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Components Involved in Peroxisome Import, Biogenesis, Proliferation, Turnover, and Movement

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Subramani, Suresh. Components Involved in Peroxisome Import, Biogenesis, Proliferation, Turnover, and Movement. *Physiol. Rev.* 78: 171–188, 1998.—In the decade that has elapsed since the discovery of the first peroxisomal targeting signal (PTS), considerable information has been obtained regarding the mechanism of protein import into peroxisomes. The PTSs responsible for the import of matrix and membrane proteins to peroxisomes, the receptors for several of these PTSs, and docking proteins for the PTS1 and PTS2 receptors are known. Many peroxins involved in peroxisomal protein import and biogenesis have been characterized genetically and biochemically. These studies have revealed important new insights regarding the mechanism of protein translocation across the peroxisomal membrane, the conservation of *PEX* genes through evolution, the role of peroxins in fatal human peroxisomal disorders, and the biogenesis of the organelle. It is clear that peroxisomal protein import and biogenesis have many features unique to this organelle alone. More recent studies on peroxisome degradation, division, and movement highlight newer aspects of the biology of this organelle that promise to be just as exciting and interesting as import and biogenesis.

I. INTRODUCTION

Peroxisomes, the last of the major subcellular organelles to be discovered, are present in virtually all eukaryotic cells. The history of their discovery was recounted a few years ago by Christian de Duve (18) at a conference focused on the biology of this organelle and its role in toxicology and disease. The volume containing the proceedings of this meeting is an excellent source of background material on the metabolic pathways residing in peroxisomes, the phenomenon of peroxisome proliferation, and the role of peroxisome proliferators in cancer, topics which are not the subject of this review. This article focuses primarily on peroxisomal protein import and bio-

genesis and their role in human peroxisomal disorders, but it also touches on subjects such as peroxisome division, turnover, and movement that have received only scanty coverage in other reviews.

II. PEROXISOMAL PROTEIN IMPORT PATHWAYS

All the protein residents of peroxisomes make their way to this organelle, which is devoid of DNA, after their synthesis in the cytoplasm (66). Like the sorting of proteins to other subcellular compartments, the targeting of proteins to the peroxisomal membrane and matrix is achieved through the interaction of specific peroxisomal targeting signals (PTSs) and their cognate receptors with the peroxisome-specific import machinery.

A. Targeting of Matrix Proteins

A decade has elapsed since the discovery of the first PTS in firefly luciferase (49, 50). Two or more PTSs are involved in the transport of proteins to the peroxisome matrix (Fig. 1). A conserved COOH-terminal tripeptide (SKL and its functional variants) found in about one-half of the mammalian peroxisomal matrix proteins, PTS1 is the most widely used sequence (34, 50, 84, 115). It is used also for the targeting of proteins to glycosomes (8) and glyoxysomes (122) which are related evolutionarily to peroxisomes. An NH_2-terminal or internal nonapeptide [(R/K)(L/V/I)X_5(Q/H)(L/A)] found in peroxisomal 3-ketoacyl CoA thiolase (89, 114), PTS2 is used by a smaller group of proteins such as watermelon glyoxysomal malate dehydrogenase (45), *Hansenula polymorpha* amine oxidase (41) and Pex8p (141), human alkyl dihydroxyacetonephosphate synthase (19), and *Trypanosoma brucei* aldolase (7). Both PTSs are conserved in evolution from yeast to humans. Other internal PTSs (33, 63, 107, 155) have been described in peroxisomal matrix proteins either lacking or containing PTS1 and/or PTS2, but distinct receptors for these PTSs have not yet been identified. In several yeasts lacking the PTS1 receptor, Pex5p, catalase is cytosolic (51, 130), suggesting either that the internal PTS identified in this protein by Kragler et al. (63) is nonfunctional or that the ability of this internal PTS to function is dependent on the PTS1 receptor. Similarly in *Saccharomyces cerevisiae*, the internal PTS in carnitine acetyltransferase requires Pex5p for the sorting of this protein to peroxisomes (33). Thus it is possible that some of these sequences defined as internal PTSs actually function not by acting as true PTSs, but by facilitating import of oligomeric proteins by association with other PTS1-containing proteins as suggested by Rachubinski and Subramani (93).

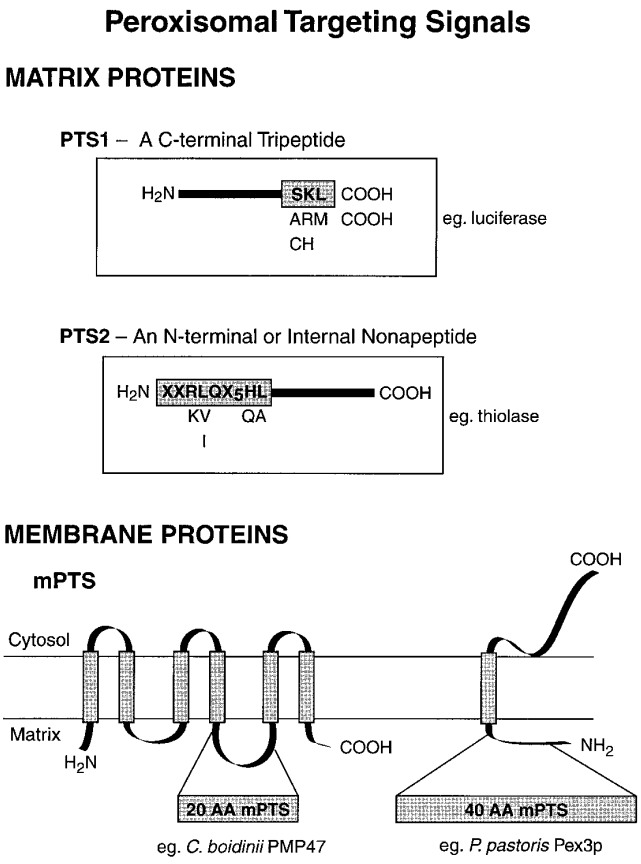

FIG. 1. Peroxisomal targeting signals (PTSs) used for import of proteins into peroxisomal matrix (PTS1 and PTS2) and those (mPTSs) used for sorting to peroxisomal membrane are shown. Proteins in which these PTSs were first described are also shown. Functional variants of consensus PTS1 (for mammalian cells) and PTS2 (for yeast) are shown below sequence of amino acids (in 1-letter code) comprising each PTS.

B. Matrix Protein Import Pathways

Two major advances in the peroxisomal import field came from the discovery that the fatty acid β-oxidation pathway in yeasts is exclusively peroxisomal (65) and from the identification of pathway-specific protein import mutants, in which either the PTS1 or the PTS2 import pathway is selectively compromised (Fig. 2). The *Pichia pastoris pex5* mutant was initially shown to have such a pathway-specific defect for only PTS1-containing proteins (77). Mutants with a similar phenotype were found in *S. cerevisiae* (130), *H. polymorpha* (88a, 128), *Yarrowia lipolytica* (116), and human patients (82, 106). Conversely, the *pex7* mutant is selectively affected only in the PTS2 import pathway (75, 82, 106, 131, 155; Y. Elgersma, M. Elgersma, T. Wenzel, and S. Subramani, unpublished data). These pathway-specific mutants not only provided genetic confirmation for the existence of at least two distinct peroxisomal matrix import pathways but also led directly to the cloning and characterization of the genes encoding the PTS1 and PTS2 receptors.

The utility of these mutants in defining the PTS receptors is exemplified by the finding that the *P. pastoris PEX5* gene encodes the PTS1 receptor (77, 119). Homologs of

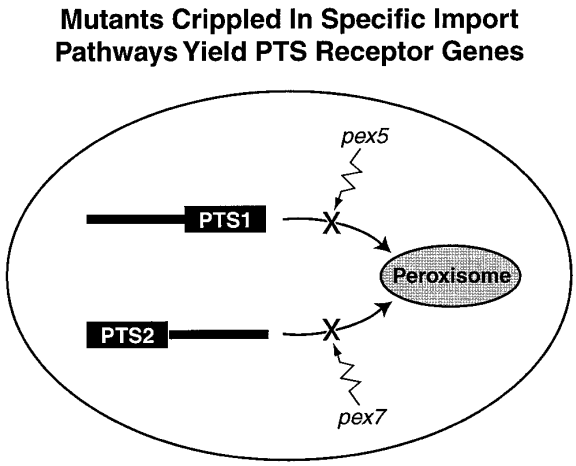

FIG. 2. Mutants crippled in specific import pathways yield PTS receptor genes. Yeast mutants, *pex5* and *pex7*, are defective in only PTS1- or PTS2-import pathways, respectively, and not in import of peroxisomal membrane proteins. Complementation of these mutants led to PTS1 and PTS2 receptor genes. In mammalian cells, certain mutations in the *PEX5* gene affect only the PTS1 import pathway, but other more severe mutations affect both PTS1 and PTS2 pathways (23, 147). Mutations in the human *PEX7* gene affect only the PTS2 import pathway (12, 83, 92). [From Subramani (112).]

this protein have been identified in several organisms (116, 128, 130), and the human *PEX5* gene is deficient in a subset of patients suffering from fatal peroxisomal disorders (23, 147, 150).

