Chapter 8

Transactions at the Peroxisomal Membrane

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1. INTRODUCTION

Peroxisomes are small organelles of eukaryotic cells with distinct contributions to cellular metabolism. Their manifestation and enzymatic content can vary depending on cell type or organism. This diversity is reflected by the different names for the organelles: peroxisomes, glyoxysomes, glycosomes. Based on conserved features of protein import and metabolic pathways, however, they are now considered to constitute one microbody family.

They were discovered by DeDuve and co-workers during biochemical fractionation studies on rat liver homogenates as small vesicles containing catalase and a number of H_2O_2 producing enzymes such as urate oxidase, D-amino acid oxidase and α-hydroxyacid oxidase (Baudhuin *et al.*, 1965).

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On the basis of this initial inventory the name peroxisomes was coined to denote the organelles. Furthermore, the enzymes were not thought to have an essential function in the cell and DeDuve even considered the possibility that peroxisomes constitute "fossil organelles" (DeDuve and Bauduin, 1966).

Our attitude towards peroxisomes is totally different now. Three important developments are responsible for this change in view:

i) In 1973 Goldfischer *et al.* reported that peroxisomes could not be detected in the cells of a patient with a severe clinical phenotype. This disease had been described before in the literature by Zellweger: the cerebro-hepato-renal or Zellweger syndrome. It underscored the importance of peroxisomal function and was the start of a still increasing list of human diseases related to peroxisome malfunction. They range from an almost total absence of the organelles to single enzyme deficiencies. The study of these diseases has been instrumental in the discovery of the various processes in human metabolism in which peroxisomes partake. These include the ß-oxidation of long chain and very long chain fatty acids, the first steps in plasmalogen biosynthesis (lipid components of membranes), bile acid and cholesterol biosynthesis etc. (Lazarow and Moser, 1994; Mannaerts and Van Veldhoven, 1993; Van den Bosch *et al.*, 1992). Apparently, the first enzymes discovered by DeDuve *et al.* with the then available enzymatic assays, were an unfortunately unrepresentative lot.

ii) Microbodies had a much better start in the research on other kingdoms of nature since clear and essential metabolic pathways were confined to the organelles. For instance, the enzymes of the glyoxylate cycle which allow fungi, protozoa, nematodes and plants to convert fat into carbohydrate, largely reside in microbodies called glyoxysomes (Beevers, 1969). Certain fungi that are specialized to grow on alcohols, alkanes or fatty acids, harbour the necessary enzymes in their microbodies. This immediately illustrates how important microbodies are to these organisms, but also demonstrates the versatility and specialization of microbodies. It makes it understandable that the unity among the microbody family was not appreciated immediately after their discovery.

iii) In recent years various yeasts, *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris* and *Yarrowia liplytica* have been used for a genetic approach to study the various processes involved in the maintenance, biosynthesis and prolifer-

ation of peroxisomes (Subramani, 1998; Elgersma and Tabak, 1996a). This has created a wealth of new information, experimental tools and mutants that can serve as models to study the various human diseases.

2. THE ISOLATION OF YEAST MUTANTS DISTURBED IN PEROXISOME FUNCTION

A significant increase in our knowledge about the biogenesis of peroxisomes came from the genetic approach using a variety of yeasts. In all these yeast species the peroxisomal compartment can enormously vary in volume and number depending on growth conditions. Because of glucose repression only a few small peroxisomes are present when cells are grown in high concentrations of glucose. Growth on non-fermentable carbon sources leads to a modest increase in the peroxisomal compartment but a spectacular increase is seen on carbon sources that require peroxisomal function such as certain alcohols, alkanes or fatty acids. For instance, when *H. polymorpha* is grown in a chemostat on a limiting amount of methanol, more than 90% of the cellular volume is occupied by large cubic sized microbodies (Veenhuis *et al.*, 1978). This versatility to switch from conditions in which peroxisomes are not essential to conditions in which they are absolutely required for growth on specific carbon sources, has been exploited for the isolation of mutants disturbed in peroxisome function.

Although all these yeasts, except *S. cerevisiae* are well adapted to growth on specific carbon sources, they were less suitable for the application of refined genetics and recombinant DNA techniques. Initially, this may have been a handicap but especially *P. pastoris* and *H. polymorpha* quickly made up their arrears. On the other hand the fatty acid oleate was discovered to induce peroxisome proliferation in *S. cerevisiae* (Veenhuis *et al.*, 1987). The generation time on this substrate is long, however, and the growth conditions compare unfavorably compared to the other yeasts. This disadvantage is outweighed by the advanced genetics and manipulations *S. cerevisiae* allows and the wealth of information already gathered for this organism. The genome project has been finished, generating many new ORFs (Goffeau *et al.*, 1996), some of which turn out to be highly relevant to peroxisome biogenesis.

