p (lanes 2 and 4) were subjected to immunoprecipitation with anti-FLAG. The immunoprecipitates were then resolved by SDS-PAGE and blotted against Pex7p (left panel, lanes 1 and 2) and ubiquitin (right panel, lanes 3 and 4). Pex18p (Fig. 4B) is ubiquitinated, which it forms a complex both in vivo and in vitro. Pex18p turnover is impaired in cells lacking the ubiquitin-conjugating enzyme Doa4p, and the congenic wild-type strain, MHY501 (bottom panel).

**Fig. 5. Pex18p turnover is impeded in strains defective in the ubiquitin pathway.** Strain Y0238 (Δube4Δube5) and its congenic wild type, Y0002, were grown and treated with (+C) or without (–C) cycloheximide, as described in the legend to Fig. 3B (top panel). Total cellular protein extracts were separated by SDS-PAGE and immunoblotted with anti-Pex18p. Similar comparisons were made between wild-type W303, W303Δpex14, and W303Δpex4 (which lacks the vacuolar proteinase A) (middle panel) and between MHY623, which is defective in the deubiquitinating enzyme Doa4p, and the congenic wild-type strain, MHY501 (bottom panel).

**Fig. 6. Pex18p is ubiquitinated.** A, total cellular protein extracts from W303Δpex18 expressing FLAG-tagged Pex18p (F-Pex18p, lanes 2 and 4) or, as a control, untagged Pex18p (lanes 1 and 3) were subjected to immunoprecipitation with anti-FLAG. The immunoprecipitates were then resolved by SDS-PAGE and blotted with antisera against Pex18p (left panel, lanes 1 and 2) and ubiquitin (right panel, lanes 3 and 4). Pex18p (Fig. 4B) is ubiquitinated, which it forms a complex both in vivo and in vitro. Pex18p turnover is impaired in cells lacking the ubiquitin-conjugating enzyme Doa4p, and the congenic wild-type strain, MHY501 (bottom panel).
abundance and turnover. This agrees with the finding that Pex7p is required for stabilization of Pex18p in generalized pex mutants and also suggests that the mechanisms of turnover of Pex18p synthesized in the absence of Pex7p and of Pex18p released from Pex7p during PTS2 import may be similar.

The rapid rate of turnover of Pex18p suggests that it is probably degraded by proteasomes, which degrade many proteins with short half-lives that are marked by polyubiquitination (46, 47). Consistent with this, a mutant strain lacking the ubiquitin-conjugating enzymes Ubc4p and Ubc5p, which are responsible for directing many proteins to proteasomal degradation (46), showed striking stabilization and accumulation of Pex18p. The alternative of vacuolar import and proteolysis appears unlikely; an import half-time of 30–40 min has been reported for vacuole-degraded aminopeptidase I (48), which is much slower than the observed Pex18p degradation rate. Moreover, we saw no change in Pex18p stability upon deletion of the PEP4 gene encoding the vacuolar proteasome A. However, we do not yet have definitive evidence for proteasomal involvement in Pex18p degradation; mutants lacking functional proteasomal subunits grow extremely poorly under peroxisome induction conditions. Also, targeting to proteasomes for degradation usually requires a chain of at least four ubiquitin moieties (49), whereas we have only detected mono- and diubiquitinated subunits.

More highly ubiquitinated forms of Pex18p may exist but be too transient for detection in our immunoprecipitation assay.

It is also possible that the observed mono- and diubiquitination of Pex18p may represent a signal distinct from, and perhaps preceding, commitment to proteasomal degradation. Ubiquitin plays a role in a remarkable variety of cellular functions and does not always do so by forming long chains. Apart from its role in targeting proteins to the proteasome (50), it affects DNA repair, ribosome function, mitochondrial DNA inheritance, and the Hsp response (51). It also contributes to the down-regulation of cell surface receptors, transporters, and ion channels (52). In S. cerevisiae, monoubiquitination of α-factor receptor has been shown to be sufficient to trigger its internalization by endocytosis, leading to its degradation in the vacuole (17). Other cellular proteins, including histone H2B (37), appear to be monoubiquitinated but not polyubiquitinated.

An involvement of ubiquitination in peroxisome biogenesis has been suspected, since the original identification of Pex4p as a member of the E2 family of ubiquitin-conjugating enzymes (35). However, thus far no Pex4p substrates have been reported. It is noteworthy that Δpex cells also show stabilization of Pex18p, raising the interesting possibility that Pex18p may be among the long sought after substrates for Pex4p-dependent ubiquitination. Consistent with this, Pex4p is localized (through its interaction with the integral membrane peroxisomal Pex22p) to the outer face of the peroxisomal membrane (45), where it might have the opportunity to interact with the docked PTS2 import complex and participate in the ubiquitination of Pex18p. Indeed, recent experiments (not shown) indicate that deletion of PEX4 alters, but does not abolish, the pattern of ubiquitination of Pex18p. This topology raises the tantalizing possibility that limited ubiquitination of Pex18p might play a role in the regulation of PTS2 targeting. However, such a role for Pex4p remains hypothetical at this time; future experiments will be required to investigate whether this might be the case.

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P. Edward Purdue and Paul B. Lazarow

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