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Williams, C.P.

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Pex5p and ubiquitin: Regulation of the PTS1 protein import receptor

Pex5p and ubiquitin: Regulation of the PTS1 protein import receptor

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. D. C. van den Boom

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Christopher Paul Williams

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Promotiecommissie:

Promotor:	Prof. dr. J. M. F. G. Aerts
Co-promotor:	Dr. B. Distel
Overige leden:	Prof. dr. R. J. A. Wanders
	Prof. dr. R. P. J. Oude Elferink
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Faculteit der Geneeskunde

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If we knew what we were doing
it wouldn't be research

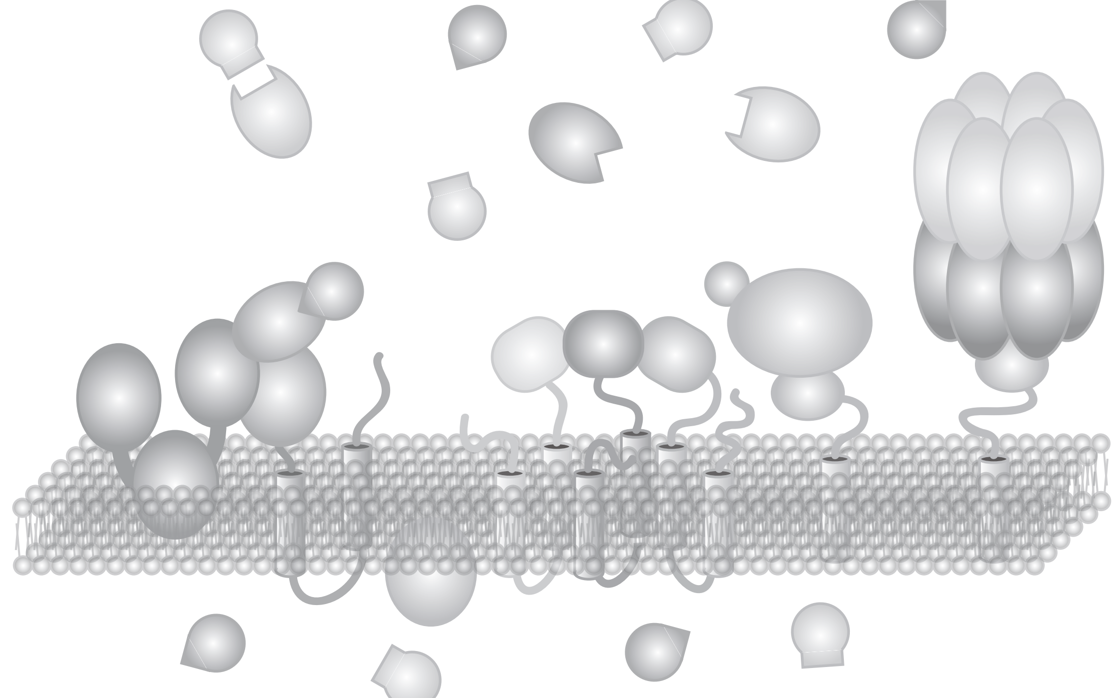
Albert Einstein

Table of contents

Chapter 1	General introduction	**9**
Addendum	Protein quality control in peroxisomes: Ubiquitination of the PTS receptors. *In preparation for publication*	**45**
Chapter 2	*S. cerevisiae* Pex14p contains two independent Pex5p binding sites, which are both essential for PTS1 protein import. *FEBS Letters* ***579****, 3416-3420 (2005)*	**63**
Chapter 3	Pex13p: Docking or cargo handling protein? *Biochimica et Biophysica Acta* ***1763****, 1585–1591 (2006)*	**77**
Chapter 4	Studies on a putative ubiquitin interacting motif present in the fourth TPR motif of *S. cerevisiae* Pex5p.	**91**
Chapter 5	A conserved cysteine is essential for Pex4p-dependent ubiquitination of the peroxisomal import receptor Pex5p. *Journal of Biological Chemistry* ***282****, 22534-22543 (2007)*	**107**
Chapter 6	Further analysis on the ubiquitination of the PTS1 receptor Pex5p.	**131**
References		**149**
Summary		**161**
Samenvatting		**163**
Dankwoord		**167**

1

General introduction



Contents

I. An introduction to peroxisomes
II. Peroxiomes and disease
III. Identification of peroxisomal biogenesis factors
IV. Peroxisome biogenesis
V. Peroxisome matrix protein import
VI. The PTS receptors
VII. Receptor docking on the peroxisomal membrane
VIII. Translocation of the PTS cargo across the peroxisomal membrane and recycling of the PTS receptors
IX. Ubiquitin and ubiquitination
X. Scope of this thesis

I. An introduction to peroxisomes

The compartmentalisation of certain cellular functions into membrane bound organelles is a general trait associated with eukaryotic cells. These different compartments are created to house specialised pathways in an optimised sub-environment within the cell. Metabolic pathways that, for example, require different conditions other than those found in the rest of the cell are often localised to organelles. Similarly, processes that result in the production of compounds that are detrimental to the cell, such as oxidising agents, can be kept under tight control in organelles. One such organelle, the peroxisome, is discussed here.

Originally identified in 1954, peroxisomes were first known as microbodies and described as small, single membrane enclosed compartments in the cytoplasm of mouse kidney cells (Rhodin, 1954). However, it took more than 10 years before these microbodies were biochemically characterised. De Duve and Baudhuin revealed that microbodies contained oxidases and catalases, enzymes that are involved in the production and degradation of hydrogen peroxide, respectively (De Duve and Baudhuin, 1966). This hydrogen peroxide respiration is common to all peroxisomes, hence the name. To date, peroxisomes have been identified in nearly every eukaryotic cell and play essential roles in the cellular metabolism. However, in certain organisms, they are referred to by different names, often depending on the specific function they fulfil. Some examples of these include glyoxysomes, which are found in germinating plant seeds and contain the glyoxylate enzymes essential for the metabolism of fatty acids stored in seeds (Breidenbach and Beevers, 1967), and glycosomes, which can be found in kinetoplastids and contain enzymes involved in glycolysis (Opperdoes and Borst, 1977). Indeed, glycosomes are absolutely essential for viability of the kinetoplastid *Trypanosoma brucei*, as certain enzymes from the glycolysis pathway are toxic when mislocalised to the cytosol (Blattner *et al.*, 1998).

Although this specialisation is observed between different species and/or cell types, peroxisomes across the evolutionary board all contain enzymes involved in the β-oxidation of fatty acids (Wanders and Waterham, 2006). In plant and yeast cells, β-oxidation is restricted to peroxisomes whereas mammalian cells have both a peroxisomal and a mitochondrial β-oxidation system that handles different substrates. For example very long chain fatty acids (VLCFA's) are shortened to long or medium chain fatty acids (LCFA's or MCFA's respectively) in peroxisomes with the subsequent steps taking place in the mitochondria. In higher eukaryotes, enzymes involved in the β-oxidation of branched

chain fatty acids and polyunsaturated fatty acids can also be found in peroxisomes. Other processes, such as the biosynthesis of bile acids, dolichol, plasmalogens, the breakdown of some amino acids and the α-oxidation of phytanic acid have been reported to take place in peroxisomes.

II. Peroxisomes and disease

The existence of several human peroxisomal disorders, caused by a deficiency in one, or sometimes a number of the metabolic pathways present in peroxisomes, emphasises the importance of peroxisomes (Gould and Valle, 2000). These disorders can be divided into two major groups; the peroxisomal biogenesis disorders (PBD's) and the single peroxisomal enzyme deficiencies (PED's).

The PBD's, which include Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD) are characterised by a general loss of peroxisome functions, due to a non-functional import system for either peroxisomal membrane or matrix proteins. This results in an increased level of VLCFA's, bile acid intermediates and phytanic acid and a decrease in the level of plasmalogens in the body. Patients typically suffer from a delay in development, facial abnormalities, hypotonia and liver disease and, in the majority of cases, die prematurely. In ZS, the most severe of the PBD's, patients usually die within the first year, whereas IRD patients can survive for up to 30 years. Another PBD is rhizomelic chondrodysplasia punctata (RCDP), where patients display a rather different phenotype to those with ZS, NALD or IRD, including the shortening of proximal limbs, a typical facial appearance and abnormal development in psychomotor. These phenotypes are caused by a defect in phytanic acid degradation and plasmalogen biosynthesis. Patients with RCDP do not usually survive the first decade of life. PBD's can be divided into 12 complementation groups, depending on the particular gene that is mutated (Steinberg *et al.*, 2006).

PED's are caused by the inactivation or mislocalisation of a single peroxisomal enzyme. One such disorder, X-linked adrenoleukodystrophy (X-ALD) results in an accumulation of VLCFA's in the patients plasma and tissue and is caused by a mutation in the gene coding for the peroxisomal membrane protein ALD, a half ABC transporter believed to be involved in the transport of VLCFA's across the peroxisomal membrane (Wanders and Waterham, 2006).

On the whole, treatment of peroxisomal defects is very difficult. This is often due to the fact that they already occur in embryogenesis and have significant consequences for further development. Therefore, therapy has mainly been aimed at the control of individual defects, such as liver failure and includes strict diets low in certain compounds, such as phytanic acid and supplementation of deficient components, including docosahexaenoic acid.

III. Identification of peroxisomal biogenesis factors

Currently there are 32 proteins that carry the name peroxin, or Pex protein (see Table I). This name implies that they play a role in peroxisomal matrix protein import, peroxisomal membrane protein import, peroxisome proliferation or peroxisome inheritance. The majority of the genes that encode these Pex proteins were originally identified in one of the many model yeast species using genetic screens, in which potential mutants were selected for their inability to grow on peroxisome-requiring carbon sources, such as oleic acid or methanol (Erdmann *et al.*, 1989; Gould *et al.*, 1992). When the sequence of these yeasts *PEX* genes were known, searches of the human expressed sequence tags (EST) database identified the first homologues in mammalian cells (Gould and Valle, 2000). Further studies using peroxisome deficient Chinese hamster ovary (CHO) cells identified more mammalian *PEX* genes (Fujiki *et al.*, 2006).