The first mutants unable to grow on oleate (Oleate Non-Utilizers or ONUs) were isolated in *S. cerevisiae* (Erdmann *et al.*, 1989). Among these were *pas* mutants (according to new nomenclature rules now called *pex* mutants (Distel *et al.*, 1996)) in which (some) peroxisomal marker enzymes

remained in the cytoplasm and (normal) peroxisomes could not be observed based on biochemical fractionation and electron microscopical experiments. These *pex* mutants were of great interest because they suggest defects in genes whose products play a direct role in the biogenesis of the organelles. The *pex* mutants are considered to be good models for the human diseases in which a similar loss of peroxisomal structures has been observed. Subsequently, positive genetic screening tests have been introduced to isolate more *pex* mutants (Lazarow, 1993; Zhang *et al.*, 1993; Elgersma *et al.*, 1993; Van der Leij *et al.*, 1992).

The excellent growth properties on one or more specific carbon sources has made it much easier to obtain a collection of mutants for *H. polymorpha* and P. *pastoris*, and the list of *pex* mutants is growing rapidly. At the moment a list of 21 *pex* mutants is available (an updated list can be viewed on the following web site: www.mips.biochem.mpg.de/proj/yeast/reviews/pex_table.html).

The mutants were used for the cloning of the corresponding wild-type genes (*PEX*) by functional complementation. In some cases intriguing gene products (called peroxins) have been identified with clear homologies to known protein families but relating them to biological function is still difficult.

3. IMPERMEABILITY OF THE PEROXISOMAL MEMBRANE

Carefully isolated mitochondria show latency: exogenously added substrates can only reach the matrix-located enzymes when the mitochondrial inner membrane is perturbed by, for instance, the addition of detergent. Despite similar care during isolation, peroxisomes did not show such latency and molecules like sucrose could freely traverse the peroxisomal membrane (Van Veldhoven *et al.*, 1983). In more extreme cases even matrix-located enzymes leaked out which is reason to still debate the location of enzymes participating in the glyoxylate cycle (McCammon *et al.*, 1990).

The permeability was suggsested to be due to the existence of integral membrane proteins resembling mitochondrial porins, which allow small molecules to cross the outer membrane. This notion was supported by patch-clamp studies carried out on membranes taken from a highly purified peroxisomal fraction (Van Veldhoven *et al.*, 1987). Now, however, we are convinced that the peroxisomal membrane, like most cell membranes, forms a closed barrier to even small molecules. The possibility to grow *H. polymorpha* cells that consist for more than 90% of peroxisomes allowed *in vivo* nuclear magnetic resonance measurements to derive the internal pH of peroxisomes (Nicolay *et al.*, 1987). It is somewhat more acidic than the

pH of the cytosol (pH 5.8–6 versus pH 7) implying that protons cannot leave the peroxisome. Using specifically engineered mutants in combination with biochemical assays we have made it plausible that metabolites like NADH, NADP and acetyl-CoA cannot diffuse out of peroxisomes, suggesting the existence of metabolite transporters (Elgersma and Tabak, 1996a; Van Roermund *et al.*, 1995; Elgersma *et al.*, 1995). A large set of transporters mediating metabolite transport across the mitochondrial inner membrane already has been described. Such information is almost completely lacking for peroxisomes. The first clue for a peroxisomal transporter came from patient studies in man. Young boys that suffer from a disease called X-linked adrenoleukodystrophy (X-ALD) displayed an elevated concentration of long-chain fatty acids in body fluids. The affected gene was identified by positional cloning and the encoded protein turned out to be an ABC (ATP Binding Cassette) half-transporter family member residing in the peroxisomal membrane (Mosser *et al.*, 1994; Mosser *et al.*, 1993). When orthologs of this protein showed up during the yeast genome sequence project (Bossier *et al.*, 1994), we and others studied the function of these peroxisomal ABC transporters in yeast (Hettema *et al.*, 1996; Shani *et al.*, 1996). The data suggest that two ABC half-transporters (Pat1p/Pat2p) combine into a functional complex which can import activated long-chain fatty acids from the cytosol into peroxisomes. Since all members of the ABC integral membrane protein family in one way or another are involved in transport of molecules across membranes, their existence in peroxisomal membranes is strong support for the notion that the peroxisomal membrane is impermeable to small molecules *in vivo*. A candidate for a second transporter is Pex11p (vide infra) and there are likely to be more.

