Receptor-mediated import of proteins into peroxisomes

Bottger, G.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 1
General Introduction

1 Introduction

2 The biogenesis of peroxisomes

3 Targeting of peroxisomal matrix proteins
   3.1 PTS1 sequences
   3.2 PTS2 sequences
   3.3 Alternative targeting sequences
   3.4 The PTS1 receptor Pex5p
   3.5 The PTS2 receptor Pex7p
   3.6 Working mechanism of the PTS1 and PTS2 receptors: the shuttle theories

4 Import of peroxisomal matrix proteins
   4.1 Saccharomyces cerevisiae
   4.2 Pichia pastoris
   4.3 Hansenula polymorpha
   4.4 Yarrowia lipolytica
   4.5 Mammalian
   4.6 Summary

5 Characterization of Pex5p

6 The SH3 domain
   6.1 SH3 domain ligand binding
   6.2 The Pex13p SH3 domain

7 Scope of this thesis
1 Introduction

Peroxisomes were initially described as small membrane enclosed compartments, called microbodies, in the cytoplasm of mouse kidney cells (Rhodin, 1954). It was not until 1966 that these microbodies were biochemically characterized in rat liver as organelles that contained enzymes that produce and degrade $H_2O_2$, hence they were given the name “peroxisomes” (De Duve and Baudhin, 1966).

Morphologically, peroxisomes are denoted as electron dense single-membrane bound organelles that vary in number and size, depending on the environmental conditions of the cell. Peroxisomes, or peroxisome-related organelles such as glyoxysomes in plants and glycosomes in kinetoplastids, are present in virtually all eukaryotic cells. The peroxisome plays a primary role in cellular metabolism, although the specific tasks of the peroxisome may differ per organism (Van den Bosch et al., 1992). For example, in yeast the $\beta$-oxidation of fatty acids is restricted to peroxisomes, whereas in mammalian cells the peroxisomes facilitate a limited number of $\beta$-oxidation cycles in the breakdown of very long chain fatty acids (VLCFA). Mammalian peroxisomes are involved in a variety of other processes including catabolism of polyamines, purines, amino acids, phytanic acid, L-pipolic acid and glyoxylate. In addition, mammalian peroxisomes play a role in plasmalogen synthesis, cholesterol and dolichol biosynthesis, fatty acid elongation and bile synthesis (Wanders and Tager, 1998). All peroxisomes are devoid of DNA or a protein synthesizing apparatus and posttranslationally import their matrix and membrane protein (Lazarow and Fujiki, 1985). The sequence homology of proteins involved in the biogenesis of peroxisomes (Pex proteins) suggests an evolutionary relation between peroxisomes from yeast to man.

2 The biogenesis of peroxisomes

2.1 Peroxisome biogenesis disorders in man

Peroxisomal disorders are recessive inheritable diseases that display a wide phenotypic heterogeneity. The most severe peroxisomal disorder in human is cerebro-hepato-renal syndrome, also called Zellweger Syndrome (ZS). ZS patients often die within their first year of life. The disease is characterized by neurological, hepatic and renal abnormalities, severe hypotonia and dysmorphic features (see for more details Lazarow, 1995 and Moser and Moser, 1996).

Biochemically, three different groups have been classified (Wanders et al., 1995): 1) peroxisomal disorders with a generalized loss of peroxisomal functions; 2) peroxisomal disorders with loss of a limited subset of peroxisomal functions; 3)
peroxisomal disorders with loss of a single peroxisomal function. Each group contains a wide variety of peroxisomal diseases that range from disorders with a severe clinical phenotype to disorders with mild clinical features. This indicates that the biochemical classification does not match the clinical classification of peroxisomal disorders. Disorders in group 3 can be explained by mutations in single genes that compromise the activity or localization of single enzymes, whereas disorders in group 1 and 2 affect the localization of multiple peroxisomal matrix proteins. For the classification of (recessive) genes responsible for peroxisomal biogenesis disorders in group 1 and 2, fibroblasts from two different patients were induced to fuse and examined for complementation of the peroxisomal defects (Brul, 1988; Roscher et al., 1989; Yajima et al., 1992). This technique has led to the identification of 12 different complementation groups, i.e. 12 different genes that are essential in the process of peroxisome assembly (Fujiki, 2000; Gould and Valle, 2000).

2.2 Peroxisome biogenesis

The biogenesis of peroxisomes can be devided into different aspects including organelle response to environmental stimuli, membrane biogenesis and import of matrix proteins. The identification of genes (PEX genes) encoding proteins that are responsible for one of the processes in peroxisome biogenesis, named peroxins, has started off by using various genetic screens in the yeasts *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha* and *Yarrowia lipolytica* (reviewed by Elgersma and Tabak, 1996). Two different approaches have led to the cloning and sequencing of mammalian PEX genes: 1) BLAST searches of mammalian sequence databases with yeast peroxins, and 2) functional complementation of human Peroxisome Biogenesis Disorder (PBD) cells and peroxisome-deficient Chinese hamster ovary (CHO) cells with mammalian cDNA expression libraries (Fujiki, 2000; Sacksteder and Gould, 2000). These combined efforts have resulted in the identification and characterization of 23 peroxins.

The first aspect in peroxisome biogenesis involves the formation of the membrane lipid bilayer and the insertion of membrane proteins into that bilayer. Peroxins that play a role in membrane biogenesis form a small group, consisting of Pex3p, Pex19p and Pex16p in mammals (Honsho et al., 1998; Matsuzono et al., 1999; Sacksteder et al., 2000; South and Gould, 1999), and Pex3p and Pex19p in yeast (Baerends et al., 1996; Höhfeld et al., 1991; Snyder et al., 1999a; Wiemer et al., 1996). Cells with major defects in these PEX genes are characterized by the absence of peroxisomal membrane remnants and rapid breakdown of mislocalized peroxisomal membrane proteins (Götte et al., 1998; Hettema et al., 2000; Matsuzono et al., 1999; Sacksteder et al., 2000). Pex19p can be farnesylated and is localized both in the cytoplasm and at the peroxisomal membrane. Pex19p binds to an array of peroxisomal membrane proteins, including several peroxins and metabolite
transporters and it is suggested that this peroxin is involved in the recruitment of newly synthesized peroxisomal membrane proteins to the peroxisome (Götte et al., 1998; Sacksteder et al., 2000; Snyder et al., 1999a; Snyder et al., 1999b; Snyder et al., 2000). The role of the integral membrane proteins Pex16p and Pex3p in peroxisomal membrane biogenesis is unclear at this moment (Muntau et al., 2000; Snyder et al., 1999b; Soukopova et al., 1999; South and Gould, 1999; South et al., 2000). Integral peroxisomal membrane proteins are generally synthesized in the cytosol and posttranslationally inserted into the peroxisomal membrane (Lazarow and Fujiki, 1985). However, certain peroxisome biogenesis and secretion mutants of the yeast *Y. lipolytica* accumulate both peroxisomal membrane proteins and secretion proteins in the ER (Titorenko et al., 1997; Titorenko and Rachubinski, 1998b). In addition, pulse-labeled peroxisomal membrane proteins can be chased from the ER to the peroxisome in wild-type cells (Titorenko et al., 1997; Titorenko and Rachubinski, 1998a; Titorenko and Rachubinski, 1998b). These findings suggest that in this organism certain peroxisomal membrane proteins are synthesized in the ER and subsequently are transported to the peroxisome in ER-derived vesicles. Although inhibition of anterograde transport from the ER by Brefeldin A results in the accumulation of peroxisomal proteins in the ER of *H. polymorpha* and tobacco plant (Mullen et al., 1999; Salomons et al., 1997), such results could not be reproduced in human fibroblasts (South et al., 2000; T. Voorn-Brouwer et al., manuscript in preparation). In addition, impairment of ER-derived traffic by a dominant negative Sar1 mutant had no effect on the assembly of peroxisomes in human fibroblasts (South et al., 2000; T. Voorn-Brouwer et al., manuscript in preparation). In addition, peroxisomal membrane proteins have not been found in the ER membrane of these organisms. It should be noted that a highly expressed form of the integral membrane protein Pex15p has been found in ER membranes in *S. cerevisiae* (Elgersma et al., 1997). The ER localization of this integral membrane protein however appeared to be an artifact caused by overexpression of the membrane protein (Hettema et al., 2000; Stroobants et al., 1999). It therefore remains unclear whether the ER is involved in the biogenesis of peroxisomes in *S. cerevisiae* and human cells.

The second aspect in peroxisome biogenesis includes the proliferation of peroxisomes upon environmental stimulation. The *PEX11* gene product has been thought to play a role in this process. This conclusion is based upon the morphological characteristics of *pex11* mutant cells, which appear to have a low number of peroxisomes that have clearly expanded in size, whereas overexpression of Pex11p results in the formation of numerous small peroxisomes (Erdmann and Blobel, 1995; Lorenz et al., 1998; Marshall et al., 1995). Two homologous *PEX11* genes in mammalian cells, *PEX11α* and *PEX11β*, are suggested to play a role in regulation of peroxisome abundance in response to extracellular stimuli and in the constitutive control of peroxisome abundance, respectively (Schrader et al., 1998). However, it has recently been shown that in *S. cerevisiae* deletion of *PEX11* primarily affects the β-oxidation of medium chain fatty acids (MCFA), which is followed in time by enlargement of peroxisomes. Targeted deletion of genes that are
directly involved in the β-oxidation of MCFA indeed resulted in enlargement of peroxisomes (Van Roermund et al., 2000). Similar observations have been made in human fibroblasts and murine hepatocytes deficient in one of the first two β-oxidation enzymes (Chang et al., 1999a; Fan et al., 1996; Poll-Thé et al., 1988; Qi et al., 1999; Suzuki et al., 1997). It is therefore suggested that the MCFA β-oxidation pathway regulates the proliferation of peroxisomes in response to extracellular stimuli.

The third aspect of peroxisome biogenesis is the import of newly synthesized peroxisomal matrix proteins. The understanding of how proteins can traverse the peroxisomal lipid membrane bilayer is still increasing. Peroxisomal protein import is a multistep process that requires targeting, docking and translocation of proteins destined for the peroxisomal matrix. These processes will be described in detail in the following sections.