IV. Peroxisome biogenesis

The biogenesis of peroxisomes can be seen as a sequential, multi-step process. Firstly, lipids are recruited to form a membrane. Next, peroxisomal membrane proteins (PMP's), which are often involved in the import of matrix enzymes, are inserted into this lipid bilayer. Finally, the enzymes essential for the metabolic function of peroxisomes are imported into the matrix, making a fully functional peroxisome. Upon completion, the size of the peroxisome can be controlled by regulating the amount of matrix enzymes and that of a specific set of peroxins at the transcriptional level, often as a direct response to environmental changes experienced by the cell.

Table I. An overview of the peroxins

Peroxin	Interacts with*	Features and (putative) functions
Pex1p	Pex6p	AAA-protein, essential for matrix protein import and receptor recycling
Pex2p	Pex5p, Pex10p, Pex12p, Pex19p	Integral PMP, contains RING domain, essential for matrix protein import and receptor recycling
Pex3p	Pex3p, Pex19p, PMP's	Integral PMP, essential for PMP import
Pex4p	Pex4p, Pex10p, Pex22p	Ubiquitin-conjugating enzyme, essential for matrix protein import and receptor recycling
Pex5p	Pex5p, Pex7p, Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, Pex17p, Pex20p, PTS1/3 proteins	PTS1/3 import receptor in yeast, required for PTS2 import in mammals, contains TPR domain and W-x-x-x-F/Y motifs
Pex6p	Pex6p, Pex15p, Pex26p	AAA-protein, essential for matrix protein import and receptor recycling
Pex7p	Pex5p, Pex13p, Pex14p, Pex18p, Pex20p, Pex21p	PTS2 import receptor, contains WD-40 repeats
Pex8p	Pex5p, Pex20p	Intra-peroxisomal protein, involved in receptor-cargo dissociation and assembly of the docking and RING complexes
Pex9p		Integral PMP, essential for matrix protein import, only in *Y. lipolytica*
Pex10p	Pex2p, Pex4p, Pex5p, Pex10p, Pex12p, Pex13p, Pex15p, Pex19p, Pex22p	Integral PMP, contains RING domain, essential for matrix protein import and receptor recycling
Pex11p	Pex11p, Pex19p	PMP, involved in peroxisome proliferation or medium chain fatty acid translocation
Pex12p	Pex2p, Pex5p, Pex10p, Pex12p, Pex13p, Pex15p, Pex19p	Integral PMP, contains RING domain, essential for matrix protein import and receptor recycling
Pex13p	Pex3p, Pex5p, Pex7p, Pex10p, Pex12p, Pex14p, Pex15p, Pex17p, Pex19p	Integral PMP, contains SH3 domain, essential for matrix protein import, involved in receptor docking
Pex14p	Pex5p, Pex7p, Pex13p, Pex14p, Pex15p, Pex17p, Pex19p	Integral PMP, phosphorylated, essential for matrix protein import, initial receptor docking site
Pex15p	Pex3p, Pex6p, Pex12p, Pex13p, Pex14p, Pex17p, Pex22p	Integral PMP, phosphorylated, essential for matrix protein import, membrane anchor for Pex6p
Pex16p	Pex19p	Integral PMP, essential for PMP import
Pex17p	Pex5p, Pex13p, Pex14p, Pex15p, Pex19p	PMP, essential for matrix protein import, involved in receptor docking

Table I. An overview of the peroxins (continued)

Peroxin	Interacts with*	Features and (putative) functions
Pex18p	Pex7p, Pex13p	PTS2 import co-receptor, partially redundant with Pex21p, only in *S. cerevisiae*, related to Pex20p
Pex19p	PMP's	Farnesylated protein, essential for PMP import
Pex20p	Pex7p, Pex8p, Pex13p, Thiolase (PTS2 protein)	PTS2 import co-receptor in yeast
Pex21p	Pex7p, Pex13p	PTS2 import co-receptor, partially redundant with Pex18p, only in *S. cerevisiae*, related to Pex20p
Pex22p	Pex4p, Pex10p, Pex15p, Pex19p	PMP, essential for matrix protein import, membrane anchor for Pex4p
Pex23p		PMP, essential for matrix protein import, only in certain yeast's
Pex24p		Integral PMP, essential for peroxisome assembly, only in certain yeast's
Pex25p	Pex11p, Pex19p, Pex25p, Pex27p	PMP, regulator of peroxisomal size and maintenance
Pex26p	Pex6p	Integral PMP, essential for matrix protein import, mammalian homologue of Pex15p
Pex27p	Pex11p, Pex25p, Pex27p	PMP, regulator of peroxisomal size and maintenance
Pex28p	Pex32p	PMP, regulator of peroxisomal size and maintenance
Pex29p	Pex30p	PMP, regulator of peroxisomal size and maintenance
Pex30p	Pex19p, Pex29p, Pex30p, Pex31p, Pex32p	PMP, regulator of peroxisomal size and maintenance
Pex31p	Pex30p	PMP, regulator of peroxisomal size and maintenance
Pex32p	Pex19p, Pex28p, Pex30p	PMP, regulator of peroxisomal size and maintenance

* Reported in literature in various organisms. Interactions are not necessarily direct.

Peroxisome formation

Lipids for the peroxisomal membrane, which is mainly made up of the phospholipids phosphatidyl choline and phosphatidyl ethanolamine (Schneiter *et al.*, 1999), are thought to derive from the endoplasmic reticulum (ER). Originally, peroxisome formation was thought to occur by budding from the ER, as peroxisomes were often found in close proximity to the ER (Novikoff and Shin, 1964). However, later studies showed that existing peroxisomes

were capable of importing newly synthesised membrane and matrix proteins and can then grow and divide to form new peroxisomes, leading to the "growth and division" model (Lazarow and Fujiki, 1985). Although this model was generally accepted, some recent observations could not be explained with this model alone. For example, mutations in certain genes result in a loss of peroxisomes or peroxisome remnants (known as ghosts) from the cell. The re-introduction of the wild type version of the gene, even after several generations without peroxisomes, results in the reappearance of normal peroxisomes (Faber *et al.*, 2002; Haan *et al.*, 2006; Tam *et al.*, 2005). This would suggest that somewhere in the cell a “pre-peroxisomal” compartment exists, that is capable of forming a peroxisome *de novo* upon the correct stimuli and that existing peroxisomes are not essential for the formation of new organelles. Several other recent observations that support the ER-peroxisome connection, making it again a hotly discussed topic, include i) several PMP’s in the yeast *Yarrowia lipolytica* are N-glycosylated, a modification that takes place exclusively in the ER (Titorenko and Rachubinski, 1998), ii) in mouse dendritic cells the PMP’s Pex13p and PMP70 could be found in special lamellar structures that were connected to the ER (Geuze *et al.*, 2003). Once these specialised sub-domains were no longer connected to the ER, they were continuous with the peroxisomal reticulum, a peroxisome precursor and iii) in the yeast *Saccharomyces cerevisiae* Pex3p, a protein involved in the early stages of peroxisome formation (see below), is targeted to a sub-domain of the ER, which then dissociates from the ER and initiates the formation of peroxisomes (Hoepfner *et al.*, 2005; Kragt *et al.*, 2005a). Further studies on this subject are needed before the role of the ER in peroxisome formation is fully understood.

Peroxisomal membrane protein (PMP) import

The targeting and insertion of PMP’s into the peroxisomal membrane occurs at an early stage in peroxisome biogenesis. This is accomplished with the aid of a membrane peroxisomal targeting signal (mPTS). However, unlike the signals responsible for the targeting of matrix enzymes (see below), mPTS’s do not contain a recognisable consensus. Instead, several features within the PMP are important for its targeting, such as a short sequence of basic amino acids and a hydrophobic membrane-spanning domain. The latter is also thought to function in anchoring the protein to the peroxisomal membrane (Wang *et al.*, 2001). Several groups have reported that PMP’s containing more than one membrane-

spanning domain. For example human Pex13p, PMP70 and *Candida boidinii* PMP47 all require multi mPTS's (Biermanns and Gartner, 2001; Jones *et al.*, 2001; Wang *et al.*, 2004). Taken together, these data may suggest that the targeting and import of PMP's is highly variable and depends very much on the PMP in question.

So far, three peroxins have been implicated in the targeting and insertion of PMP's into the peroxisomal membrane: Pex3p, Pex16p and Pex19p. These three proteins play an essential role early in peroxisomal membrane biogenesis, as many groups have reported that in the absence of one of them peroxisomal structures are missing, although this seems not always to be the case (Baerends *et al.*, 1996; Götte *et al.*, 1998; Hazra *et al.*, 2002; Otzen *et al.*, 2004). So far, both Pex3p and Pex19p have been identified in all organisms containing peroxisomes. Pex16p, on the other hand, is only present in mammalian cells and the yeast *Y. lipolytica*.

Pex19p is an acidic protein that exhibits a broad range of binding specificity for PMP's. Its localisation is predominantly cytosolic, although a small but significant amount localises to the peroxisomal membrane. Most Pex19p's contain a C-terminal C-A-A-X box, a well-characterised farnesylation consensus motif. The exact role the farnesylation plays is unknown, but it may allow Pex19p to associate with the peroxisomal membrane or allow conformational changes in Pex19p to occur (Götte *et al.*, 1998; Sacksteder *et al.*, 2000; Snyder *et al.*, 2000).