4. IMPORT OF PROTEINS INTO PEROXISOMES

Growth and maintenance of the peroxisomal compartment requires the specific targeting of proteins and lipid components to the organelles. The current view is that proteins are synthesized in the cytoplasm, folded to a large extent and subsequently translocated across or inserted into the peroxisomal membrane. There are only vague ideas how integral membrane proteins reach the peroxisome. It probably requires a different set of proteins, like in mitochondria (Adam *et al.*, 1999; Köhler *et al.*, 1998) because it is not dependent on components of the pathway responsible for the import of matrix proteins. Possible candidates under investigation are Pex3p, Pex16p and Pex19p (South and Gould, 1999; Götte *et al.*, 1998; Baerends *et al.*, 1996; Wiemer *et al.*, 1996). Comparison of primary amino acid sequences of membrane proteins has pinpointed consensus motifs that

might be used for targeting of these proteins to peroxisomes (Dyer *et al.*, 1996). Even less is known of how lipids are recruited to the membranes of enlarging peroxisomes.

Major progress in understanding protein targeting has been made especially for peroxisomal matrix proteins. Serendipity was very helpful when it was observed that the well-known reporter protein luciferase, expressed in African Green Monkey cells, showed punctate fluorescence coinciding with peroxisomes. Indeed, it was shown that luciferase is a peroxisomal matrix protein in the lantern organ of fireflies (Keller *et al.*, 1987). The targeting signal is a tripeptide SKL located at the C-terminal end of luciferase (Gould *et al.*, 1987). At the moment a lot of peroxisomal matrix proteins are known that have this peroxisomal targeting sequence (PTS1) or a conserved derivative thereof (Elgersma *et al.*, 1996c; Gould *et al.*, 1989). Later, a different, N-terminally located sequence was discovered that is responsible for import of thiolase into peroxisomes (PTS2) (Osumi *et al.*, 1991; Swinkels *et al.*, 1991). There is only a limited number of proteins that follow the PTS2 pathway.

The existence of two import signals suggests the presence of specialized components of the import machinery to accommodate the import of both PTS1 and PTS2 containing proteins. This is borne out by the occurrence of two *S. cerevisiae* mutants: *pex5* and *pex7* (Van der Leij *et al.*, 1992). Pex5p and Pex7p are soluble proteins that specifically recognise PTS1 respectively PTS2 import signals (reviewed in Erdmann *et al.*, 1997). Although there is still debate as to whether Pex7p is a cytosolic or peroxisomal protein or whether it functions at both locations (Lazarow and Kunau, 1997), a general working model is that Pex5p and Pex7p pick up their corresponding peroxisomal matrix proteins in the cytosol and deliver them to docking proteins located in the peroxisomal membrane. After delivery they cycle back to pick up their next cargo (Figure 1).

Identification of the components of the membrane-located translocation machinery turned out to be more difficult. A number of *pex* mutants are integral membrane or membrane associated proteins but it is difficult to establish whether they have a direct or indirect role in protein import. In certain cases peroxisomes showed altered morphology, ranging from still recognizable organelles to residual peroxisomal remnants ("ghosts"). In other cases such remnants are even absent. The import of PTS1 and/or PTS2 proteins or GFP derivatives is the only specific assay we can rely on to distinguish between protein import per se or other aspects of peroxisome biogenesis. An extreme example of how difficult it is to identify the correct function of a membrane protein is *PEX11*. *Pex11*, although considered a *pex* mutant, was not found in the genetic screens for the isolation of *pex* mutants. Instead, it was isolated as a major membrane constituent of

