Pex5p, a guide for import of proteins into peroxisomes
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Chapter 1

General Introduction
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I. Peroxisomes: an introduction

A hallmark of the eukaryotic cell is the presence of different subcellular membrane-bounded compartments, the organelles. This compartmentalization allows the formation of specialized subenvironments inside the cell, which is necessary for optimal performances of many different (biochemical) processes.

For instance, in the endoplasmic reticulum (ER) the first sorting steps take place of proteins transported through the secretory pathway. It contains its own set of chaperones, proteins that assist in the folding of the secretory proteins, which are located inside this organelle. Furthermore, the ER forms a unique, oxidizing environment in the cell, which allows the formation of disulfide bonds in a protein during folding. In the context of this thesis, an important feature of a number of organelles is that they form the sites where specialized metabolic pathways take place. For instance, lysosomes specialize in the intracellular digestion of macromolecules, while mitochondria harbor the enzymes of the citric acid cycle and the respiratory chain, and thus produce ATP.

Another group of organelles is formed by the microbodies, which were first identified in mouse kidney cells by electron microscopy (Rhodin, 1954). Microbodies are bounded by a single membrane and filled with a dense granular matrix. Biochemical characterization of these organelles resulted in the identification of a number of enzymes involved in the production and degradation of hydrogen peroxide (H$_2$O$_2$), which led to the name peroxisome (De Duve and Baudhuin, 1966). Microbodies are found in almost every eukaryotic cell and in most cases they are referred to as peroxisomes. However, in some species the presence of a very characteristic metabolic pathway inside the microbodies has resulted in a different name. Examples are glyoxysomes in germinating plant seeds, which were found to contain the glyoxylate cycle enzymes (Breidenbach and Beevers, 1967), and glycosomes in kinetoplastids, which were found to contain several glycolytic enzymes (Oppendoes and Borst, 1977). Despite this variety in nomenclature, all microbodies are characterized by the presence of the β-oxidation pathway for fatty acids. In the yeast *Saccharomyces cerevisiae*, the entire breakdown of fatty acids takes place inside peroxisomes, while in mammalian cells the first steps of the β-oxidation of very long chain fatty acids (VLCFA) are located inside peroxisomes and the next steps take place inside mitochondria. In mammalian cells, other processes in which peroxisomes are involved are the synthesis of bile acids, dolichol, cholesterol, and glycerol lipids such as plasmalogens. The α-oxidation of 3-methyl-branched fatty acids and the breakdown of polyamines, purines and some amino acids such as L-lysine also occur.
inside peroxisomes (reviewed by van den Bosch et al., 1992; Mannaerts and Van Veldhoven, 1993; Wanders and Tager, 1998).

The number and size of peroxisomes in eukaryotic cells are variable and depend on environmental conditions. In rat liver, which is active in lipid metabolism, thousands of peroxisomes are present as spheres with a diameter of 0.2 - 1 μm, whereas in other tissues they are considerably smaller: 0.1 - 0.2 μm. In the yeast S. cerevisiae, only two or three peroxisomes are present under normal growth conditions, such as growth on glucose-containing medium. The number of peroxisomes increases to approximately ten when yeast is grown on the fatty acid oleic acid, circumstances under which the β-oxidation inside peroxisomes is essential for energy generation. Although normally spherical, under certain growth conditions peroxisomes are reported to be connected to each other and to form a tubular network also referred to as a peroxisomal reticulum. These structures were observed both in yeast and in liver tissue (Lazarow and Fujiki, 1985; Yamamoto and Fahimi, 1987; Schrader et al., 1998a).

In contrast to other subcellular organelles such as mitochondria and chloroplasts, peroxisomes do not contain DNA. This implies that nuclear genes encode all proteins that are necessary for peroxisomes to function correctly. Synthesis of both peroxisomal membrane proteins and peroxisomal matrix proteins occurs in the cytosol, after which they are post-translationally imported (reviewed by Lazarow and Fujiki, 1985).

To study peroxisome biogenesis and function, the yeast S. cerevisiae has proven to be an excellent model system since its entire pathway of fatty acid β-oxidation is confined to peroxisomes. During growth on glucose medium peroxisomes are dispensable, but when shifted to oleic acid medium, the presence of functional peroxisomes is essential to survive. Many genetic tools are available for S. cerevisiae and the stable haploid phase of this yeast allows rapid isolation of mutants and cloning of the genes of interest by functional complementation. Furthermore, these genetic tools make it possible to create targeted deletions of genes. Therefore, S. cerevisiae and other yeast species have been used in a variety of positive and negative selection screens to isolate genes involved in peroxisome biogenesis and function (Erdmann et al., 1989; Cregg et al., 1990; Gould et al., 1992; Liu et al., 1992; Van der Leij et al., 1992; Elgersma et al., 1993; Nuttley et al., 1993; Zhang et al., 1993; Kalish et al., 1996). The identified genes were named PEX genes and the proteins they encode were called peroxins (Distel et al., 1996).

Since peroxisomal functions have been conserved in different eukaryotes, the sequences of these yeast PEX genes were used to screen human expressed sequence tag (EST) databases to identify the human orthologs (reviewed by Gould and Valle,
2000). Furthermore, Chinese hamster ovary (CHO) cells were isolated with a defect in peroxisome biogenesis, and rat cDNA libraries were used to complement the peroxisomal defect in these cells (Tsukamoto et al., 1991). This resulted in the identification of a number of mammalian \textit{PEX} genes (reviewed by Gould and Valle, 2000). Together these combined approaches have led to the identification of 25 \textit{PEX} genes (Table I).

II. Peroxisomes and their relationship to human disease

Several severe genetic disorders are caused by a deficiency in one or more peroxisomal functions (reviewed by Wanders et al., 1995; Gould and Valle, 2000). Based upon the level to which peroxisomal functions are affected, these diseases can be classified as three groups.

The first group consists of most of the peroxisome biogenesis disorders (PBDs). Examples of these disorders are the Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD). These diseases are characterized by a general loss of peroxisomal functions, resulting in an increase in the levels of very-long-chain fatty acid and phytanic acid in body fluids, and in a decrease in the level of plasmalogens (reviewed by Wanders et al., 1995). Patients suffering from these diseases are clinically characterized by a wide range of abnormalities, such as delayed development, typical craniofacial abnormalities (high forehead, broad nasal bridge, shallow orbital ridges), abnormalities of the eyes, profound neurological abnormalities, hypotonia, and liver disease. The distinction between the disorders is based on severity. ZS is the most severe and patients normally die within the first year after birth, whereas NALD patients and IRD patients can survive up to one and three decades, respectively.

PBDs are genetically heterogeneous, which means that they can be caused by mutations in different \textit{PEX} genes. For instance, ZS can be caused by mutations in \textit{PEX1}, \textit{PEX2}, \textit{PEX3}, \textit{PEX5}, \textit{PEX6}, \textit{PEX10}, \textit{PEX12}, \textit{PEX16} or \textit{PEX19}, NALD by mutations in \textit{PEX1}, \textit{PEX5}, \textit{PEX6}, \textit{PEX10}, \textit{PEX12} or \textit{PEX13}, and IRD by mutations in \textit{PEX1} or \textit{PEX12} (reviewed by Gould and Valle, 2000). These examples also show that mutations in a certain \textit{PEX} gene are not linked to a specific disease. Different alleles of the same gene encode a variety of mutant proteins, which are differently affected in their function.
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<th>Peroxin</th>
<th>Interacting partner(s)</th>
<th>Features and / or Possible Function</th>
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<tr>
<td>Pex1p</td>
<td>Pex6p</td>
<td>AAA-protein required for import of peroxisomal matrix proteins or vesicle fusion</td>
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<tr>
<td>Pex2p</td>
<td></td>
<td>RING zinc finger protein, integral PMP, required for import of peroxisomal matrix proteins</td>
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<td>Pex3p</td>
<td>Pex19p</td>
<td>Integral PMP, marking of peroxisomal membranes, required for the insertion of PMPs</td>
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<td>Pex4p</td>
<td>Pex22p</td>
<td>E2 ubiquitin-conjugating enzyme required for import of peroxisomal matrix proteins</td>
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<td>Pex5p</td>
<td>Pex7p, Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, Pex21p, Pex19p, PTS1 and PTS3 proteins</td>
<td>TPR domain-containing receptor for PTS1 proteins, required for import of PTS1 proteins into peroxisomes. In mammals also required for PTS2 import</td>
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<td>Pex6p</td>
<td>Pex1p, Pex15p</td>
<td>AAA-protein required for import of peroxisomal matrix proteins or vesicle fusion</td>
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<tr>
<td>Pex7p</td>
<td>Pex5p, Pex13p, Pex14p, Pex18p, Pex21p, Pex20p, PTS2 proteins</td>
<td>WD-40 repeat-containing receptor for PTS2 proteins, required for import of PTS2 proteins into peroxisomes</td>
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<td>Pex8p</td>
<td>Pex5p, Pex20p</td>
<td>Intra-peroxisomal localization, contains a PTS1 and a PTS2, required for import of peroxisomal matrix proteins</td>
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<td>Integral PMP required for import of peroxisomal matrix proteins</td>
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<td>Pex5p, Pex12p, Pex19p</td>
<td>RING zinc finger protein, integral PMP, required for import of peroxisomal matrix proteins</td>
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<td>Pex11p</td>
<td>Pex19p</td>
<td>PMP involved in peroxisome proliferation or translocation of MCFA</td>
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<th>Peroxin</th>
<th>Interacting partner(s)</th>
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<td>Pex12p</td>
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<td>RING zinc finger protein, integral PMP, required for import of peroxisomal matrix proteins</td>
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<td>Pex13p</td>
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<td>SH3 domain containing integral PMP, required for import of peroxisomal matrix proteins, involved in receptor docking</td>
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<td>Pex14p</td>
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<td>integral PMP required for import of peroxisomal matrix proteins, membrane anchor for Pex6p</td>
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<td>Pex16p</td>
<td>Pex19p</td>
<td>integral PMP required for PMP import</td>
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<td>Pex7p</td>
<td>required for import of PTS2 proteins</td>
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<td>Pex19p</td>
<td>many PMPs</td>
<td>required for import of PMPs</td>
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<td>Pex20p</td>
<td>thiolase</td>
<td>required for import of thiolase in Y.lipolytica</td>
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<td>Pex21p</td>
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<td>required for import of PTS2 proteins</td>
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<td>Pex4p, Pex19p</td>
<td>PMP required for import of peroxisomal matrix proteins, membrane anchor for Pex4p</td>
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<td>Pex23p</td>
<td></td>
<td>PMP required for import of peroxisomal matrix proteins</td>
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<tr>
<td>Pex24p</td>
<td></td>
<td>PMP involved in the targeting and / or assembly of PMPs</td>
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<tr>
<td>Pex25p</td>
<td></td>
<td>PMP required for regulating peroxisomal size and maintenance</td>
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<tr>
<td>Djpl</td>
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<td>DnaJ-like protein, chaperone required for import of peroxisomal matrix proteins</td>
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* interactions reported in literature in different organisms
Rhizomelic chondrodysplasia punctata (RCDP) and Zellweger-like syndrome form the second group of peroxisomal diseases. Patients with these diseases are characterized by impairment of several, but not all peroxisomal functions. Peroxisomes are still present and have a normal appearance. RCDP is caused by mutations in *PEX7* and is clinically characterized by severe shortening of the proximal limbs (rhizomelia), a typical facial appearance, and mental retardation. In most cases patients do not survive their second year. Biochemically these patients are characterized by a defect in plasmalogen synthesis and phytanic acid breakdown.