3 Targeting of peroxisomal matrix proteins

Peroxisomal matrix proteins are synthesized on free polyribosomes in the cytosol and post-translationally imported into the peroxisome (Lazarow and Fujiki, 1985). The peroxisomal matrix protein is equipped with a specific targeting sequence that determines transport of the protein from the cytoplasm to the peroxisomal lumen. Two types of Peroxisomal Targeting Signals (PTS) can direct a protein to the peroxisome: PTS1 and PTS2. The two targeting signals are composed of distinct sequences that are recognized by their cognate receptor: Pex5p for the PTS1 and Pex7p for the PTS2.

3.1 PTS1 sequences

The peroxisomal targeting signal type 1 (PTS1) was first identified in firefly luciferase and appeared to consist of the carboxyterminal tripeptide Ser-Lys-Leu (S-K-L). This tripeptide was shown to be both necessary and sufficient for import of luciferase into peroxisomes of mammalian cells (Gould et al., 1987). A consensus PTS1 sequence for peroxisomal proteins in mammalian cells was determined by permutation of the S-K-L tripeptide and was defined as S/C/A-K/R/H-L/M (Gould et al., 1989; Swinkels et al., 1992). The consensus PTS1 allows import of proteins into peroxisomes in organisms varying from yeast to man, indicating the evolutionary conservation of PTS1-mediated peroxisomal import (reviewed by Subramani, 1993). An array of studies have shown that the composition of the PTS1 sequence can display large variations that do not fit the consensus PTS1 and that certain PTS1 sequences are not functional when fused to a heterologous protein (Elgersma et al., 1996b; Kragler et al., 1998; Motley et al., 1995; Purdue and Lazarow, 1994). The
requirement for a specific combination of a PTS1 and a peroxisomal protein indicates that amino acids adjacent to the PTS1 contribute to the peroxisomal import of the protein either by increasing the affinity for the PTS1 receptor Pex5p or by stimulating another step in the import pathway. Screening of a two-hybrid random peptide library revealed that human and yeast PTS1 receptors bind the same peptide with different affinities. These differences in affinity appeared to rely on amino acids upstream of the PTS1 sequence (Lametschwandtner et al., 1998). In line with this, quantitative two-hybrid interaction data showed that the binding affinity of *S. cerevisiae* Pex5p for the homologous protein Mdh3pSKL is much higher than that for the heterologous protein GFP-SKL (Klein et al., 2001). However, a specific sequence, other than the PTS1, that participates in increasing the binding affinity for Pex5p has not been defined. Cooperation in the peroxisomal import process by amino acids upstream of the PTS1 sequence has been demonstrated for human and cottonseed catalase A. The carboxyterminal three amino acids of both catalases (Ala-Asn-Leu in human and Pro-Ser-Ile in cottonseed) are unable to target chloramphenicol acetyl transferase to peroxisomes, unless the first amino acid N-terminal to the tripeptide (a positively charged amino acid; Lys in human catalase and Arg in cottonseed catalase) is included in the targeting sequence (Mullen et al., 1997; Purdue and Lazarow, 1996). Whether the fourth amino acid in the targeting signal of catalase A contributes to higher affinity binding with the PTS1 receptor remains however to be established.

### 3.2 PTS2 sequences

The peroxisomal targeting signal 2 (PTS2) is present in only a few peroxisomal proteins. The PTS2 is located at the N-terminus of the protein and is composed of a bipartite sequence spaced by 5 amino acids (consensus R/K-L/V/I - X₅ - H/Q-L/A; Subramani, 1996). Reporter proteins carrying a PTS2 are efficiently targeted to peroxisomes, even when the targeting sequence is placed internally of the protein (Kato et al., 1996; Osumi et al., 1991; Rehling et al., 1996; Swinkels et al., 1991). Human thiolase, a PTS2-containing protein, was correctly imported into peroxisomes of the yeast *S. cerevisiae*, implying evolutionary conservation of the PTS2 targeting pathway (Rehling et al., 1996). The PTS2 of human and plant peroxisomal proteins is a cleavable sequence, although signal cleavage is not linked with peroxisomal import (Gietl et al., 1994; Kato et al., 1996; Motley et al., 1994; Osumi et al., 1991; Preisig-Muller and Kindl, 1993; Swinkels et al., 1992). Interaction of PTS2 sequences and PTS2-containing proteins with the receptor Pex7p have been shown both *in vivo* and *in vitro* (Elgersma et al., 1998; Otera et al., 2000; Rehling et al., 1996; Zhang and Lazarow, 1996). Interestingly, the genome of the nematode *Caenorhabditis elegans* lacks any PTS2-containing proteins or a *PEX7* ortholog. Indeed, a PTS2-fused GFP reporter was mistargeted in *C. elegans*, underlining the absence of a PTS2 pathway in this organism (Motley et al., 2000). Standard PTS2 proteins such as 3-ketoacyl-CoA thiolase and alkyldihydroxy-acetonephosphate synthase have a PTS1 sequence.
which is likely exchanged for the PTS2 sequence during evolutionary development (Motley et al., 2000).

3.3 Alternative targeting sequences

A number of (yeast) proteins that do not contain an identifiable targeting signal can be correctly imported into peroxisomes. For several of these proteins it has been shown that, in spite of the absence of a recognizable PTS1 sequence, their import depends on a functional PTS1 receptor, Pex5p. To date, proteins of this category comprise the *Y. lipolytica* acyl-CoA oxidase isoenzyme 3 (C-terminal tripeptide Asp-Glu-Glu; Szilard et al., 1995; Wang et al., 1998), *S. cerevisiae* acyl-CoA oxidase (C-terminal tripeptide Ile-Asn-Lys; chapter 5 of this thesis), *H. polymorpha* malate synthase (C-terminal tripeptide Ser-Leu-Lys; Bruinenberg et al., 1990; Van der Klei et al., 1995) and the PTS1 deleted version of *S. cerevisiae* carnitine acetyl transferase (CAT ΔPTS1; Elgersma et al., 1995). Yeast two-hybrid experiments with a pex5 mutant (Pex5pN393D) that has specifically lost PTS1 interaction showed that *S. cerevisiae* acyl-CoA oxidase and CAT ΔPTS1 are still able to bind Pex5p(N393D), which suggested that these proteins do not use the classical PTS1 binding site on Pex5p (Klein et al., unpublished results). An alternative targeting sequence has however not been identified in these proteins. Peroxisomal import of *S. cerevisiae* Dci1p, a protein of the isomerase/hydratase family (Geisbrecht et al., 1999; Gurvitz et al., 1999), appeared to be unaffected in the absence of the PTS2 receptor Pex7p and was not dependent of its carboxy-terminal PTS1 (Karpichev and Small, 2000), indicating that import of Dci1p does not require the PTS1 or PTS2 protein import routes. It is noteworthy that subcellular localization of Dci1p in a pex5∆ strain has not been shown and it is therefore not excluded that Dci1p, just like the peroxisomal matrix proteins mentioned above, requires Pex5p for its import into peroxisomes. An *in vitro* import assay with fragments of the *Candida tropicalis* acyl-CoA oxidase, Pox4p (C-terminal tripeptide Leu-Lys-Ser; Okazaki et al., 1987) showed that two internal regions in this oxidase could be imported into isolated peroxisomes (Small et al., 1988), suggesting the existence of alternative targeting sequences. It remains to be investigated whether CtPox4p depends on either Pex5p or Pex7p for import into peroxisomes *in vivo* and whether the internal regions required for peroxisomal targeting can form a binding site for one of the import receptors.

3.4 The PTS1 receptor Pex5p

PTS1 sequences are recognized by and bound to Pex5p, the receptor for PTS1 proteins. Pex5p has been identified and cloned in a large variety of organisms. In yeast and plant, a defect in Pex5p specifically interferes with the import of PTS1-containing proteins (Terlecky et al., 1995; Van der Klei et al., 1995; Van der Leij et
General introduction

The C-terminal half of Pex5p contains 7 tetratricopeptide (TPR) motifs, which are highly conserved in all Pex5 proteins identified. Binding studies with deleted versions of Pex5p have shown that these TPR domains are both necessary and sufficient for the interaction with PTS1 sequences (Brocard et al., 1994; Dodt et al., 1995; Szilár and Rachubinski, 2000). Selection for pex5 mutants that are disturbed in PTS1 binding, and recent co-crystallization of human Pex5p bound to a PTS1 pentapeptide have revealed that all TPR motifs are involved in the interaction with the PTS1 sequence (Gatto et al., 2000b; Klein et al., 2001). In human and CHO cells, Pex5p is expressed as two isoforms, which is the result of alternative splicing of the PEX5 gene. The long form of Pex5p (Pex5pL) distinguishes itself from the short form of Pex5p (Pex5pS) by an insertion of 37 amino acids adjacent to Glu<sup>215</sup> (in human and in Chinese hamster). Both isoforms are fully capable of binding PTS1 sequences (Matsumura et al., 2000). Human Peroxisome Biogenesis Disorder (PBD) cell lines and CHO cells containing a defective PEX5 gene can phenotypically be devided into two distinct groups (Dodt et al., 1995; Otera et al., 1998; Shimozawa et al., 1999b; Slawecki et al., 1995): the first group is only impaired in the import of PTS1 proteins and the second group displays an import defect for both PTS1 and PTS2 proteins (reviewed in Fujiki, 2000; Gould and Valle, 2000). Complementation analysis of PEX5 defective cells revealed that Pex5pS is only capable to restore PTS1 protein import, whereas Pex5pL can complement import of both PTS1 and PTS2 proteins (Braverman et al., 1998; Otera et al., 1998). Co-immunoprecipitation experiments revealed that Pex5pL interacts with the PTS2 receptor Pex7p and that this interaction is essential for import of PTS2 containing proteins (Matsumura et al., 2000). The PEX5 gene products in mammalian cells therefore participate in the import of both PTS1 and PTS2 proteins.