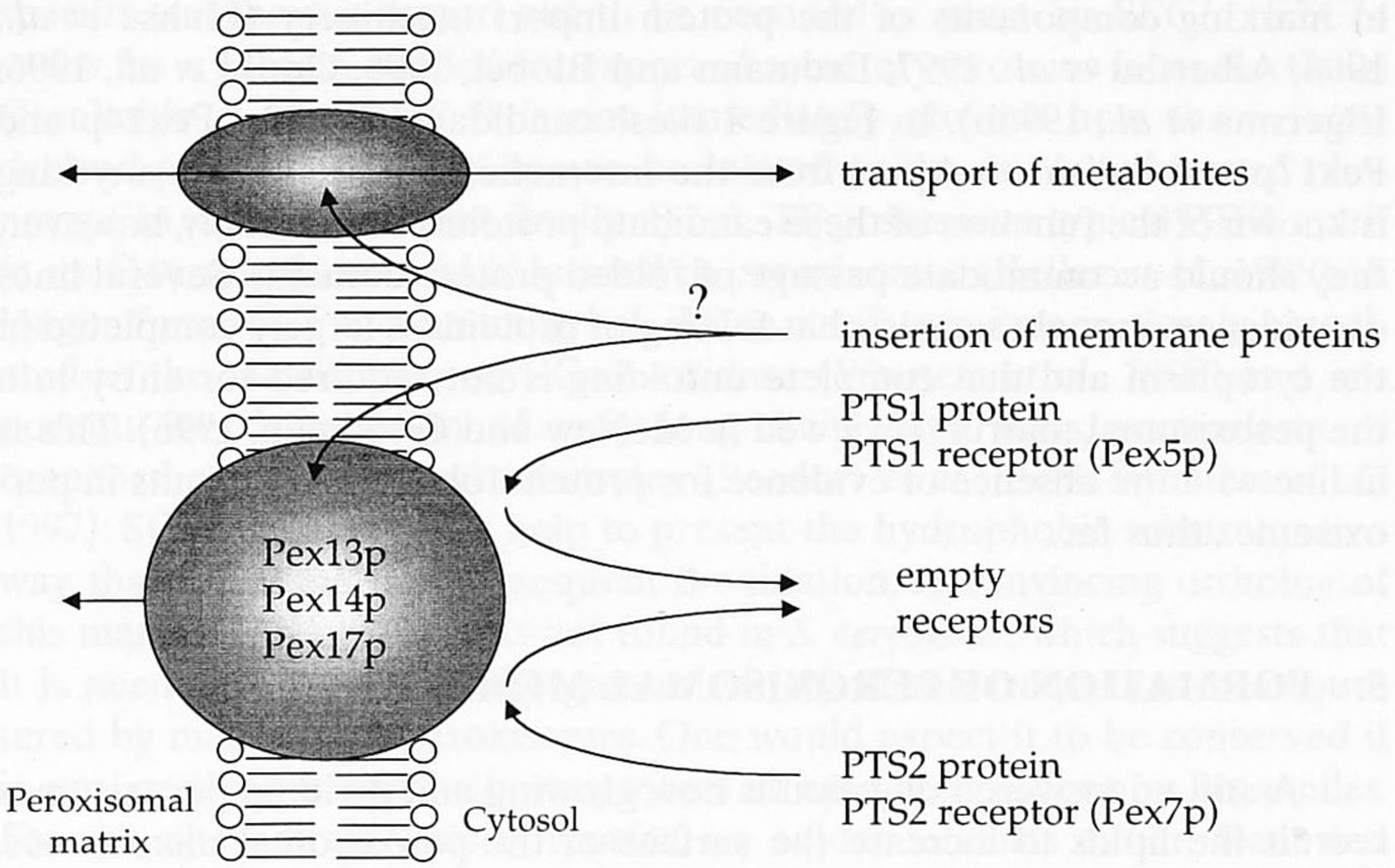

FIGURE 1. Translocation of proteins and metabolites across the peroxisomal membrane. Peroxisomal matrix and membrane proteins are targeted to the peroxisome via separate pathways. PTS containing proteins bind their cognate receptors, Pex5p and Pex7p, in the cytosol and are shuttled to the peroxisomal membrane. The membrane located docking complex for the receptors consists of at least three proteins: Pex13p, Pex14p and Pex17p of which Pex13p is the only bona fide integral membrane protein. After delivery of their cargo the receptors cycle back to the cytosol to pick up new cargo. Components required for peroxisomal membrane protein targeting and insertion have not yet been identified. Metabolite carriers are present in the peroxisomal membrane to shuttle metabolites across the membrane. For further details see text.

peroxisomal membranes and the gene was cloned after determination of part of its amino acid sequence (Marshall *et al.*, 1995; Erdmann and Blobel, 1995). The *pex11* mutant is not disturbed in the import of matrix proteins but shows enlarged peroxisomes. Overexpression of Pex11p in yeast as well as in mammalian cells resulted in large numbers of small peroxisomes (Schrader *et al.*, 1998; Erdmann and Blobel, 1995; Marshall *et al.*, 1995). Moreover, mammalian Pex11p interacted with a component of the coatomer complex (see below). These observations were interpreted to suggest a role for Pex11p in division of peroxisomes. Upon closer examination, our evidence suggests that it is involved in metabolism by transporting metabolites across the peroxisomal membrane (Hettema and Van Roermund, personal communication).

Two hybrid interaction analysis between the PTS1 and PTS2 receptor proteins and peroxisomal membrane proteins has provided the best results

in marking components of the protein import machinery (Huhse *et al.*, 1998; Albertini *et al.*, 1997; Erdmann and Blobel, 1996; Gould *et al.*, 1996; Elgersma *et al.*, 1996b). In Figure 1 these candidates, Pex13p, Pex14p and Pex17p, are indicated. Apart from the interaction studies hardly anything is known of the function of these candidate proteins. Remarkably, however, they should accommodate passage of folded protein domains. Several lines of evidence strongly suggest that folding of proteins is largely completed in the cytoplasm and that complete unfolding is not required for entry into the peroxisomal matrix (reviewed in McNew and Goodman, 1996). This is in line with the absence of evidence for protein folding components in peroxisomes thus far.