Current opinion is somewhat divided with respect to the precise function of Pex19p in PMP import. Several groups have suggested that, based on its predominantly cytosolic localisation and its high affinity for a broad range of PMP's, Pex19p is a chaperone for newly synthesised PMP's in the cytosol. In the absence of Pex19p, PMP's synthesised in a cell-free system formed aggregates, whereas in the presence of Pex19p, the same PMP's are soluble. In addition, Pex19p binding increased not only the stability of PMP's but also allowed them to retain their membrane insertion-conformation (Halbach *et al.*, 2006; Halbach *et al.*, 2005; Rottensteiner *et al.*, 2004; Sacksteder *et al.*, 2000; Shibata *et al.*, 2004). Others have gone further in their interpretation of the role of Pex19p, stating that the protein not only functions as a chaperone, but also as a PMP import receptor. This was based on the observation that a small amount of Pex19p was associated with the peroxisomal membrane at any one time, coupled with the fact that RNA interference (RNAi) of PEX19, which results in a transient depletion of Pex19p, caused a PMP targeting

and import deficiency. Furthermore, *in vitro* import assays showed that Pex19p was able to transport newly synthesised PMP's to the peroxisomal membrane and then recycle back to the cytosol (Götte *et al.*, 1998; Jones *et al.*, 2004; Matsuzono and Fujiki, 2006; Sacksteder *et al.*, 2000).

Fransen *et al*. (2004) proposed an alternative role for Pex19p, namely as an assembly or disassembly factor for membrane associated protein complexes, as Pex19p is capable of competing with the PTS1 receptor Pex5p (see below) and the PMP Pex13p for binding to Pex14p (Fransen *et al.*, 2004). In addition, the Pex19p binding site and the mPTS in certain PMP's do not overlap (Fransen *et al.*, 2001). It is evident that the role of Pex19p in peroxisome biogenesis is far from clear.

Pex3p is a peroxisomal membrane protein essential for the import of other PMP's. Although Pex3p interacts with Pex19p, this binding is not to the mPTS of Pex3p, contained within the first 46 amino acids of the protein (Fransen *et al.*, 2004; Sacksteder *et al.*, 2000; Snyder *et al.*, 2000). In addition, Pex3p can target to mature peroxisomes in the absence of Pex19p, suggesting that Pex3p targeting is Pex19p independent (Jones *et al.*, 2001). This led to the theory that two independent mPTS pathways exist: one dependent of Pex19p (class I) and a second, independent of Pex19p (class II). So far, only one class II protein has been identified, Pex3p.

There is considerable evidence to support the idea that Pex3p is the docking factor for Pex19p on the peroxisomal membrane. Indeed, transient inhibition of Pex3p by RNAi blocks the recruitment of Pex19p to the peroxisomal membrane and the interaction with Pex3p is essential for its peroxisomal docking (Fang *et al.*, 2004; Fransen *et al.*, 2005; Matsuzono *et al.*, 2006). In addition, a tertiary complex consisting of Pex3p, Pex19p and PMP's can assemble *in vitro* (Matsuzono and Fujiki, 2006; Shibata *et al.*, 2004). However, the actual process of PMP insertion into the peroxisomal membrane remains unknown. We are, therefore, left with a model for the import of PMP's where Pex19p binds newly synthesised PMP's in the cytosol, in order to protect the hydrophobic domains. Pex19p then transports its cargo to the peroxisomal membrane, where it docks with the aid of Pex3p. PMP's are then inserted into the membrane and Pex19p returns to the cytosol to repeat the process. This model is reminiscent of the model proposed for the PTS1 receptor Pex5p (see below).

V. Peroxisome matrix protein import

Once the membrane and PMP's are in place, the peroxisome can begin with the import of matrix enzymes. Proteins destined for the peroxisomal matrix are synthesised in the cytosol on free polyribosomes and post-translationally imported (Lazarow and Fujiki, 1985) (Fig. 1). This is achieved with the aid of a peroxisomal targeting signal (PTS). Currently, two PTS's have been identified: type I (PTS1) and type II (PTS2). There is also evidence for a third signal, often referred to as type III (PTS3), although this signal is ill defined. In turn, these PTS's are recognised by a cycling receptor and are directed to the peroxisome. Pex5p has been identified as the receptor for PTS1- (and PTS3-) containing proteins, Pex7p for the PTS2 proteins.

The peroxisomal targeting signal type I

The discovery of the first peroxisomal targeting signal, the PTS1 was somewhat coincidental. Keller and co-workers (Keller *et al.*, 1987) noticed that recombinant luciferase from the firefly *Photinus pyralis* co-localised with catalase in peroxisomes when expressed in monkey kidney cells. Following this observation, luciferase was found to localise to peroxisomes present in the lantern organ of the firefly (Gould *et al.*, 1987). Genetic manipulations led to the identification of the domain responsible for luciferase's peroxisomal targeting, which consisted of the last three amino acids. These residues, serine, lysine and leucine (S-K-L), when introduced onto the C-terminus of a non-peroxisomal protein, were sufficient to target it to the peroxisome (Gould *et al.*, 1989). The introduction of amino acid substitutions into the PTS1 yielded the consensus S/A/C-K/R/H-L or in other words a small neutral residue at position -3, a positively charged residue at -2 and finally a leucine at the carboxyl terminus position (Swinkels *et al.*, 1992). However, as more and more PTS1 proteins were identified, it became clear that not only was this original consensus a little on the strict side, but also that changes to the PTS1 are quite well tolerated, depending on the organism. The PTS1 of *S. cerevisiae* malate dehydrogenase (Mdh3p) for example, can cope with the introduction of residues that do not conform to the consensus, especially at the positions -2 and -3, and still target the protein to peroxisomes (Elgersma *et al.*, 1996b). A similar observation was reported for the PTS1 (K-K-L) of human alanine-glyoxylate aminotransferase (AGT), suggesting that considerable variation within the PTS1 consensus is not uncommon (Motley *et al.*, 1995). Taking it one step

further, catalase, the prototype of a peroxisomal matrix enzyme requires the C-terminal tetrapeptide sequence K-A-N-L in humans and R-P-S-I in cottonseed for its peroxisomal targeting; the tripeptides A-N-L and P-S-I alone are not sufficient (Mullen *et al.*, 1997; Purdue *et al.*, 1996).

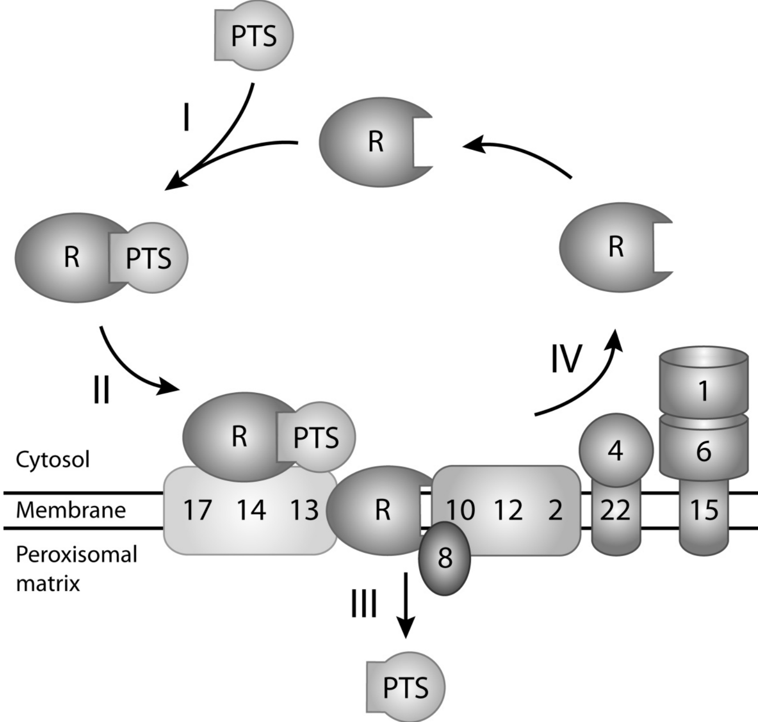


Fig. 1 Model for the import of peroxisomal matrix proteins and receptor recycling
Peroxisomal matrix proteins containing a peroxisomal targeting signal (**PTS**) are synthesised in the cytosol and recognised by an associated cycling receptor (**I**). The receptor-cargo complex then docks on the peroxisomal membrane (**II**). Next, the PTS cargo is dissociated from the receptor and translocated into the peroxisome (**III**) and the receptor is recycled to the cytosol for another round of import (**IV**). R represents the cycling receptors and numbers indicate specific peroxins. See text for details.

These examples show that to define the PTS1 as a simple C-terminal tripeptide sequence is an oversimplification. Furthermore, the presence of a PTS1 sequence does not necessarily guarantee peroxisomal targeting. The multifunctional enzyme in the yeast *C. tropicalis* contains a PTS1 with the consensus A-K-I. This sequence was also essential for targeting to peroxisomes when the same protein was expressed in the yeasts *Candida albicans* and *S. cerevisiae*. Substitution of this consensus with either G-K-I or A-Q-I

abolished peroxisomal targeting in *S. cerevisiae* but not in *C. albicans* (Aitchison *et al.*, 1991). Similarly, the PTS1 of human AGT, K-K-L is not sufficient to target luciferase to peroxisomes, suggesting that efficient targeting requires more than just the PTS1 alone (Motley *et al.*, 1995). Indeed, several groups have reported the importance of residues upstream of the PTS1 sequence, which was often species-specific. Although both human and *S. cerevisiae* had a preference for a lysine or arginine at position -4, they had different preferences for the position -5, with *S. cerevisiae* preferring a hydrophilic or polar residue as opposed to a hydrophobic residue in humans (Lametschwandtner *et al.*, 1998). Currently, a PTS1 prediction program, developed by Neuberger and co-workers predicts potential PTS1 sequences based on a region of 12 amino acids at the C-terminal end of the protein (Neuberger *et al.*, 2003). These 12 amino acids are divided into 3 regions; the last 3 amino acids, representing the actual tripeptide consensus that fits into the PTS1 binding cavity on Pex5p (see below), a region of around 4 amino acids directly upstream, which may partake in additional binding to Pex5p and, further upstream, a region of around 5 amino acids that acts as a hinge and allows the sequence to be flexible.