Similarly, the *PEX7* gene of *S. cerevisiae*, identified by its ability to complement the *pex7* mutant, encodes the PTS2 receptor (75, 96, 155). Its homolog has the same function in *P. pastoris* (Elgersma et al., unpublished data). The human homolog of the gene is mutated in the disease rhizomelic chondrodysplasia punctata (12, 83, 92).

C. Targeting of Membrane Proteins

The details regarding the targeting of membrane proteins have been unraveled more slowly. Targeting sequences, termed mPTSs, have been described in two proteins *Candida boidinii* PMP47 (26, 76) and *P. pastoris* Pex3p (149) (Fig. 1). An mPTS resides in a 20-amino acid loop in PMP47 and is located between putative transmembrane domains 4 and 5 in a protein presumed to have six transmembrane segments. In PpPex3p, it lies in the NH_2-terminal 40-amino acid segment. Neither mPTS includes a transmembrane domain, both face the peroxisome matrix, and both have a charged stretch of five basic amino acids (KIKKR in PMP47 and RNKKK in PpPex3p). There is no other striking homology outside this region. It is interesting to note that although these two mPTSs do not need the adjacent transmembrane domains to function, fusion proteins containing the mPTS are not only targeted to the peroxisomal membrane, but the mPTS is sufficient to make passenger proteins to which it is fused become resistant to alkaline carbonate extraction. This would argue that the mPTS is tightly associated with one or more components of the lipid bilayer.

A thorough analysis of the mPTS in the 20-amino acid loop (sequence EQLKSPIVKIKKRNITPVDA) of PMP47 shows that it is composed of three blocks (I, II, and III), with the sequence KIKKR comprising block II, the sequences preceding this consisting of block I, and those after the basic cluster forming block III (26). Block I is the least conserved in both sequence content and length and contains little sorting information. Block II is the most conserved and important sequence, with a string of basic residues. Either block II or III is sufficient for insertion of passenger proteins into the peroxisomal membrane, so both must contain some sorting information. It has been proposed that blocks I and III form an antiparallel β-sheet with the intervening block II displayed as a positively charged loop. The bipartite sorting signal consisting of blocks II and III may serve to orient the protein in the membrane.

In vitro studies (20) indicate that a two-step mechanism operates in the assembly of proteins into the peroxisomal membrane. The first step proceeds at 4°C and involves binding but no insertion into the membrane. The second step requires higher temperature and includes insertion of the protein into the lipid bilayer. Both steps are affected by pretreatment of the peroxisomes with proteases, suggesting that proteinaceous receptors are involved, but these have not been identified.

D. Membrane Protein Import Pathways

Until other mPTSs are identified, it is difficult to conclude whether the two mPTSs described to date are similar or distinct. Because some emerging evidence points to the possibility that at least some peroxisomal membrane proteins may be targeted to the peroxisomes via the endoplasmic reticulum (ER) (see sect. VII), it seems likely that there are two classes of mPTS (111). One class (mPTS1) might target proteins from the cytosol directly to the peroxisomal membrane. The second type (mPTS2) might be involved in directing membrane proteins from the ER to the peroxisome. The topology of mPTS2 relative to the membrane (i.e., does it reside in the ER lumen or the cytosol) is a key aspect relevant to its mechanism of action.

III. GENETIC ANALYSIS OF PEROXISOMAL PROTEIN IMPORT AND BIOGENESIS

The discovery of the different PTSs and the recognition that they are evolutionarily conserved led naturally to an interest in the mechanism of import, a problem that has been addressed using genetic as well as biochemical

strategies. Many laboratories have exploited yeast and cultured mammalian cells as model systems, and a collection of *pex* mutants affected in peroxisome protein import, biogenesis, proliferation, and inheritance now exists in *S. cerevisiae*, *P. pastoris*, *H. polymorpha*, *Y. lipolytica*, *C. boidinii* (reviewed in Ref. 31), and Chinese hamster ovary (CHO) cells.

A. Strategies for Isolation of *pex* Mutants

These mutants have been isolated using multiple strategies that are summarized below.

1) Yeast strains require peroxisomes and certain peroxisomal enzymes for growth on some carbon sources such as oleic acid (for most yeasts) and methanol (for methylotrophic yeasts). Several *pex* strains were obtained by screening for their failure to grow on these carbon sources, followed by biochemical and morphological analyses of the mutants for deficiencies in peroxisomal import and/or biogenesis (17, 38, 51, 70, 88, 117).

2) An H_2O_2-suicide selection scheme was developed in *S. cerevisiae* based on the principle that when catalase, the peroxisomal enzyme that degrades toxic H_2O_2, is inhibited either by 3-aminotriazole or by mutation, the production of H_2O_2 by the peroxisomal β-oxidation enzymes is lethal. However, in *pex* mutants, no H_2O_2 production occurs in the cytosol because the β-oxidation pathway is not functional, and hence these mutants survive (131, 153).

3) Mistargeting of bleomycin^r-PTS fusions to the peroxisome renders the bleomycin^r protein unable to bind cytosolic bleomycin and confers sensitivity to this drug. Mutagenesis of such yeast strains yields *pex* mutants in which resistance to bleomycin is restored because of retention of the bleomycin^r-PTS fusions in the cytosol (32).

4) Green fluorescence protein (GFP)-PTS fusions are targeted to the peroxisome where they produce punctate fluorescence. In yeast and mammalian *pex* mutants, this fluorescence is diffuse and cytosolic (60).

5) Zoeller and Raetz (158) adapted colony autoradiography to screen for CHO mutants deficient in the enzyme dihydroxyacetonephosphate acyltransferase. This enzyme is required for plasmalogen synthesis. Most of the mutants turned out to be deficient not only in this enzyme but also in the assembly of functional peroxisomes and had the same cellular protein import defects seen in Zellweger syndrome cells (156). Morand et al. (79) developed a direct selection procedure for peroxisome biogenesis mutants using CHO cells. This is based on the idea that wild-type CHO cells incorporate the pyrene-labeled fatty acid analog 9-(1′-pyrene)nonanol into other lipids as the fatty acid alcohol and consequently become susceptible to free radicals and singlet oxygen species produced by excitation of the pyrene with ultraviolet light. Plasmalogen-deficient cells do not incorporate the pyrene-labeled analog and are resistant to ultraviolet light (157). The use of these two mutant screens has led to the description of at least three complementation groups of CHO mutants deficient in peroxisome biogenesis. These mutants correspond to the human complementation groups 1, 4, and 10 (in the notation used in USA) (43).

B. *PEX* Genes and the Peroxins They Encode

The analysis of the phenotypes of these mutants and the characterization of the proteins (peroxins) encoded by the *PEX* genes (21) has led quickly to the description of about 17 genes involved in these processes (Table 1). Included in this group are the PTS1 receptors (23, 42, 77, 116, 119, 128, 130, 147) and PTS2 receptors (12, 75, 83, 92, 96, 154), a peroxisome-associated docking protein (Pex13p) for the PTS1 receptor (Pex5p) (30, 36, 48), as well as another docking protein (Pex14p) that binds to the PTS1 (Pex5p) and PTS2 receptors (Pex7p) (1). Three Pex proteins (Pex2p, Pex10p, and Pex12p) contain zinc fingers and behave as peroxisomal integral membrane proteins (5, 15, 60, 61, 118, 124), two (Pex1p and Pex6p) are ATPases of the AAA family (53, 87, 109, 125, 135, 140, 152), one (Pex4p) is a ubiquitin-conjugating enzyme (16, 146), one (PxF) is a farnesylated protein associated with peroxisomes (59, 64), one (Pex11p) is involved in peroxisome proliferation (35, 72, 73), one matrix protein (Pex8p) contains both PTS1 and PTS2 sequences and plays a role in biogenesis (141), two peripherally associated peroxisomal membrane proteins (Pex16p and Pex17p) may be involved directly or indirectly in the import of a subset of matrix proteins (28, 108), and several others encode membrane proteins that are excellent candidates for the protein translocation machinery (27, 54, 64, 69). Detailed insights into the biochemical functions of these proteins are accumulating steadily through the analysis of the phenotypes of mutants, the use of yeast two-hybrid systems, and the development of biochemical assays for these constituents.