The third group patients are affected in the function of a single peroxisomal enzyme due to inactivation or mislocalization. Many diseases caused by a peroxisomal defect belong to this group, including acyl-CoA oxidase deficiency and X-linked adrenoleukodystrophy (X-ALD), but these diseases do not belong to the spectrum of PBDs.

The fact that a severe deficiency in peroxisomal function is still compatible with (cellular) life makes it most likely that more genes might be present than have been identified in relation to disease. Mutations in some of these genes might not result in a noticeable loss of peroxisomal function. However, if they would be present in combination with subtle mutations in other genes, mutations in the as yet unknown genes might give rise to a disease (multifactorial diseases). Therefore, some multifactorial diseases may partly be caused by mutations in genes coding for peroxisomal proteins and thus may contribute to peroxisomal dysfunction.

### III. Peroxisome biogenesis

Most peroxins identified (Table I) play a role in either the targeting of peroxisomal membrane proteins or the import of peroxisomal matrix proteins. This separation is mainly based on the phenotypes found for yeast and mammalian cells with a mutated or deleted *PEX* gene. Normal peroxisomes are not present in these cells. However, in most cases peroxisomal ghosts could be detected. Peroxisomal ghosts are remnants of peroxisomal membranes that still contain some peroxisomal membrane proteins but lack most of their internal content, *i.e.* the peroxisomal matrix proteins. These ghosts could not be detected in yeast cells with a mutation in or a deletion of *PEX3* or *PEX19*, and in mammalian cells lacking functional Pex3p, Pex16p, or Pex19p (Shimozawa *et al.*, 1998; South and Gould, 1999; Hettema *et al.*, 2000). In these cells the peroxisomal membrane proteins are rapidly degraded after their synthesis in the cytosol. The exact role of Pex3p and Pex16p is still unclear. Both proteins are peroxisomal membrane
proteins (Hohfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996; Honsho et al., 1998; Kammerer et al., 1998) and the fact that no peroxisomal ghosts are detectable in the absence of these proteins suggests that they are involved in one of the early stages of peroxisome formation. Reintroduction of the wild-type gene in these mutant cells led to reappearance of peroxisomes.

An important question deals with how peroxisomes are formed. Different models were designed to explain the origin of this organelle (reviewed by Titorenko and Rachubinski, 1998; Tabak et al., 1999; Purdue and Lazarow, 2001; Stroobants, 2001). After cell division, new peroxisomes might be formed by vesicle budding from the ER. This early model was suggested since in observations obtained by electron microscopy peroxisomes were found to be in close proximity to the ER (Novikoff and Shin, 1964). However, studies on Pex3p could not show a connection with the ER (South et al., 2000; Voorn-Brouwer et al., 2001). These studies showed that if peroxisomes arose from the ER, their formation did not follow the rules established for vesicular transport in the secretory pathway, which is dependent on budding of small vesicles mediated by COPI and COPII. In these studies several methods were used to block the formation of the ER-derived vesicles in human fibroblasts and the consequences of such a block on the localization of newly synthesized Pex3p were assessed. COPI vesicle transport was inhibited by Brefeldin A and COPII transport was inhibited in cells expressing a dominant negative mutation in the Sar1p protein required in the budding process. However, no effect on the transport of Pex3p to peroxisomes was observed under these conditions (South et al., 2000; Voorn-Brouwer et al., 2001). Although these results do not support the view that Pex3p reaches peroxisomes via the ER, the experiments do not exclude the existence of another, as yet unidentified, exit pathway out of the ER. However, another experiment that does support the ER-peroxisome connection was performed in mouse dendritic cells. By immuno electron microscopy it was shown that the peroxisomal membrane protein Pex13p is located in specialized extensions of the ER (Stroobants, 2001). Furthermore, Pex13p was also found to be located in tubules that have a morphology similar to that of these ER extensions. These tubules were often found to surround mature peroxisomes, and 3-D analysis using electron tomography showed connections between these tubules and mature peroxisomes (A.K. Stroobants, personal communication). These observations suggest that peroxisomes originate from the ER.

As mentioned before, the exact role for Pex3p in the formation of peroxisomes is still unclear. South et al. (2000) suggested that this protein might function as a factor that is inserted into membranes and in this way marks them as being peroxisomal membranes. Whether these are the same specialized regions of the ER as were observed in mouse dendritic cells is not known. Indeed, although Pex3p contains a
peroxisomal targeting signal located in its amino terminus (Wiemer et al., 1996; Kammerer et al., 1998), the exact mechanism for Pex3p insertion into peroxisomal membranes is still unclear. The carboxyl terminus of Pex3p interacts with Pex19p, another protein involved in the early steps of peroxisome formation, and deletion of the carboxyl terminus of Pex3p disrupts its function (Soukupova et al., 1999; Ghaedi et al., 2000).

Pex19p is a farnesylated protein located mainly in the cytosol, only a small fraction is associated with the peroxisomal membrane (James et al., 1994; Snyder et al., 1999a; Sacksteder et al., 2000). The exact function of the farnesylation is not known, but it might aid association with the peroxisomal membrane. In S. cerevisiae, the farnesylation of Pex19p is essential for its function (Gotte et al., 1998), but this is not the case in H. polymorpha (Snyder et al., 1999a) and in humans (Sacksteder et al., 2000). Pex19p interacts with a large number of peroxisomal membrane proteins, ranging from peroxins to transporters of metabolites (Sacksteder et al., 2000; Snyder et al., 2000). This broad range of interactions with peroxisomal membrane proteins has resulted in a model in which Pex19p functions as a mobile receptor or chaperone for these proteins. This proposed receptor function was supported by elegant experiments in which Pex19p was directed to the nucleus by fusing it to a nuclear localization signal (Sacksteder et al., 2000). Together with Pex19p many peroxisomal membrane proteins were found to be targeted to the nucleus. Furthermore, for several peroxisomal membrane proteins the region involved in Pex19p interaction also proved to be necessary for their targeting, which supported the role of Pex19p as a mobile receptor to target membrane proteins to the peroxisomal membrane (Sacksteder et al., 2000).

In human cells and in S. cerevisiae cells deprived of functional Pex19p no peroxisomal ghosts could be detected (Gotte et al., 1998; Matsuzono et al., 1999; Hettema et al., 2000). However, in Pichia pastoris pex19-deficient cells, vesicles and tubules were observed by deconvolution immunofluorescence microscopy with anti-Pex3p antibodies (Snyder et al., 1999a). This indicates that, although Pex3p interacts with Pex19p, this function is not necessary for membrane targeting of Pex3p itself and suggests that Pex3p acts before Pex19p. Based on these and other observations a model can be proposed for the possible function of these proteins in peroxisome biogenesis. The first step involves the insertion of Pex3p into a membrane, the origin of which is unknown but, as mentioned before, this might be a specialized region of the ER. In this way the membrane is marked as a peroxisomal membrane. Subsequently, Pex19p targets other peroxisomal membrane proteins to this membrane, possibly by using Pex3p as a docking site. After the assembly of these peroxisomal membrane proteins and the formation of a competent protein-import complex, this pre-
peroxisome can grow further by importing peroxisomal matrix proteins. The attractiveness of this speculative model is that it provides an explanation for the origin of the peroxisomal membrane.

In the second model of peroxisome formation, peroxisomes are maintained by growth and division of pre-existing peroxisomes, and thus follow the same mechanisms of organelle inheritance as mitochondria and chloroplasts. This process of peroxisome maintenance in eukaryotic cells can be divided into three major steps:

1. Enlargement of the peroxisomal membrane and import of peroxisomal membrane proteins
2. Import of peroxisomal matrix proteins from the cytosol, across the membrane, into the peroxisomal lumen
3. Proliferation of peroxisomes and distribution of peroxisomes over mother and daughter cells during cell division

In this model, Pex3p and Pex19p are also involved in the first step, i.e. enlargement of the peroxisomal membrane, which is achieved by the combined import of peroxisomal membrane proteins and lipids into existing peroxisomes. The membrane proteins are synthesized on polyribosomes in the cytosol and are subsequently targeted to the peroxisome. How lipids are recruited to support enlargement of the growing peroxisomal membrane remains unclear. In the second step, the peroxisomal compartment increases in size by the import of peroxisomal matrix proteins. As to the third step, in *S. cerevisiae*, several proteins have been suggested to be involved in the process of peroxisome division and segregation of peroxisomes from mother to bud. These are Vps1p, a dynamin-like protein that regulates the number of peroxisomes, and Myo2p, a myosin motor protein that is involved in the active movement of peroxisomes to the bud along actin cables (Hoepfner et al., 2001). Another protein that is probably involved in peroxisome proliferation is Pex11p, a peroxisomal membrane protein. In *S. cerevisiae* or *Candida boidinii* cells that are disturbed in Pex11p function either by mutation or deletion, the number of peroxisomes does not increase when the cells are grown on oleic acid, but giant peroxisomes can be observed. The opposite phenotype is observed when Pex11p is overproduced: the number of peroxisomes per cell is increased while the size of peroxisomes is decreased (Erdmann and Blobel, 1995; Marshall et al., 1995; Sakai et al., 1995).

In humans, two forms of Pex11p exist, Pex11pα and Pex11pβ. Overproduction of Pex11pβ resulted in the same phenotype as that observed in yeast, i.e. proliferation of peroxisomes (Abe and Fujiki, 1998; Abe *et al.*, 1998; Passreiter *et al.*, 1998; Schrader
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et al., 1998b). The overproduction of Pex11pα also led to peroxisome proliferation, but this was observed at a much lower frequency (Schrader et al., 1998b). The α and β forms of Pex11p differ in their expression patterns. The expression levels of Pex11pβ are the same in different tissues and are not influenced by peroxisome proliferating drugs, but Pex11pα expression varies in different tissues and is highly increased by peroxisome-proliferating drugs (Schrader et al., 1998b). These results indicate that the function of Pex11pβ is to control constitutive peroxisome proliferation, which is for instance necessary to guarantee the presence of peroxisomes in both cells after cell division. Pex11pα might be involved in the adaptation to environmental changes, which requires a greater number of peroxisomes.

An alternative model to the one described above, was put forward by van Roermund et al. (2000). Based on their observations in S. cerevisiae, they suggested that Pex11p is involved in the translocation of medium chain fatty acids (MCFA) across the peroxisomal membrane. This would ensure delivery of new substrates and continuation of the β-oxidation pathway. The authors postulated that during the β-oxidation a signaling molecule is produced that modulates the proliferation of peroxisomes. This would explain why in pex11-deficient cells first a deficiency in MCFA oxidation is seen, followed by the formation of giant peroxisomes (van Roermund et al., 2000). Although this model also suggests that Pex11p is involved in the proliferation of peroxisomes, this involvement would be indirect.