It is clear that the definition of a PTS1 sequence is far from complete and factors such as the accessibility of the sequence play a crucial role in the targeting of the protein to the peroxisome.

The peroxisomal targeting signal type II

Although the majority of peroxisomal matrix enzymes contain a PTS1 signal, a small but significant number contain a PTS2, present at the N-terminus of the protein. This type of signal was originally identified in rat 3-ketoacyl-CoA thiolase and later in thiolase from other mammals as well as yeasts, plants and kinetoplastids (Erdmann, 1994; Osumi *et al.*, 1991; Swinkels *et al.*, 1991). The PTS2 consensus is highly variable and depends very much on the organism under study. However, bioinformatics and mutagenesis studies have given us a consensus sequence for the most common PTS2 variants: R/K-L/V/I/Q-x-x-L/V/I/H-L/S/G/A/K-x-H/Q-L/A/F (Petriv *et al.*, 2004). This rather ungainly consensus can be helpful in predicting potential PTS2 proteins but, since a number of PTS2 sequences do not conform to this consensus, its use remains limited. The ability to predict PTS2 sequences will be greatly enhanced by the identification of more PTS2 proteins. This will not only give us more PTS2 sequences with which to refine the consensus but, in

combination with alternative approaches such as NMR and X-ray crystallography, will allow wide scale searches for PTS2 proteins based on a number of criteria, such as consensus plus structural considerations.

An interesting observation regarding PTS2 proteins is that the entire PTS2 pathway is missing from *Caenorhabditis elegans*. This means that thiolase, a well-characterised PTS2 protein in other organisms, contains a PTS1 in *C. elegans* (Motley *et al.*, 2000). The reason for this anomaly is unknown but it seems likely that the PTS2 pathway was lost during evolution, as a number of “PTS2 like” sequences have been identified in this organism. Expression of these proteins in an organism containing a PTS2 pathway does not result in their targeting to peroxisomes, which may suggest that being redundant, these PTS2 signals have mutated over time.

Alternative targeting signals

A number of proteins that are imported into peroxisomes lack a recognisable PTS1 or PTS2 consensus or contain a redundant PTS, which is not essential for their import. How these proteins are targeted to the peroxisome remains a point of discussion. Several groups have suggested that this occurs via “piggy backing”, a process where matrix enzymes form hetero-oligomeric complexes in the cytosol with at least one of the proteins containing a PTS signal, which is recognised by the cycling receptor and allows import of the whole complex. The import of an N-terminal truncated version of thiolase, lacking a PTS2, was only observed when full-length thiolase was co-expressed, indicating that the truncated version could only be imported by ‘tagging along’ with the full-length protein (Glover *et al.*, 1994). Similar results were obtained with *S. cerevisiae* Dci1p and Eci1p, two PTS1 proteins that form hetero-oligomers (Yang *et al.*, 2001). However, “piggy backing” import has only ever been shown with artificial substrates and not with proteins that simply lack a PTS, leaving us to conclude that the import of non-PTS containing proteins is likely to occur in a different manner. One theory suggested the existence of a third import pathway, separate from either the PTS1 or PTS2 pathway and with its own import receptor. Studies in the yeast *S. cerevisiae* showed that a peroxisome biogenesis (PEB) mutant strain (*peb5*) was impaired in the import of the PTS1 protein catalase A but not in the import of the PTS2 protein thiolase and acyl CoA oxidase (AOX). The authors concluded that the peroxisomal targeting of AOX was independent of either the PTS1 or PTS2 pathway (Zhang *et al.*,

1993). Later studies however, showed that the import of AOX is dependent on the PTS1 receptor Pex5p and therefore, the existence of another import receptor is unlikely (Klein *et al.*, 2002).

Indeed, several matrix enzymes rely on Pex5p for their peroxisomal targeting but this targeting does not require a PTS1. *S. cerevisiae* carnitine acetyltransferase (Cat2p) and alcohol oxidase (AO) from the yeast *Hansenula polymorpha* are two such enzymes. Both of these proteins contain PTS1-like sequences at the C-terminal end, but these sequences are redundant for targeting (Elgersma *et al.*, 1995). Remarkably, HpPex5p is unable to bind the PTS1 of AO. Attempts to identify the signal responsible for the targeting of these Pex5p mediated non-PTS1 proteins (often referred to as PTS3) have been unsuccessful, which may suggest that the signals are based on tertiary structure rather than on one set of amino acids or may comprise two or more separate regions.

VI. The PTS receptors

As PTS containing proteins are synthesised in the cytosol, they need to be targeted to their place of action, the peroxisome. This is achieved with the aid of a mobile receptor. Currently two such receptors have been identified, Pex5p and Pex7p. A number of co-receptors for Pex7p, the Pex20p family of proteins, have also been identified.

The PTS1 receptor Pex5p

Pex5p was originally identified in the yeast *Pichia pastoris* using functional complementation of a mutant deficient in the import of PTS1 but not PTS2 proteins (McCollum *et al.*, 1993). Since this time, Pex5p orthologs have been identified in other yeasts, plants, mammals, nematodes and kinetoplastids and have been studied in numerous ways. The current model for Pex5p mediated PTS1 (and PTS3) protein import runs as follows (Fig. 1): Pex5p binds to a newly synthesised PTS1 protein in the cytosol and transports it to the peroxisomal membrane, where docking takes place. The cargo is then released into the peroxisomal matrix and Pex5p recycles back to the cytosol for another round of import (Purdue and Lazarow, 2001a). Several groups have suggested that Pex5p inserts into the peroxisomal membrane or even enters completely into the peroxisome but this observation is yet to be confirmed (Dammai and Subramani, 2001; Kerssen *et al.*, 2006).

The protein Pex5p can be divided into two main regions. The C-terminal region, which consists of 6-7 tetratricopeptide repeat (TPR) motifs, is responsible for the recognition and binding of PTS1 proteins (Brocard *et al.*, 1994; Dodt *et al.*, 1995). TPR motifs are well-characterised protein-protein interaction mediators. Their presence in proteins involved in processes such as nuclear import as well as in chaperones bears witness to this (D'Andrea and Regan, 2003). Although the sequence similarity between two independent TPR motifs is often very low, these motifs fold in a distinct manner, in the form of two anti-parallel helices (A and B) separated by a single loop region. At least three individual TPR motifs are required to make up a typical TPR “domain”. These individual motifs stack on top of each other, with all the A helices forming the inner concave surface. In the case of Pex5p, two such domains are formed, each consisting of 3 TPR motifs (TPR1-3 and 5-7) that form a ring-like structure (Gatto *et al.*, 2000; Stanley *et al.*, 2006). These two TPR clusters are linked by a distorted TPR motif, TPR4 that may act as a flexible "hinge" between the two separate domains. The PTS1 binding site can be found in a groove between the two TPR domains. Several residues in TPR 2 and 3 are important for the interaction between Pex5p and the PTS1 in both yeast and humans, namely an asparagine, a large hydrophobic residue and a number of glutamic acid residues (Klein *et al.*, 2001). These residues are very well conserved throughout the Pex5p family, indicating that the manner of the Pex5p-PTS1 interaction is likely to be similar in all organisms.

Upon binding of the PTS1 protein, the two TPR domains of Pex5p undergo considerable conformational change. In the absence of a PTS1 cargo, these two TPR domains form an open ring-like structure, which close upon binding of a PTS1 protein. A change in the conformation of the PTS1 protein is also observed. This takes place in the form of “unwinding” the PTS1 sequence from the surface of the protein, presumably to allow the sequence to be accessible for Pex5p (Stanley *et al.*, 2006).

Although generally not well conserved, the N-terminal region of Pex5p does have some conserved features, the most notable of these are multiple W-x-x-x-F/Y motifs (Saidowsky *et al.*, 2001). Several of these motifs are found in the N-terminal region of all Pex5p’s identified to date, ranging from two in *S. cerevisiae* to seven in mammalian and even twelve in watermelon Pex5p. These motifs are involved in the interaction between Pex5p and the docking factors Pex13p and Pex14p (see below), although considerable variation is seen in both the specificity and the interaction strength, depending on the motif

in question (Bottger *et al.*, 2000; Otera *et al.*, 2002; Saidowsky *et al.*, 2001). Curiously, several yeast Pex5p's contain reverse W-x-x-x-F/Y motifs and at least one of these reverse motifs plays a role in the interaction with Pex14p (Kerssen *et al.*, 2006; Williams *et al.*, 2005, Chapter 2). Another conserved region of Pex5p is the N-terminal 20-30 amino acids (Fig. 2). Sequence alignments show the presence of several conserved residues, most notably a cysteine, a proline, several lysines and a number of large hydrophobic and large polar residues. This region of Pex5p is predicted to be of a helical nature and, in mammals, is essential for the recycling of Pex5p from the peroxisomal membrane (Costa-Rodrigues *et al.*, 2004 and see below).

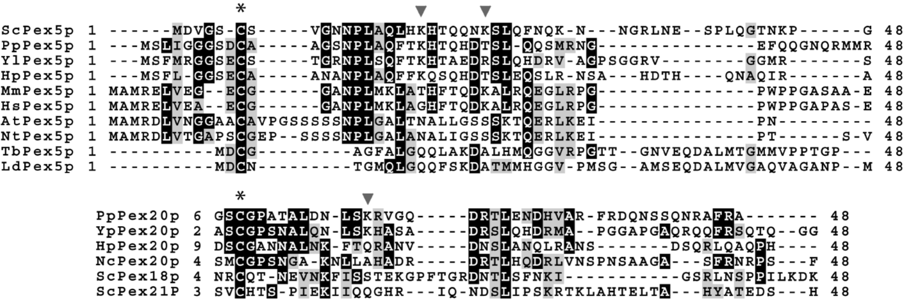


Fig. 2 Sequence alignment showing the N-terminal 48 amino acids of a number of Pex5 (*upper panel*) and Pex18/20 (*lower panel*) proteins from different species

* Indicates the conserved cysteine residue. Arrowheads indicate lysine residues shown to be involved in poly-ubiquitination (for Pex5p, lysines 18 and 24 in *S. cerevisiae* and 21 in *H. polymorpha* and for Pex20p lysine 19 in *P. pastoris*, see Addendum). Sc; *Saccharomyces cerevisiae*, Pp; *Pichia pastoris*, Yl; *Yarrowia lipolytica*, Hp; *Hansenula polymorpha*, Mm; *Mus musculus*, Hs; *Homo sapiens*, At; *Arabidopsis thaliana*, Nt; *Nicotiana tabacum*, Tb; *Trypanosoma brucei*, Ld; *Leishmania donovani*, Nc; *Neurospora crassa*.