IV. BIOCHEMICAL ANALYSIS OF PEROXISOMAL PROTEIN IMPORT

A. Import of Matrix Proteins

The development of several in vitro peroxisomal protein import assays has facilitated a biochemical dissection of the proteins and small molecules required. Although the import of peroxisomal matrix proteins into purified rat liver (58) and yeast (121) peroxisomes was developed first, it has not been used to identify proteins necessary for import. Difficulties with these assays relate to the fragility of purified peroxisomes, the differential leakage of several matrix proteins out of the organelle, the lack of

TABLE 1. *Conservation of peroxins and their role in human diseases*

PEX Genes	Function
PpPEX1	117- to 127-kDa AAA family ATPase; cytosolic and vesicle associated; involved in biogenesis
ScPEX1	
PpPEX2	35- to 52-kDa C_3HC_4 zinc-binding IMP; human *PEX2* complements CG10
RnPEX2	
PaPEX2	
HsPEX2	
PpPEX3	51- to 52-kDa IMP involved in biogenesis; mPTS in first 40 amino acids
ScPEX3	
HpPEX3	
PpPEX4	21- to 24-kDa peroxisome-associated ubiquitin-conjugating enzyme
ScPEX4	
PpPEX5	64- to 69-kDa PTS1 receptor; 7 TPR domains; peroxisome associated and cytosolic; human *PEX5* complements CG2
ScPEX5	
HpPEX5	
YlPEX5	
HsPEX5	
PpPEX6	112- to 127-kDa AAA family ATPase; cytosolic and vesicle associated; involved in biogenesis; human *PEX6* complements CG4
ScPEX6	
YlPEX6	
RnPEX6	
HsPEX6	
ScPEX7	37- to 42-kDa peroxisomal PTS2 receptor; 7 WD repeats; human *PEX7* complements RCDP/CG11 lines
KlPEX7	
MmPEX7	
HsPEX7	
PpPEX8	71- to 81-kDa peroxisome-associated protein; has a PTS1
HpPEX8	
YlPEX9	42-kDa peroxisomal integral membrane protein
PpPEX10	34- to 48-kDa C_3HC_4 zinc-binding IMP
HpPEX10	
ScPEX11	27- to 32-kDa peroxisome-associated protein involved in peroxisome proliferation
CbPEX11	
PpPEX12	40- to 48-kDa C_3HC_4 zinc-binding IMP; human *PEX12* complements CG3
ScPEX12	
HsPEX12	
PpPEX13	40- to 43-kDa SH3 domain-containing IMP; yeast Pex13p binds Pex5p
ScPEX13	
HsPEX13	
HpPEX14	39-kDa peroxisomal membrane protein; yeast Pex14p interacts with Pex5p, Pex7p, Pas9p, and Pex13p (1)
ScPEX14	
HsPEX14	
YlPEX16	45.5-kDa protein residing on matrix face of peroxisomes; required for import of certain matrix proteins; overexpression causes accumulation of enlarged peroxisomes (28)
YlPEX17	75.6-kDa protein peripherally associated with the peroxisome membrane; cells lacking Pex17p have large multimembrane structures; selective impairment of matrix protein import (108)

IMP, integral membrane protein. *Pp*, *Pichia pastoris; Sc*, *Saccharomyces cerevisiae; Hp*, *Hansenula polymorpha; Cb*, *Candida boidinii; Yl*, *Yarrowia lipolytica; Kl*, *Kluyveromyces lactis; Pa*, *Podospora anserina; Mm*, *Mus musculus; Hs*, *Homo sapiens; Rn*, *Rattus norwegicus.* [Adapted from Distel et al. (21) and Subramani (112).]

cytosol dependence of the assay, and uncertainties regarding the relative import competencies of peroxisome subpopulations.

These difficulties led to the use of microinjection into living mammalian cells as an approach to study the import of proteins into peroxisomes. Although the import of proteins into peroxisomes after their microinjection into mammalian cells is difficult to quantitate and is not dependent on exogenously added cytosol, the use of this technique led to the discovery that a 70-kDa heat shock protein (HSP70) is required for import of PTS1-containing proteins (138), demonstrated a PTS1-protein import deficiency in a Zellweger syndrome cell line, and showed that protein unfolding is not a prerequisite for peroxisomal matrix protein import (136, 137).

The most robust in vitro import assays currently in use depend on semipermeabilized mammalian cells in which holes in the plasma membrane, but not the peroxisomes, allow the diffusion of the import substrate, antibodies, and cofactors into the intracellular milieu surrounding the peroxisomes. In one of these systems, developed by Wendland and Subramani (144) and Rapp et al. (95), the import is time, temperature, signal, ATP, and cytosol dependent. Import substrates include matrix proteins such as luciferase or human serum albumin coupled to a PTS1 (SKL) peptide. Import using this assay has been quantitated by counting the percentage of cells that exhibit peroxisomal targeting of the substrate (M. Fransen and S. Subramani, unpublished data).

An alternative method used by Terlecky and Subramani (unpublished data) is one in which biotinylated luciferase is used as the import substrate with semi-permeabilized human cells. After import of the substrate into peroxisomes, the biotin groups on unimported luciferase are sequestered with avidin, and the excess avidin is bound to biocytin. Both these reagents cannot penetrate the peroxisomal membrane. The luciferase is released from the cells and captured in microtiter wells using antiluciferase antibodies, and the biotin groups remaining on luciferase (corresponding to the imported substrate) are quantitated by an enzyme-linked immunoabsorbent assay system employing streptavidin conjugated to horseradish peroxidase.

The ability to quantitate import has led to the discovery that mammalian 40-kDa heat shock protein (HSP40), HSP70, and Pex5p are cytosolic components necessary for import of PTS1-containing proteins into peroxisomes. Membrane components include Pex2p (PAF1), a zinc-binding protein, deficient in certain human patients with Zellweger syndrome (120). Although mammalian homologs have been identified in expressed sequence tag databases for docking proteins (Pex13p and Pex14p) that bind Pex7p and/or Pex5p, the roles of these mammalian proteins have not yet been demonstrated in vitro.

Import of matrix proteins has been demonstrated to be a two-step process. Binding of import substrates occurs at low temperature, in the absence of ATP, and translocation into the organelle requires higher temperature and ATP hydrolysis (58, 144).

B. Import of Membrane Proteins

The import of membrane proteins into peroxisomes has also been examined in vitro using purified rat liver

peroxisomes (20, 57). Two rat proteins, PMP22 and PMP70, radiolabeled with [^{35}S]methionine by translation in vitro, have been shown to bind to these purified peroxisomes, and they then get inserted into the membrane over a 30-min period as judged either by resistance to protease digestion or to extraction by alkaline sodium carbonate. For PMP22, 75% of the radiolabeled protein was bound to peroxisomes, and about one-half of this was inserted into the membrane (20) at 26°C. Import was dependent on time and temperature, but not on ATP, GTP, or *N*-ethylmaleimide. Low temperature allowed binding but no membrane insertion. Pretreatment of the cells with proteases reduced binding to a low level and completely inhibited membrane insertion. A proteinaceous receptor is presumed to be involved in the insertion of peroxisomal membrane proteins into the organelle membrane, but it has not been identified.

The question of topology of the protein in the membrane has not been addressed in vivo or in vitro. This is an important issue that deserves further attention. Is it the mPTS or the transmembrane segments that serve as the primary determinants of topology in the peroxisomal membrane? One hypothetical model is that the mPTS essentially acts like a regular PTS in that it is translocated to the matrix side of the peroxisome (111). Starting from this nucleation point, transmembrane domains that act essentially as stop-transfer sequences might get inserted in the membrane moving outward toward the NH_2 and COOH termini of the protein. A specific prediction of this model is that it is the relative location of the mPTS in the protein, and not that of the transmembrane segments, that plays a key role in the overall topology of the protein in the peroxisomal membrane.