3.5 The PTS2 receptor Pex7p

Both in yeast and mammalian cell lines, the absence of the receptor for PTS2-containing peroxisomal matrix proteins, Pex7p, results in selective loss of PTS2 protein import (Braverman et al., 1997; Elgersma et al., 1998; Marzioch et al., 1994; Motley et al., 1997; Purdue et al., 1997). In addition, the specific interaction of Pex7p with PTS2 sequences (Elgersma et al., 1998; Rehling et al., 1996; Zhang and Lazarow, 1996) defines Pex7p as the PTS2 receptor. Pex7p contains 6 WD40 repeats, which are protein motifs involved in protein-protein interactions, that show up as highly conserved regions distributed over the entire Pex7 protein. The binding region in Pex7p for PTS2 sequences has, however, not been identified yet. Elgersma et al. (1998) showed that S. cerevisiae Pex7p can functionally complement a pex7 mutant of P. pastoris, showing a high degree of conservation of the PTS2 protein import pathways between the two yeasts.
Figure 1. Proposed model for the function of Pex5p in peroxisomal import of PTS1-containing proteins. Pex5p (5) interacts with a PTS1-containing protein (P1) in the cytoplasm and directs P1 to the peroxisome. The Pex5p-P1 complex associates with a docking factor (D) at the peroxisomal membrane, followed by either handing over of P1 to the translocation apparatus (T), or by transport of the Pex5p-P1 complex to the peroxisome lumen (extended shuttle, boxed area), where P1 is released. Pex5p cycles back to the cytoplasm to pick up new P1 cargo. It should be noted that it is unknown whether the same translocation apparatus (T) is used to export Pex5p from the peroxisome lumen to the cytoplasm. The model may also apply to Pex7p.

3.6 Working mechanism of the PTS1 and PTS2 receptors: the shuttle theories

The interaction between Pex5p and the PTS1 cargo is the initial step in the process of PTS1-mediated protein import. In most organisms Pex5p is predominantly localized in the cytoplasm and partially associated to peroxisomes (Dodt and Gould, 1996; Elgersma et al., 1996a; Gould et al., 1996; Gouveia et al., 2000; Wiemer et al., 1995; Wimmer et al., 1998). In human and in the yeasts *Y. lipolytica* and *H. polymorpha* Pex5p can (occassionally) be detected in the peroxisomal matrix (Dodt
and Gould, 1996; Szilard et al., 1995; Van der Klei et al., 1995). Based upon the dual localization of Pex5p two shuttle theories have been put forward that describe the overall function of Pex5p in peroxisomal protein import (Figure 1) (Dodt and Gould, 1996; Erdmann et al., 1997). According to the first shuttle theory Pex5p picks up its PTS1 cargo in the cytoplasm, from where the receptor-cargo complex travels to the peroxisome. Cargo-loaded Pex5p transiently associates with docking proteins (D) at the peroxisomal membrane, delivers the PTS1 cargo to the translocation machinery (T) and the unloaded Pex5p is released back into the cytoplasm. In the second (extendend) shuttle theory cargo-loaded Pex5p enters the peroxisomal matrix (Figure 1, boxed area), where the PTS1 cargo is released, and the unloaded Pex5p re-enters the cytoplasm. To establish PTS1 receptor mobility between cytoplasm and peroxisome, Dodt and Gould (1996) have performed fluorescence experiments that show a temporary redistribution of Pex5p from the cytoplasm to the peroxisome under protein translocation-inhibiting conditions. A surprising exception is formed by *Y. lipolytica* Pex5p, which is exclusively localized inside the peroxisome. In *Y. lipolytica* Pex5p is present at the luminal side of the peroxisomal membrane, where it may bind translocating PTS1 proteins, and in the peroxisome matrix, where Pex5p may release its PTS1 protein cargo (Szilard et al., 1995). A factor that is responsible for the recruitment of cytosolic PTS1-containing proteins to the peroxisomal membrane has not been identified in *Y. lipolytica*. The PTS2 receptor Pex7p is localized in the cytoplasm and at the peroxisome (Braverman et al., 1997; Elgersma et al., 1998, Marzioch et al., 1994; Otera et al., 2000; Purdue et al., 1998). The bimodal localization of Pex7p suggests that the mode of action of this receptor in peroxisomal protein import can be compared with that of Pex5p (see Figure 1). In contrast to *S. cerevisiae* and *P. pastoris* Pex5p, that associate with the cytoplasmic face of the peroxisomal membrane, an overexpressed form of Pex7p was shown to reside inside the peroxisome matrix of these yeasts (Elgersma et al., 1998; Zhang and Lazarow, 1996). It remains to be established whether the endogenous Pex7p also enters the peroxisome lumen.

In human and in rat it was shown that Pex5p forms a stable complex with the integral peroxisomal membrane protein Pex14p (Gouveia et al., 2000; Schliebs et al., 1999). In rat, the resistance of pex5p to be extracted from peroxisome membranes by carbonate indicated that Pex5p is tightly associated with the peroxisomal membrane, likely via the interaction with Pex14p (Gouveia et al., 2000). Resistance of Pex5p to carbonate extraction has also been observed in *Trypanosoma brucei* and in the yeast *P. pastoris* (de Walque et al., 1999; McCollum et al., 1993; Terlecky et al., 1995; Wiemer et al., 1995). It should be noted that in the yeast *S. cerevisiae*, a significant amount of Pex5p from the organellar pellet cannot be extracted with carbonate. However, the inability to extract Pex5p with carbonate was also found in cells lacking peroxisomal membrane remnants (the *pex3Δ* strain), suggesting that resistance to carbonate extraction may not be related to strong association with peroxisomal membranes or with proteins at the peroxisomal membrane (G. Bottger, unpublished results).
Table 1: Cloned PEX genes from different organisms and their proposed function in peroxisome biogenesis

<table>
<thead>
<tr>
<th>X ) Cloned and sequenced PEX gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>a ) Genes involved in peroxisomal protein import. Defects in these genes are characterized by mistargeting of peroxisomal matrix proteins, whereas peroxisomal membranes containing membrane proteins are still present.</td>
</tr>
<tr>
<td>b ) Genes involved in peroxisomal membrane biogenesis. Defects in these genes are characterized by the absence of detectable peroxisomal membrane remnants.</td>
</tr>
<tr>
<td>c ) Genes involved in peroxisome proliferation</td>
</tr>
<tr>
<td>d ) Genes involved in PTS1 receptor stability</td>
</tr>
<tr>
<td>e ) Genes involved in vesicle fusion</td>
</tr>
<tr>
<td>f ) Genes affecting protein exit from the ER and formation of peroxisomes</td>
</tr>
<tr>
<td>g ) Genes of unknown function in peroxisome biogenesis</td>
</tr>
</tbody>
</table>

4 Import of peroxisomal matrix proteins

The evolutionary conservation of PTS1 and PTS2 sequences and peroxisomal import of proteins bearing such sequences indicates that the initial step in protein import, i.e. the recognition of peroxisomal proteins by the PTS receptor and subsequent delivery to the peroxisomal translocation apparatus is universal in most organisms. Despite increasing efforts to unravel the mechanism of peroxisomal matrix protein import, only little of this process is known. The translocation event does not require unfolding of the matrix protein, since chemically cross-linked proteins and PTS1-coated 9 nm gold particles are able to traverse the peroxisomal membrane (Häusler et al., 1996; Walton et al., 1995). The ability of several peroxisomal matrix enzymes to form oligomers in the cytoplasm allows PTS-containing proteins to serve as a template for peroxisomal “piggy-back” import of PTS-less forms of these proteins. These findings suggest that oligomeric protein complexes can be delivered to the peroxisomal matrix (Elgersma et al., 1996b; Glover et al., 1994; Lee et al., 1997; Leiper et al., 1996; McNew and Goodman, 1994; Smith et al., 2000). In vitro import assays and studies with semi-permeabilized cells have shown that peroxisomal protein import requires cytosolic components and ATP hydrolysis (Imanaka et al., 1987; Lopez-Huertas et al., 1999; Walton et al., 1992; Wendland and Subramani, 1993).

The identification of several PEX genes have started to shed some light on the
### Table 1

<table>
<thead>
<tr>
<th>PEX gene</th>
<th>Saccharomyces cerevisiae</th>
<th>Pichia pastoris</th>
<th>Hansenula polymorpha</th>
<th>Yarrowia lipolytica</th>
<th>Chinese hamster</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEX1</td>
<td>Xa</td>
<td>Xa,e</td>
<td>Xa</td>
<td>Xe,f</td>
<td>Xa,d</td>
<td>Xa,d</td>
</tr>
<tr>
<td>PEX2</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xf</td>
<td>Xa,</td>
<td>Xa,</td>
</tr>
<tr>
<td>PEX3</td>
<td>Xb</td>
<td>Xb</td>
<td>Xb</td>
<td>Xb</td>
<td>Xb</td>
<td>Xb</td>
</tr>
<tr>
<td>PEX4</td>
<td>Xa</td>
<td>Xa,d</td>
<td>Xa</td>
<td>Xe,f</td>
<td>Xa,d</td>
<td>Xa,d</td>
</tr>
<tr>
<td>PEX5</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX6</td>
<td>Xa</td>
<td>Xa,d</td>
<td>Xa</td>
<td>Xe,f</td>
<td>Xa,d</td>
<td>Xa,d</td>
</tr>
<tr>
<td>PEX7</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xb</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX8</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX9</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xb</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX10</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX11</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX12</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX13</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX14</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX15</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX16</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX17</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX18</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX19</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX20</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX21</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX22</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>PEX23</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td>Xb</td>
<td>Xb</td>
<td>Xb</td>
</tr>
</tbody>
</table>

process of peroxisomal protein import. Table 1 shows the currently cloned and characterized PEX genes of the most popular species for research in peroxisome biogenesis: the yeasts *S. cerevisiae*, *P. pastoris*, *H. polymorpha*, *Y. lipolytica* and Chinese hamster and human. Amino acid sequence homology combined with certain functional similarity determines whether a newly identified peroxin is the ortholog of a previously characterized peroxin of another species. However, the functional conservation of peroxins between two species is usually too low to allow this peroxin to restore peroxisome biogenesis in a mutant of the other species. Peroxin orthologs also can display species-dependent variations in protein-protein interactions and in subcellular localization. Given these differences, the role of peroxins in the import of peroxisomal proteins in five different organisms will be described separately. Since most PEX genes isolated from CHO cells, mouse and rat are able to functionally complement peroxisome defects in human PBD cells and vice versa, peroxisome biogenesis is likely to be strictly conserved in these mammals. Therefore, the import of peroxisomal proteins in human, Chinese hamster, mouse and rat is discussed here as one system.