In contrast to its C-terminal TPR domain, which adopts a well-defined structure, the N-terminal domain of Pex5p lacks a recognisable secondary or tertiary structure and can be described as "intrinsically unstructured" (Carvalho *et al.*, 2006; Costa-Rodrigues *et al.*, 2005). Intrinsically unstructured proteins are defined as proteins that are "natively unfolded" and challenge the theory that in order to be functional a protein must adopt a well-defined three-dimensional structure. Originally it was thought that regions lacking a secondary or tertiary structure were merely "spacers" between folded domains. However, more and more natively unfolded proteins have been reported in literature and their importance in biology is now being recognised. Being a natively unfolded protein has several functional benefits, one of which is extreme flexibility. Many natively unfolded

proteins have a large number of binding partners, due to their flexibility. The binding of a specific ligand often induces a transition from unfolded to folded of a certain domain and might represent a simple mechanism for the regulation of distinct processes. Natively unfolded proteins play important roles in numerous processes including the regulation of transcription and translation, endocytosis and cell cycle control (Tompa, 2002). It is highly likely that, being an intrinsically unstructured region allows the N-terminal region of Pex5p to perform multiple tasks, including the docking of Pex5p on the peroxisomal membrane, as well as being involved in its recycling from the peroxisomal membrane to the cytosol (see below) and may even have other, as yet unidentified, functions.

The PTS2 receptor Pex7p

Not long after the PTS1 receptor Pex5p was identified, a protein responsible for the import of PTS2 proteins, Pex7p, was discovered. Pex7p was originally identified in the yeast *S. cerevisiae*, but later orthologs in other yeasts as well as mammals were found (Braverman *et al.*, 1997; Elgersma *et al.*, 1998; Marzioch *et al.*, 1994). Deletion of Pex7p only causes mislocalisation of PTS2 and not of PTS1 proteins, indicating that Pex7p is indeed specific for PTS2 proteins. Pex7p contains a distinct N-terminal region, followed by six WD-40 repeats. These repeats consist of 40 amino acids and contain a central tryptophane-aspartic acid (WD) motif and are only found in eukaryotes. Currently, the structures of only a handful of WD-40 repeat containing proteins have been solved but all show that WD-40 repeats adopt a β-propeller fold. Each WD-40 repeat constitutes a “blade” of the propeller, which is in turn, made up of 4 anti-parallel β-strands. Similar to TPR repeats, WD-40 repeats are found in proteins involved in numerous cellular processes, such as signal transduction, transcription regulation, cell cycle control and apoptosis. The role WD-40 repeat proteins fulfil in these processes appears to be highly similar, namely the coordination of multi-protein complex assembly, by providing a rigid scaffold for protein interactions to take place. Typically, the specificity of the proteins is determined by sequences outside the WD-40 repeats found, for example, in the loop regions that link the individual WD-40 repeats (Van der Voorn and Ploegh, 1992).

When compared to Pex5p, our knowledge of Pex7p is limited. For example, the recognition site for the PTS2 sequence in Pex7p remains elusive. The structural nature of Pex7p is partially to blame for this. The production of deleted and/or mutated versions of

Pex7p has proved to be nearly impossible, as they generally end up being unstable (Lazarow, 2006). However, it would appear that Pex7p-mediated PTS2 protein import resembles that of its PTS1 counterpart, with Pex7p recognising and binding newly synthesized PTS2 proteins in the cytosol, transporting them to the peroxisomal membrane, inserting the PTS2 protein into the peroxisome and recycling back to the cytosol for another round of import (Fig. 1). Pex7p can bind to both Pex13p and Pex14p, docking factors present on the peroxisomal membrane (Girzalsky *et al.*, 1999; Johnson *et al.*, 2001). Interestingly, several reports suggest that Pex7p can also enter the peroxisome during its import cycle, in much the same way as Pex5p (Nair *et al.*, 2004) (see above).

PTS2 co-receptors: the Pex20p family and mammalian Pex5pL

One interesting observation concerning Pex7p and PTS2 import is that, unlike the PTS1 receptor Pex5p, which is capable of carrying out all the steps associated with a cycling receptor, Pex7p requires additional proteins for the import of PTS2 proteins. These proteins, known as the Pex20p family are often referred to as PTS2 "helper", "accessory" or "auxiliary" proteins, but their actual function is better described as PTS2 co-receptors, due to their essential role in the import of PTS2 proteins (Einwächter *et al.*, 2001; Purdue *et al.*, 1998; Titorenko *et al.*, 1998). The Pex20p family has only been identified in yeasts. Interestingly, whereas most yeasts contain only one PTS co-receptor, *S. cerevisiae* contains two, partially redundant Pex20p-like proteins, referred to as Pex18p and Pex21p. While deletion of one of the two proteins only partially affects PTS2 import, the double mutant is no longer able to import PTS2 proteins (Purdue *et al.*, 1998). Why *S. cerevisiae* requires two PTS2 co-receptors is unknown, although their gene expression profiles are quite different, suggesting a functional difference between the two proteins. The expression of Pex18p is repressed on glucose containing media, where peroxisomes are not needed for growth and is induced on media containing oleic acid. Pex21p on the other hand, does not show oleic acid inducible expression (Smith *et al.*, 2002).

The PTS2 co-receptors function in a way similar to the N-terminal region of Pex5p. Indeed, Schäfer and colleagues reported that a chimeric protein, consisting of ScPex18p fused to the TPR domain of ScPex5p could import PTS1 proteins (Schafer *et al.*, 2004). In addition, Pex20p's share several common features with this N-terminal region of the PTS1 receptor, including at least one W-x-x-x-F/Y motif as well as a conserved N-

terminal domain of around 20-30 amino acids, complete with cysteine and lysine residues (Fig. 2). Pex20p's also interact with the docking factors Pex13p and Pex14p and partially localise to peroxisomes, much the same as both Pex5p and Pex7p (Leon *et al.*, 2006b; Stein *et al.*, 2002).

In higher eukaryotes, Pex20p-like proteins appear to be absent. Instead, their PTS2 co-receptor function is performed by a variant of Pex5p, known as Pex5p Long isoform (Pex5pL). Pex5pL contains a 37 amino acid insertion, the product of alternative splicing. This region binds to Pex7p and its presence is essential for PTS2 protein import. Expression of the short isoform of Pex5p (Pex5pS) alone cannot restore PTS2 import in *pex5* deficient cells (Braverman *et al.*, 1998; Otera *et al.*, 2000). Sequence alignments indicate that this Pex7p binding region of Pex5pL is highly homologous with the Pex7p binding site found in the Pex20p family, indicating a conserved function for these proteins (Dodt *et al.*, 2001).

A question that remains unanswered is why Pex7p mediated PTS2 import requires co-receptors, since Pex7p can interact with both its PTS2 cargo and the docking factors Pex13p and Pex14p independently of its co-receptors. In addition, in the yeast *P. pastoris*, both Pex7p and Pex20p associate with the peroxisomal membrane irrespective of the presence of the other protein. Therefore, a role in the enhancement and/or stabilisation of the PTS2-Pex7p interaction has been suggested with the PTS2 co-receptors acting in a chaperone-like manner (Schliebs and Kunau, 2006).

VII. Receptor docking on the peroxisomal membrane

After the soluble cycling receptor has bound its cargo in the cytosol, the next step on its long journey is the association with the peroxisomal membrane. To date, three proteins have been directly implicated in this docking step: Pex13p, Pex14p and Pex17p. Pex13p and Pex14p can be found in all organisms, whereas Pex17p has only been identified in yeast.

The identification of Pex13p, the first peroxisomal membrane protein involved in the import of PTS1 proteins represented a considerable advance in peroxisome research. Originally defined as *pas20* in *S. cerevisiae* and *pas6-1* in *P. pastoris*, Pex13p contains two transmembrane domains as well as a Src homology 3 (SH3) domain (Elgersma *et al.*, 1996a; Gould *et al.*, 1996). In addition, Pex13p was also found to be essential for the

import of PTS2 proteins (Elgersma *et al.*, 1996a; Erdmann and Blobel, 1996). Pex13p interacts with both PTS receptors Pex5p and Pex7p and with its fellow peroxisomal membrane protein Pex14p (Albertini *et al.*, 1997; Brocard *et al.*, 1997). Other than being involved in a step essential for the docking of both Pex5p and Pex7p on the peroxisomal membrane, little is known about the function of Pex13p (for a full review of Pex13p, see Chapter 3 "Pex13p: Docking or bridging protein", Williams and Distel, 2006).