IV. Import of peroxisomal matrix proteins

Peroxisomal matrix proteins are synthesized on free polyribosomes in the cytosol and are posttranslationally imported into the peroxisome (Figure 1) (for the first and now classical review see Lazarow and Fujiki, 1985). To reach their correct cellular location, i.e. the peroxisomal matrix, these proteins contain a specific peroxisomal targeting signal (PTS). Two of these PTSs, type I (PTS1) and type II (PTS2), have been well characterized for more than a decade (reviewed below). More recently, alternatives on this basic principle were identified and we were able to show that an additional PTS3 route exists for the import of matrix proteins into peroxisomes (Klein et al., 2002). Receptor proteins bind these PTSs in the cytosol and direct the proteins to the peroxisome. Pex5p is the receptor for proteins containing a PTS1 or a PTS3 and Pex7p is the receptor for proteins containing a PTS2.
Figure 1. Model for the import of matrix proteins into peroxisomes in *S. cerevisiae*.
Peroxisomal matrix proteins containing either a PTS1 or a PTS2 are synthesized in the cytosol. After synthesis the PTS1 and PTS2 are recognized and bound by the receptors Pex5p and Pex7p, respectively. The receptor-cargo complex docks at the peroxisomal membrane via the interaction with the Pex13p-Pex14p-Pex17p complex. Subsequently the PTS1 cargo is translocated into the peroxisome and Pex5p recycles back into the cytosol. In mammals Pex5p interacts with Pex7p and is involved in the import of PTS2 proteins (some of the interactions could not be fitted into this figure, see text for more details).
Peroxisomal targeting signal type 1 (PTS1)
The presence of a PTS1 was first noticed by serendipity in firefly luciferase, a well-known reporter protein in transcriptional studies (Gould et al., 1987). The three carboxyl-terminal amino acids of this protein, *i.e.* serine, lysine, leucine (S-K-L in single letter codes), were shown to be both necessary and sufficient for directing non-peroxisomal proteins to peroxisomes (Gould et al., 1989). By introducing amino acid substitutions at each position of the PTS1, a consensus sequence was defined as S/A/C-K/R/H-L/M (Gould et al., 1989; Swinkels et al., 1992), or in words: a small uncharged residue at the -3 position, a basic residue at the -2 position, and a non-polar residue at the -1 position. This consensus sequence was based on the import of a heterologous protein into peroxisomes of mammalian cells. Subsequent studies have shown that, especially in a homologous context, *i.e.* the protein is expressed in the organism it is derived from, even more variations of this tripeptide may occur, although this also depends on the protein and organism studied. For *S. cerevisiae* peroxisomal malate dehydrogenase (Mdh3p), it was shown that in a homologous context many amino acid substitutions at the -3 and -2 positions of the PTS1 that do not comply with the consensus sequence are tolerated without severely affecting peroxisomal targeting (Elgersma et al., 1996b). A similar observation was reported for the human peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT) (Motley et al., 1995). The carboxyl-terminal K-K-L sequence, which does not comply with the PTS1 consensus sequence, directs this protein to peroxisomes but is insufficient for targeting of the proteins luciferase and chloramphenicol acetyltransferase (CAT) to peroxisomes.

These examples show that the classical definition of a PTS1 as being both necessary and sufficient to target a protein to peroxisomes is too strict in some cases, especially proteins in a homologous context. The carboxyl-terminal tripeptides are necessary for the targeting of homologously expressed proteins, but they are not sufficient to direct a heterologously expressed protein to peroxisomes. For AGT it was postulated that other sequences in this protein might enable the K-K-L to function as a PTS1 (Motley et al., 1995). These ‘enabling sequences’ or ‘accessory sequences’ were not present in the reporter proteins to which the carboxyl-terminal K-K-L was fused, which would explain why these proteins are not directed to peroxisomes. The presence of accessory sequences could also explain the many variations in the PTS1 of Mdh3p (Elgersma et al., 1996b). The question arose as to the nature of these accessory sequences and where are they located in a peroxisomal matrix protein. One clue comes from an elegant study of Lametschwandtner et al. (1998). By screening a library of random hexadecapeptides fused to the Gal4p activation domain in a two-hybrid setup against human and *S. cerevisiae* Pex5p, interacting peptides were identified in which the three
carboxyl-terminal amino acids deviated from the consensus PTS1. Interestingly, it was shown that residues just upstream of the carboxyl-terminal tripeptide were able to influence the interaction with Pex5p, although some differences were noted between S. cerevisiae and human Pex5p. Although both species prefer an arginine or lysine at the -4 position immediately preceding the carboxyl-terminal tripeptide, S. cerevisiae Pex5p had a preference for a polar or a hydrophilic residue at the position two amino acids upstream of the PTS1. At this position and five amino acids upstream of the PTS1, human Pex5p preferred hydrophobic residues. These accessory sequences or upstream residues became increasingly important when the carboxyl-terminal tripeptide deviated more from the consensus PTS1 sequence (Lametschwandtner et al., 1998). Also in plants, residues upstream of the PTS1 tripeptide were shown to contribute to targeting to glyoxysomes, a specialized type of peroxisomes (Mullen et al., 1997a).

The above-mentioned results were all obtained with artificially created peptides or with reporter proteins. An example of an endogenous peroxisomal matrix protein in which a residue upstream of the carboxyl-terminal tripeptide plays a critical role in the targeting to peroxisomes is human catalase. The four carboxyl-terminal amino acids of this enzyme are K-A-N-L. Clearly, the last three amino acids do not comply with the PTS1 consensus sequence, previously mentioned, since a basic amino acid is absent at the -2 position. When the tripeptide A-N-L was fused to the carboxyl terminus of the CAT protein, the fusion protein failed to target to peroxisomes in yeast and human fibroblasts (Purdue and Lazarow, 1996). However, fusing K-A-N-L to this reporter protein resulted in a peroxisomal localization. Substitutions of this lysine at the -4 position resulted in either a complete loss of or a reduction in peroxisomal targeting (Purdue and Lazarow, 1996). A similar observation was made for the PTS1 of cottonseed catalase. The last three amino acids of this protein (P-S-I) were not sufficient to direct the CAT protein to glyoxysomes. However, when the last four amino acids of cottonseed catalase (R-P-S-I) were fused to CAT, import of this reporter protein into glyoxysomes was observed (Mullen et al., 1997b).

These examples show that the original definition of a PTS1 needs some adjustment. The fact that a reporter protein cannot be directed to peroxisomes does not necessarily mean that a certain amino acid sequence is not a PTS1. When a carboxyl-terminal sequence is needed to direct a peroxisomal matrix protein to peroxisomes in the homologous context, it can be regarded as a PTS1. Also, the PTS1 is not limited to the carboxyl-terminal three amino acids since residues upstream might be involved as well.
Peroxisomal targeting signal type 2 (PTS2)
The PTS2 was first identified in the amino terminus of rat peroxisomal 3-ketoacyl-CoA thiolase and was shown to be a cleavable presequence (Osumi et al., 1991; Swinkels et al., 1991). Subsequent sequence alignments and mutagenesis studies showed that the PTS2 is a bipartite amino acid motif with the consensus sequence R/K-L/V/I/-X5-H/Q-L/AA (Osumi et al., 1992; Gietl et al., 1994; Glover et al., 1994b; Tsukamoto et al., 1994; Kato et al., 1996; Flynn et al., 1998). The PTS2 pathway has been identified in other mammals, plants, trypanosomes and yeasts, but the number of proteins shown to possess a PTS2 is very limited compared to the number of PTS1-containing proteins. PTS2-containing proteins are *S. cerevisiae* 3-ketoacyl-CoA thiolase (Erdmann, 1994; Glover et al., 1994b), *Hansenula polymorpha* amine oxidase (Faber et al., 1995), watermelon glyoxysomal malate dehydrogenase (Gietl et al., 1994), *Trypanosoma brucei* aldolase (Blattner et al., 1995), human alkyl-dihydroxyacetonephosphate synthase (de Vet et al., 1997), and human phytanoyl-CoA hydroxylase (Jansen et al., 1997).

A remarkable exception with regard to the import of peroxisomal matrix proteins is *Caenorhabditis elegans* in which the PTS2-targeting pathway is apparently absent. The *C. elegans* orthologs of 3-ketoacyl-CoA thiolase, alkyl-dihydroxyacetonephosphate synthase and phytanoyl-CoA hydroxylase do not contain a PTS2, but instead have acquired a PTS1 (Motley et al., 2000). Furthermore, *C. elegans* has no homolog of Pex7p. Probably the PTS2-targeting pathway was lost during evolution after switching targeting signals for the above-mentioned proteins.

Peroxisomal targeting signal type 3, or alternative targeting signals
A number of peroxisomal matrix proteins are imported into peroxisomes independently of a PTS1 and PTS2. An example is *Candida tropicalis* acyl-CoA oxidase, a protein without a recognizable PTS1 and PTS2. Two alternative regions in this protein have been identified that proved to be able to direct a reporter protein into peroxisomes (Small et al., 1988). Another example is *S. cerevisiae* acyl-CoA oxidase, which also lacks a PTS1 and PTS2, and instead uses another internal peroxisomal targeting signal (PTS3) that has not been characterized yet. For its targeting to peroxisomes, *S. cerevisiae* acyl-CoA oxidase is dependent on the PTS1-receptor Pex5p. The interaction of acyl-CoA oxidase with Pex5p is direct but the binding site on the receptor is clearly distinct from the PTS1-binding site (Klein et al., 2002). A similar behavior was found for *S. cerevisiae* carnitine acetyltransferase (Cat2p) from which the PTS1 had been deleted. This protein was still targeted to peroxisomes in a Pex5p-dependent manner (Elgersma et al., 1995). For that reason we concluded that the accessory sequences, which are present in a number of peroxisomal matrix proteins
and contribute to the interaction with Pex5p, can even function as a peroxisomal targeting signal in the case of Cat2p (Klein et al., 2002).

**Import of folded proteins**

Accumulating data show that some peroxisomal matrix proteins can be imported after folding and/or oligomerization has taken place. This was shown for the first time for chloramphenicol acetyltransferase, which was found to be imported after oligomerization (McNew and Goodman, 1994). Other examples are thiolase in *S. cerevisiae* (Glover et al., 1994a) and *Y. lipolytica* (Titorenko et al., 1998), malate dehydrogenase in *S. cerevisiae* (Elgersma et al., 1996b), dihydroxyacetone synthase in *C. boidinii* (Stewart et al., 2001), isocitrate lyase in glyoxysomes of plants (Lee et al., 1997) and alanine:glyoxylate aminotransferase 1 in mammals (Leiper et al., 1996). In a number of the above-mentioned examples, the experimental design was based on the fact that many matrix proteins are homo-multimers. PTS-less variants of a protein were expressed together with a PTS-containing version. Formation of an oligomer between these subunits allowed the PTS-less version to hitch a ride into peroxisomes. Also other approaches showed that stably folded proteins could be imported into peroxisomes. Folded proteins were either stabilized by disulfide bonds and chemical cross-linkers (Walton et al., 1995) or by the addition of a ligand analog (Hausler et al., 1996) without affecting import into peroxisomes or glycosomes.

For a number of proteins it has been shown that proteins without a PTS are directed to peroxisomes by hetero-oligomerization. *S. cerevisiae* $\Delta^3,\Delta^2$-enoyl-CoA isomerase (Eci1p), for instance, contains H-R-L as a PTS1. However, this tripeptide is much weaker PTS1 than the classical S-K-L (Yang et al., 2001). Even after deletion of its PTS1, Eci1p is still imported into peroxisomes but only when $\Delta^3,\Delta^2$-dienoyl-CoA isomerase (Dci1p), another PTS1-containing protein, is present. Eci1pPTS1 oligomerizes with Dci1p, which results in peroxisomal import via the Pex5p-dependent PTS1 pathway (Yang et al., 2001). In the absence of Dci1p, there is only a partial import of Eci1p into peroxisomes (Geisbrecht et al., 1999), indicating that oligomerization is probably important for peroxisomal import of Eci1p under normal circumstances, *i.e.* in the presence of its PTS1. Hetero-oligomerization also precedes the peroxisomal import of acyl-CoA oxidase in the yeast *Y. lipolytica* (Titorenko et al., 2002): five different acyl-CoA oxidase subunits form a heteropentameric complex, which is assembled in the cytosol together with the cofactor FAD. Formation of this complex is required for peroxisomal import.