### 4.1 *Saccharomyces cerevisiae*

The application of several genetic screens in *S. cerevisiae* and the completion of the *Saccharomyces* genome sequencing project have resulted in the identification of a large number of PEX genes (Elgersma et al., 1993; Erdmann et al., 1989; Van der Leij et al., 1992; Zhang et al., 1993). In addition, the yeast two-hybrid assay has largely contributed to the identification of dimeric and even trimeric protein interactions and has extended our insight into the peroxisomal protein import complex. A predominantly cytoplasmic protein specifically involved in peroxisomal protein import is the *S. cerevisiae* DnaJ-like protein Djp1p (Hettema et al., 1998) (Figure 2). *Djp1Δ* cells partially mislocalize PTS1 and PTS2-containing proteins to the cytoplasm. The degree of mislocalization is depending on the PTS protein investigated and on the culture conditions (Hettema et al., 1998). The presence of the DnaJ domain in ScDjp1p suggests that this protein can physically interact with proteins from the Hsp70 family and therefore act as a potential cofactor for chaperones. However, the Hsp70 partner for ScDjp1p has not been identified, and therefore the role of ScDjp1p as a chaperone in peroxisomal protein import remains speculative (Hettema et al., 1998). Pulse-chase experiments have shown that peroxisomal targeting of PTS1 and PTS2 proteins is delayed in *djp1Δ* cells (C. Ruigrok, manuscript in preparation), suggesting that ScDjp1p may play a role in an early step of protein import. The receptors for PTS1 and PTS2 protein import, ScPex5p and Pex7p can function independent of each others presence (Marzioch et al., 1994; Van der Leij et al., 1993; Zhang and Lazarow, 1994).
Two weakly homologous peroxins, ScPex18p and ScPex21p were identified in a two-hybrid screen with ScPex7p as bait (Purdue et al., 1998). More detailed two-hybrid analysis showed that PTS2 cargo-loaded ScPex7p can exist in complex with ScPex18p or ScPex21p. While single gene deletion of PEX18 or PEX21 hardly affects peroxisome biogenesis, the deletion of both PEX18 and PEX21 blocks import of thiolase (a PTS2 protein) and abolishes the peroxisomal targeting of ScPex7p. These observations define ScPex18p and ScPex21p as the first proteins to play a role in PTS receptor targeting (Purdue et al., 1998). Although a small fraction of the otherwise cytoplasmic ScPex18p and ScPex21p colocalizes with peroxisomes, it remains to be investigated whether ScPex18p and ScPex21p also participate in the docking of ScPex7p at the peroxisomal membrane.

At the cytoplasmic face of the peroxisome, both ScPex5p and ScPex7p interact with the peripheral membrane protein Pex14p (Albertini et al., 1997; Brocard et al., 1997). ScPex14p can bridge the two-hybrid interaction between ScPex5p and ScPex7p and is required for the import of both PTS1 and PTS2-containing proteins (Albertini et al., 1997). ScPex14p is therefore proposed to be the point of convergence in the PTS1 and PTS2 protein import pathways. In the absence of ScPex14p, Pex5p was not associated with peroxisomes (Chapter 4 of this thesis) and the amount of organellar ScPex7p appeared to be decreased (Girzalsky et al., 1999), which underlines the role for ScPex14p as the primary docking factor for ScPex5p.
and ScPex7p. In addition, ScPex5p directly interacts with the SH3 domain of the integral membrane protein ScPex13p (Barnett et al., 2000; Bottger et al., 2000; Elgersma et al., 1996a; Erdmann and Blobel, 1996). Disruption of the Pex5p-Pex13-SH3 interaction by a specific mutation in ScPex5p selectively interferes with PTS1 protein import. This mutated form of ScPex5p is still associated with peroxisomes, likely via the interaction with ScPex14p (Bottger et al., 2000). ScPex7p also interacts with ScPex13p, however the Pex7p binding site in ScPex13p, which is not the SH3 domain, has not yet been characterized (Girzalsky et al., 1999). The interaction of ScPex5p and ScPex7p with ScPex13p is probably involved in a step in protein import following the docking event. The SH3 domain of ScPex13p also binds ScPex14p. Specific disruption of the Pex14p-Pex13p-SH3 contact by a mutation in the SH3 domain, or disturbing the stoichiometry by overexpression of either ScPex14p or ScPex13p seriously affects PTS1 and PTS2 protein import, showing that the two proteins act in complex with each other (Bottger et al., 2000; Girzalsky et al., 1999). Pex5p and Pex14p have different contact sites on the SH3 domain of Pex13p (Barnett et al., 2000; Bottger et al., 2000). A Pex5p peptide containing the SH3 binding site and Pex14p do not compete for binding to the Pex13p-SH3 domain, suggesting that the three proteins can form a trimeric complex (Barnett et al., 2000). ScPex13p plays a significant role in anchoring ScPex14p to the peroxisomal membrane, since deletion of the PEX13 gene results in partial cytoplasmic localization of ScPex14p (Girzalsky et al., 1999 and our unpublished observations). Interestingly, the SH3 domain mutation that disrupts the interaction with ScPex14p does not affect the peroxisomal localization of ScPex14p, suggesting that the full length ScPex13p, and not just the SH3 domain takes part in attachment of ScPex14p to the peroxisomal membrane (Girzalsky et al., 1999 and our unpublished observations). ScPex14p has been shown to interact with itself in the two-hybrid system (Albertini et al., 1997) and forms a homomeric complex in isolated peroxisomal membranes (G. Bottger, unpublished results). The third peroxisomal membrane component involved in PTS1 and PTS2 protein import is ScPex17p (Albertini et al., 1997). This peroxin shows the characteristics of a peripheral membrane protein and interacts with ScPex14p and indirectly with ScPex5p in a Pex14p-dependent manner (Albertini et al., 1997; Huhse et al., 1998). In addition to ScPex14p, ScPex17p is required for peroxisomal localization of ScPex5p (Chapter 4 of this thesis). Immunprecipitation of myc-tagged ScPex7p coprecipitates a complex of ScPex13p, ScPex14p, ScPex17p, ScPex5p, Sc(myc)Pex7p, PTS1 and PTS2 proteins. These proteins are predominantly maintained in complex by the presence of ScPex14p (Girzalsky et al., 1999; Huhse et al., 1998).

ScPex5p also interacts with ScPex8p, a peripheral peroxisomal membrane protein that is essential for peroxisomal import of PTS1 and PTS2-containing proteins (Rehling et al., 2000). The ScPex8 protein is localized to the luminal face of the peroxisomal membrane. ScPex8p contains a PTS1, but does not require this
sequence for its interaction with ScPex5p, for its intraperoxisomal localization, or for its function in peroxisome biogenesis (Rehling et al., 2000). The localization of ScPex8p in pex5Δ cells has not yet been determined, and it is therefore not ruled out that the interaction of ScPex5p with ScPex8p plays a role in peroxisomal targeting of ScPex8p. Although ScPex5p has never been found inside peroxisomes of S. cerevisiae, it is possible that the interaction of ScPex5p with ScPex8p occurs at the lumenal side of the peroxisomal membrane. If the interaction with ScPex8p is not a rate-limiting step, the levels of intraperoxisomal ScPex5p might be too low to detect. In pex8Δ cells the complex of ScPex13p, ScPex14p, ScPex17p and the two PTS cargo-loaded receptors can still be coprecipitated, suggesting that ScPex8p plays a role downstream of import receptor docking (Rehling et al., 2000).

The S. cerevisiae ubiquitin conjugating (UBC) enzyme Pex4p is a peroxisomal protein that is essential for import of PTS1 and PTS2 proteins. Substitution of the active-site cysteine, which abolishes UBC enzyme activity, inhibits the import of peroxisomal matrix proteins, although the cysteine mutation does not interfere with the peroxisomal localization of ScPex4p (Wiebel and Kunau, 1992). The target protein for Pex4p-mediated ubiquitination in S. cerevisiae remains to be identified. ScPex4p interacts with the S. cerevisiae protein Yaf5p, which is a homolog of P. pastoris Pex22p and is here named ScPex22p. S. cerevisiae pex22Δ cells mislocalize GFP-PTS1 to the cytoplasm and are unable to grow on oleate, suggesting a role for ScPex22p in peroxisome biogenesis (Koller et al., 1999). In P. pastoris, PpPex22p anchors PpPex4p to the peroxisomal membrane and is, together with PpPex4p, required for PpPex5p stability (Koller et al., 1999). Deletion of PEX4 in S. cerevisiae does not result in ScPex5p instability, but does seem to lead to a posttranslational modification of ScPex5p (T. Voorn-Brouwer and A. Klein, pers. communication). The function of ScPex4p and ScPex22p in peroxisomal protein import therefore will require further investigation.

4.2 Pichia pastoris

Genetic screens in the yeast P. pastoris have resulted in the identification of several Pex proteins essential for PTS1 and PTS2 protein import (Gould et al., 1992; Liu et al., 1992). Like in S. cerevisiae, the PTS1 and PTS2 import pathways can be functionally separated (Elgersma et al., 1998; Terlecky et al., 1995). P. pastoris Pex5p interacts with the SH3 domain of the integral peroxisomal membrane protein PpPex13p (Gould et al., 1996; Urquhart et al., 2000) and with the membrane protein PpPex14p (a protein that as yet has not fully been characterized in P. pastoris) (Figure 3). In vitro interaction and competition studies have shown that a PTS1 cargo-loaded PpPex5p binds PpPex14p with higher affinity than does the unloaded PpPex5p. In addition, the binding affinity of cargo-loaded PpPex5p for the PpPex13-SH3 domain was reduced compared to that of unloaded PpPex5p (Urquhart et al.,
Figure 3. Subcellular localization and interactions (indicated with arrows) of Pex proteins participating in peroxisomal protein import in *P. pastoris*. The question mark indicates that it is uncertain whether Pex1p and Pex6p are able to associate with the peroxisomal membrane. The number refers to the number of the Pex protein. The SH3 domain is indicated in italic. PTS1 and PTS2-containing proteins are indicated as P1 and P2, respectively. See text for details.