Pex14p was originally identified in yeast as a peroxisomal membrane-associated protein, essential for the import of both PTS1 and PTS2 proteins, giving us the first indication that the two pathways may overlap (Albertini *et al.*, 1997). Later, Pex14p was identified in all organisms that contain peroxisomes. Depending on the organism, the protein is around 150 amino acids in length and contains some interesting features, including a coiled-coil region and a rather distinctive N-terminal domain. Pex14p is associated with the peroxisomal membrane, very likely as an integral membrane protein, although one report suggests that Pex14p does not insert into the peroxisomal membrane but is, instead, peripheral (Albertini *et al.*, 1997; Komori *et al.*, 1999; Will *et al.*, 1999). Concerning its topology, epitope tagging of Pex14p along with protease protection assays suggested that the C-terminal two-thirds of the protein are exposed to the cytosol (Johnson *et al.*, 2001; Shimizu *et al.*, 1999). Results on the N-terminal region, however, were not so clear. Using an immunufluorescence assay and antibodies raised against the N-terminal 134 amino acids of Pex14p, Will *et al.* reported that Pex14p could only be detected when the peroxisomal membrane was permeabilised, suggesting that the N-terminal region is not exposed to the cytosol (Will *et al.*, 1999). Similar results were obtained using an N-terminally epitope-tagged version of Pex14p (Shimizu *et al.*, 1999). Interestingly, protease protection assays indicated that, even when protease was allowed access to the peroxisomal matrix, a domain corresponding to the first 130 amino acids of Pex14p was completely resistant, suggesting that at least part of this domain is protected from the protease by the membrane itself (Oliveira *et al.*, 2002; Shimizu *et al.*, 1999).

Pex14p is capable of directly interacting with several proteins involved in peroxisomal matrix protein import, including itself, its fellow peroxisomal membrane protein Pex13p (and Pex17p in yeast) as well as the PTS1 receptor Pex5p and the PTS2 receptor Pex7p (Albertini *et al.*, 2001; Albertini *et al.*, 1997; Brocard *et al.*, 1997; Snyder *et al.*, 1999). Pex14p also interacts with the PMP importer Pex19p, although this interaction is

likely to be involved in Pex14p's membrane targeting (Fransen *et al.*, 2001 and see above). The interaction between Pex5p and Pex14p is of considerable interest as it is likely to represent the first contact between elements essential for the docking and translocation of PTS1 proteins and the cargo-laden receptor Pex5p. Originally, Saidowsky and co-workers reported that the N-terminal region of mammalian Pex14p binds to W-x-x-x-F/Y motifs present in Pex5p. Interestingly, not all of these motifs could interact with the N-terminal region of Pex14p and considerable variation was observed in the binding affinity of those motifs that could bind, suggesting that sequences outside the motif may influence the interaction (Otera *et al.*, 2002; Saidowsky *et al.*, 2001). Why Pex5p would need so many Pex14p binding sites remains unknown, since several reports suggest that only one is sufficient for functionality. One possible explanation is that the presence of several potential Pex14p binding sites in Pex5p may result in an improvement in the docking efficiency of the receptor. However, in several yeasts W-x-x-x-F/Y motifs appear to play no role in the binding to Pex14p (Barnett *et al.*, 2000; Bottger *et al.*, 2000; Urquhart *et al.*, 2000). Pex5p from the yeast *S. cerevisiae*, contains two W-x-x-x-F/Y motifs, at positions 120 and 204, but neither of these two motifs are essential for the binding to the N-terminal of Pex14p (Bottger *et al.*, 2000; Williams *et al.*, 2005, Chapter 2). Instead, the motif present at position 204 plays an important role in the interaction between Pex5p and Pex13p (Barnett *et al.*, 2000; Bottger *et al.*, 2000). The N-terminal region of Pex14p in *S. cerevisiae* binds to a region in Pex5p that contains a reverse W-x-x-x-F/Y motif (Williams *et al.*, 2005, Chapter 2). Mutational analysis indeed confirmed that the tryptophane residue in this reverse W-x-x-x-F/Y motif is essential for the interaction (Kerssen *et al.*, 2006). Sequence alignments show that reverse W-x-x-x-F/Y motifs can be found in other yeast Pex5p's as well as in Pex5p from *Leishmania donovanii*. This, coupled with the observation that a fragment of *P. pastoris* Pex5p containing all three of its W-x-x-x-F/Y motifs is unable to bind Pex14p, suggests that we are far from understanding all the details concerning the Pex14p-Pex5p interaction (Urquhart *et al.*, 2000).

Another interesting observation concerning the Pex5p-Pex14p interaction in yeast is the presence of a separate binding site for Pex5p in the C-terminal region of Pex14p, which was identified using the two-hybrid system (Williams *et al.*, 2005). Removal of this region of Pex14p results in a PTS1 import defect, indicating that the interaction is essential for Pex14p's function. Later, it was shown that this interaction is direct (Niederhoff *et al.*,

2005). The function of this interaction is unknown and has not been found in the mammalian system.

It is a common belief that Pex14p is the initial docking site for both the PTS1 and PTS2 import receptors on the peroxisomal membrane. This is based on several observations; i) Pex5p localises to the cytosol in CHO cells lacking Pex14p (Otera *et al.*, 2000), ii) Pex5p accumulates on the peroxisomal membrane in CHO cells overexpressing Pex14p (Otera *et al.*, 2000) and iii) ScPex5p, tagged at its N-terminus with GFP does not associate with peroxisomes in a *pex14Δ* strain (Bottger, 2001). These effects were not observed with cells lacking Pex13p; in contrast, a build up of Pex5p on the peroxisomal membrane was seen, indicating that Pex14p acts upstream of Pex13p (Otera *et al.*, 2000). The same group reported that Pex14p interacts with Pex5p bound to a PTS1 protein, whereas Pex13p preferentially interacts with unbound Pex5p, again indicating that Pex5p initially contacts Pex14p and is then “handed over” to Pex13p (Otera *et al.*, 2002).

However, several groups have suggested that Pex14p’s role goes further than just receptor docking, with the protein possibly being involved in the actual translocation of PTS proteins into the peroxisomal matrix. Evidence for this role in translocation comes from *in vitro* import assays, where protease resistant forms of Pex5p, very likely representing import intermediates, co-immunoprecipitated with Pex14p when Pex5p antibodies were used (Gouveia *et al.*, 2003). In addition, the binding affinities observed between Pex14p and Pex5p are so high that, as no energy is needed for the actual import of PTS1/2 proteins, it seems unlikely that Pex14p “lets go” of Pex5p until import is complete as the energy input required to dissociate the Pex14p-Pex5p complex would be considerable.

Many similarities are observed when comparing the role of Pex14p in the two import cycles. Removal of the N-terminal region of Pex14p in *S. cerevisiae* blocks not only PTS1 but also PTS2 protein import (Williams *et al.*, 2005, Chapter 2). The binding site for Pex7p has been reported to be in the C-terminal region of Pex14p and overlaps somewhat with that of the secondary binding site for Pex5p (Niederhoff *et al.*, 2005; Williams *et al.*, 2005, Chapter 2). Indeed, further deletion from the C-terminus gives not only a PTS1 but also a PTS2 import defect (Niederhoff *et al.*, 2005). It seems likely, therefore, that the N-terminus of Pex14p binds to the PTS2 co-receptors Pex21p and Pex18p. Both of these proteins contain W-x-x-x-F/Y motifs, however, as these motifs play no role in the

interaction between the N-terminal of Pex14p and Pex5p, further investigation is needed to clarify this.

Little is known about the transmembrane domain-containing protein Pex17p other than that it is only found in yeast, that it localises to the peroxisomal membrane and that it interacts with Pex14p (Huhse *et al.*, 1998; Smith *et al.*, 1997). Reports suggested that Pex17p may be involved in the targeting of PMP's to the peroxisomal membrane (Snyder *et al.*, 1999). However, others have reported that PMP targeting, including that of Pex14p, is unaffected in a *pex17Δ* strain. Similar to the results obtained with *pex14Δ* cells, GFP-Pex5p can no longer localise to the peroxisomal membrane in a *pex17Δ* strain, indicating an early role for Pex17p in the docking step (Bottger, 2001).

VIII. Translocation of the PTS cargo across the peroxisomal membrane and recycling of the PTS receptors

After docking of the cargo-laden receptor on the peroxisomal membrane, two important steps need to occur before the cycle is complete: the translocation of the PTS protein into the peroxisomal matrix and the recycling of the receptor to the cytosol, preparing it for another round of import. A number of peroxins have been implicated in these steps, though the actual role of some of them play is poorly understood.

Pex8p, the only intra-peroxisomal peroxin

Pex8p is a peripheral membrane protein tightly associated with the inner side of the peroxisomal membrane that has, to date, only been identified in yeast (Liu *et al.*, 1995; Rehling *et al.*, 2000). All Pex8p orthologs contain a C-terminal PTS1 sequence and several Pex8p's also contain a PTS2 sequence. Interestingly, the PTS2 sequences from *S. cerevisiae* and *P. pastoris* are not present at the N-terminus but are, instead, internal (Rehling *et al.*, 2000). Recent data from Zhang *et al*. suggest that *P. pastoris* Pex8p's PTS1 and PTS2 sequences are functional in the targeting of Pex8p to the peroxisome, but are redundant (Zhang *et al.*, 2006). Removal of Pex8p's PTS1 sequence had no effect on its localisation when expressed in a *pex8Δ* strain. However, when Pex8pΔPTS1 was expressed in a *pex8Δpex20Δ* strain, the protein remained cytosolic. Similar results were obtained when the internal PTS2 in Pex8p was mutated and expressed in a *pex8Δpex5Δ* strain.

Pex8p can interact with the PTS1 receptor Pex5p and the PTS2 co-receptor Pex20p (Rehling *et al.*, 2000; Smith and Rachubinski, 2001). The PTS1 sequence in Pex8p is not essential for the interaction with full-length Pex5p (Rehling *et al.*, 2000). This result would appear to be contradictory to the observation that the PTS1 in Pex8p can function as a targeting signal (see above). However, truncated versions of *P. pastoris* Pex5p, when tested in the two-hybrid suggested that Pex5p might bind Pex8p in at least two different ways. An interaction was observed between the TPR domain of Pex5p and Pex8p and this interaction was dependent on the presence of Pex8p's PTS1. On the other hand, Pex8p interacted with the N-terminal region of Pex5p and this interaction did not depend on the presence of Pex8p's PTS1 (Rehling *et al.*, 2000). Why this interaction is insufficient for Pex8p targeting in the absence of a PTS1 is unknown.