Although there is sufficient evidence that import of oligomerized proteins into peroxisomes can occur, oligomerization is not a prerequisite for import. For instance,
alcohol oxidase is imported as a monomer in peroxisomes of *S. cerevisiae*, *P. pastoris* and *C. boidinii* (Distel et al., 1987; Waterham et al., 1997; Stewart et al., 2001). Assembly of the individual subunits into the homo-octameric form takes place in the peroxisomal matrix after import (Waterham et al., 1997; Stewart et al., 2001).

### V. The import receptors

**Pex5p: the receptor for PTS1 proteins**

Pex5p is the best-studied protein of all peroxins. It was first identified in the yeast *P. pastoris* (McCollum et al., 1993) by functional complementation of a mutant deficient in the import of proteins with a PTS1, but not a PTS2. Following this initial characterization, orthologs of Pex5p were identified in other yeast species such as *S. cerevisiae* (Van der Leij et al., 1993), *H. polymorpha* (Nuttley et al., 1995), *Yarrowia lipolytica* (Szilard et al., 1995), *Candida albicans* (Navarro-Garcia et al., 1998) and *Schizosaccharomyces pombe* (Wood et al., 2002). Pex5p was also identified in the plants *Citrullus lanatus* (Wimmer et al., 1998), *Nicotiana tabacum* (Kragler et al., 1998), and *Arabidopsis thaliana* (Brickner et al., 1998), in the nematode *C. elegans* (Consortium, 1998), in the trypanosomes *T. brucei* (de Walque et al., 1999) and *Leishmania donovani* (Jardim et al., 2000), and in the mammals *Homo sapiens* (Dodt et al. 1996) and *H. polymorpha* (van der Klei et al., 1995). However, also in humans (Dodt and Gould, 1996) and in *Cricetulus griseus* (Ito et al., 2001).

Pex5p functions as a mobile receptor for peroxisomal matrix proteins that contain a PTS1. Binding of the receptor to the PTS1 is the first step in the targeting of matrix proteins to peroxisomes. Pex5p does not contain a hydrophobic domain that would be able to anchor the protein to the peroxisomal membrane. Studies on the subcellular localization of Pex5p have generated different results. In most organisms, Pex5p is located mainly in the cytosol and only a small fraction is associated with peroxisomes (Dodt et al., 1995; Wiemer et al., 1995; Dodt and Gould, 1996; Elgersma et al., 1996a; Gould et al., 1996; Wimmer et al., 1998; Gouveia et al., 2000; Jardim et al., 2000). An exception is the yeast *Y. lipolytica* where Pex5p is mainly located inside peroxisomes (Szilard et al., 1995). However, also in humans (Dodt and Gould, 1996) and in *H. polymorpha* (van der Klei et al., 1995) a small fraction of Pex5p was sometimes found in the peroxisomal matrix. This dual localization of Pex5p has led to a model for protein import into peroxisomes in which the first step, i.e. binding of cargo to the receptor protein, takes place in the cytosol. The cargo is subsequently delivered to the
peroxisome and translocated across the peroxisomal membrane, followed by recycling of the receptor back to the cytosol (Figure 1).

A central issue in this model of a shuttling receptor is whether Pex5p itself enters the peroxisome (extended shuttle), followed by dissociation of the receptor-cargo complex and export of the receptor to the cytosol, or whether dissociation of the receptor-cargo complex already occurs at or in the peroxisomal membrane (simple shuttle). To address this question, elegant experiments were performed by Dammai and Subramani (2001). By fusing the minimal pre-thiolas e processing site and a tag to the amino terminus of human Pex5p, they were able to show that this artificially created Pex5p was processed. Since the protease activity that is responsible for the maturation of the precursor thiolas e is located in the luminal space, these experiments showed that Pex5p was exposed to the peroxisomal matrix. The processed Pex5p was also recovered in the cytosol, indicating that it cycled back to the cytosol after docking at the peroxisome. Although this example clearly showed that Pex5p is exposed to the peroxisomal matrix, it is still unclear whether the complete Pex5p molecule enters the peroxisome. For rat-liver Pex5p it was shown that the fraction that is associated with peroxisomes had the characteristics of a transmembrane protein (Gouveia et al., 2000). Maybe Pex5p functions in a similar way as SecA, which cycles between membrane-inserted and deinserted states, and in this way guides preproteins across the bacterial membrane (Economou and Wickner, 1994).

TPR motifs
Pex5p belongs to the family of tetratricopeptide repeat (TPR)-containing proteins, which are characterized by a highly degenerate, repetitive sequence of 34 amino acids (Goebl and Yanagida, 1991; Lamb et al., 1995). TPRs are found as tandem arrays of 3-16 motifs in a wide variety of proteins involved in many different cellular processes, including cell-cycle regulation, chaperone functions and protein phosphorylation (Blatch and Lassle, 1999). TPR motifs are present in a variety of organisms ranging from bacteria to mammals.

The crystal structure of the three TPR motifs in protein phosphatase 5 (PP5) showed that each TPR motif consists of a pair of antiparallel α-helices, named helices A and B, separated by an intra-repeat loop that forms a turn (Figure 2A) (Das et al., 1998). TPR motifs are organized in a parallel arrangement such that sequentially adjacent α-helices are antiparallel. Small hydrophobic amino acids such as alanine and glycine are located at the positions 8, 20 and 27 within a TPR motif (Figure 3). These are the positions of closest contact between adjacent α-helices and only limited space is available for amino acid side chains. The spatial and angular arrangement of the
helices A and B is the same within and between adjacent TPR motifs. This results in
the formation of a regular right-handed superhelix, which contains an amphipathic
groove (Figure 2B). This amphipathic groove probably forms the binding surface for
proteins that interact with PP5 (Das et al., 1998). However, the structural basis for the
PP5 TPR-mediated protein-protein interaction remains unclear, since no structure of
the PP5 TPRs with a bound peptide is available as yet. For the TPR-containing protein
Hop (Hsp70 and Hsp90 organizing protein) it was shown that binding of peptides did
indeed occur in this general TPR groove, which is cradle shaped (Scheufler et al.,
2000). The nine TPR motifs of Hop form two TPR domains, i.e. TPR domain-1
consisting of three TPR motifs and TPR domain-2 consisting of six TPR motifs. The
TPR domain-1 of Hop interacts with the carboxyl terminus of Hsp70 (Figure 2C) and
the TPR domain-2A interacts with the carboxyl terminus of Hsp90. Hop mediates the
association of Hsp70 and Hsp90, which is important for the transfer of substrate from
Hsp70 to Hsp90. Both Hsp70 and Hsp90 possess a conserved E-E-V-D motif at the
end of their sequence. Peptides ending on E-E-V-D and corresponding to the carboxyl
termini of Hsp70 or Hsp90 are bound in the grooves of the TPR domain-1 and TPR
domain-2A. Electrostatic interactions mediate the binding of the E-E-V-D motifs to
the side chains of amino acids that are located in the helices A of the TPR motifs.

The TPR domain of Pex5p consists of six or seven of these TPR motifs present in
the carboxyl-terminal part of the protein (Figure 3). The TPR domain mediates the
interaction of Pex5p with PTS1-containing proteins (Brocard et al., 1994; Dodt et al.,
1995; Terlecky et al., 1995). The interaction of the TPR domain of Pex5p with the
PTS1 has been studied in detail in two organisms. The X-ray structure of human
Pex5p in complex with the PTS1 peptide Y-Q-S-K-L was solved (Gatto et al., 2000),
and for S. cerevisiae Pex5p, the amino acids involved in the interaction with PTS1
were identified by combining an extensive mutagenesis analysis with molecular
modeling (Klein et al., 2001). The carboxyl-terminal part of Pex5p proved to form two
entities, each consisting of three TPR motifs (TPR1-3 and TPR5-7). ‘TPR4’, a
questionable TPR motif that forms a flexible hinge, connects these two entities (Gatto
et al., 2000). The PTS1 is bound between these two TPR clusters and corresponding
amino acids in the human and the yeast Pex5p were found to contact the PTS1 (Figure
4) (Gatto et al., 2000; Klein et al., 2001, chapter 3 of this thesis). Thus these two
independent studies showed that the manner of Pex5p-PTS1 interaction is similar in
both human and S. cerevisiae.

The mode of interaction between the PTS1 and the TPR5-7 (Figure 4B) is
comparable to the interaction of the Hop adaptor protein with the carboxyl termini of
Hsp70 and Hsp90 (Figure 2C) (Scheufler et al., 2000).
Figure 2. Structure of TPR domains.
A) Structure of the TPR domain of PP5 (PDB ID: 1A17), which consists of three TPR motifs. The antiparallel α-helices A and B of each TPR motif are indicated. Black arrows mark the loops between different TPR motifs (inter-repeat loop) and gray arrows mark the loops within a TPR motif (intra-repeat loop). B) Molecular surface representation of the TPR domain of PP5 (same orientation as in panel A). The groove that is present in the TPR domain is indicated. C) Structure of the TPR domain-1 of Hop (gray) in complex with the C-terminal heptapeptide of Hsp70 (black) (PDB ID: 1ELW). The peptide is bound in the groove of the TPR domain. Electrostatic interactions mediate the binding of the peptide E-E-V-D motif to the sidechains of amino acids located in the A helices of the TPR domain. D) Structure of the TPR domain of p67phox (gray) in complex with RacGTP (black) (PDB ID: 1E96). Interactions are mediated by the loops that connect TPR1 with TPR2, TPR2 with TPR3, and an insertion that is located in the loop that connects TPR3 with TPR4. For RacGTP only the residues involved in complex formation are shown.
The general TPR groove forms the interaction site and residues from the helices A of TPR6 and TPR7 mediate most contacts (Gatto et al., 2000; Klein et al., 2001, chapter 3 of this thesis). The interaction between the PTS1 and TPR1-3 does not involve the general TPR groove. Instead, several residues from the intra-repeat loops of TPR2 and TPR3 are involved (Figure 4A) (Gatto et al., 2000; Klein et al., 2001, chapter 3 of this thesis). This is a novel mode of interaction between TPR domain and peptide. The two entities of three TPRs almost completely surround the PTS1 peptide and in this way form a single binding site for the PTS1 peptide (Figure 4C) (Gatto et al., 2000).

<table>
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Figure 3. Sequence alignment of different TPR motifs.

The TPR motifs of PP5, Hop, p67phox (all human), and S.cerevisiae Pex5p are aligned and the small hydrophobic amino acids at positions 8, 20, and 27, which are important for the folding of a TPR domain are highlighted. At position 32 often a proline is located. Indicated above the sequences are the regions that form the α-helices A and B and the intra-repeat loop.

The interaction of TPR1-3 with the PTS1 is not the only example of a TPR domain using regions outside the general TPR groove to bind its substrate. For instance, the NADPH oxidase that is involved in the generation of reactive oxygen species as a defence against microbial infection consists of several subunits. One of these is
p67phox, which contains four TPR motifs in its amino terminus (Ponting, 1996). Binding of p67phox to the small GTPase Rac-GTP is an important step in the assembly of the NADPH oxidase. The interaction with Rac is mediated by the amino-terminal part of p67phox, which contains four TPR motifs (Koga et al., 1999). The TPR-mediated interaction of p67phox with Rac does not involve the general TPR groove but instead is mediated by an insertion of 20 amino acids between TPR3 and TPR4 and by residues in the loops that connect TPR1 with TPR2 and TPR2 with TPR3 (Figure 2D) (Lapouge et al., 2000). Thus, the initial prediction that the general TPR groove forms the ligand-binding site in an array of TPR motifs (Das et al., 1998) proved not to be the only way of interaction. The examples discussed above show that at least three different modes of interaction exist for TPR motifs and their ligands.