2000). This suggests that the PpPex5p-PTS1 cargo complex docks at the peroxisomal membrane via the interaction with PpPex14p, and that after release of the PTS1 cargo Pex5p is handed over to the SH3 domain of Pex13p. The *in vitro* studies also showed that PpPex13-SH3 and PpPex14p directly interact with each other (Urquhart et al., 2000). An overexpressed form of the *P. pastoris* PTS2 receptor Pex7p is localized both in the cytoplasm and in the peroxisomal matrix (Elgersma et al., 1998). Interactions of PpPex7p with peroxisomal proteins other than PTS2-containing proteins have however not been reported yet. In a recent review (Subramani et al., 2000) it has been described that PpPex14p can be phosphorylated, and that phosphorylation of PpPex14p is required for its interaction with PpPex13p, whereas either phosphorylated or dephosphorylated PpPex14p binds the peroxisomal membrane protein PpPex17p. However, experimental data substantiating these findings have not been published yet.

*P. pastoris* Pex17p is an integral peroxisomal membrane protein that interacts with PpPex14p and can be coimmunoprecipitated with antisera directed against PpPex14p, PpPex5p and PpPex7p, suggesting that PpPex17p is present in protein import complexes (Snyder et al., 1999b). In addition, pex17Δ cells are deficient in peroxisomal PTS1 and PTS2 protein import. PpPex17p was initially isolated in a screen for proteins involved in peroxisomal membrane protein targeting, and pex17 mutant cells mistarget several peroxisomal membrane proteins to the cytosol (Snyder et al., 1999b). It is therefore assumed that PpPex17p has a dual function in both
peroxisomal matrix and membrane protein import. PpPex17p is weakly homologous to *S. cerevisiae* Pex17p (18% amino acid sequence identity). *S. cerevisiae pex17Δ* cells are not affected the localization of peroxisomal membrane proteins (Hetteama et al., 2000; Huhse et al., 1998), which indicates that the effect of Pex17p on membrane protein import is limited to the yeast *Pichia pastoris*.

PpPex2p, PpPex10p and PpPex12p are integral membrane proteins that contain a zinc-binding RING domain that is essential for proper peroxisome assembly (Kalish et al., 1996; Kalish et al., 1995). *Pex2, pex10* and *pex12* mutants specifically interfere with peroxisomal matrix protein import. However, a specific role for these proteins in the process of protein import has not been defined yet.

PpPex8p is an ill-defined peroxisomal membrane protein that, just like ScPex8p, contains a PTS1 sequence (Liu et al., 1995). A *pex8* mutant (*per3-l*) disturbs the import of PTS1 proteins but allows import of thiolase, whereas *pex8Δ* cells mislocalize both PTS1 and PTS2 proteins (Liu et al., 1995). Subramani et al. (2000) have described that Pex8p can interact with Pex14p, however experiments providing evidence for this interaction have not been published yet.

Deletion of the *P. pastoris* ubiquitin-conjugating enzyme Pex4p results in a partial PTS1 and PTS2 protein import defect (Collins et al., 2000; Crane et al., 1994). A fraction of PpPex4p is ubiquitinated and site-directed mutagenesis of the active site cysteine, required for ubiquitination, abolishes PpPex4p function in peroxisomal protein import (Crane et al., 1994). PpPex4p interacts with the cytosolic C-terminus of PpPex22p, which is an integral peroxisomal membrane protein involved in PTS1 and PTS2 protein import (Koller et al., 1999). In *pex22Δ* cells, PpPex4p is localized in the cytosol and is highly unstable. It has therefore been suggested that PpPex22p anchors PpPex4p to the peroxisomal membrane (Koller et al., 1999). The absence of either PpPex22p or PpPex4p results in a dramatic destabilization of PpPex5p, which is virtually undetectable in these cells (Collins et al., 2000; Koller et al., 1999). Mutation of the active site cysteine of PpPex4p also results in a low steady-state protein level of PpPex5p, indicating that PpPex4p UBC enzyme activity is essential for the stability of Pex5p (Collins et al., 2000). Surprisingly, protein levels of the PTS2 receptor PpPex7p are not altered, suggesting that the import defect of PTS1 and PTS2 proteins in *pex4Δ* and *pex22Δ* cells is not necessarily a direct result of PTS receptor instability (Collins et al., 2000). The breakdown of PpPex5p in *pex4Δ* and *pex22Δ* cells is suppressed when other *PEX* genes are also deleted, which strongly suggests that PpPex4p and PpPex22p act in one of the terminal steps in peroxisome assembly (Collins et al., 2000).

*Pichia pastoris* Pex1p and Pex6p are both members of the AAA protein family, meaning that they are capable of binding and hydrolyzing ATP, and share a high degree of amino acid sequence identity (29%). The two proteins interact with each other in an ATP-dependent manner (Faber et al., 1998). Mutated versions of PpPex1p and PpPex6p still display some residual peroxisomal protein import (Heyman et al., 1994; Spong and Subramani, 1993). Interestingly, in *pex1* and *pex6* mutant cells an accelerated turnover of PpPex5p has been observed, although basal protein levels of
PpPex5p in \textit{pex1\Delta} and \textit{pex6} mutant cells are still higher than PpPex5p levels in \textit{pex4\Delta} and \textit{pex22\Delta} cells (Collins et al., 2000; Yahraus et al., 1996). Epistasis analysis using the PpPex5p protein levels as a reference revealed that PpPex1p and PpPex6p act before PpPex4p and PpPex22p but after all the other known peroxins in \textit{Pichia pastoris}. Given the late step in protein import in which the two peroxins act, PpPex1p and PpPex6p are proposed to play a role in PTS receptor recycling (Collins et al., 2000). The function of PpPex1p and PpPex6p is however a matter of debate. Both proteins are found in the supernatant fraction after differential centrifugation at 27,000 \(x\) g but sediment after centrifugation at 100,000 \(x\) g (Faber et al., 1998). PpPex1p and PpPex6p migrate into a flotation gradient, which is indicative for the association with membranous structures. Since proteins of AAA family comprise \textit{N}-ethylmalmeimide-sensitive factor (NSF) and NSF-like ATPases, and Pex1p and Pex6p in the yeast \textit{Yarrowia lipolytica} are necessary to initiate membrane fusion of two different preperoxisomal compartments (Titorenko et al., 2000; Titorenko and Rachubinski, 2000), it has been proposed that the two proteins are involved in vesicle fusion (Faber et al., 1998). The exact function of the two AAA proteins in \textit{Pichia pastoris} will require further investigation.

\subsection*{4.3 \textit{Hansenula polymorpha}}

In the methylotrophic yeast \textit{H. polymorpha} a subset of \textit{PEX} genes involved in peroxisomal protein import have been characterized (Cregg et al., 1990). At least two different Pex proteins are required for functional PTS1 protein import. The first protein is the PTS1 receptor, Pex5p. HpPex5p is specifically involved in import of PTS1-containing proteins and of malate synthase, that does not contain a recognizable PTS1 or PTS2. HpPex5p is localized both in the cytoplasm and inside the peroxisomal matrix (Van der Klei et al., 1995) (Figure 4). The second gene acting specifically in PTS1 protein import is HpPex4p, a protein that, just like its orthologs in \textit{S. cerevisiae} and \textit{P. pastoris}, belongs to the protein family of ubiquitin conjugating enzymes. In contrast to the \textit{S. cerevisiae} and \textit{P. pastoris} orthologs, HpPex4p is localized in the cytosol. Target protein(s) for HpPex4p-mediated ubiquitination have as yet not been identified. Interestingly, the total amount of HpPex5p and HpPex14p has increased in cells lacking HpPex4p (Van der Klei et al., 1998), which contrasts with the strongly decreased Pex5p levels in \textit{P. pastoris pex4\Delta} cells (Collins et al., 2000; Koller et al., 1999). In addition, overexpression of HpPex5p suppresses the inhibition of PTS1 protein import in \textit{pex4\Delta} cells, suggesting that increase in newly synthesized HpPex5p can rescue a defect caused by the absence of HpPex4p. Since HpPex5p accumulates inside peroxisomes in \textit{pex4\Delta} cells, it is suggested that HpPex4p is involved in recycling of HpPex5p to the cytoplasm (Van der Klei et al., 1998). \textit{H. polymorpha} also contains proteins bearing a PTS2 sequence that can target a fused reporter protein to the peroxisome (Faber et al., 1994; Faber et al., 1995;
Waterham et al., 1994). However, a candidate PTS2 receptor (Pex7p) has not been isolated yet.

The membrane protein HpPex14p, which shows 35% sequence identity with ScPex14p, is required for import of PTS1 and PTS2 proteins in H. polymorpha. Overproduction of HpPex14p interferes with protein import resulting in the disappearance of mature peroxisomes and in the accumulation of numerous vesicles that are positive for the peroxisomal membrane protein HpPex10p (Komori et al., 1997). Shifting growth conditions from glucose to methanol media induces phosphorylation of HpPex14p (Komori et al., 1999), suggesting a regulatory role for HpPex14p in protein import. In contrast to ScPex14p and PpPex14p, an interaction of HpPex14p with HpPex5p has not been reported yet and it is therefore unknown whether Pex14p plays a role in receptor docking. Interestingly, overexpression of HpPex5p in a pex14Δ strain can partially restore peroxisomal import of certain PTS1 proteins (alcohol oxidase and dihydroxyacetone synthase). In these transformed pex14Δ cells HpPex5p is not localized inside the peroxisome, but at the peroxisomal membrane facing the cytosol (Salomons et al., 2000). These findings suggest that HpPex14p not directly mediates docking of Pex5p, as is the case for the other known Pex14p orthologs, but may play a role in shuttling HpPex5p across the membrane. However, these localization experiments were performed with an excess of HpPex5p and it would therefore be worth while to investigate the subcellular localization of endogenous HpPex5p in pex14Δ cells.