5. FORMATION OF PEROXISOMAL MEMBRANES

A still unanswered question is how growing and dividing peroxisomes recruit the lipids to increase the surface of the peroxisomal membranes. Three different options come to mind: i) close association with the ER, the major site of phospholipid biosynthesis in the cell, allowing transfer of lipids from ER to peroxisomes via such contact sites, ii) the use of phospholipid transfer proteins and iii) vesicular transport. Evidence for contact sites between ER and peroxisomes was found by electronmicroscopy (Novikoff and Shin, 1964) but the functional significance could not be substantiated by biochemical experiments (but see section THE ER TO PEROXISOME CONNECTION). Note, however, that the existence of contact sites between ER and mitochondria was suggested recently based on both biochemical and morphological data (Rizzuto *et al.*, 1998; Vance, 1990). Lipid transfer proteins are soluble proteins capable of transferring labeled phospholipids one at a time from a donor membrane to an unlabeled acceptor membrane in a time dependent manner. Two of these activities show phospholipid specificity against phosphatidylcholine (PC-TP) and phosphatidylinositol (PI-TP), respectively, while a third one transfers all common diacyl phospholipids, glycolipids and cholesterol (nsL-TP)(Wirtz, 1991). Based on their *in vitro* characterisation a role in transfer of phospholipids between membranes was proposed but direct evidence is lacking. Recent observations actually argue against such a role: i) these proteins mediate no net transfer from donor to acceptor membrane, ii) it is not clear how a single protein can display organelle membrane specificity considering the number of proteins needed to guide vesicles to their specific targets, iii) since they transfer a single lipid molecule at a time it is questionable whether they can mediate the large amount of lipids needed to support the biogenesis of organelles (Futerman, 1998), iv) the phosphatidyl inositol

specific transporter turned out to be encoded in yeast by *SEC14*. *SEC14* codes for a protein needed for export of secretory proteins from the Golgi (Bankaitis *et al.*, 1990). It is not immediately obvious how the *in vitro* derived properties of Sec14p can be related to the *in vivo* observed phenotype in the *sec14* mutant, finally, v) nsL-TP possesses a typical PTS1 motif at its C-terminal end and is located in peroxisomes (Keller *et al.*, 1989). A longer form, SCPx, containing nsL-TP in its C-terminus is closely associated in the matrix with acyl-CoA oxidase (Wouters *et al.*, 1998) and important for degradation of very long-chain fatty acids, particularly of branched-chain fatty acyl substrates (Seedorf *et al.*, 1998; Wanders *et al.*, 1997). SCPx/nsL-TP might help to present the hydrophobic substrates in a way that is optimal for subsequent ß-oxidation. A convincing ortholog of this mammalian protein was not found in *S. cerevisiae*, which suggests that it is needed to cope with the greater variety of substrates that is encountered by mammalian peroxisomes. One would expect it to be conserved if it was involved in such a basic process as lipid recruitment by organelles. For the alternative, vesicle transfer, no evidence exists either. Renewed interest in this possibility recently arose, however, when suggestive evidence was put forward that peroxisomes or peroxisomal pre-structures might be derived from the ER.

6. THE ER TO PEROXISOME CONNECTION

The observations made in the mid-eighties that peroxisomal matrix and membrane proteins are synthesized on free polysomes and post-translationally imported into peroxisomes has led to the view that peroxisomes are autonomous organelles: they multiply by growth and division of pre-existing peroxisomes and recruit their proteins directly from the cytosol (Lazarow and Fujiki, 1985). However, recent observations challenge this model and invoke the involvement of the endoplasmic reticulum in peroxisome biogenesis. One of the first pieces of evidence referred to nowadays came from a study on the site of synthesis of peroxisomal integral membrane proteins in rat liver. Bodnar *et al.* (1991) showed that a 50 kDa peroxisomal integral membrane protein is synthesized preferentially on ER bound ribosomes. More recently, studies in yeast suggest that some peroxisomal integral membrane proteins can be found in the ER under certain conditions. Overexpression of Pex15p, a tail-anchored type II integral membrane protein, causes a profound proliferation of membranes that contain Pex15p (Elgersma *et al.*, 1997). The continuity of the proliferated membranes with the nuclear envelope suggests that they most likely originated from the ER. Final proof for the ER origin of these membranes was