V. PROTEINS AND COFACTORS REQUIRED FOR IMPORT OF PEROXISOMAL TARGETING SIGNAL 1-CONTAINING PROTEINS

Hydrolyzable ATP, the PTS1 sequence, as well as cytosolic and membrane components are necessary for import. The functions of the cytosolic and peroxisome-associated components are described below.

A. HSP70 and HSP40

These are the *Escherichia coli* DnaK and DnaJ homologs in mammalian cells. Evidence for their requirement comes from experiments in which specific antibodies directed against these proteins inhibit import of HSA-SKL into peroxisomes of semipermeabilized cells. The traditional roles of these proteins are to keep other proteins unfolded as they are translated so that they can be transported in the unfolded state across organellar membranes. However, because the import of peroxisomal matrix proteins can occur even when unfolding is prevented (see sect. VI), the functional requirement for HSP70 and HSP40 in peroxisomal protein import must be different from that for protein import into other organelles.

Several suggestions have been made for the action of these chaperones (110). *1*) The chaperones may stabilize the exposed PTS1 sequence during thermal fluctuations of the polypeptide chain that unfold the COOH-terminal end of the protein, without unfolding the entire polypeptide. The PTS1/HSP complex may then render the PTS1 sequence more accessible to the PTS1 receptor. *2*) The chaperones may serve to facilitate assembly of the PTS1/Pex5p complex with docking proteins on the surface of the peroxisome. *3*) HSP70 and HSP40 may act, in a manner analogous to clathrin-uncoating ATPase, to disassemble the PTS1/Pex5p/docking protein complex on peroxisomes. Specific tests are being designed to determine whether any of these models is correct.

In an *S. cerevisiae* mutant lacking Pas22p (Pex no. not yet assigned), matrix protein import is affected (31). This protein has a domain with homology to DnaJ. It is unclear whether this protein functions like the HSPs mentioned above or whether it acts in a different manner to facilitate import.

B. PTS Receptors

The PTS1 receptor, encoded by the *PEX5* gene, is a member of a family of proteins containing one or more tetratricopeptide (34 amino acid) repeats (TPR). It has been characterized from *P. pastoris*, *S. cerevisiae*, *H. polymorpha*, *Y. lipolytica*, *C. boidinii*, and *Homo sapiens* (see Ref. 110). It binds the PTS1 sequence specifically and directly with a dissociation constant of ~450–500 nM (119). The binding occurs either when the receptor is soluble (or cytosolic) or when it is bound to peroxisomes, and the binding of the PTS1 sequence is to the TPR repeats (119). Although Pex5p from different species contains seven or eight TPR motifs, only three of these repeats suffice for the binding of the PTS1 sequence (119). The same receptor binds to the canonical SKL peptide, as well as PTS1 variants (42). The receptor also interacts with the PTS1 sequence in the yeast two-hybrid system (13, 42). Its involvement in import is shown by the fact that antibodies to the human Pex5p inhibit the import of PTS1-containing proteins in an in vitro import assay using streptolysin O-permeabilized CHO cells (147).

In mammalian cells, the PTS1 receptor is 90–95% cytosolic and 5–10% peroxisome associated (23, 150). This observation and the ability of in vitro-translated human Pex5p to bind the PTS1 peptide in the absence of peroxisomes led to the suggestion that the receptor binds its cargo in the cytosol and then shuttles from the cytosol to the peroxisome. The receptor shuttling was shown by the accumulation of human Pex5p on peroxisomes under

conditions that inhibit peroxisomal protein translocation (16°C or ATP depletion). The receptor returns to the cytosol when translocation is restored (at 37°C or by ATP addition) and reaccumulates on the peroxisomes when translocation is inhibited. This accumulation of Pex5p on the peroxisomes is dependent on peroxisomes because it fails to occur in a human patient cell line (CG9) that lacks peroxisomes. These results provide strong support for receptor cycling between the cytosol and the peroxisomes during the import of PTS1-containing proteins (24).

What mediates the association of the receptor with the surfaces of the peroxisome? Once associated with the peroxisomal membrane, the yeast and mammalian receptors are tightly bound and cannot be extracted even by alkaline sodium carbonate, suggesting either insertion in the membrane or tight association with peroxisomal integral membrane proteins (42, 119, 147, 150). Supporting the latter is the fact that a docking protein, Pex13p, has been identified for Pex5p in *S. cerevisiae* (34, 36) and in *P. pastoris* (48). Pex13p is a peroxisomal integral membrane protein. A human homolog of this protein has been found, but its function has not been studied (48). However, evidence has been gathered for receptor accumulation on the surface of the peroxisomes during in vitro import in permeabilized human cells (S. Terlecky and S. Subramani, unpublished data), as well as in cultured human cells (24). Whether this is mediated by the human homolog of Pex13p or some other protein (e.g., Pex14p homolog) is unclear at present.

One of the puzzling observations relates to the exact subcellular location of Pex5p. It has been reported to be associated predominantly with the cytosolic face of the peroxisomes (119) and primarily in the cytosol in *P. pastoris* (48). Its location is very much dependent on the growth state of the cells and the precise methods and conditions used for the preparation of organelle pellets, which often assume, without confirmation, that anything that remains in the supernatant after a 27,000-*g* spin is cytosolic (48). There is clearly a cytosolic as well as a peroxisome-associated pool, consistent with the concept of a shuttling receptor, but the relative fractions in these two pools are what is under debate, but this can vary. In *S. cerevisiae* and *H. polymorpha*, the receptor is mainly cytosolic (30, 128), and in *Y. lipolytica*, it is mostly intraperoxisomal (116). The multiple locations of the receptor were rationalized by a model explaining the dynamics of the receptor (93). Hypothetical proteins were proposed to function as "gatekeepers" to obstruct the entry of receptors into the matrix in association with their cargo, or as "recyclers" to facilitate the return of the receptor to the cytosol. Physiological conditions affecting the relative concentrations of receptor, receptor-docking proteins, and the gatekeeper or recycler proteins, could then affect the localization of the receptor such that it might appear cytosolic, or on the peroxisome membrane, or even within the organelle. Support for such a dynamic receptor model has been provided by the experiments of Dodt and Gould (24) using a variety of human patient cell lines (e.g., CG7). What remains unanswered is what happens to the receptor if and when it enters the peroxisome lumen. Is it degraded, or is it actively reexported out of the peroxisome to the cytosol for another round of import?

The PTS2 receptor, encoded by the *PEX7* gene, has been identified from *S. cerevisiae* (75, 96, 154, 155), *Kluyveromyces lactis* (83), *P. pastoris* (Elgersma et al., unpublished data), mice (12), and humans (12, 83, 92). It contains characteristic repeats that justify its classification as a member of the family of proteins containing WD repeats. The receptor binds the PTS2 sequence specifically as suggested by multiple lines of evidence (96, 155). A temperature-sensitive mutation in the PTS2 sequence of peroxisomal thiolase in *S. cerevisiae* is suppressed by overexpression of ScPex7p, the PTS2 sequence interacts with ScPex7p in the yeast two-hybrid system, the receptor coimmunoprecipitates with proteins containing the PTS2 sequence, and the receptor binds to a GST-PTS2 fusion in vitro. Furthermore, like the PTS1 receptor, Pex7p also binds its cargo in the absence of peroxisomes.

The subcellular location of this receptor has been determined in *S. cerevisiae* (75, 155) and in human cells (12) only with epitope-tagged Pex7p constructs and is controversial. Marzioch et al. (75) claimed that the receptor is principally cytosolic and that the small amount that is associated with peroxisomes could be increased by the overexpression of thiolase, a PTS2-containing protein. This observation formed the basis of a model in which the PTS2 receptor recruits cargo in the cytosol and shuttles it to the peroxisome. Consistent with this is the fact that human Pex7p, epitope-tagged at its NH_2 terminus and expressed from the strong cytomegalovirus promoter is also cytosolic (12). The alternative model, proposed by Zhang and Lazarow (154, 155), is that most of the Pex7p (tagged with a different epitope) is intraperoxisomal and that the NH_2-terminal 56 amino acids of the protein contain a new PTS. This result suggests that the PTS2 receptor is first targeted to the peroxisome matrix and then acts from within the matrix to pull PTS2-containing proteins into the organelle.