Another protein involved in PTS1 and PTS2 protein import is HpPex8p, an intraperoxisomal protein carrying both a PTS1 and a PTS2 signal (Waterham et al., 1994). The function of this protein in peroxisomal protein import is however unknown.
4.4 *Yarrowia lipolytica*

Like in the yeasts mentioned above, mutants have been selected that are disturbed in the assembly of peroxisomes in *Y. lipolytica* (Nuttley et al., 1993). Although peroxisomal matrix proteins in *Y. lipolytica* contain functional PTS1 and PTS2 sequences, it is not certain whether the traditional PTS1 and PTS2 pathways are employed in peroxisomal protein import. Unlike Pex5 proteins from other organisms, YlPex5p is exclusively localized in the peroxisomal matrix and associated with the luminal side of the peroxisomal membrane (Szilard et al., 1995) (Figure 5). The intraperoxisomal localization of YlPex5p is maintained for a *pex5* mutant that has lost the ability to bind PTS1 proteins, although this *pex5* mutant is found only in the peroxisomal matrix and does no longer associate with the peroxisomal membrane (Szilard and Rachubinski, 2000; Szilard et al., 1995). The localization of YlPex5p is apparently not related to peroxisomal targeting of PTS1-containing proteins and its function may therefore be different from other Pex5p orthologs.

The *Y. lipolytica* homolog of the PTS2 import receptor, Pex7p, has not yet been identified. The *Y. lipolytica pex20* mutant strain is however selectively disturbed in peroxisomal import of thiolase (a PTS2 protein) (Titorenko et al., 1998). YlPex20p interacts with thiolase independent of the presence of a PTS2 sequence (Titorenko et al., 1998). In the absence of YlPex20p, thiolase fails to assemble into homodimers and is retained in the cytoplasm. The finding that Pex20p and thiolase are present in cytoplasmic heterotetramers, suggests that Pex20p assists in the dimer formation of thiolase (Titorenko et al., 1998). YlPex20p is predominantly localized in the cytoplasm, whereas a fraction of 4-8% is associated with peroxisomes (Titorenko et al., 1998). YlPex20p also interacts with YlPex8p (formerly YlPex17p; Smith and Rachubinski, 2000). YlPex8p is an intraperoxisomal peripheral membrane protein.

**Figure 5.** Subcellular localization and interactions (indicated with arrows) of Pex proteins participating in peroxisomal protein import in *Y. lipolytica*. The question mark indicates that the interaction between Pex8p and Pex20p is not certain to occur at that subcellular location. The number refers to the number of the Pex protein. PTS1 and PTS2-containing proteins are indicated as P1 and P2, respectively. See text for details.
that plays a role in peroxisomal import of PTS1 and PTS2 proteins (Smith et al., 1997). Just like the known Pex8p orthologs, YLPex8p bears a PTS1 sequence that is not required for peroxisomal targeting of the protein (Smith et al., 1997). In the absence of YLPex8p the majority of YLPex20p colocalizes with peroxisomes, whereas thiolase is mainly localized in the cytoplasm (Smith and Rachubinski, 2000). YLPex8p likely assists in the recycling of peroxisomal YLPex20p, which on its turn can act in the cytoplasm in the dimerization and subsequent peroxisomal targeting of thiolase. A peroxisomal protein that resembles a docking factor such as Pex14p has not yet been identified in Y. lipolytica.

4.5 Mammalian cells

Characterization of genes involved in peroxisome function in human peroxisome biogenesis disorder (PBD) cells and in mutagenized CHO cells have greatly enhanced the elucidation of the biogenesis of peroxisomes in mammals.

Peroxisomal import of PTS1-containing proteins in mammalian cells is regulated by two isoforms of Pex5p (Pex5pS and Pex5pL; Dodt et al., 1995; Otera et al., 1998), whereas import of PTS2-containing proteins requires, unlike the known PTS2 import pathways in yeast, both Pex5pL and Pex7p (Braverman et al., 1998; Braverman et al., 1997; Matsumura et al., 2000; Motley et al., 1997; Otera et al., 2000; Otera et al., 1998; Purdue et al., 1997). Recently, Fujiki and coworkers found that Pex5pL, but not Pex5pS, coprecipitates with PTS2 cargo-loaded Pex7p (Otera et al., 2000) (Figure 6) and that the interaction between Pex5pL and Pex7p is disrupted by the Ser214Phe mutation in Pex5pL. Interestingly, this mutant selectively inhibits the import of PTS2 proteins, but not the import of PTS1 proteins (Matsumura et al., 2000), which is a phenotype that previously has been observed only in pex7 mutant cells (Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997). Pex5pL and Pex5pS can form homo and heterodimers in vivo (Otera et al., 2000).

Pex5pL and Pex5pS interact in vivo and in vitro with mammalian Pex14p, which is, in contrast to yeast Pex14p, characterized as an integral peroxisomal membrane protein (Fransen et al., 1998; Gouveia et al., 2000; Otera et al., 2000; Schliebs et al., 1999; Shimizu et al., 1999; Will et al., 1999). Immunoprecipitation studies in CHO cell lysates showed that a complex of Pex14p, Pex5pL, Pex5pS and PTS1 proteins (Shimizu et al., 1999), and of Pex14p, Pex5pL and Pex7p (Otera et al., 2000) could be coprecipitated. In addition, using immunofluorescence on CHO cells overexpressing Pex14p, Otera et al. (2000) showed that Pex5p accumulates at peroxisomes in these cells whereas in the absence of Pex14p Pex5p is localized in the cytosol. Pex14p is therefore suggested to be the docking factor for PTS1 cargo-loaded Pex5p and for the complex of Pex5pL with PTS2 cargo-loaded Pex7p. The requirement for Pex5pL in PTS2 protein import suggests that the interaction via Pex5pL is primarily responsible for docking of Pex7p at the peroxisomal membrane. In addition, overexpression of Pex5pL recruits Pex7p from the cytoplasm to the
Chapter 1

Figure 6. Subcellular localization and interactions (indicated with arrows) of Pex proteins participating in peroxisomal protein import in mammalian cells. The long and short forms of Pex5p are indicated with 5L and 5S, respectively. The question mark indicates that the interactions have been shown only in vitro. The number refers to the number of the Pex protein. Domains (SH3 and RING) are indicated in italic. PTS1 and PTS2-containing proteins are indicated as P1 and P2, respectively. See text for details.

peroxisomal membrane (Otera et al., 2000). The complex of Pex5p and Pex14p is very stable and appears to contain several molecules of Pex14p to one molecule of Pex5p (Gouveia et al., 2000; Schliebs et al., 1999). Whether this complex is directly involved in translocation or membrane insertion of Pex5p, as is suggested by Gouveia et al., remains to be investigated. In human and Chinese hamster, overexpression of Pex14p results in a general defect in peroxisomal protein import, accompanied with aberrant peroxisome morphology (Otera et al., 2000; Will et al., 1999). Similar phenotypes are observed in H. polymorpha and S. cerevisiae overexpressing Pex14p and are caused by disturbance of the stoichiometry in the Pex14p-Pex13p protein complex. Mammalian Pex14p also interacts with the SH3 domain of mammalian Pex13p (Fransen et al., 1998; Otera et al., 2000). The isolated CHO mutants and the identified PBD cells that carry a defective PEX13 gene all display either mutations in, or truncations of the C-terminal SH3 domain (Liu et al., 1999; Shimozawa et al., 1999a; Toyama et al., 1999), underlining a role for the SH3 domain of Pex13p in peroxisomal protein import. Although Pex5p and Pex13p-SH3 can be coprecipitated in the absence of Pex14p, there is no evidence for a direct interaction of mammalian Pex13p-SH3 with Pex5p (Otera et al., 2000), as is observed in the yeasts S. cerevisiae and P. pastoris.

Pex5p binds to the RING finger domain of the integral peroxisomal membrane protein Pex12p (Chang et al., 1999b; Okumoto et al., 2000) and to the RING finger domain of the integral peroxisomal membrane protein Pex10p (Okumoto et al.,
The latter interaction has only been observed \textit{in vitro}. The RING finger domain of Pex12p is essential for the function of this peroxin in peroxisome biogenesis (Chang et al., 1999b; Okumoto et al., 1998) and this domain directly interacts with the RING finger domain of the integral peroxisomal membrane protein Pex10p and with the TPR domains of Pex5p (Chang et al., 1999b; Okumoto et al., 2000). The \textit{in vivo} interaction of Pex5p and Pex10p with the Pex12-RING finger domain has become evident since overexpression of Pex10p and Pex5p can rescue peroxisomal import in PBD cells containing the RING mutant Pex12p(S320F). The combination of two RING mutant alleles Pex12p(S320F) and Pex10p(H290Q) results in dominant negative inhibition of peroxisomal protein import (Chang et al., 1999b), underscoring the importance of Pex10p-Pex12p interaction in peroxisome biogenesis.

\textit{In vitro}, the RING finger domain of Pex10p binds the RING finger domain of Pex2p (Okumoto et al., 2000), another peroxisomal integral membrane protein involved in peroxisomal import of PTS1 and PTS2 containing proteins (Shimozawa et al., 1992; Tsukamoto et al., 1991). Mutations in \textit{PEX2} cause differential import defects, in particular insufficient import of catalase A and other PTS1 proteins, suggesting a function for this peroxin in peroxisomal protein import (Fujiwara et al., 2000; Huang et al., 2000; Imamura et al., 1998; Shimozawa et al., 2000).

Immunofluorescence and biochemical studies have shown that mutations in \textit{PEX2}, \textit{PEX10}, \textit{PEX12} and \textit{PEX13} can cause accumulation of Pex5p at the peroxisomal membrane and in the matrix of the peroxisome (Chang et al., 1999b; Dodt and Gould, 1996; Otera et al., 2000). Pex2p, Pex10p, Pex12p and Pex13p are therefore suggested to play a role downstream of (PTS1) receptor docking.