In line with the data above is the identification of pex5 mutant cells with an import defect in PTS1 proteins. A cell line from a patient suffering from NALD was shown to carry a mutation in the sixth TPR repeat, in which asparagine 526 (or asparagine 489 in Pex5pS) is mutated to a lysine (Dodt et al., 1995), which results in a 1000-fold reduction in the affinity for PTS1 proteins (Gatto et al., 2000). The N526K substitution is predicted to disturb the interaction with the carboxyl-terminal carboxylate anion of the PTS1, which would explain this phenotype. Another cell line from a patient suffering from Zellweger syndrome contained a mutation resulting in a premature stop codon at position 427 (R427ter) of Pex5p (Dodt et al., 1995). This gave rise to a truncated protein completely lacking TPR5-7, which would explain the inability to import PTS1 proteins.

**Pex7p: the receptor for PTS2 proteins**

Pex7p was first identified in *S. cerevisiae* by functional complementation of a mutant that was selectively defective in the import of thiolase into peroxisomes (Marzioch et al., 1994; Zhang and Lazarow, 1995; Zhang and Lazarow, 1996). After this initial characterization, Pex7p was identified in the yeast *P. pastoris* (Elgersma et al., 1998) and in mammals (Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997). Pex7p binds to the amino-terminal PTS2 of thiolase (Zhang and Lazarow, 1996; Elgersma et al., 1998) and this interaction is essential for the import of a PTS2-containing protein into peroxisomes. The absence of Pex7p does not influence the targeting of PTS1-containing proteins.

Pex7p is a member of the WD-40 repeat family, which can be recognized by the presence of a repeated domain of 40 amino acids containing a central tryptophan-asparagine (WD) motif (van der Voorn and Ploegh, 1992). Seven of these WD-40 repeats are present in Pex7p, but which part of Pex7p forms the binding site for PTS2 is not yet known.
The subcellular location of Pex7p is still controversial since conflicting results were reported. Amino-terminally tagged with a Myc-epitope, Pex7p was found predominantly in the cytosol of human fibroblasts (Braverman et al., 1997) and S. cerevisiae (Marzioch et al., 1994; Rehling et al., 1996), whereas carboxyl-terminally HA-tagged Pex7p was found to be entirely located inside peroxisomes in S. cerevisiae (Zhang and Lazarow, 1995). This variation in localization of Pex7p might be caused by the different tags that were fused to the protein. Also the position of the tag, fused to either the amino terminus or the carboxyl terminus, could have influenced the localization. In P. pastoris and mammals, untagged Pex7p was found to be located both in the cytosol and inside peroxisomes (Elgersma et al., 1998; Mukai et al., 2002).

The difference in subcellular localization has led to different models of how Pex7p directs PTS2 proteins to peroxisomes. For example, intraperoxisomal Pex7p might be involved in pulling PTS2 proteins into the peroxisomes (Zhang and Lazarow, 1996). If this is true and Pex7p is located inside peroxisomes from where it pulls PTS2 proteins, then another factor must exist that directs these PTS2 proteins from the cytosol to the peroxisome. However, such a factor has so far not been identified. Moreover, the domains of the peroxisomal membrane proteins that bind to Pex7p are located on the cytosolic face of the peroxisomal membrane (see below). Hence, these findings support another model, i.e. the shuttling receptor model, in which Pex7p travels between the cytosol and the peroxisome and thus delivers PTS2 proteins at the peroxisomal membrane (Figure 1) (Rehling et al., 1996; Elgersma et al., 1998).

**PTS2 import in mammals depends on both Pex5p and Pex7p**

As mentioned before, Pex5p is involved in the import of PTS1 proteins in all species studied so far. However, in mammals two different phenotypes have been reported as a consequence of a *pex5* deficiency. The import of PTS1 alone or that of both PTS1 and PTS2 were disturbed in Chinese hamster ovary (CHO) mutant cells (Otera et al., 1998) and in fibroblasts from patients with peroxisome biogenesis disorders (Dodt et al., 1995; Braverman et al., 1998). The first phenotype can be explained in the same way as for yeast *pex5* mutants: a mutation in the PTS1 binding site abolishes the import of PTS1 proteins. Examples of this type of mutation are the G522E mutation in Chinese hamster Pex5p (Otera et al., 1998; Otera et al., 2000) and the N526K mutation in...
human Pex5p (N489K in Pex5pS) (Dodt et al., 1995). However, in mutants where Pex5p is completely absent, both the import of PTS1 and that of PTS2 are disturbed. This suggests that some function of Pex5p is essential for PTS2 import.

Two isoforms of Pex5p exist in mammals: a long form (Pex5pL) and a short form (Pex5pS). Pex5pL contains an additional internal region of 37 amino acids that is positioned between amino acids 214 and 215 (human) or 215 and 216 (Chinese hamster) of the short form (Pex5pS) (Figure 5) (Dodt et al., 1995; Braverman et al., 1998; Otera et al., 1998). This additional internal region of Pex5p is encoded by exon 8 and its presence is regulated by alternative splicing. In pex5-deficient CHO cells that were disturbed in both PTS1 and PTS2 import, Pex5pS only restored the import of PTS1 proteins, but Pex5pL restored both PTS1 and PTS2 import into peroxisomes in the same cells (Otera et al., 1998). Also in fibroblasts from a complementation group 2 (CG2) patient with NALD only Pex5pL restored both PTS1 and PTS2 import (Braverman et al., 1998). The explanation for these findings is the involvement of Pex5pL in the transport of Pex7p to peroxisomes, together with its PTS2-containing cargo protein (Figure 1). In line with this suggestion, a direct interaction between Pex5pL and Pex7p was found (Otera et al., 2000); the region in Pex5pL necessary for this interaction was mapped using truncated versions of Pex5pL. Approximately the same regions in human Pex5p (amino acids 191-222) and Chinese hamster Pex5p (amino acids 190-233) were shown to be sufficient for the interaction with Pex7p (Figure 5 and Figure 6) (Dodt et al., 2001; Otera et al., 2002). This sequence includes the amino-terminal amino acids of the Pex5pL-specific 37 amino acid insertion, together with amino acids lying outside this insertion. The S214F mutation in this area disrupted binding to Pex7p (Otera et al., 2002) and resulted in a specific PTS2-import defect, while PTS1 import was not affected (Matsumura et al., 2000).

The role of Pex5pL in the import of PTS2 proteins in mammals proved to be independent of its role in the import of PTS1 proteins. Expressing a truncated version of Pex5pL, containing only the amino-terminal half of the protein without the TPR motifs, complemented the PTS2 import defect in pex5-deficient mammalian cells (Dodt et al., 2001; Otera et al., 2002). In yeast, PTS2 import is not dependent on Pex5p. Recently, a peptide motif in this 37 amino acid insertion of Pex5pL was shown to be highly conserved in S. cerevisiae Pex18p and Pex21p and in Y. lipolytica Pex20p (Figure 7) (Dodt et al., 1995; Einwachter et al., 2001; Otera et al., 2002). ScPex18p and ScPex21p are cytosolic proteins that are necessary for the formation of an import-competent complex containing Pex7p and thiolase (Purdue et al., 1998; Stein et al., 2002). YTPex20p binds thiolase and is necessary for its dimerization and targeting to the peroxisomal membrane (Smith and Rachubinski, 2001). This finding suggests similar roles for Pex18p and Pex21p in S. cerevisiae, for Pex20p in Y. lipolytica, and
for mammalian Pex5pL in the targeting of PTS2-containing proteins to peroxisomes. This is supported by the partial complementation of the *S. cerevisiae pex18Δpex21Δ* mutant by *YpPex20p* (Einwachter et al., 2001). Since Pex7p has not been identified in *Y. lipolytica*, Pex20p might contain the functions of Pex7p, Pex18p, and Pex21p.

The presence of this conserved peptide motif in Pex5p of *H. sapiens, M. musculus, Cricetulus longicaudatus, T. brucei, L. donovani,* and *A. thaliana* (Figure 5) indicates that Pex5p is necessary for the import of PTS2 proteins in mammals, protozoa and plants (Dodt et al., 2001). The absence of the conserved peptide motif in fungi fits with the finding that the PTS2-import pathway was independent of Pex5p. The complete lack of the PTS2-import pathway in *C. elegans* (Motley et al., 2000) could explain the absence of the conserved peptide motif in this organism.

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**Figure 5. Sequence alignment of Pex5p.**

Aligned are the Pex5p sequences of *Saccharomyces cerevisiae* (Sc), *Candida albicans* (Ca), *Pichia pastoris* (Pp), *Hansenula polymorpha* (Hp), *Yarrowia lipolytica* (Yl), *Homo sapiens* (Hs), *Mus musculus* (Mm), *Cricetulus longicaudatus* (Ci), *Trypanosoma brucei* (Tb), and *Leishmania donovani* (Ld). Indicated above the sequence are the different W-X-X-F/Y repeats that are involved in the interaction with Pex14p in mammals and the regions mediating the interaction with Pex13p and Pox1p in *S. cerevisiae*. Also the 37 amino acid insert specific for mammalian Pex5p is indicated.

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continued on next two pages
VI. Docking of the receptors

**Pex5p interacts with Pex13p and Pex14p**

Binding of Pex5p at the peroxisomal membrane takes place through interaction with the peroxisomal membrane proteins Pex13p (Elgersma et al., 1996a; Erdmann and Blobel, 1996; Gould et al., 1996; Barnett et al., 2000; Bottger et al., 2000; Urquhart et al., 2000; Otera et al., 2002) and Pex14p (Albertini et al., 1997; Brocard et al., 1997; Fransen et al., 1998; Schliebs et al., 1999; Shimizu et al., 1999; Will et al., 1999; Urquhart et al., 2000; Otera et al., 2002) (Figure 1).

Pex14p is a peroxisomal membrane protein that is required for the import into peroxisomes of both PTS1 and PTS2 proteins. It is tightly associated with the peroxisomal membrane either as an integral membrane protein (Brocard et al., 1997; Shimizu et al., 1999; Will et al., 1999) or as a peripheral membrane protein (Albertini et al., 1997). The topology of the protein in the peroxisomal membrane is still controversial, since different results were reported regarding the localization of its amino terminus: it has been found to be exposed to the cytosol (Brocard et al., 1997; Shimizu et al., 1999) and located inside peroxisomes (Will et al., 1999).

Pex14p does not only interact with Pex13p but can also bind directly to Pex5p (Fransen et al., 1998; Bottger et al., 2000; Otera et al., 2000; Urquhart et al., 2000) and Pex7p (Shimizu et al., 1999; Otera et al., 2000). When the sites in Pex14p and Pex5p that are involved in the Pex14p-Pex5p interaction were determined, the amino-terminal 78 amino acids of human Pex14p were found to bind to the amino-terminal half of Pex5p with a very high affinity (Schliebs et al., 1999). Multiple binding sites for Pex14p were shown to be present in the amino terminus of Pex5p and a detailed analysis showed that the pentapeptide W-x-x-x-F/Y repeats were involved in binding Pex14p in mammals and in plants (Saidowsky et al., 2001; Nito et al., 2002; Otera et al., 2002). Multiples of these pentapeptide W-x-x-x-F/Y repeats were detected in the amino terminus of Pex5p and the number varied depending on the organism (Figure 5). Two repeats were found in S. cerevisiae Pex5p, four in P. pastoris Pex5p, seven in the long form of mammalian Pex5p, and even nine in higher plants such as watermelon and A. thaliana. The overall sequence conservation in the amino-terminal half of Pex5p is low compared to the carboxyl-terminal half, which contains seven TPR motifs, but some of the few conserved residues in the amino-terminal part are part of the pentapeptide W-x-x-x-F/Y repeats.