The function of Pex8p is likely to involve the dissociation of cargo from the PTS receptors. Indeed, addition of purified Pex8p to a Pex5p-PTS1 complex results in dissociation of the PTS1 protein from Pex5p and the formation of a Pex8p-Pex5p complex (Wang *et al.*, 2003). However, other functions have been attributed to Pex8p. Agne and co-workers reported that Pex8p was essential for the formation of a large complex, named the importomer (Agne *et al.*, 2003). In this model, Pex8p acts as a bridge between two sub-complexes, one consisting of the docking factors Pex13p, Pex14p and Pex17p and the other consisting of the RING finger proteins Pex2p, Pex10p and Pex12p (see below). These two potential functions are not necessarily mutually exclusive and Pex8p may function both as a bridge and a cargo release factor.

The E_2 Pex4p and its membrane anchor Pex22p

Pex4p, also known as Ubc10p, is a member of the ubiquitin-conjugating enzyme (E_2) family of proteins (Crane *et al.*, 1994; van der Klei *et al.*, 1998; Wiebel and Kunau, 1992). Ubiquitination is the covalent linkage of the 7-kDa protein ubiquitin to a lysine residue in a substrate (see below). Pex4p has been identified in yeast and plants but not, surprisingly, in mammals. It is essential for the import of both PTS1 and PTS2 proteins. The expression of Pex4p is dramatically upregulated when cells are shifted to media in which peroxisomes are essential for growth (Crane *et al.*, 1994). This behaviour is more reminiscent of a matrix enzyme, as the majority of the peroxins are not dramatically upregulated under these conditions. Epistasis analysis, performed by Collins and co-workers suggested that Pex4p

acts very late in the import cycle (Collins *et al.*, 2000). This conclusion was largely based on the observation that in *pex4Δ* cells in the yeast *P. pastoris*, Pex5p is much less abundant than in the wild-type strain and that Pex5p can be stabilised by deletion of Pex14p, Pex10p or Pex6p in the *pex4Δ* strain. However, the authors assumed the degradation of Pex5p to be the penultimate step in the import cycle and did not consider a potential role for the peroxins Pex10p and Pex6p in this degradation process (see Addendum).

The presence of an ubiquitin conjugating enzyme at the peroxisomal membrane, which is essential for the import of matrix enzymes, suggests an important role for ubiquitin in import. However, it took more than 10 years after the identification of Pex4p before an ubiquitinated peroxin was discovered. Recent reports have indicated that Pex5p and members of the Pex20p family are all post-translationally modified by ubiquitin (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Leon *et al.*, 2006b; Platta *et al.*, 2004; Purdue and Lazarow, 2001b). However, the ubiquitination of these PTS (co-) receptors does not always depend on the presence of Pex4p. In certain *pex* deletion strains, namely *pex1Δ*, *pex4Δ*, *pex6Δ*, *pex15Δ* and *pex22Δ*, these three PTS (co-) receptors are ubiquitinated in an Ubc4p-dependent manner. It has been suggested that two independent forms of ubiquitination can occur on the PTS (co-) receptors: Pex4p-mediated and Ubc4p-mediated ubiquitination. The different details concerning these independent modifications are discussed later (See Addendum).

Pex4p directly interacts with the peroxisomal membrane protein Pex22p. Pex22p is a transmembrane domain-containing protein required for the import of both PTS1 and PTS2 proteins (Koller *et al.*, 1999). Like Pex4p, Pex22p has been identified in yeasts and plants but not in mammals. In the absence of Pex22p, Pex4p cannot associate with peroxisomes, leading Koller and co-workers to suggest that it may function as a membrane anchor for Pex4p. The interaction between the two proteins was mapped in *P. pastoris*. A region of Pex4p, consisting of the C-terminal 79 amino acids was required for binding. The residues 26-88 in Pex22p, just downstream of the transmembrane domain were required for Pex4p binding.

Current thinking suggests that Pex4p's role in the import recycle is the ubiquitination of the PTS (co-) receptors and this modification acts as a signal for their removal from the peroxisomal membrane to allow another round in import (Platta *et al.*, 2007). However, the mechanistic details of such an action remain speculative.

Pex2p, Pex10p and Pex12p: three RING finger proteins on the peroxisomal membrane

Apart from the docking complex, formed by Pex13p and Pex14p (and Pex17p in yeast), another protein complex involved in the import of PTS1 and PTS2 proteins has been identified: the RING complex. The three proteins that make up this complex, Pex2p, Pex10p and Pex12p all contain two transmembrane domains as well as a so-called RING (really interesting new gene) finger domain at their C-terminus (Albertini *et al.*, 2001; Chang *et al.*, 1999; Fujiki *et al.*, 2000). RING finger domains coordinate zinc ions with a set of eight well-conserved cysteine and histidine residues, with a typical RING domain coordinating two zinc ions (Borden and Freemont, 1996). Zinc binding has only been shown for Pex10p (Kalish *et al.*, 1995). Pex2p and Pex12p, although predicted to contain RING finger domains, lack a complete set of cysteine/histidine residues, suggesting that they may not bind zinc. However, the SMART sequence analysis program (http://smart.embl-heidelberg.de) predicts that both Pex2p and Pex12p contain a U-box, instead of a RING domain. U-box domains are very similar to RING domains but lack the cysteine and/or histidine residues essential for the coordination of zinc (Hatakeyama and Nakayama, 2003). They rely on intra-molecular hydrogen bonds for their structure (see below).

The topology is similar for all three RING proteins, with the N- and C-terminus (containing the RING domain) exposed to the cytosol and a small region between the two transmembrane domains that is exposed to the peroxisomal matrix. The RING domain of Pex10p can interact with the RING domains of both Pex2p and Pex12p, but no interaction between Pex2p and Pex12p was seen. In mammals, both Pex10p and Pex12p RING domains can interact with Pex5p (Albertini *et al.*, 2001; Chang *et al.*, 1999; Okumoto *et al.*, 2000). Using the *in vivo* split-Ub system, it was shown that the C-terminal region of Pex12p (containing the RING domain) interacted with Pex15p, Pex13p and Pex10p. In the same assay, the C-terminal region of Pex10p not only interacted with the same peroxins as Pex12p but also with Pex4p, indicating that several late acting peroxins are in close proximity on the peroxisomal membrane (Eckert and Johnsson, 2003).

RING finger domains can be found in a subset of ubiquitin ligases (E_3's), proteins that catalyse the last step in the ubiquitination cascade (see below). Indeed, the RING domain of Pex10p is highly homologous to that of c-Cbl, a well-characterised E_3 ligase (Joazeiro *et al.*, 1999). U-box domains are also found in E_3 ligases. The observation that

Pex10p and Pex4p can interact in the split-Ub system may indicate that Pex10p is the RING E_3 ligase for Pex4p. However, the presence of all three RING proteins is needed for Pex4p mediated ubiquitination of Pex5p, suggesting that, directly or indirectly, they all play a role in this process (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). Pex10p is also essential for the Ubc4p-dependent ubiquitination of Pex5p and can indeed function as an E_3 ligase in the presence of Ubc4p, indicating that further study is essential before we fully understand the role of the RING proteins in matrix protein import (Kiel *et al.*, 2005a and Chapter 6).

Pex1p, Pex6p and Pex15p, the final peroxins

The import of peroxisomal matrix proteins is dependent on energy, in the form of ATP (Imanaka *et al.*, 1987). The peroxins Pex1p and Pex6p are members of the type II AAA (ATPases associated with various cellular activities) protein family. Type II AAA-proteins are characterised by the presence of one or two ATPase domains, referred to as D1 and D2. These domains each contains a Walker A and Walker B motif, responsible for the binding and hydrolysis of ATP, respectively (Iyer *et al.*, 2004; Ogura and Wilkinson, 2001). Structurally, these domains are well conserved and undergo considerable conformational change upon ATP binding and hydrolysis. These conformational changes are responsible for the effects of these proteins on their substrates (Hanson and Whiteheart, 2005). The large majority of AAA proteins are involved in the unfolding and/or dissociation of proteins and protein complexes. For example, the AAA protein NSF (N-ethylmaleimide-sensitive factor) is involved in the dissociation of SNARE (soluble NSF attachment receptor) complexes, an important step in intracellular membrane fusion (Whiteheart *et al.*, 2001).

Pex1p and Pex6p are able to associate with the peroxisomal membrane via a transmembrane domain-containing protein, known as Pex15p in yeast and Pex26p in mammals (Birschmann *et al.*, 2003; Matsumoto *et al.*, 2003). Pex6p interacts with Pex15p and with Pex1p. So far, Pex6p is the only identified interacting partner for Pex1p (Birschmann *et al.*, 2005; Birschmann *et al.*, 2003). Both Pex1p and Pex6p require the ability to bind and hydrolyse ATP, as mutations in their AAA domains cause phenotypes similar to a complete knock out. Interestingly, ATP hydrolysis is required to disconnect Pex6p from Pex15p in *S. cerevisiae*, leading to the suggestion that Pex6p functions in an ATP-dependent cycle of recruitment to and release from Pex15p. In addition, the

interaction between Pex1p and Pex6p also relies on ATP and it has been suggested that Pex1p and Pex6p can form a large hetero-oligomeric complex, although the exact molecular constitution of such a complex remains unclear (Birschmann *et al.*, 2005; Faber *et al.*, 1998; Kiel *et al.*, 1999).