Approximately one third of all the patients suffering from peroxisome biogenesis disorders contain a mutated form of the \textit{PEX1} gene. HsPex1p is 21\% homologous to HsPex6p (Tamura et al., 1998). Just like the yeast orthologs, HsPex1p and HsPex6p interact with each other (Geisbrecht et al., 1998; Tamura et al., 1998). This interaction is partially disrupted in the HsPex1p(G843D) mutant, that accounts for more than 50\% of the mutated \textit{PEX1} alleles identified so far (Geisbrecht et al., 1998; Portsteffen et al., 1997; Reuber et al., 1997). Pex1 and pex6 mutants are phenotypically characterized by a partial defect in PTS1 and PTS2 protein import and by a markedly decreased stability of HsPex5p (Dodt and Gould, 1996; Geisbrecht et al., 1998; Imamura et al., 2000; Yahraus et al., 1996). The disturbance of protein import in \textit{pex1} and \textit{pex6} mutant cells can not be rescued by overexpression of Pex5p (Dodt et al., 1995), which indicates that the instability of Pex5p is not the primary cause of the peroxisomal defect. Given the similarities with the phenotype observed for \textit{Pichia pastoris pex1} and \textit{pex6} mutants, it is suggested that HsPex1p and HsPex6p play a role in HsPex5p receptor recycling (Dodt and Gould, 1996).
4.6 Summary

The current knowledge of peroxins involved in peroxisomal protein import suggests a certain sequence of events between the synthesis of peroxisomal matrix proteins in the cytoplasm and the appearance of such a protein in the peroxisomal matrix. Although there are marked differences in the protein import systems between several species, a general mechanism of peroxisomal protein import can be summarized as follows: 1) folding of the newly synthesized protein and oligomer formation, a process that may be assisted by Djp1p in \textit{S. cerevisiae} and that requires Pex20p in \textit{Y. lipolytica}; 2) recognition and binding of the peroxisomal targeting signal to the cognate receptor in the cytoplasm, Pex5p or Pex7p; 3) targeting of the receptor-cargo complex to the peroxisomal membrane, which in the \textit{S. cerevisiae} PTS2 pathway requires Pex18p and Pex21p; 4) binding of the import receptor-cargo complex at the peroxisomal membrane to Pex14p, that is likely complexed with Pex17p in \textit{S. cerevisiae} during the docking event; 5) translocation of either the receptor-cargo complex, or of the cargo protein alone, via an unknown mechanism. This event requires the interaction between Pex14p and the SH3 domain of Pex13p in \textit{S. cerevisiae} and in \textit{P. pastoris} and is accompanied by handing over of Pex5p from Pex14p to the SH3 domain of Pex13p. Additionally, the interaction between RING finger domains of Pex10p and Pex12p is essential in mammals. In general, the presence of Pex2p, Pex10p, Pex12p and (in yeast) Pex8p is required for the translocation step; 6) recycling or breakdown of Pex5p, which is regulated by Pex11p and Pex6p in human and \textit{P. pastoris} and requires the interaction between these two proteins. In a later step in the process, receptor recycling or breakdown may further be mediated by Pex4p and Pex22p in \textit{P. pastoris}.

5 Characterization of Pex5p

The PTS1 receptor Pex5p is one of the best characterized peroxins in \textit{S. cerevisiae}. ScPex5p has at least four interaction partners: the PTS1 cargo protein, the docking factor Pex14p and the protein import factors Pex13p and Pex8p. The C-terminal half of Pex5p consists of two clusters each comprising three TPR domains that are linked with a hinge region, which has been denoted as TPR repeat motif 4. The TPR domains participate in the special folding structure that allows the interaction with the PTS1 tripeptide that appears to be embraced by all the TPR motifs (Gatto et al., 2000a; Gatto et al., 2000b; Klein et al., 2001).

In contrast with the C-terminal TPR region, the N-terminus of Pex5p is very poorly conserved. Multiple sequence alignments of the N-terminal half of yeast, plant and mammalian Pex5p reveal a conserved region localized near the extreme N-terminus consisting of a patch of 10 amino acids. Other regions of sequence identity are formed by pentapeptide motifs composed of the sequence Trp-X-X-X-Phe/Tyr.
**General introduction**

### Pex5p

<table>
<thead>
<tr>
<th>1</th>
<th>612</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pex13-SH3</td>
<td>PTS1</td>
</tr>
</tbody>
</table>

**Figure 7.** Domain structure *Saccharomyces cerevisiae* Pex5p. The arrows indicate binding regions for Pex13-SH3, Pex14p and PTS1.

*S. cerevisiae* and *H. polymorpha* Pex5p contain two pentapeptide motifs, *P. pastoris* and *Y. lipolytica* Pex5p contain three pentapeptide motifs, human, mouse and Chinese hamster contain seven pentapeptide motifs and tobacco Pex5p and watermelon Pex5p even contain ten and eleven of these motifs, respectively. The pentapeptide motifs of *S. cerevisiae* Pex5p are composed of the amino acid sequences W\textsubscript{120}SQEF\textsubscript{124} and W\textsubscript{204}TDQF\textsubscript{208}. The latter pentapeptide motif, and probably the homologous motif (W\textsubscript{196}EDQF\textsubscript{200}) in *P. pastoris*, is part of the Pex13p-SH3 domain binding region of Pex5p (Barnett et al., 2000; Bottger et al., 2000; Urquhart et al., 2000). PHD secondary structure predictions of this region in *S. cerevisiae* Pex5p showed that the pentapeptide motif is part of an \(\alpha\)-helix (Barnett et al., 2000). Binding studies with Pex13p-SH3 and a combination of alanine scanning of amino acids in and adjacent to the motif and substitution of Lys\textsubscript{210} to a helix-breaking proline confirmed that this region in Pex5p adopts an \(\alpha\)-helical conformation that forms the contact site for binding *S. cerevisiae* Pex13-SH3 (Barnett et al., 2000). In human Pex5p these motifs have been implicated in binding of Pex14p (Schliebs et al., 1999), although double amino acid substitutions of both conserved tryptophans of the pentapeptide repeat motifs in *S. cerevisiae* did not abolish binding activity with ScPex14p. The double mutant however did lose two-hybrid interaction with the PTS1-deleted version of ScPex8p (A. Stein and G. Bottger, unpublished observations).

*S. cerevisiae*, *P. pastoris* and human Pex5p require only their N-terminus for interaction with Pex14p (Schliebs et al., 1999; Urquhart et al., 2000)(our unpublished observations).
observation). It is has however not been possible to identify a point mutation in Pex5p that selectively inhibits Pex14p binding (A. Stein and G. Bottger, unpublished observations), which may support the suggestion that Pex5p contains multiple binding sites for Pex14p (Schliebs et al., 1999). Figure 7 schematically represents the domain structure of Pex5p and binding regions for Pex13p, Pex14p and the PTS1.

Pex5p chimeras have been able to successfully rescue peroxisomal protein import defects in yeast and have emphasized that the C-terminus of yeast Pex5p can functionally be replaced by the C-terminus of a plant or human Pex5p (Dodt et al., 1995; Kragler et al., 1998; Wiemer et al., 1995). A Pex5p chimera consisting of only the first 37 amino acids of Pex5p from S. cerevisiae fused to the carboxyterminal 553 amino acids from Tobacco Pex5p was capable to (partially) restore the PTS1 protein import defect in S. cerevisiae pex5Δ cells, whereas the full length Tobacco plant Pex5p was unable to rescue PTS1 protein import in these cells (Kragler et al., 1998). In addition, a Pex5p chimera comprising the amino terminal 124 amino acids of P. pastoris Pex5p fused to the C-terminal 388 amino acids from human Pex5p, comprising the complete TPR domain region, restores growth of P. pastoris pex5Δ cells on oleate (but not on methanol), whereas full length human Pex5p does not (Dodt et al., 1995). These studies indicate that the N-terminus of Pex5p determines the species specificity of the protein. It would be of interest to test whether these Pex5 chimeric proteins have retained the ability to interact with the known Pex5p partners.

6 The SH3 domain

6.1 SH3 domain ligand binding

The peroxisomal membrane protein Pex13p contains a conserved region in the C-terminal part of the protein encoding a Src Homology 3 (SH3) domain. Truncation of the SH3 domain abolishes the function of Pex13p in peroxisome biogenesis (Elgersma et al., 1996a; Gould et al., 1996; Shimozawa et al., 1999a; Toyama et al., 1999), but does not interfere with the subcellular localization of the protein (Elgersma et al., 1996a). Pex13p is the first SH3 domain-containing protein identified in an organellar protein import complex. Furthermore, the Pex13p SH3 domain displays an unusual property: it harbours binding sites for two different proteins: the PTS1 receptor Pex5p and the PTS receptor docking protein Pex14p (Barnett et al., 2000; Urquhart et al., 2000).

SH3 domains are found in a variety of proteins that play a role in dynamic events such as signal transduction, cytoskeletal rearrangement and assembly of the phagocyte NADPH oxidase complex. It has been well established that the function of the SH3 domain is to mediate protein-protein interactions. The cellular events
regulated by SH3 domain interactions are however very diverse. For example, the signaling molecules Grb2 (growth factor receptor bound) and phospholipase-γ and the component of the NADPH oxidase p47phox require their SH3 domain for correct targeting to specific subcellular locations (Bar-Sagi et al., 1993; Sumimoto et al., 1994). SH3 domains in the oncoproteins Src, Hck and Abl regulate the enzyme activity of these proteins through an intramolecular interaction, which induces the protein to adopt an inactive conformation (Barila and Superti-Furga, 1998; Moarefi et al., 1997; Sicheri et al., 1997; Xu et al., 1997).