The affinity for binding Pex14p differs for each individual W-x-x-x-F/Y repeat, as was shown for human Pex5p (Saidowsky et al., 2001). It is not known whether in yeast these repeats are also involved in binding Pex14p. For example, a mutation of
the conserved tryptophan (W204A) in the second pentapeptide repeat in *S. cerevisiae* Pex5p did not affect the interaction with Pex14p (Bottger et al., 2000). Similar results were found for the conserved tryptophan (W120A) in the first pentapeptide repeat and for the double mutant (W120A, W204A) (G. Bottger, personal communication). However, mutating the conserved tryptophan 261, which is not part of a W-x-x-x-F/Y repeat, did result in a loss of Pex14p interaction (Klein et al., 2002). This loss of interaction is probably caused by misfolding of Pex5p and further research should determine whether or not the region surrounding W261 is involved in binding Pex14p in *S. cerevisiae*.

Pex13p is a peroxisomal membrane protein with two membrane-spanning domains. The amino terminus and the carboxyl terminus, which contains a Src homology 3 (SH3) domain, are both located in the cytosol. SH3 domains are found in a wide variety of proteins involved in many different processes ranging from signal transduction to regulation of cytoskeleton assembly. These domains consist of 60-70 amino acids and have a high sequence similarity. They adopt similar folds: two
antiparallel β-sheets and three variable loops, named RT loop, n-Src loop and distal loop (Figure 8A) (for review see Mayer and Eck, 1995). The ligands of SH3 domains contain a conserved proline-rich sequence, which forms a polyproline type II (PP-II) helix. The core element of this PP-II helix is the P-x-x-P sequence (in which x can be any amino acid). Different classes of ligands can bind to SH3 domains: class I ligands with the consensus sequence R-x-x-P-x-x-P, and class II ligands with the consensus sequence P-x-x-P-x-R (Ren et al., 1993; Feng et al., 1994; Lim et al., 1994; Mayer and Eck, 1995). The SH3 domain forms a relatively flat, hydrophobic ligand-binding surface that is located between the RT and n-Src loops (Figure 8B) (Weng et al., 1995; Arold et al., 1998). Three shallow pockets are present for binding the ligand, two of which are occupied by the conserved prolines of the P-x-x-P motif and the third is the ‘(acidic) specificity pocket’ formed by residues from the RT and n-Src loops. This pocket interacts with a basic residue located either amino- or carboxyl-terminally of the P-x-x-P sequence. Residues in the variable RT and n-Src loops of the SH3 domain contribute to ligand recognition and specificity of binding by interacting with residues located outside the P-x-x-P motif of the ligand (Feng et al., 1995; Lee et al., 1995; Wu et al., 1995; Lee et al., 1996).

![Sequence Alignment](image)

**Figure 7.** Sequence alignment of a conserved region that is present in HsPex5p, ScPex18p, ScPex21p, and YpPex20p.

This region is possibly involved in the interaction with Pex7p. A line above the sequence marks the region in human Pex5p that is encoded by the extra exon. The serine 214 position in mammalian Pex5p is indicated by an asterisk. A mutation to phenylalanine at this position (S214F) disrupts the interaction with Pex7p.

The SH3 domain of Pex13p interacts with Pex14p (Brocard et al., 1997; Girzalsky et al., 1999). This interaction is direct (Fransen et al., 1998; Barnett et al., 2000; Bottger et al., 2000; Urquhart et al., 2000) and involves a class II P-x-x-P motif (PPTLPHRDW) in Pex14p, as was shown for *S. cerevisiae* (Girzalsky et al., 1999). In several yeast species the SH3 domain of Pex13p was shown not only to bind Pex14p,
Figure 8. Structure of the SH3 domain of mouse c-CRK in complex with a proline-rich peptide (PDB ID: 1CKA).

The SH3 domain is shown in gray and the class II peptide (sequence: P-P-P-A-L-P-P-K-K) in black.

A) Indicated are the β-strands (large arrows in structure), the RT-loop, n-Src loop, and distal loop.

B) Molecular surface representation of the SH3 domain. The binding surface for the class II peptide, which is located between the RT- and the n-Src loops, is shown. Only the residues P-A-L-P-P-K of the peptide are shown.
but also to interact directly with Pex5p (Elgersma et al., 1996a; Erdmann and Blobel, 1996; Barnett et al., 2000; Bottger et al., 2000; Urquhart et al., 2000). The interacting domain of Pex5p comprises amino acids 203-227 in S. cerevisiae (Figure 5) (Barnett et al., 2000; Bottger et al., 2000) and amino acids 100-213 in P. pastoris (Urquhart et al., 2000). However, this interaction does not involve a P-x-x-P sequence; instead, one of the W-x-x-x-F/Y motifs of S. cerevisiae Pex5p was shown to be involved (Barnett et al., 2000; Bottger et al., 2000). Binding of Pex5p to the SH3 domain of Pex13p does not occur at the PP-II binding face but at a novel interaction site (Barnett et al., 2000). Pex5p and Pex14p can simultaneously bind to Pex13p-SH3 and there is no competition between these two proteins for binding to Pex13p-SH3. The presence of separate binding sites for the two ligands was underscored by the differential effect of mutations in Pex13-SH3 on the interaction with Pex14p and Pex5p. S. cerevisiae Pex13p(E320K) and Pex13p(W349A) were disturbed in the interaction with Pex14p, whereas Pex5p could still bind (Barnett et al., 2000; Bottger et al., 2000). The existence of two functionally and structurally independent binding sites on the SH3 domain of Pex13p was confirmed by using X-ray crystallography and NMR spectroscopy (Douangamath et al., 2002). Also in Pex13p-SH3 of P. pastoris, mutations with a differential effect on the Pex5p and Pex14p interaction were isolated, confirming the presence of different binding sites (Urquhart et al., 2000). However, these binding sites might partially overlap in P. pastoris since the E318K mutation in the n-Src loop abolished binding to both Pex5p and Pex14p (Urquhart et al., 2000).

There is a significant difference in the Pex13p-Pex5p interaction between mammals and yeast. Although the interaction between mammalian Pex13p and Pex5p is direct, it does not involve the carboxyl-terminal SH3 domain of Pex13p. Instead, the amineterminal region of Pex13p, upstream of the first transmembrane domain, interacts with Pex5p in mammals (Otera et al., 2002). Mutational analysis indicated that it is most likely that the W-x-x-x-F/Y motifs 2 to 4 of Pex5p mediate this interaction with Pex13p (Otera et al., 2002). The exact interaction site on Pex13p has not been mapped yet.

**Pex7p interacts with Pex13p and Pex14p**

Pex14p is not only essential for the import of PTS1 proteins but also for the import of PTS2 proteins (Figure 1) (Albertini et al., 1997; Shimizu et al., 1999; Will et al., 1999). Pex7p interacts with Pex14p in yeast and mammals, and this interaction proved to be direct (Albertini et al., 1997; Girzalsky et al., 1999; Shimizu et al., 1999; Otera et al., 2000; Stein et al., 2002). It is, however, still unclear which domains mediate this interaction. In yeast and mammals it was also shown that there is a direct interaction between Pex7p and Pex13p (Girzalsky et al., 1999; Otera et al., 2002; Stein et al.,
The amino terminus of Pex13p, upstream of the first transmembrane domain, mediates this interaction. This is the same region that is also involved in the interaction with Pex5p in mammals.

**Mechanism of receptor docking**

Since both import receptors interact with Pex13p and Pex14p, it is likely that these proteins form the point where the PTS1 and PTS2 import pathways for peroxisomal matrix proteins converge (Figure 1). However, despite the many interaction data and genetic experiments with Pex13p and Pex14p, the exact function of these proteins has not been elucidated yet. Two observations suggest that Pex14p is the initial and main site of docking of the PTS1-receptor Pex5p. First, Pex5p was shown to be mainly located in the cytosol of CHO cells by immunofluorescence microscopy and subcellular fractionation experiments (Otera et al., 2000). Overexpression of Pex14p in these cells resulted in accumulation of Pex5p on peroxisomes and an import defect of PTS1 and PTS2 proteins. These effects were not seen when Pex13p was overexpressed. In contrast, in the absence of functional Pex13p accumulation of Pex5p on peroxisomal remnants was observed.

Second, experiments in which the localization of GFP-tagged Pex5p (GFP-Pex5p) was studied in different *S. cerevisiae* pex-deletion strains showed that in wild-type cells and in *pex13A* cells GFP-Pex5p was targeted to peroxisomes. Only in *pex14Δ* and *pex17Δ* cells was GFP-Pex5p completely mislocalized to the cytosol. Furthermore, by using an inducible promoter to regulate the expression of Pex14p, it was shown that GFP-Pex5p redistributed to peroxisomes in *pex14Δ* cells upon Pex14p expression (Bottger, 2001).

Even though these results obtained *in vivo* are based on steady-state observations, they suggest that Pex14p is the site where the initial docking of Pex5p on the peroxisomal membrane takes place. Pex13p might be involved in a subsequent step involving the release of Pex5p from the peroxisomal membrane after PTS1 cargo has been imported (Otera et al., 2000). This model is supported by *in vitro* binding experiments with PTS1-loaded or unloaded mammalian Pex5p (Otera et al., 2002): Pex14p bound to PTS1-loaded Pex5p, while Pex13p only bound to unloaded Pex5p. Similar observations were made in *P. pastoris* (Urquhart et al., 2000). Loading of Pex5p with a PTS1 peptide had opposite effects on the interaction with Pex13p and Pex14p: binding of Pex5p to Pex14p was enhanced, while interaction of Pex5p with Pex13p was decreased. The results discussed above indicate that Pex14p forms the docking site on the peroxisomal membrane for Pex5p carrying PTS1 cargo. The next important step would be to reconstitute and study these reactions *in vitro*, but despite
some claims (Small and Lazarow, 1987; Gao et al., 1996) such an *in vitro* protein-import system is generally not available.

The early observations that a *P. pastoris* pex13Δ mutant was affected in the association of Pex5p with the peroxisomal membrane (Gould et al., 1996) led to a model in which Pex13p functions as the initial docking factor for Pex5p. These results might also be explained by impaired association of Pex14p with the peroxisomal membrane in the absence of functional Pex13p, as was shown for *S. cerevisiae* (Girzalsky et al., 1999). It is, however, not the direct interaction between Pex13p and Pex14p that is necessary for the peroxisomal localization of Pex14p, since mutations in either the SH3 domain of Pex13p or the P-x-x-P motif of Pex14p that disrupted the interaction between these two proteins did not affect the peroxisomal localization of Pex14p. Maybe Pex14p is bound to the peroxisomal membrane by a protein complex consisting of several components. Pex13p might then be an essential factor for the formation of this complex and when Pex13p is absent the complex might disintegrate. Other proteins that are part of this complex, such as Pex17p (see below), might be directly involved in binding Pex14p to the peroxisomal membrane (Girzalsky et al., 1999).