An unambiguous determination of the subcellular location of the *S. cerevisiae* PTS2 receptor awaits the generation of antibodies to the native protein. In *P. pastoris*, antibodies to the endogenous, wild-type Pex7p show that 10–30% of this protein is intraperoxisomal and the rest is cytosolic. Efforts are underway to confirm whether the peroxisome-associated Pex7p is in the matrix or on the cytosolic face of the peroxisome (Elgersma et al., unpublished data).

Many proteins of the TPR and WD families interact with each other. Such an interaction was proposed to explain why a human patient lacking Pex5p was deficient

not only in import via the PTS1-dependent pathway as expected, but also for import by the PTS2-dependent pathway (93). Evidence for such interactions between the PTS1 and PTS2 receptors has been provided in *S. cerevisiae* using the yeast two-hybrid system (96), but we do not know whether this interaction is direct or requires a bridging protein such as Pex14p (see below), which is a docking protein for both Pex5p and Pex7p. The two-hybrid interaction could be tested in a *pex14* mutant background to shed light on this question. In yeasts, the physiological role for an interaction between Pex5p and Pex7p is unclear because deletion of each of the genes encoding these receptors affects only PTS1-specific or PTS2-specific import, but not both.

C. Peroxisomal Docking Proteins for PTS Receptors

Two classes of docking proteins have been described in yeast. As mentioned in section v*B*, Pex13p is a peroxisomal integral-membrane protein that interacts with Pex5p in a yeast two-hybrid system and using a ligand-blot format (30, 36, 48). This protein contains an SH3 domain that faces the cytosol and is required for the binding of Pex5p. No interactions have been detected between Pex13p and Pex7p, yet *pex13*Δ strains are affected in import via both the PTS1- and PTS2-import pathways, but not in the import of peroxisomal membrane proteins. There is no explanation, at present, for why the PTS2 pathway is affected in *pex13*Δ cells.

Another peroxisomal peripheral membrane protein, Pex14p, interacts with both the PTS1 and PTS2 receptors, with Pex13p through the SH3 domain on the latter, and with Pas9p (Pex# not yet assigned), another peroxisomal integral membrane protein (1) (Fig. 3). Mutations in Pex14p affect both matrix protein-import pathways, as expected (1, 62). Surprisingly, overproduction of Pex14p in wild-type *H. polymorpha* cells resulted in a peroxisome-deficient phenotype. Numerous small vesicles containing the membrane components Pex14p and Pex3p but devoid of matrix contents accumulated in cells, and most of the matrix proteins were found in the cytosol. Thus the stoichiometry of Pex14p relative to other components is important for peroxisome biogenesis and/or protein import (62).

VI. PROTEIN UNFOLDING DURING PEROXISOMAL MATRIX PROTEIN IMPORT

In cells expressing subunits (containing or lacking either the PTS1 or PTS2 sequences) of an oligomeric protein, multimeric species with subunits lacking a PTS have been found inside the peroxisomes in association with subunits containing a PTS (33, 46, 78) (Fig. 3). Completely

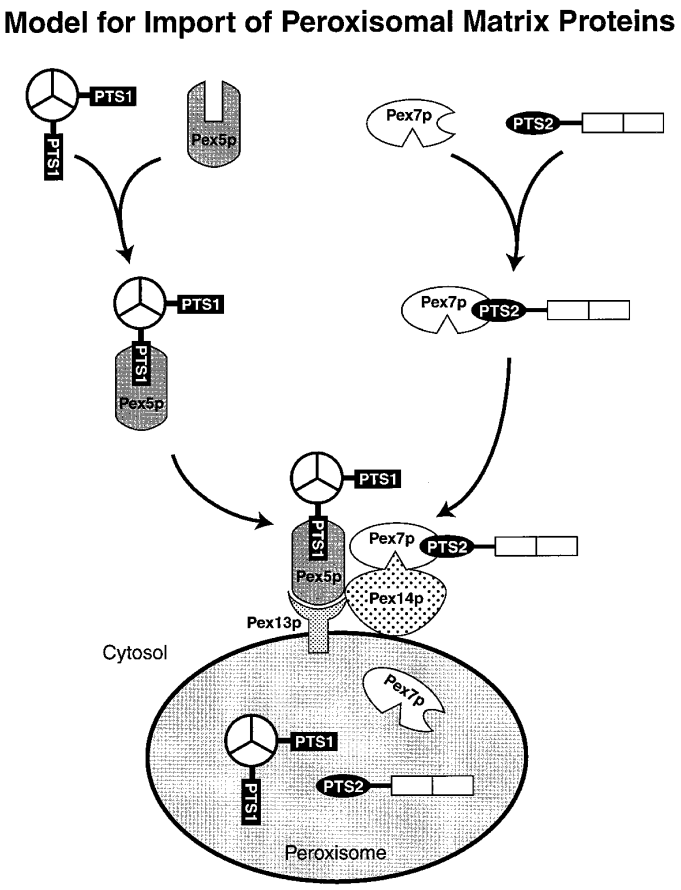

FIG. 3. Model for import of peroxisomal matrix proteins. Proteins with COOH-terminal PTS1 and NH_2-terminal PTS2 sequences are recognized by their cognate PTS receptors, Pex5p and Pex7p, respectively, in cytosol. These PTS receptors bound to cargo then interact with docking proteins in peroxisomal membrane. Pex5p interacts with Pex7p, Pex13p, and Pex14p (1, 30, 36, 48, 96). Pex7p interacts with Pex5p and Pex14p (1, 96). Because Pex7p is also found within peroxisomes in *Saccharomyces cerevisiae*, it is also shown in organelle matrix, where it might act to facilitate import of PTS2-containing proteins as suggested by Zhang and Lazarow (154, 155). In *Yarrowia lipolytica*, a homolog of Pex5p is primarily intraperoxisomal (not shown in figure; Ref. 116). Protein unfolding is not required for import of matrix proteins into peroxisomes. This is depicted by transport of mixed oligomers (trimers in case of chloramphenicol acetyltransferase, or dimers in case of thiolase), either containing or lacking PTS1 or PTS2 sequences (46, 78).

folded polypeptides, disulfide-bonded and chemically cross-linked proteins or HSA-SKL conjugated to gold particles are imported into the peroxisome matrix (137). Protein unfolding is also not required for glycosomal protein import because the import of a dihydrofolate reductase (DHFR)-phosphoglycerate kinase fusion protein into peroxisomes is not inhibited by the folate analog aminopterin, which stabilizes the folded conformation of DHFR (52). Similar "piggyback" import of oligomeric proteins has been reported recently during the targeting of PTS1-containing proteins into plant glyoxysomes (68). These experiments demonstrate convincingly that protein unfolding is not a prerequisite for the import of proteins into the microbody (peroxisomes, glyoxysomes, and glycosomes) matrix.

This result, which is distinct from that for import of proteins across the ER and mitochondrial membranes, does not necessarily imply that all peroxisomal multimeric proteins enter the organelle only as preassembled oligomeric complexes. A substantial number of peroxisomal matrix proteins are oligomeric (cited in Ref. 66), with some (e.g., alcohol oxidase) being as large as 640 kDa. There is, in fact, evidence in *H. polymorpha* and in *C. boidinii* that monomers of alcohol oxidase are imported into peroxisomes (4), where they assemble with FAD, in the presence of an undefined peroxisome assembly factor (39, 40), to form enzymatically active octamers.

VII. NEW INSIGHTS INTO THE BIOGENESIS OF PEROXISOMAL MEMBRANE PROTEINS

The biogenesis of peroxisomal integral membrane proteins is an area that requires a lot more attention. Novikoff and Shin (86) suggested, based mainly on the juxtaposition of ER and peroxisomal membranes, that all peroxisome membrane and matrix proteins were derived from the ER. This model was revised by Lazarow and Fujiki (66), who recognized that many peroxisomal matrix proteins were made in the cytosol and transported directly to the peroxisome posttranslationally. Furthermore, no glycosylated peroxisomal membrane or matrix proteins have been found, and a few membrane proteins are synthesized in the cytoplasm and imported posttranslationally to peroxisomes (9, 44). These results led to the suggestion that all peroxisomal membrane and matrix proteins were imported from the cytosol to the peroxisomes (66). Consistent with a part of this model is the fact that matrix proteins lacking PTSs are cytosolic.