obtained by double labeling experiments. Antibodies against Pex15p and an ER marker protein decorated the same membranes in yeast cells overproducing Pex15p (Stroobants, Van den Berg and Hettema, personal communication). The observation that overexpressed Pex15p is O-glycosylated, a sugar modification that in *S. cerevisiae* occurs exclusively in the ER or in ER-derived compartments, provided additional evidence for trafficking of this protein to the ER (Elgersma *et al.*, 1997). Similar results have been reported for the integral peroxisomal membrane protein Pex3p. In yeast and in mammalian cells overexpression of Pex3p leads to proliferation of ER derived membranes (Kammerer *et al.*, 1998; Elgersma *et al.*, 1997). Moreover, the first 16 amino acids of yeast Pex3p were sufficient to target a reporter protein to the ER (Baerends *et al.*, 1996). Thus, Pex3p is a possible candidate to take the ER dependent route to peroxisomes. Although suggestive, the above mentioned experiments do not exclude an artificial mislocalization to the ER caused by overexpression of the proteins. Whether the endogenous Pex15p and Pex3p travel through the ER remains to be shown.

The *PEX1* and *PEX6* genes encode proteins that belong to the so called triple A (AAA) family of proteins (reviewed in Patel and Latterich, 1998). All of them contain one or two ATP binding pockets and the most well-known examples of this family are the NSFs (NEM-sensitive factors). These proteins are implicated in vesicle trafficking between ER, Golgi and plasma membrane (Rothman and Wieland, 1996) and it is tempting to deduce a similar role for the peroxins Pex1p and Pex6p. Indeed, suggestive evidence for the presence of these proteins in small vesicles that differ from mature peroxisomes was reported in *P. pastoris* but it is not yet clear how these vesicles feature in a peroxisome biogenesis or maturation pathway (Faber *et al.*, 1998). In contrast, in mammalian cells a cytosolic location for Pex6p was found (Yahraus *et al.*, 1996).

Additional evidence implicating the ER in peroxisome assembly came from studies in the yeast *Y. lipolytica*. Titorenko *et al.* (1997) identified and characterized mutants that affect both protein secretion and peroxisome biogenesis. Some of these mutants were originally isolated by the group of Ogrydziak (Ogrydziak *et al.*, 1982). Although the nomenclature is similar to the secretion mutants collected in *S. cerevisiae* by the Schekman group, they do not necessarily have the same properties since *Y. lipolytica* differs from *S. cerevisae* in a number of aspects. For instance, three different secretion routes have been postulated to exist based on the analysis of the various secretion mutants (Titorenko *et al.*, 1997). The *sec238* mutant is disturbed in the secretion of an extra cellular protease while the *srp54* causes abnormal function of the signal recognition particle (Lee and Ogrydziak, 1997; Ogrydziak *et al.*, 1982).

Mutations in the *SEC238*, *SRP54*, *PEX1* and *PEX6* genes not only cause defects in the exit of secretory proteins from the ER but also compromise the biogenesis of peroxisomes. More interestingly, mutations in these four genes significantly delay or prevent the exit of two peroxisomal membrane proteins, Pex2p and Pex16p, from the ER. Peroxisomal matrix proteins did not associate with the ER under these conditions. Although these results suggest that the ER is required for the assembly of functional intact peroxisomes one could argue that the observed accumulation of Pex2p and Pex16p in the ER reflects an indirect effect of the mutations. However, the same group has shown that in wild-type yeast cells endogenously expressed Pex2p and Pex16p transiently associate with the ER en route to the peroxisome (Titorenko and Rachubinski, 1998). Pulse-chase experiments followed by subcellular fractionation and immunoprecipitation revealed that Pex2p and Pex16p are located in ER enriched fractions during the pulse, and then are chased into fractions enriched in peroxisomes. In these experiments Pex2p and Pex16p were never found in Golgi enriched fractions suggesting direct ER-to-peroxisome transport of these proteins.

Further evidence for trafficking of Pex2p and Pex16p via the ER is provided by N-linked core glycosylation of both proteins. Protease protection experiments of ER enriched and peroxisome enriched fractions showed that the glycans on Pex2p and Pex16p remained intact indicating that core glycosylation of these proteins occurs in the ER lumen and that the glycosylated forms are delivered to the peroxisome. *Y. lipolytica* Pex2p contains one canonical Asn-Xaa-Thr sequence for N-linked glycosylation. This N-glycosylation site is not conserved in any of the eight Pex2p orthologues, which could provide an explanation for the fact that glycosylation of Pex2p has not been reported before. The human ortholog of *Y. lipolytica* Pex16p also lacks a consensus sequence for N-linked glycosylation (South and Gould, 1999). Furthermore, microinjection experiments in human fibroblasts failed to provide evidence for an ER role in Pex16p biogenesis: Pex16p was always detected in peroxisomes, even at the earliest time points after microinjection, and Pex16p transport to peroxisomes was not blocked or delayed in fibroblasts with an inactive *PEX1* gene or upon incubation at 15°C, a treatment that blocks exit of proteins from the intermediate compartment between the ER and Golgi (South and Gould, 1999). In addition to these differences in Pex16p biogenesis, Pex16p seems to have disparate roles in different organisms. In *Y. lipolytica* it plays a role in import of a subset of peroxisomal matrix proteins, but it is not required for membrane biogenesis (Eitzen *et al.*, 1997). In contrast, human Pex16p is required for peroxisomal membrane protein import and peroxisome synthesis (South and Gould, 1999). Caution is needed, therefore, in extrapolating the functions of a particular peroxin to its homologs in other species.