*In vitro* binding studies with purified mammalian Pex proteins further showed that the Pex13p-Pex14p interaction was also affected by binding of Pex5p in a cargo-dependent manner. Binding of unloaded Pex5p to Pex14p blocked the Pex13p-Pex14p interaction, while this was not observed in the case of PTS1-loaded Pex5p (Otera et al., 2002). These findings led to a model for receptor docking in mammals, in which Pex5p bound to a PTS1 protein first binds to the Pex13p-Pex14p complex via interaction with Pex14p. The PTS1 protein is released from Pex5p during translocation into the peroxisome, followed by dissociation of the Pex13p-Pex14p complex. The unloaded Pex5p is transferred to Pex13p and shuttles back to the cytosol (Otera et al., 2002). This model implies that Pex13p and Pex14p form functionally distinct subcomplexes, which are both involved in the import process of peroxisomal matrix proteins. Indeed, by isolating peroxisomes from rat liver, followed by solubilization with a mild detergent, it was shown that different high-molecular-weight complexes exist in the peroxisomal membrane (Reguenga et al., 2001). Pex14p proved to be part of a complex together with Pex5p and the two peroxisomal membrane proteins Pex2p and Pex12p. Only a very small fraction of Pex13p was associated with this complex, which might be caused by the experimental conditions used. Especially the type of detergent that is used in these experiments can influence the results, as was shown by the differences in composition of the high-molecular-weight complexes isolated from peroxisomes treated with Nonidet P-40 or digitonin (Reguenga et al., 2001). If Pex13p is only weakly associated with the Pex14p-Pex5p-Pex2p-Pex12p complex, it can
easily dissociate due to the treatment with detergent. Also a second high-molecular-weight complex was isolated from peroxisomes after detergent treatment (Reguenga et al., 2001). Pex13p was shown to be the major component of this complex, which might be involved in the recycling of Pex5p from the peroxisome to the cytosol. The Pex14p-Pex5p-Pex2p-Pex12p complex is most likely involved in the docking and translocation of peroxisomal matrix proteins. An interesting question remaining to be solved is whether this Pex13p-containing complex can associate and dissociate with the Pex14p-containing complex during the import cycle.

In yeast, overexpression of either Pex13p or Pex14p resulted in impaired growth of *S. cerevisiae* in oleate-containing medium, *i.e.* circumstances under which peroxisomes are needed. Overexpression of both Pex13p and Pex14p did not inhibit growth (Bottger et al., 2000). Since disrupting the Pex13p-Pex14p interaction by a mutation in the SH3 domain of Pex13p also resulted in impaired growth on oleic acid (Elgersma et al., 1996a; Girzalsky et al., 1999) it is most likely that Pex13p and Pex14p form a well-defined pair in yeast (Bottger et al., 2000). For correct functioning it is important that these two proteins and maybe also the different complexes of which they are part are present in the right stoichiometry.

**Other peroxins involved in the import of peroxisomal matrix proteins**

Different (genetic) screens have identified 25 peroxins that are essential for peroxisome biogenesis (Table I) (Sacksteder and Gould, 2000; Purdue and Lazarow, 2001; Smith et al., 2002; Tam and Rachubinski, 2002). Many of these peroxins are involved in the import of matrix proteins into peroxisomes, but most of them have not been studied as extensively as the import receptors Pex5p and Pex7p and the peroxisomal membrane proteins Pex13p and Pex14p. Furthermore, the lack of a good *in vitro* import system makes it difficult to dissect the different steps in peroxisomal protein import and to elucidate the role of individual peroxins. Therefore the exact function of these proteins is still not completely solved.

**Pex17p: another component of the docking complex?**

Pex17p was identified in *S. cerevisiae* (Huhs et al., 1998) and *P. pastoris* (Snyder et al., 1999b). In *S. cerevisiae*, Pex17p behaves as a peripheral membrane protein that is tightly bound to the outer face of the peroxisomal membrane, while in *P. pastoris* it is as an integral membrane protein with the carboxyl terminus facing the cytosol. The amino acid identity between ScPex17p and PpPex17p is very low, with the exception of the carboxyl-terminal coiled-coil domains (Snyder et al., 1999b). Different models exist for the function of Pex17p. In *S. cerevisiae*, Pex17p is thought to be part of the docking complex for the protein import receptors Pex5p and Pex7p, and to interact
with Pex14p and Pex5p in the two-hybrid system. However, the Pex17p-Pex5p interaction was only detectable in the presence of Pex14p and therefore can be considered to be an indirect interaction (Huhse et al., 1998). In *S. cerevisiae pex17Δ* cells, GFP-Pex5p was found only in the cytosol, which indicates that Pex17p is important for the association of Pex5p with the peroxisomal membrane (Bottger, 2001). A *S. cerevisiae pex17Δ* mutant was found to be affected in the import of both PTS1 and PTS2 proteins, and morphologically detectable peroxisomes were lacking as determined by electron microscopy (Huhse et al., 1998). However, indirect immunofluorescence microscopy on *S. cerevisiae pex17Δ* cells showed that peroxisomal membrane proteins were still present in peroxisomal ghosts (Huhse et al., 1998; Hettema et al., 2000). Also in *P. pastoris*, Pex17p interacted with Pex14p and therefore appeared to be part of the docking complex for the import of peroxisomal matrix proteins. However, different results were found for the import of peroxisomal membrane proteins in a *P. pastoris pex17Δ* mutant (Snyder et al., 1999b): a fraction of the peroxisomal membrane proteins Pex3p and Pex22p was found in the cytosol instead of in peroxisomal remnants. This in turn resulted in a model in which *PpPex17p* is involved in the import of peroxisomal membrane proteins (Snyder et al., 1999b). A subsequent study in *P. pastoris* by another group could not confirm these findings (Harper et al., 2002), they found that *P. pastoris pex17Δ* cells were able to import peroxisomal membrane proteins. Together with the results obtained in *S. cerevisiae* this finding suggests that Pex17p is only involved in the import of peroxisomal matrix proteins and not in the import of peroxisomal membrane proteins.

**VII. Translocation over the peroxisomal membrane**

**The RING finger proteins: Pex2p, Pex10p and Pex12p**

Three peroxins belong to the family of RING zinc finger proteins, namely Pex2p, Pex10p and Pex12p, and they have been identified in different yeast species and mammals. All three are integral peroxisomal membrane proteins and have a carboxyl-terminal RING finger domain located in the cytosol. Pex10p and Pex12p interact with each other and both proteins can also directly bind the PTS1 receptor Pex5p. These interactions are all mediated by the carboxyl-terminal parts of Pex10p and Pex12p, containing the RING domains (Chang et al., 1999b; Okamoto et al., 2000; Albertini et al., 2001). Cells deficient in *pex2, pex10* or *pex12* are disturbed in the import of peroxisomal matrix proteins, while the import of peroxisomal membrane proteins is not affected (Kalish et al., 1995; Kalish et al., 1996; Waterham et al., 1996; Chang et
General Introduction

When fibroblasts from PBD patients deficient in pex2, pex10 or pex12 were compared to normal fibroblasts, accumulation of Pex5p was found at peroxisomes (Dodt and Gould, 1996; Chang et al., 1999b). This indicates that these RING finger proteins are not involved in receptor docking but in one of the subsequent steps of protein import.

Differential permeabilization immunofluorescence experiments, in which only the plasma membrane is disrupted and the peroxisomal membrane is kept intact, showed that in fibroblasts deficient in pex2 and pex12 the accumulation of Pex5p is on or near the outer surface of the peroxisome. However, in one of the pex12 mutant fibroblasts the accumulated Pex5p could not be detected in differential permeabilization experiments. This indicates that Pex5p was not accessible and probably was located in the peroxisomal lumen (Dodt and Gould, 1996). Protease protection experiments on the organellar pellet of these pex12 mutant cells showed that they contained more protease-resistant Pex5p than normal fibroblasts (Chang et al., 1999b). These findings led to a model in which Pex2p, Pex10p and Pex12p are involved in the translocation of proteins across the peroxisomal membrane (Figure 1). Evidence to support this model is, however, still lacking.

Pex8p, an intra-peroxisomal peroxin

Currently Pex8p is the only identified peroxin with an intra-peroxisomal localization. PEX8 has been cloned in several yeast species but no mammalian homolog is known at the present time. The protein behaves as a peripheral membrane protein that is tightly associated with the peroxisomal membrane in P. pastoris (Liu et al., 1995), Y. lipolytica (Smith et al., 1997) and S. cerevisiae (Rehling et al., 2000). In H. polymorpha, Pex8p was shown to be localized at the periphery of peroxisomes, between the alcohol oxidase crystalloids and the peroxisomal membrane (Waterham et al., 1994). Pex8p interacts with the PTS1 receptor Pex5p (Rehling et al., 2000; Smith and Rachubinski, 2001). This last finding might indicate that Pex5p enters peroxisomes during the import cycle or at least that Pex5p is exposed to the peroxisomal lumen. However, it is important to realize that it has not been shown that the Pex5p-Pex8p interaction really occurs inside peroxisomes. Each of the orthologs of Pex8p contains a PTS1 at the carboxyl terminus, although the consensus sequence varies. The PTS1 is dispensable for the association of Pex8p with Pex5p, which suggests that Pex8p contains an additional Pex5p binding site (Rehling et al., 2000). Furthermore, Pex8pΔPTS1 is still targeted to peroxisomes and complements the pex8Δ mutant. In H. polymorpha, Pex8p also possesses a functional PTS2 in its amino terminus (Waterham et al., 1994). Although not located at the amino terminus but
more internally, also in ScPex8p a PTS2 consensus sequence is present (Rehling et al., 2000).

Pex8p is required for the import of PTS1 and PTS2 proteins but even in the absence of Pex8p in *S. cerevisiae* the PTS receptors could still bind to the peroxisomal docking complex (Rehling et al., 2000). In the *Y. lipolytica* pex8Δ mutant, a much larger fraction of Pex20p, which is necessary for peroxisomal targeting of the PTS2 protein thiolase, is associated with peroxisomes than that of wild-type cells. The peroxisome-associated Pex20p in *pex8A* cells was also protected from external proteases, which suggests that Pex20p is imported into peroxisomes (Smith and Rachubinski, 2001). For those reasons, Pex8p is probably involved in a step of the import process following docking of the receptors. Since Pex8p contains both a PTS1 and a PTS2, it has been suggested that it is involved in the dissociation of PTS1 and PTS2 cargo from the receptors Pex5p and Pex7p, respectively. However, biochemical evidence to support this model is still lacking.

**VIII. Receptor recycling: the role of Pex1p, Pex4p and Pex6p**

The peroxins Pex1p and Pex6p are members of the large family of AAA proteins (ATPases associated with a wide range of cellular activities) (Erdmann et al., 1991; Spong and Subramani, 1993). The AAA domain consists of 220-230 amino acids and contains two motifs named Walker A and B, which bind and hydrolyse ATP, respectively (Patel and Latterich, 1998; Vale, 2000).

Pex1p and Pex6p interact with each other in an ATP-dependent manner (Faber et al., 1998; Geisbrecht et al., 1998; Tamura et al., 1998; Kiel et al., 1999). In *S. cerevisiae*, Pex6p also interacts with Pex15p, an integral peroxisomal membrane protein (Elgersma et al., 1997; Stroobants, 2001), which interaction targets Pex6p to the peroxisomal membrane. Pex6p contains two AAA domains and ATP binding to the first of these domains stimulates the binding of Pex6p to Pex15p; binding of ATP to the second AAA domain, followed by hydrolysis, stimulates the release of Pex6p from Pex15p (Stroobants, 2001). However, Pex15p has only been identified in *S. cerevisiae* and whether or not Pex6p is targeted to peroxisomes in other species in the same manner is unknown. Even more striking is that the subcellular localization of both Pex1p and Pex6p seems to vary among different species. In *S. cerevisiae*, Pex6p is mainly located in the cytosol and a small amount is associated with peroxisomes (Stroobants, 2001), whereas in humans, both Pex1p and Pex6p are cytosolic (Yahraus
et al., 1996; Tamura et al., 1998). However, these results were obtained by overexpressing Pex1p and Pex6p, which might have influenced the localization of these proteins. If only limited docking sites for Pex1p and Pex6p would be available on the peroxisomal membrane, any extra Pex1p or Pex6p would automatically end up in the cytosol. Indeed, in S. cerevisiae the presence of the docking factor Pex15p seems to be the limiting factor for association of Pex6p with the peroxisomal membrane, since overexpression of Pex15p resulted in increased membrane association of Pex6p (Stroobants, 2001). In rats and H. polymorpha, Pex1p and Pex6p are associated with the peroxisomal membrane (Tsukamoto et al., 1995; Kiel et al., 1999), but in P. pastoris and Y. lipolytica they seem to be associated with vesicles distinct from mature peroxisomes (Faber et al., 1998; Titorenko et al., 2000; Titorenko and Rachubinski, 2000).