Similar experiments performed with membrane proteins do not produce the unambiguous results observed with matrix proteins. As mentioned earlier, the *C. boidinii* has an mPTS located between amino acid 225 and 244 of the 423-amino acid protein (76). This segment lies in a loop between transmembrane segments 4 and 5 of the protein. Deletion mutants fused to DHFR, such as 1–199DHFR or DHFR268–423, lacking the mPTS and transmembrane domains 4 and 5 are cytosolic. Likewise, a mutant lacking amino acids 200–267 is cytosolic and mitochondrial, and a fusion protein consisting of amino acids 1–224 of PMP47 with the epitope (HA) from influenza virus hemagglutinin is also cytosolic. These results are consistent with the import of PMP47 from the cytosol directly to the peroxisome.

Another peroxisomal membrane protein, ScPex15p from *S. cerevisiae*, behaves quite differently. It is a 383-amino acid protein with an mPTS-like residing between amino acids 354 and 372. Deletion of the last 30 amino acids of this protein targets the resulting polypeptide to the ER, but removal of the last 12 amino acids still targets it to the peroxisomes. Furthermore, wild-type Pex15p may be targeted to the peroxisome via the ER (Elgersma et al., unpublished data), and the COOH-terminal 34 amino acids of this protein are not sufficient to target passenger proteins to the peroxisome. Thus this protein differs from PMP47 in that removal of the mPTS targets it to the ER rather than stranding the protein in the cytosol.

The different behaviors of PMP47 and ScPex15p lacking their respective mPTSs suggest strongly that there are two distinct classes of mPTS (111). The first one, referred to hereafter as mPTS1 and exemplified by the mPTS in PMP47, would target proteins from the cytosol directly to the peroxisome. The second, called mPTS2 and exemplified by that found in ScPex15p, would target proteins to the peroxisome from the ER. The topology of this mPTS2 is such that it faces the lumen of the ER and eventually resides in the matrix of the peroxisome (Elgersma et al., unpublished data).

The notion that certain peroxisomal membrane proteins may travel to this organelle via the ER is not contradicted by the existing data. Formal proof exists, in the form of pulse-chase experiments, for only a single mammalian protein, PMP70, showing that it appears first in the cytosol and can subsequently be chased to the peroxisomes (57). One other mammalian protein, PMP22, appears to be imported posttranslationally into purified peroxisomes in vitro (20). In this case, assuming that the peroxisomes are free of ER-derived microsomes, one can conclude that the protein goes from the cytosol to the peroxisome. For a few other membrane proteins, translation on cytosolic polysomes has been used to infer import from the cytosol directly to the peroxisome (9, 44, 113), but this may or may not be true.

An equivalent amount of circumstantial evidence shows that some peroxisomal membrane proteins may go to the peroxisome via the ER. Part of the evidence comes from the data that in *S. cerevisiae*, Pex15p, may go to the peroxisomes via the ER (Elgersma et al., unpublished data). Another protein, rat PMP50, is made on membrane-bound polysomes and is found associated with both the ER and the peroxisomes (9). These data could be perfectly consistent with the two-mPTS hypothesis, which suggests two distinct pathways for the import of membrane proteins to peroxisomes, one directly from the cytosol and the other via the ER.

The current view, that peroxisomes are derived from preexisting ones, predicts that in mutant cells lacking all peroxisome remnants it should be impossible to regenerate a peroxisome de novo. This prediction is apparently violated by the experimental observation that several yeast mutants (*pex3* of *S. cerevisiae*, *H. polymorpha*, and *P. pastoris*; and type I mutants of *S. cerevisiae*) lacking morphologically and biochemically detectable peroxisomes can still be complemented by the respective genes to restore peroxisomes (3, 37, 54, 149). A *H. polymorpha* mutant temperature sensitive for peroxisome assembly

has also been shown to recover peroxisomes upon shift from the nonpermissive to permissive temperature (142). Although one can argue that technical limitations may have hampered the detection of peroxisome remnants in these mutants, the alternative possibility is that peroxisomes may have been regenerated from some other reservoir of intracellular membranes, such as a segment of the ER.

Two proteins, Pex1p and Pex6p, involved in peroxisome biogenesis in *P. pastoris* are localized to vesicular structures distinct from peroxisomes (K. Faber, J. Heyman, and S. Subramani, unpublished data). These proteins interact with each other in the yeast two-hybrid system, and physical evidence for complex formation has been obtained in the presence of ATP, whose hydrolysis is not required for the formation of the complex. The two proteins coimmunoprecipitate with each other, and temperature-sensitive mutations in Pex1p are suppressed by overexpression of Pex6p. It has been proposed that these two proteins interact with each other to cause fusion of vesicles that import other proteins to become peroxisomes, or which fuse with preexisting peroxisomes to allow their growth. The origin of the vesicles is unknown, but one source that has been suggested is the ER (111). According to this view, peroxisomes do not arise simply by budding and fission of preexisting organelles. It remains to be seen whether the involvement of the ER and vesicular structures are required only for peroxisome proliferation in response to nutritional cues, or whether such a mechanism also operates during constitutive division for the organelle (see sect. XI).

VIII. HUMAN PEROXISOMAL DISORDERS

The disorders of peroxisome biogenesis are defined as a genetically heterogeneous subset of autosomal recessive diseases characterized by multiple defects in peroxisome function (reviewed by Refs. 67 and 148). These diseases are classified into three groups. The first one, *group A*, comprising Zellweger syndrome, infantile Refsum disease, and neonatal adrenoleukodystrophy, is characterized by severe neurological and hepatic dysfunction, craniofacial abnormalities, and hypotonia, leading inevitably to early death. Patients accumulate phytanic acid and very-long-chain fatty acids (VLCFA) in the circulation and are deficient in the synthesis of plasmalogens, a class of ether phospholipids. The medical prognosis for affected individuals is bleak, with little chance of survival beyond the age of ten. Patients in *group B*, exemplified by the disease rhizomelic chondrodysplasia punctata, display severe growth defects, rhizomelia, cataracts, epiphysial calcifications, and ichthyosis. These individuals accumulate higher levels of phytanic acid and have normal levels of VLCFA, but lack plasmalogens. The *group C* disorders are characterized by a range of milder symptoms depending on the gene affected. *Group C* diseases can be explained by mutations in single genes compromising the activity or localization of single enzymes. In contrast, the disorders in *groups A* and *B* affect the location of multiple peroxisomal matrix proteins. Although patients with many of the disorders die, the ability to propagate their cells in the laboratory has provided insight into the cellular defects. Somatic cell hybridization studies place *group A* disorders into nine complementation groups (CG1-CG4 and CG6-CG10), and most *group B* cells belong to CG11 (14, 81, 90, 98, 105).

IX. PROTEIN IMPORT DEFICIENCIES IN HUMAN PEROXISOMAL DISORDERS

Although patients with Zellweger were first described as early as 1964, the first inkling that this was a disease connected with peroxisomes came from the observation of Goldfischer et al. in 1973 (46a). However, it was not until the late 1980s that it became clear that many of these patient cell lines contained peroxisome membrane "ghosts" while lacking several matrix proteins (101). Direct evidence for a protein import defect in these cells did not come until Walton et al. (136) and Wendland and Subramani (145) used microinjection and in vitro assays using SLO-permeabilized cells to show a defect in the import of PTS1-containing proteins in many of these cell lines. These studies were subsequently extended in a more comprehensive manner for both PTS1- and PTS2-containing proteins by Motley et al. (84) and then by Slawecki et al. (106). These studies revealed that, like the situation with the yeast *pex* mutants, the human cells could be divided into four groups. These include *1*) those deficient in PTS1 import alone (CG2); *2*) those affected in PTS2 import only (CG11); *3*) those impaired in import of both PTS1- and PTS2-containing proteins, but not peroxisomal membrane proteins (CG1, CG3, CG4, CG6-CG8, CG10); and *4*) those affected in the biogenesis of peroxisomal membrane, as well as PTS1- and PTS2-containing proteins (CG9). Thus there is an excellent parallel between the cellular import-deficiency phenotypes of the yeast mutants and the human patient cell lines, and the paradigm that the yeast mutants are excellent models of the human peroxisomal disorders is well established (112).