SH3 domains exhibit high sequence homology and among those proteins for which structures have been determined, high structural homology. The SH3 fold consists of two antiparallel β sheets and three variable loops, named the RT, n-src and distal loops. A number of studies using λ-cDNA libraries and combinatorial peptide libraries revealed that the SH3 binding ligand is composed of a short proline-rich peptide. The minimal consensus sequence for this peptide is Pro-X-X-Pro, where X is any amino acid, and an additional basic amino acid located C-terminally (class I: PXXPXR) or N-terminally (class II: RXXPXXP) of the PXXP core (Feng et al., 1994; Lim et al., 1994; Ren et al., 1993). The proline-rich peptide segment adopts a left-handed polyproline-type helix (PPII) that, depending on the class of the ligand, can bind in two orientations with respect to the SH3 domain (Feng et al., 1994; Lim et al., 1994). The prolines in the PPII helix form a hydrophobic surface, whereas the peptide backbone can facilitate hydrogen bonding (MacArthur and Thornton, 1991). The proline-rich region of the ligand fits into a shallow hydrophobic pocket of the SH3 domain (Lim et al., 1994) while the basic amino acid of both class I and II ligands contacts residues outside the PXXP binding cleft in a pocket that is formed by the RT and n-Src loops (Feng et al., 1995). The relative rigidity of the PPII helix makes this SH3 ligand moiety easily accessible for binding. Since SH3 domains are primarily found in proteins that participate in dynamic cellular processes, it can be suggested that SH3 domain interactions play a role in transient contacts with ligand proteins rather than in stable protein complexes.

Identification of natural SH3 domain ligands revealed that the SH3 ligand binding pocket does not only accomodate peptides that contain the canonical PXXP motif. For example, residues that are brought together by the specific fold of the ligand are capable of interacting with the PXXP binding cleft of the SH3 domain, as is the case for the interaction of p53 and p53BP2-SH3 (Gorina and Pavletich, 1996). Other sequences that lack a PXXP motif but that do interact with the hydrophobic ligand binding pocket of the SH3 domain are identified in the Src and Hck proteins (Sicheri et al., 1997; Xu et al., 1997). In both proteins the SH3 domain has an intramolecular interaction with a linker region that contains only one proline. Other SH3 ligand proteins lacking a canonical PXXP motif are Eps8 and SKAP55, which contain the ligand sequences PXXDY (Mongiovi et al., 1999) and RKXXXYYY (Kang et al., 2000), respectively.

Binding studies with the Hck SH3 domain revealed that $K_d$ values for the interaction with a full length ligand protein HIV Nef were considerably lower than
the $K_d$ values for the interaction with synthetic ligand peptides (Lee et al., 1995). The differences between these binding affinities could be explained by an additional contact site for the ligand in the RT loop of the SH3 domain (Lee et al., 1996). This contact determines the binding affinity and specificity of the ligand with the SH3 domain (Arold et al., 1998; Lee et al., 1996).

### 6.2 The Pex13p SH3 domain

Of the two proteins Pex5p and Pex14p, that interact with the SH3 domain of *S. cerevisiae* Pex13p only Pex14p contains a canonical PXXP motif, composed of the sequence PTLPHR. Site-directed mutation of the two prolines confirmed that this motif is involved in the interaction with the Pex13p SH3 domain (Girzalsky et al., 1999). In addition, mutations in the RT loop (Glu$_{320}$Lys) and of the conserved aromatic residue in the PXXP binding cleft (Trp$_{349}$Ala) of the Pex13p SH3 domain abolished the interaction with Pex14p (Bottger et al., 2000; Girzalsky et al., 1999). These mutants however did not affect the interaction with Pex5p. In *P. pastoris* it has been shown that mutants of the SH3 domain of PpPex13p are specifically disturbed the interaction of one ligand, whereas binding to the other ligand remained, suggesting that also in this organism Pex5p and Pex14p interact at different sites of the SH3 domain (Urquhart et al., 2000).

A two-hybrid screen selecting for *pex5* mutants that have lost the interaction with the SH3 domain in *S. cerevisiae* revealed that the SH3 contact site on Pex5p consisted of a stretch of amino acids W$_{204}$TDQFEKLEKE$_{214}$ that are predicted to form an $\alpha$ helix instead of the classical PPII helix (Barnett et al., 2000; Bottger et al., 2000). Alanine scanning of this region in Pex5p revealed that all residues involved in SH3 domain contact (Trp$_{204}$, Phe$_{208}$, Leu$_{211}$, Glu$_{212}$ and Glu$_{214}$) were predicted to face one side of this $\alpha$ helix (Barnett et al., 2000). Peptides including this region of Pex5p specifically bound to the SH3 domain and did not compete with Pex14p (Barnett et al., 2000). By contrast, the presence of Pex5p increased the binding affinity of Pex13p-SH3 and Pex14p in the two-hybrid assay (Bottger et al., 2000). A screen selecting for Pex13p SH3 domain mutants that regained interaction with the *pex5* mutants mentioned above, identified mutations in the RT and distal loops. Viewed in the predicted 3D structure of the *S. cerevisiae* Pex13-SH3 domain, the contact site for Pex5p may be situated in a pocket between the RT and distal loops (Barnett et al., 2000). The Pex13p SH3 domain of *S. cerevisiae* therefore shows a novel mode of binding to an SH3 domain ligand, which may establish simultaneous contact of the Pex13p SH3 domain with Pex5p and Pex14p proteins. Simultaneous binding of two proteins with the SH3 domain of Pex13p might regulate transition of Pex5p from Pex14p to Pex13p SH3, a step that is suggested to occur during or after the delivery of PTS1 cargo to the peroxisomal matrix (Urquhart et al., 2000). Disrupting the interaction of Pex13p SH3 with Pex5p by specific *pex5* mutants partially affects the import of PTS1 containing proteins. However, loss of interaction with Pex14p
disturbs both PTS1 and PTS2 protein import and affects the viability of yeast cells grown on oleate (Bottger et al., 2000; Elgersma et al., 1996a). Physical contact between Pex13p SH3 and Pex14p is therefore a crucial step in the process of peroxisomal import. Whether or how this step is regulated is not known, although Pex14p has been reported to be phosphorylated in *H. polymorpha* (Komori et al., 1999) and possibly also in *P. pastoris* (Subramani et al., 2000). Since most peroxins involved in peroxisomal protein import are able to interact with one or more other peroxins, the actual protein import process may be regulated by the assembly of a transient protein complex. The dynamic character of SH3 domain interactions suggests that the contact of Pex14p with the Pex13p SH3 domain acts as a regulatory switch, allowing fast regulation of one of the steps in the peroxisomal protein import system. It is tempting to speculate that, based on the receptor docking function of Pex14p, the SH3-Pex14p interaction regulates the step between docking and translocation.

7 Scope of this thesis

Proteins that are part of the peroxisomal matrix are synthesized in the cytoplasm and posttranslationally imported into peroxisomes. The import process involves a set of proteins (Pex proteins) that act in targeting, docking and membrane translocation of peroxisomal matrix proteins. A defect in one of these Pex proteins can affect the functioning of the complete peroxisomal protein import machinery. In humans such defects can result in various peroxisomal disorders.

This thesis focuses on three Pex proteins that are required for peroxisomal protein import: Pex5p, Pex13p and Pex14p. Pex13p and Pex14p are both peroxisomal membrane proteins that interact with the mainly cytoplasmic receptor for newly synthesized peroxisomal matrix proteins carrying a Peroxisomal Targeting Sequence type 1 (PTS1), Pex5p. Pex13p contains an SH3 domain that forms the binding surface for Pex14p, a canonical SH3 ligand, and Pex5p. It is therefore suggested that Pex13p and Pex14p provide the docking sites for Pex5p.

In chapter 2 we have addressed this issue by making use of mutants that specifically disrupt the interaction between these Pex proteins. These studies revealed that complex formation of Pex13p with Pex14p plays a key role in peroxisomal protein import, whereas the interaction between Pex13p and Pex5p only partially affects the import of PTS1-containing proteins. *In vivo*, Pex5p mutants that had lost interaction with Pex13p were still found to be associated with peroxisomes, indicating that other proteins are able to bind Pex5p at the peroxisome membrane. Using a fluorescent Pex5p fusion protein we could show that two peroxisomal membrane proteins, Pex14p and Pex17p are absolutely required for association of Pex5p with the peroxisome membrane, whereas Pex13p is dispensable for this process (chapter 4). We propose that Pex5p docks on the peroxisome via the interaction with a complex of Pex14p and Pex17p. Interaction of Pex5p with the SH3...
domain of Pex13p is an event that follows docking of Pex5p and is suggested to occur after complex formation of Pex14p and Pex13p.

The interaction between Pex5p and Pex13-SH3 was examined in closer detail in chapters 2 and 3. Using random and site-directed mutagenesis, in vitro interaction analysis and homology modeling we show that Pex5p is a novel SH3 ligand that contacts the Pex13-SH3 domain at a site distinct from the canonical ligand binding site, which is reserved for Pex14p association.

Finally, in chapter 5 it is shown that targeting of the peroxisomal matrix protein acyl-CoA oxidase requires the PTS1 receptor Pex5p, although the oxidase does not require a functional PTS1 binding site on Pex5p for import. This suggests that Pex5p also contains alternative PTS binding sites. Characterization of this binding site on Pex5p and the PTS in oxidase awaits further analysis.

Our studies show that the analysis of a mutant protein that is specifically affected in one interaction, while other protein interactions are not affected, can provide detailed information about the role of this protein in peroxisomal protein import and may eventually lead to the dissection of the import process in defined steps.
REFERENCES


General introduction


Rhodin, J. 1954. Correlation of ultrastructural organisation and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney. *(Thesis).*


sensitive mutations in *PEX13* are the cause of complementation group H of peroxisome biogenesis disorders. *Hum. Mol. Genet.* 8:1077-1083.


Waterham, H.R., V.I. Titorenko, P. Haima, J.M. Cregg, W. Harder, and M. Veenhuis. 1994. The *Hansenula polymorpha* PER1 gene is essential for peroxisome biogenesis and encodes a


Zhang, J.W., and P.B. Lazarow. 1994. PEB1 (PAS7) in Saccharomyces cerevisiae encodes a hydrophilic, intraperoxisomal protein which is a member of the WD repeat family and is essential for the import of thiolase into peroxisomes. *J. Cell Biol.* 129:65-80.

Zhang, J.W., and P.B. Lazarow. 1996. PEB1 (Pas7p) is an intraperoxisomal receptor for the NH2-terminal, type 2, peroxisomal targeting sequence of thiolase: PEB1p itself is targeted to peroxisomes by an NH2-terminal peptide. *J. Cell Biol.* 132:325-334.