When one considers the possibility of a connection between ER and peroxisomes then vesicle transport would be a likely mechanism to establish such communication. Two observations suggest a role for ARF (ADP ribosylation factor) and coatomer in peroxisome biogenesis. Salomons *et al.* (1997) studied the effect of brefeldin A (BFA) on peroxisome biogenesis in the yeast *H. polymorpha*. BFA is a fungal toxin that prevents coatomer assembly onto membranes by inhibiting the GDP/GTP exchange activity of ARF. Immunocytochemical analysis of BFA treated cells suggested that newly synthesized peroxisomal membrane and matrix proteins accumulated at membranes that could be of ER-origin based on their continuity with the nuclear envelope. These results suggested that inhibition of recruitment of coat proteins onto membranes could interfere with peroxisome biogenesis (Salomons *et al.*, 1997). In human cells, however, peroxisome synthesis is insensitive to BFA (South and Gould, 1999).

Passreiter *et al.* (1998) found that purified rat liver peroxisomes incubated with bovine brain cytosol could recruit ARF and coatomer in a GTP-γ-S dependent manner. Further support for the involvement of coatomer in peroxisome biogenesis came from the observation that CHO cells expressing a temperature sensitive version of the ε-subunit of the coatomer showed elongated tubular peroxisomes when incubated at the non-permissive temperature. The authors suggested that Pex11p, an abundant peroxisomal membrane protein that contains a cytoplasmically exposed KXKXX motif at its C-terminus, could mediate coatomer binding. It is remarkable, however, that this putative coatomer binding motif and by inference this crucial function is not conserved in the *S. cerevisiae* Pex11p ortholog. Moreover, recent observations suggest that in yeast Pex11p is involved in metabolism (see above).

7. DO PEROXISOMES POSSESS UNIQUE FEATURES?

The PTS1 and PTS2 receptors (Pex5p and Pex7p) are hydrophilic proteins and they are not associated permanently with the peroxisomal membrane. The escort model shown in Figure 1 gives for now the best explanation for their function. The concept of soluble receptors initially was received with certain reservations. Comparison with other eukaryotic protein import systems shows that this is the rule rather than the exception. The cytosolic soluble signal recognition particle picks up most of the ER targeted proteins (Walter and Johnson, 1994). Nuclear proteins are escorted to nuclear pores by the soluble receptors importin α and importin β (Görlich and Mattaj, 1996), and even for mitochondria soluble cytosolic factors for efficient protein import were found (Hachiya *et al.*, 1995). In

mitochondria they were overlooked for a long time probably because of the success of the *in vitro* import system that used purified mitochondria and because a genetic analysis of mitochondrial protein import only lately has showed more promise (Maarse *et al.*, 1992).

The concept that (partially) folded proteins can enter the peroxisome is not unique either. Folded proteins can traverse nuclear pores and pass through the plant chloroplast thylakoid membrane and bacterial inner-membrane (reviewed in Settles and Martienssen, 1998). Careful measurement of the water-filled space in the Sec61 pore of the ER indicates that folded domains in principle can be accommodated (Hamman *et al.*, 1997). The original concept that only a completely unfolded peptide chain can be threaded through a proteinaceous pore of a translocation complex is still upheld in mitochondria only (Neupert, 1997).

Finally, the observation in *Y. lipolytica* that glycosylated proteins end up in peroxisomes is not unique. Also in mitochondria a 45 kDa glycosylated protein was found (Chandra *et al.*, 1998). Pulse-chase experiments indicated that it acquired its modification in the ER before arriving in mitochondria. The paradigm is that mitochondria form by growth and division of pre-existing organelles. Because of the double membrane of the mitochondrion vesicular traffick, suggesting mitochondria to be part of the ER, is less likely to be involved. However, there may be reasons to rethink standard views on organelle biogenesis on the basis of new provocative experimental data (for a heretic opinion see Glick and Malhotra, 1998).