X. CONSERVATION OF *PEX* GENES AND THEIR ROLE IN HUMAN DISEASE

Despite the fact that the first couple of *pex* mutants isolated in *S. cerevisiae* appeared to reflect the protein import defects of the human disorders, a disturbing feature was the lack of peroxisomal ghosts or remnants in several type I mutants (37). This concern vanished, however, when it became apparent that most *pex* mutants

(*pex1*, *pex2*, *pex5*, *pex6*, *pex7*, *pex10*, *pex12*, *pex13*) of *P. pastoris* did indeed exhibit peroxisome ghosts analogous to those seen in human patient cell lines (48, 51, 53, 60, 61, 70, 77, 109, 140). Subsequently, the use of antibodies to peroxisomal membrane, rather than matrix, markers provided better reagents for the detection of organelle remnants, and some *S. cerevisiae* mutants (e.g., *pex1*) that lacked detectable remnants using electron microscopy (153) were found to contain such structures using fluorescence-based assays for membrane markers (91). Similar remnants were found in several mutants (*pex1*, *pex5*, *pex6*, and *pex14*) of *H. polymorpha*, and these could be detected more readily by overproduction of HpPex10p (62, 128, 134), which promotes peroxisome proliferation (118).

The yeast *PEX* genes (Table 1) showed a remarkable conservation in evolution, suggesting immediately that the search for human homologs of the yeast *PEX* genes might lead the way to the genes compromised in the human peroxisomal disorders. What drove home the link between the yeast and human genes was the discovery that, as predicted from the phenotype of the appropriate *pex5* mutants in the two systems, the human *PEX5* gene (23, 42, 147) identified by its homology to *PpPEX5*, and by its interaction with the PTS1 sequence, was deficient in human CG2 (23, 147).

Progress in the cloning of *PEX* genes from several yeasts and in the identification of human ESTs homologous to the yeast counterparts has led very quickly to additional human genes affected in peroxisomal disorders (12, 15, 83, 92, 152; reviewed in Ref. 112). Not surprisingly, all the human genes affected in peroxisomal disorders have yeast homologs. Because at least 17 *PEX* genes have already been described in the literature and several other are being characterized in multiple labs, we are faced with the exciting prospect that in the next 3–5 years the yeast genes will lead to identification of most of the genes involved in human peroxisomal disorders. Cloning of human genes involved in peroxisomal disorders began several years ago by direct complementation of CHO mutants deficient in peroxisome assembly (124, 125) but is likely to be superseded by homology-based cloning strategies that identify and track down mammalian EST homologs of yeast genes known to be involved in peroxisome biogenesis (22). This approach is especially valuable because the small sizes of the families affected with peroxisomal disorders make linkage mapping and positional cloning impossible.

XI. CONSTITUTIVE AND REGULATED DIVISION OF PEROXISOMES

Like all other subcellular organelles, peroxisomes must divide and be segregated to daughter cells during cell division. Organelles such as the peroxisome have the capacity to divide and proliferate, or be degraded in response to nutritional and environmental cues. These processes have been proposed to be constitutive or regulated (72). Constitutive division is required for maintenance of organelle homeostasis (in terms of number and volume) during cell division or as organelles age and undergo autophagy. Regulated division refers to the proliferation of an organelle, in response to external signals, and need not be associated with mitosis.

One protein involved in regulation of peroxisomal division has been defined in *C. boidinii* and *S. cerevisiae* (35, 47, 73, 80, 100). This protein, recently renamed Pex11p, is associated with the matrix face of the peroxisome membrane but is not an integral membrane polypeptide. Disruption of this gene results in fewer and larger peroxisomes per cell, rather than several smaller ones. As a result, peroxisome inheritance to daughter cells is affected. Yeast cells grown on peroxisome-proliferating media accumulate with multiple buds, but the buds have no peroxisomes. However, there appears to be no peroxisome inheritance defect upon growth on glucose because the peroxisomes are invariably fewer and smaller under these conditions. This suggests that constitutive peroxisome division is normal. It is interesting to note that *pex11*Δ strains have no peroxisomal protein import defect.

Overproduction of Pex11p results in the hyperproliferation of peroxisomes, all of which are smaller, less dense, and more abundant than normal. It has been demonstrated recently that this protein can cause the fragmentation of large peroxisomes to smaller ones. The protein exists as a monomer in proliferating peroxisomes, and primarily as a dimer in mature peroxisomes. It has been proposed that it functions as a monomer, playing a role in regulated peroxisome division. It homodimerizes in a redox-sensitive manner, acquires intermolecular disulfide bonds, and becomes nonfunctional. Consistent with this idea is the fact that mutation of a specific cysteine, believed to be involved in disulfide-bond formation, increases peroxisome proliferation. However, the peroxisomal environment may not be an oxidizing one, leaving open the possibility that the disulfide bonds were generated during the experimental procedures used and that the cysteines may play some other essential role in the function of Pex11p (J. Goodman, personal communication). This new evidence regarding the function of Pex11p suggests that the peroxisome inheritance defect observed in *pex11* mutants grown on oleate, and not on glucose, is a secondary consequence of an impairment in regulated division. The precise mechanism by which Pex11p regulates peroxisome division from the inside of the organelle is unknown and remains a topic of great interest.

Another protein, Pex16p, from *Y. lipolytica* produces enlarged peroxisomes upon overexpression, which is the exact opposite of what happens with Pex11p (28).

XII. PEROXISOME DEGRADATION BY AUTOPHAGY

In the last decade, much has been learned about the biogenesis of most subcellular organelles, including the peroxisome. However, little is known about the selective degradation of organelles. The elucidation of the mechanism of organelle degradation is essential for an understanding of the process of organelle homeostasis, a process that is likely to ensure that a cell directs its energies optimally toward specialized functions (as in differentiated cells) or for survival.

Protein degradation in eukaryotic cells occurs in the cytosol and in virtually all the subcellular compartments. Lysosomes (or vacuoles in yeast) are involved in two types of autophagic phenomena (25, 74). The first, called microautophagy, is the sequestration of small portions of cytoplasm (including, for example, glycogen granules and ribosomes) by invagination of the lysosomal membrane or by wrapping of a flaplike protrusion (25). The second process, called macroautophagy, refers to the sequestration of organelles and cytosol within vesicles of the lysosomal system. These processes are present in virtually all eukaryotic cells and constitute the major pathway for the degradation of cellular proteins. The sequestration of organelles by macroautophagy requires ATP and can be inhibited in mammalian cells by 3-methyladenine, okadaic acid, and phosphodiesterase inhibitors, such as theophylline (55, 56, 102, 104).

Both microautophagy and macroautophagy are stimulated by nutrient deprivation and may be a mechanism for recycling redundant cellular materials for survival and growth. In general macroautophagy, autophagosomes (probably derived from the ER membrane) first sequester peroxisomes, mitochondria, ER, ribosomes, and cytosolic components for degradation. The autophagic process of degradation consists of sequestration of the cytosol and organelles by autophagosomes (74). This is followed by the fusion of the autophagosomes with late endosomes or lysosomes. Finally, degradation of the vacuolar contents is observed to form a residual body (25).

In addition to this general macroautophagy, selectivity of organelle degradation has also been observed. For example, phenobarbital-induced ER or clofibrate-induced peroxisomes in mammalian cells are selectively degraded by macroautophagy upon removal of the drug (10, 71). It is unclear at present whether selective and general macroautophagy represent different manifestations of the same overall process.

The autophagic degradation of organelles has been demonstrated in *H. polymorpha*, *P. pastoris*, and *C. boidinii* (11, 127, 132). The autophagosomes involved in macroautophagy have been characterized ultrastructurally in *S. cerevisiae* (2). In this yeast, general macroautophagy is induced by nutrient deprivation and inhibited by phenylmethylsulfonyl fluoride or by mutations in genes affecting lysosomal proteinases (e.g., proteinase A and B). The distinct morphology of the autophagic bodies that accumulate under nutrient-deprivation conditions has been used to isolate *S. cerevisiae* mutants that are deficient in the accumulation of these autophagic bodies. These autophagy (*apg*) mutants fall into at least 15 complementation groups and are distinct in their phenotypes from the *vps* mutants affecting vacuolar biogenesis (123). Cloning of some of the *APG* genes, however, suggested that many of them may be involved in cellular signaling mechanisms rather than in autophagy per se. Consequently, a new assay was devised for monitoring autophagy. This method utilizes a modified vacuolar alkaline phosphatase expressed as an inactive precursor in the cytosol (85). Under starvation conditions, this precursor is taken up by autophagy into the vacuoles, and the processing of the precursor in the vacuole results in the accumulation of phosphatase activity, providing thereby a facile assay for autophagy. This scheme could be used to identify new autophagy mutants.