Various functions have been ascribed to Pex1p and Pex6p. Both proteins belong to the AAA family of ATPases, of which several members are required for different membrane fusion processes (Patel and Latterich, 1998). Therefore it was suggested that Pex1p and Pex6p in P. pastoris and Y. lipolytica might be involved in the fusion of the small vesicles in which they are located, leading to the maturation of peroxisomes (Faber et al., 1998; Titorenko et al., 2000; Titorenko and Rachubinski, 2000). Another possibility is that Pex1p and Pex6p play a role in the import pathway of peroxisomal matrix proteins, since Pex1p and Pex6p are the only peroxins known to bind ATP and the import of matrix proteins into peroxisomes is ATP-dependent (Bellion and Goodman, 1987; Imanaka et al., 1987; Wendland and Subramani, 1993; Dott and Gould, 1996). This second model is supported by a number of observations. First, import of peroxisomal matrix proteins was affected in cells deficient in pex1 and pex6, although residual levels were imported (Spong and Subramani, 1993; Heyman et al., 1994; Yahraus et al., 1996; Reuber et al., 1997; Kiel et al., 1999). Second, the presence of Pex1p and Pex6p proved to be important for the stability of the PTS1 receptor Pex5p. In cells deficient in pex1 and pex6 from both humans and P. pastoris, the steady-state levels of Pex5p were reduced (Dott and Gould, 1996; Yahraus et al., 1996). Finally, after an epistasis analysis in P. pastoris it was suggested that Pex1p and Pex6p play a role in the import of peroxisomal matrix proteins. These proteins function in the terminal steps of this pathway, after Pex2p, Pex10p and Pex12p, but before Pex4p and Pex22p (Figure 1) (Collins et al., 2000).

Pex4p belongs to the E2 family of ubiquitin-conjugating enzymes. Ubiquitination is an important step in the proteasome-mediated degradation of proteins (for reviews see Hershko and Ciechanover, 1998; Pickart, 2001). Ubiquitin is conjugated to a substrate protein via a number of enzymatic steps. First, the ubiquitin-activating E1 enzyme activates ubiquitin in an ATP-dependent manner. In the next step, ubiquitin is
transferred to an ubiquitin-conjugating E2 enzyme, which binds the activated ubiquitin via a conserved active-site cysteine. In the final step, an E3 enzyme, a ubiquitin-protein ligase, transfers ubiquitin from the E2 enzyme to the amino group of a lysine residue of the substrate. If several (more than four) ubiquitin molecules are conjugated in a chain to a protein, this will result in targeting to the proteasome, followed by degradation. Addition of either one or several ubiquitin molecules is a way to regulate a variety of cellular processes, such as for instance endocytosis (Hicke and Riezman, 1996) and sorting into multivesicular bodies (Katzmann et al., 2001).

Pex4p has been identified in *S. cerevisiae*, *P. pastoris* and *H. polymorpha* (Wiebel and Kunau, 1992; Crane et al., 1994; van der Klei et al., 1998). In each of these yeast species the active-site cysteine is conserved; for ScPex4p and *Pp*Pex4p this cysteine was shown to be essential for the function of the protein. Furthermore, ubiquitin was shown to be conjugated to *Pp*Pex4p (Crane et al., 1994). These findings indicate that Pex4p does indeed function as an E2 ubiquitin-conjugating enzyme. The substrate that receives the ubiquitin still has to be identified, although we have indications that Pex5p is ubiquitinated (T. Voorn-Brouwer, unpublished results). It is not known whether one of the peroxins functions as an E3 enzyme. Many of the proteins with E3 ligase activity belong to the family of RING finger proteins (for review see Pickart, 2001). Since Pex10p and Pex12p contain a RING domain and both proteins bind to Pex5p, it is tempting to speculate about a possible involvement of these proteins in the ubiquitination of Pex5p.

Pex4p is a peripherally associated peroxisomal membrane protein, located at the cytosolic face. The protein is probably kept at the peroxisomal membrane via interaction with Pex22p, an integral peroxisomal membrane protein (Koller et al., 1999). Both Pex4p and Pex22p are involved in the import of peroxisomal matrix proteins (Wiebel and Kunau, 1992; Crane et al., 1994; van der Klei et al., 1998; Koller et al., 1999). Although residual amounts of PTS1-containing proteins can still be imported in *pex4*-deficient cells, the major fraction is mislocalized to the cytosol in *H. polymorpha* and *P. pastoris* (van der Klei et al., 1998; Collins et al., 2000). In *H. polymorpha* this phenotype could be partially suppressed by overexpression of *PEX5* (van der Klei et al., 1998). Due to accelerated turnover, steady-state levels of Pex5p were severely reduced in *P. pastoris pex4* or *pex22*-deficient cells. These Pex5p levels were even lower than in *pex1* or *pex6*-deficient cells (Collins et al., 2000). In *S. cerevisiae* *pex4*-deficient cells, Pex5p stability does not seem to be affected (T. Voorn-Brouwer and A.T.J. Klein, unpublished results). In the absence of Pex4p, the residual Pex5p was associated with peroxisomes in *H. polymorpha* and *P. pastoris* (van der Klei et al., 1998; Collins et al., 2000). These observations made in *pex4*-deficient cells suggest that Pex4p might be involved in the recycling of Pex5p from the peroxisomal
membrane to the cytosol. The exact mechanism is, however, still unclear. Pex4p might exert this function in collaboration with the AAA proteins Pex1p and Pex6p, since it is known that certain members of this protein family are involved in dissociation of protein complexes (Neuwald et al., 1999).

IX. Concluding remarks

During the last 10-15 years, the combined efforts of many scientists have resulted in a great increase in our knowledge about peroxisomes. In different organisms, ranging from fungi to mammals, mutant cell lines disturbed in peroxisomal function have been isolated. Complementation of the mutant phenotype has resulted in identification of many PEX genes and the proteins (peroxins) they encode that are essential for the correct functioning of peroxisomes. Protein-interaction studies with these peroxins have revealed the function of the various domains that are present in these proteins, and have illustrated how they communicate with each other. However, many of these data remain largely descriptive without real mechanistic insight. This is in part due to the lack of success in *in vitro* reconstitutions of the partial reactions that for instance occur during the import process of peroxisomal matrix proteins. Although Pex5p and Pex7p, the import receptors for peroxisomal matrix proteins, are among the best-studied peroxins and much is known about their function, also here important questions remain to be solved. For instance: where does the dissociation of the receptor-cargo complex take place, is this inside the peroxisomal lumen (extended shuttle) or at the peroxisomal membrane (simple shuttle)? Also the mechanism that ensures the presence of peroxisomes in mother and daughter cells after cell division is still a subject of debate: are peroxisomes derived from the ER or do they form from pre-existing peroxisomes by growth and division? This second option is associated with an additional question: what is the origin of the membrane lipids and how are they recruited? Trying to resolve these issues will be a major challenge for the coming years.

X. Scope of this thesis

Pex5p plays a central role in the import of the majority of peroxisomal matrix proteins. It functions as the import receptor for PTS1 proteins by binding these proteins in the cytosol and directing them to the peroxisome. During this process, Pex5p interacts with many different proteins. In the experiments described in this thesis the domain
organization of *S. cerevisiae* Pex5p was investigated by locating the different binding sites that are present in this protein. We focussed on the interactions that take place during the first two steps of the import process, *i.e.* binding of Pex5p to peroxisomal matrix proteins in the cytosol and docking of Pex5p on the peroxisomal membrane.

In chapter 2 the interaction is described of Pex5p with the peroxisomal targeting signal type I (PTS1), which is present in most peroxisomal matrix proteins. Many different studies already showed that the carboxyl-terminal TPR domain of Pex5p, consisting of six or seven TPR motifs, mediates this interaction. However, this TPR domain forms a large part of the protein and we were interested in determining the exact binding site for PTS1 proteins. We therefore decided to use a combination of random and site-directed mutagenesis to isolate Pex5p mutants affected in the interaction with PTS1 proteins. The position of these mutations in combination with a structural model we created for the TPR domain of Pex5p revealed that two clusters of three TPR motifs bind to the PTS1. Remarkably, these two TPR clusters have different modes of interaction with the PTS1: TPR1-3 use the intra-repeat loops of TPR2 and TPR3 to contact the PTS1, while TPR5-7 use the general TPR groove formed by residues in the α-helices A of TPR6 and TPR7.

The details of the Pex5p-PTS1 interaction as found for *S. cerevisiae* and *H. sapiens* were compared in chapter 3. In both species, similar residues in Pex5p proved to be involved in contacting the PTS1, which indicated that the mode of interaction is conserved.

Pex5p functions as a multi-functional peroxisomal import receptor, since it not only directs proteins with a PTS1 to peroxisomes but also certain proteins that do not contain a PTS1. An example is *S. cerevisiae* acyl-CoA oxidase, a peroxisomal matrix protein without a PTS1 or PTS2. In chapter 4 we describe the targeting of acyl-CoA oxidase to peroxisomes and show that this is dependent on Pex5p, but uses another binding site on Pex5p than PTS1-containing proteins. Since there is a direct interaction between Pex5p and acyl-CoA oxidase, the results in chapter 4 indicate that a third import route (PTS3-route) into peroxisomes exists.

Docking of Pex5p on the peroxisomal membrane involves interaction of Pex5p with two peroxisomal membrane proteins, Pex13p and Pex14p. The Src homology 3 (SH3) domain of Pex13p plays a central role in this process, since it interacts with Pex5p and Pex14p. This feature of the Pex13p-SH3 domain makes it an exception in the SH3 field, since it was found that most SH3 domains have only one binding partner. We therefore focussed on the Pex13p-SH3 domain in order to locate the binding sites for Pex5p and Pex14p. Homology modeling was used to generate a structural model for the SH3 domain of Pex13p (chapter 5). The classical P-x-x-P binding pocket of Pex13p-SH3, located between the RT and n-Src loops, was used for binding of
Pex14p, which possesses a P-x-x-P motif. Since this motif is not present in Pex5p, we used a combination of random and site-directed mutagenesis to scan for residues in Pex5p involved in the binding of Pex13p-SH3. In this way we found that the binding site for Pex13p-SH3 is formed by an α-helical region in the amino terminus of Pex5p (chapter 5 and Bottger et al., 2000). Several of the Pex5p mutants were subsequently used in a screen for suppressor mutants in the Pex13p-SH3 domain and revealed the binding site for Pex5p on Pex13p-SH3. This study showed that a site other than the Pex14p binding site is used for the interaction of Pex13p-SH3 with Pex5p, and hence showed the existence of a novel mode of SH3 interaction. Furthermore, Pex5p and Pex14p did not compete for binding to the Pex13p-SH3 domain.

XI. References


Chapter 1


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Chapter 1


General Introduction


Chapter 1