8. TECHNICAL SHORTCOMINGS IN THE PEROXISOME FIELD

A major handicap of peroxisomes is that they loose some of their properties after isolation. Particularly latency, a characteristic feature they display *in vivo*, is absent. The unknown causes of this damage prevented development of a reliable *in vitro* protein import system. In addition, the absence of efficient post-translational modifications of proteins upon import, such as cleavage of targeting signals, makes it even more difficult to design good import assays. As a consequence, a number of neat tricks as developed for other protein import systems cannot be applied. Attempts to translate such manipulations to the *in vivo* situation were not successful thus far. For instance, no one succeeded in designing protein constructs that would jam the peroxisomal import machinery which would allow identification of components of a protein translocation pore via chemical cross-linking with the jammed construct (Häusler *et al.*, 1996, and our own unpublished results). Digitonin or SLO permeabilized cells have been used as a compromise since (Wendland and Subramani, 1993; Rapp *et al.*, 1993).

Recently, a breakthrough was reported by the group of A. Baker using plant glyoxysomes (Pool *et al.*, 1998). The protein substrate was imported in a time-dependent and ATP-dependent manner in freshly isolated organelles as judged from acquisition of resistance to protease. Interestingly, a protein A fusion construct got stuck during membrane translocation. This would be a great tool for further studies to unravel the properties and features of protein import into peroxisomes.

An important aspect in applying the yeast "two-hybrid" protein interaction technique is that validation is obtained for a direct interaction between 'bait' and 'prey'. When the technique was used in the homologous context with yeast peroxisomal proteins it became clear that this was not an academic issue. For instance, the yeast PTS1 and PTS2 receptors (Pex5p and Pex7p) were originally suggested to interact with each other (Rehling *et al.*, 1996). This seemed reasonable since proteins with TPR repeats (Pex5p) and WD-40 repeats (Pex7p) were reported to interact with each other using genetically based experiments. Later, however, the interaction between Pex5p and Pex7p was demonstrated to be indirect and to be mediated by Pex14p, a protein associated with the peroxisomal membrane (R. Erdmann, personal communication). By carrying out the two-hybrid interactions in yeast strains with specific deletions in genes coding for peroxisomal proteins, this initial setback was turned into an advantage. For instance, in a *pex14Δ* strain the interaction between Pex5p and Pex7p is lost. By performing the interaction studies in various combinations in different genetic backgrounds the "two-hybrid" technique can be modified to act as a "three-hybrid" reporter. It is remarkable that even integral membrane proteins can act as "go betweens" in such "three-hybrid" assays.

An obvious approach to follow in studying a possible relationship between ER and peroxisomes is the application of a thermosensitive set of mutants (*sec* mutants) isolated by the Schekman group in *S. cerevisiae*. In practice this turned out to be rather difficult and meaningful results were not yet obtained: i) The parent strain used for making the *sec* mutants cannot grow on fatty acids as their sole carbon source. This is often the case with laboratory strains and is caused by mutations in genes coding for peroxisomal proteins. Probably such strains accumulated cryptic mutations in genes that are never challenged for function under growth conditions practised in most laboratories. ii) At the non-permissive temperatures required to call up the phenotype, *S. cerevisiae* anyhow grows miserably in the detergent-like fatty acid containing growth medium. iii) It is difficult to separate possible peroxisome-related events from the already traumatic events caused by a block in such an essential pathway as secretion.

A difficulty in comparing literature data is that differences in opinion are encountered whether proteins are associated with the peroxisomal

membrane or whether they are integral membrane proteins. Although all groups use the same technique, extraction of membranes with Na_2CO_3 at alkaline pH, the outcome often is different. We have observed cases in which mitochondrial membrane proteins were not extractable at all and were completely confined to the pellet fraction, whereas peroxisomal membrane proteins such as Pex15p and the ABC transporter Pat1p were 50% extractable and only 50% remained in the pellet fraction. (Elgersma *et al.*, 1997; Hettema, personal communication). The reason for such aberrant behaviour of peroxisomal membrane proteins is not clear but it does give rise to confusion in the literature.

9. OUTLOOK

The field of peroxisome biogenesis is rapidly expanding and the application of yeast genetics has unearthed many new genes coding for proteins involved in this process. Although this provides us with essential tools allowing us to catch up with the more advanced state of the art already attained with other organelles such as mitochondria, nucleus and ER, very basic questions still remain to be answered. For instance, the simple concept that peroxisomes multiply by growth and division of pre-existent ones is debated again.

How proteins are imported into peroxisomes is still a complete mystery and the first intrinsic component of the peroxisomal protein import machinery still needs to be identified. Here the advantage of an *in vitro* protein import system is desperately needed to make progress.

These critical remarks illustrate that we still have a number of challenges ahead. However, the development and application of yeast genetics to unravel aspects of peroxisome function and biogenesis has stimulated the field considerably and has already paid off for further understanding of the pathology of peroxisomes in man.

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