In both *H. polymorpha* and *P. pastoris* there is a massive proliferation of peroxisomes upon growth on methanol. Under these conditions, the peroxisomes occupy 50–80% of the total cell volume. Concomitant with this is the induction of peroxisomal enzymes, alcohol oxidase, dihydroxyacetone synthase (DHAS) and catalase, involved in methanol utilization. Alcohol oxidase alone constitutes 25–50% of the total cell protein under these conditions. It is transported into peroxisomes where it forms the crystalline core. When the carbon source is changed from methanol to glucose or ethanol, there is a rapid loss of peroxisomes. In *H. polymorpha*, this leads to a full repression of alcohol oxidase synthesis. In *P. pastoris*, the synthesis of alcohol oxidase is depressed 10-fold when the methanol-grown cultures attain the stationary phase of growth. No further reduction of the synthesis of this protein is observed upon shift of the cultures to glucose medium. The half-life of DHAS and alcohol oxidase is >3 h in stationary phase cultures maintained in methanol. Upon shift to glucose, however, as much as 80% of alcohol oxidase and DHAS is degraded from both yeasts in <3 h, and the process is selective for peroxisome degradation (127). Ultrastructural studies have revealed that in both yeasts, the degradation of peroxisomes occurs in vacuoles, the yeast equivalent of mammalian lysosomes (51, 127, 132, 133).

The morphological and biochemical events of selective peroxisome degradation have been analyzed in detail (127, 132, 133). At present, two conditions are known to lead to a rapid turnover of alcohol oxidase-containing peroxisomes in *H. polymorpha*. This occurs *1*) after a shift from methylotrophic conditions to nonmethylotrophic conditions (132) and *2*) after irreversible inactivation of peroxisome function (99, 129).

The degradation process appears to be energy depen-

dent (132). The peroxisomes are degraded individually by means of a highly selective autophagic process, and the steps in degradation appear to be identical under both of the above conditions.

Peroxisome degradation in *H. polymorpha* is a rapid process. Generally, the total turnover of a single organelle is accomplished in 20–45 min (132). However, not all organelles of the peroxisome population present in a single cell are targeted for degradation. The large "mature" peroxisomes are rapidly degraded. The few import-competent "immature" peroxisomes escape degradation and subsequently appear to serve as the target organelle for newly synthesized peroxisomal proteins essential for growth in a new environment (133). These results strongly suggest that the organelles to be degraded are specifically tagged. Peroxisomes, although individually degraded, are not destroyed synchronously. In *pim* mutants of *H. polymorpha*, the cytosolic alcohol oxidase is not degraded, but the organelles are, suggesting that the tagging of the organelle for degradation must occur on some component of the peroxisomal membrane (129). Selective peroxisome degradation in *H. polymorpha* includes three distinct steps: *1*) sequestration of the organelle to be degraded by ER-derived membranous layers, *2*) fusion with the vacuoles, and *3*) degradation of the organellar contents in the vacuole.

P. pastoris grows on multiple carbon sources such as glucose, ethanol, and methanol. Upon transfer from glucose to methanol medium, cytosolic enzymes, such as formate dehydrogenase, as well as peroxisomal proteins (e.g., alcohol oxidase and DHAS), are induced to facilitate assimilation of methanol. Interestingly, upon shift back to glucose or ethanol, two distinct autophagic pathways of degradation are activated (126). These pathways differ in the signals they respond to, the mode of sequestration of peroxisomes (micro- vs. macroautophagy), requirement for protein synthesis, and ability to degrade cytosolic enzymes. A shift from methanol to ethanol medium results in the induction of a degradative pathway that is protein synthesis independent; involves the sequestration, fusion, and degradative steps defined earlier for macroautophagy; and degrades only peroxisomes and not cytosol. In contrast, the switch from methanol to glucose triggers an alternative process that is protein synthesis dependent, degrades cytosolic and peroxisomal enzymes, and involves engulfment of peroxisomes by fingerlike projections of the vacuolar membrane, in a process resembling microautophagy. Thus, among the methylotrophic yeasts, macroautophagy is involved in peroxisome turnover in both *H. polymorpha* and *P. pastoris*, but microautophagy is restricted to *P. pastoris*. Mutants deficient in glucose-stimulated autophagy (*gsa1* and *gsa2*) are unable to sequester peroxisomes in glucose-adapted cells, but proficient in the macroautophagic process in ethanol-adapted cells. However, mutants lacking the vacuolar proteinases A and B (PrA and PrB) are deficient in the vacuolar degradation steps common to both pathways (126).

XIII. MODE OF PEROXISOME INHERITANCE DURING MITOSIS

Two general modes of organelle inheritance have been recognized in previous studies on organelle inheritance.

The first of these is termed "stochastic" and is dependent on the existence of multiple, randomly dispersed copies of the organelle in the cytoplasm of the dividing cell. During cytokinesis, a certain number of organelles are distributed randomly to the two daughter cells (6, 139). Once cytokinesis is completed, the process of constitutive division would restore the organelle balance in each daughter cell. Mitochondria of many, but not all, organisms are segregated to daughter cells by this mechanism (6, 97).

The alternative strategy termed "ordered inheritance" is one in which the organelle is associated with some other cytoskeletal or structural element (e.g., the centriole or the mitotic spindle) and is partitioned along with the division and segregation of the underlying structure. Division of the vacuole in yeast obeys this mode of inheritance (143).

Recent data on the inheritance of peroxisomes labeled via the import of GFP-SKL fusions show that inheritance of this organelle in mammalian cells is stochastic and not ordered (151). Although all the peroxisomes in interphase cells are associated with microtubules, at the time of mitosis, the peroxisomes are not microtubule associated but are randomly dispersed in the cytoplasm. This causes their random distribution to daughter cells at the time of cytokinesis.

XIV. MICROTUBULE-DEPENDENT PEROXISOME MOVEMENT IN MAMMALIAN CELLS

Several recent studies using different mammalian cells have demonstrated that most peroxisomes are closely associated with microtubules in HepG2 (103), CHO (94), and CV1 cells (151). The microtubules may serve to disperse the peroxisomes uniformly in the cells and perhaps to restrict their movement.

The dynamic behavior of peroxisomes in living cells has been examined either by the microinjection of fluorescein-labeled luciferase into cultured CHO cells (94) or by expression of GFP-SKL fusions in monkey kidney cells (151). Two types of peroxisome movement distinguished by their velocities, net organelle displacement, energy dependence, and requirement for microtubules have been defined using CHO (94) and CV1 cells (151). About 90–95% of the organelles exhibit a slow, energy- and microtubule-

independent intracellular movement resulting in little or no net displacement. The average velocity observed is 0.013 μm/s, with a range of 0.009–0.02 μm/s. The remaining 5–10% of the organelles exhibit rapid, saltatory movements that are energy and microtubule dependent (94, 151). These exhibit average velocities of 0.12 μm/s, but the range varies from 0.05 to 0.9 μm/s. Peak velocities of as much as 0.75 μm/s and sustained velocities of up to 0.45 μm/s over 11.5 μm have been seen. The two populations of moving peroxisomes are interchangeable. The physiological relevance of the organelle movement, as well as the molecular basis of the movement (e.g., motor proteins involved), are not understood. It is also unclear why only 5–10% of the organelles move when essentially all of them are associated with microtubules.

XV. FUTURE PROSPECTS

It is remarkable how much progress has been made in our understanding of peroxisomal protein import and biogenesis in the last decade. What is very clear is that this organelle has many features about its biogenesis that make it unique. The role of the ER in peroxisome biogenesis and the details of the mechanism of protein import are areas of great promise. It is very likely that in the next 5 years the molecular mutations in most of the human peroxisomal disorders will be defined. The role of peroxisomes in mammalian development, the development of animal models of human peroxisomal disorders, and the investigation of the pathogenesis of these diseases will occupy us for another decade. The biochemical functions of the various peroxins will still take some time to understand. It will also be interesting to investigate how this organelle proliferates, how its homeostasis is maintained, how it moves, and why its intracellular movement is necessary.

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