The function of Pex1p and Pex6p has been a hotly discussed topic. Recent data has suggested that they play an important role in the removal of the PTS (co-) receptors from the peroxisomal membrane, either for recycling back to the cytosol, for another round of import or for the degradation of the protein (Platta *et al.*, 2007; Platta *et al.*, 2005). In this potential function, several parallels can be drawn with another AAA protein: Cdc48p (known as p97 or VCP mammals). Cdc48p is functional in the ERAD (ER associated degradation) pathway, where misfolded proteins are removed from the ER membrane and targeted for degradation. Cdc48p forms a ring-shaped hexameric complex that docks on the ER membrane, via its interaction with VIMP (VCP-interacting membrane protein) and, with the aid of the heterodimeric cofactor Ufd1p/Npl4p, pulls the emerging substrate out of the membrane (Woodman, 2003). Although ubiquitination of the substrate plays an important role in this process, the mechanistic details are not fully understood. Rapoport and co-workers suggested that as Cdc48p removes the misfolded substrate from the membrane, it is ubiquitinated by an ER-associated E_3 ligase, with this modification acting as a degradation signal (Tsai *et al.*, 2002). In contrast, the group of Jentsch reported that Cdc48p recognises ubiquitinated substrates in the ER membrane and, with the aid of Ufd1p/Npl4p, removes them from the membrane. Subsequently, a co-factor called Ufd2p extends the ubiquitin chain on the substrate and the modified substrate is targeted for degradation by the 26S proteasome (Richly *et al.*, 2005)

If the Pex1p-Pex6p complex were to function in a way similar to the Cdc48p/Ufd1p/Npl4p complex, then it seems likely that, as initial reports from Platta and co-workers suggested, the ubiquitination of the PTS (co-) receptors may act as a signal for their Pex1p-Pex6p dependent removal from the peroxisomal membrane (Kragt *et al.*, 2006; Platta *et al.*, 2007).

IX. Ubiquitin and ubiquitination

Around 30 years ago, Goldstein and co-workers identified a small, ubiquitous, well-conserved protein and called it ubiquitin (Goldstein *et al.*, 1975). Since that time, ubiquitin,

true to its name, has been shown to have a hand in numerous cellular processes and the number of ubiquitinated substrates increases on a daily basis. The conjugation of ubiquitin was seen as the equivalent of a death sentence for the substrate, as ubiquitination was thought to be solely for protein degradation via the 26S proteasome (Thrower *et al.*, 2000). At present, however, ubiquitination is known to play important roles in a number of non-proteolytic processes as well as retaining its role in degradation (Johnson, 2002).

The conjugation of the 76 amino acid protein ubiquitin to a substrate is a three step, ATP-requiring process. A separate enzyme controls each of the three steps. Initially, the ubiquitin is activated by the action of the ubiquitin-activating enzyme (E_1). The E_1 forms a thioester bond with its active site cysteine residue and the carboxyl group of glycine 76 in ubiquitin (its extreme C-terminal residue) in an ATP-dependent manner. Next, the E_1 enzyme transfers the ubiquitin to the active site cysteine of an ubiquitin-conjugating enzyme (E_2) by transthiolation. Finally, the attachment of ubiquitin to the substrate occurs in one of two ways. Either the E_2 transfers ubiquitin to the active site cysteine of an E_3, which then attaches the ubiquitin to the substrate or the E_2 enzyme transfers the ubiquitin to the substrate directly, with the E_3 acting as a bridge between the E_2 and the substrate. The three enzymes in the ubiquitin cascade are organised in an hierarchical manner: a single E_1 enzyme, a small but significant number of E_2 enzymes and a large number of E_3's (reviewed in Pickart, 2001).

In the yeast *S. cerevisiae*, the gene UBA1 encodes the E_1. Cells lacking UBA1 are inviable, much the same as cells lacking ubiquitin, reasserting the importance of ubiquitin and ubiquitination in the cell (McGrath *et al.*, 1991). Two similar genes, UBA2 and UBA3 encode the activating enzymes for the ubiquitin-like proteins SUMO (small ubiquitin-like modifier) and Nedd8 (neural precursor cell expressed, developmentally down-regulated), respectively (Johnson *et al.*, 1997; Liakopoulos *et al.*, 1998).

E_2 enzymes are encoded by UBC (ubiquitin conjugating) genes, 13 of which have been identified in the yeast *S. cerevisiae* and over a hundred in mammals. All E_2 enzymes share a conserved UBC domain consisting of around 150 amino acids and including the active site cysteine residue. The 3D structure of a typical E_2 enzyme contains four helices, a four-stranded antiparallel β-sheet and a short 3_{10} helix (VanDemark and Hill, 2002). Many E_2 enzymes consist of a UBC domain alone, for example Ubc4p and Ubc5p in *S. cerevisiae* and UbcH5a in humans (Fig. 3). However, a number of E_2's contain extra sequences, which

may be involved in the enzymes specificity. For example, the specific E_2 for Nedd8 in humans, Ubc12p, contains an N-terminal extension of 12 amino acids, which allows it to interact with the E_1 of Nedd8 but not with that of ubiquitin (Huang *et al.*, 2005). Interestingly, Pex4p (Ubc10p) in *S. cerevisiae* also contains such an N-terminal extension and *P. pastoris* Pex4p has an insertion of 32 amino acids before its UBC domain (Fig. 3). The functions of these extra sequences are not known (Crane *et al.*, 1994).

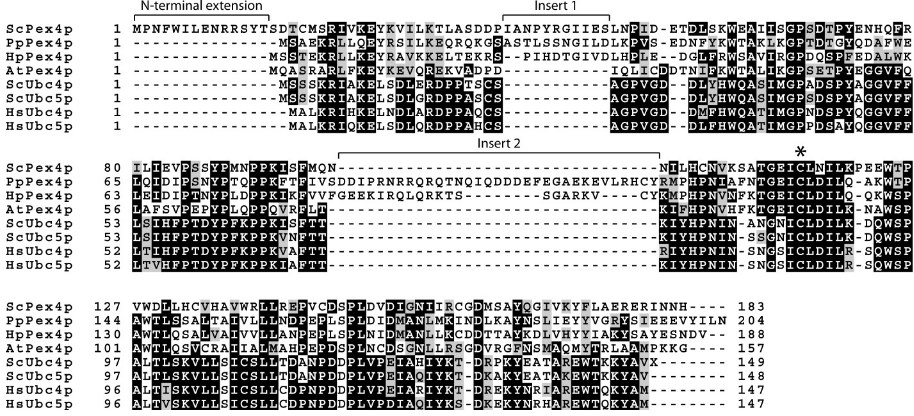


Fig. 3 Sequence alignment of a number of Pex4 proteins from different species and members of the Ubc4/5 family of ubiquitin conjugating enzymes (Ubc's)
* Indicates the position of the active site cysteine residue. The position of an N-terminal extension, specific for *S. cerevisiae* Pex4p as well as two inserts that are only found in certain Pex4p's are indicated. Sc; *Saccharomyces cerevisiae*, Pp; *Pichia pastoris*, Hp; *Hansenula polymorpha*, At; *Arabidopsis thaliana*, Hs; *Homo sapiens*.

E_3 ligases are important for substrate specificity. There are three types of E_3 ligase domains currently known: the HECT (homologous to E6-AP carboxyl terminus) domain, the RING domain (see above) and the U-box domain. HECT domains are around 350 amino acids in length and contain a catalytic cysteine residue near the C-terminal end of the domain that receives the ubiquitin from the E_2 before conjugating it to a substrate (reviewed in Kee and Huibregtse, 2007). RING and U-box E_3's, unlike HECT E_3's, do not conjugate ubiquitin. Instead, they act as a bridge between the ubiquitin-laden E_2 and the substrate, allowing direct transfer of ubiquitin from the E_2 to the substrate (Aravind and Koonin, 2000; Joazeiro and Weissman, 2000) (Fig. 4). RING and U-box domains are closely related and adopt similar folds. One significant difference, however, is the absence of zinc coordinating residues in the U-box domain. Instead, these domains rely on hydrogen bonding for their folding (Ohi *et al.*, 2003). As mentioned above, the peroxins Pex2p and

Pex12p lack a complete set of zinc coordinating residues, which may suggest that they contain a U-box domain, rather than a RING domain. A number of RING E_3's are not capable of catalysing the ubiquitination of a substrate alone, but instead require the presence of one or more co-factors. Some examples of such a multisubunit E_3 include SCF (skip1-Cul-F-box) and CBC/VCB (elongin C-elongin B-Cul2/Von Hippel-Lindau-elongin C/B) (Fang and Weissman, 2004). Similarly, the activity of a fourth enzyme is sometimes required for the ubiquitination of substrates. This enzyme, known as an E_4 elongates the ubiquitin chain (see below) and is essential for the degradation of certain substrates by the proteasome (Koegl *et al.*, 1999).

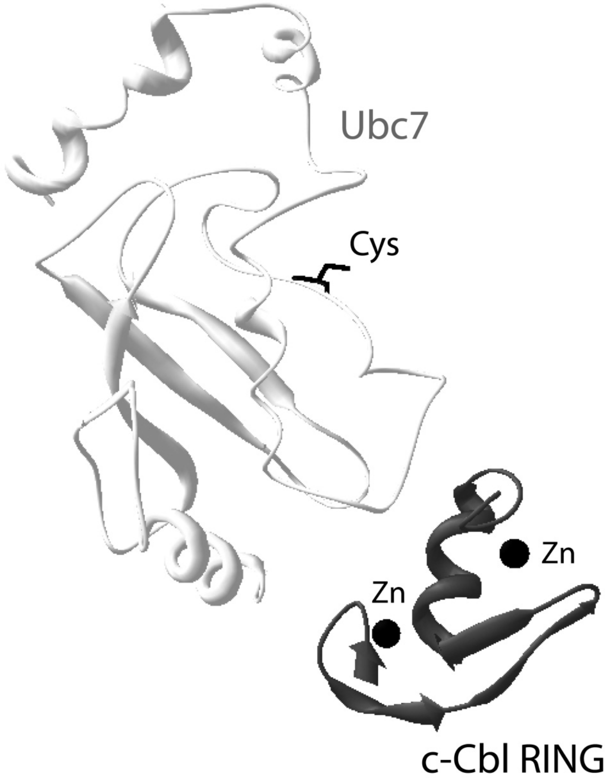


Fig. 4 Crystal structure of the ubiquitin-conjugating enzyme UbcH7p in complex with the RING domain of c-Cbl (PDB accession code 1FBV)
UbcH7p adopts the characteristic E_2 α/β structure and binds to c-Cbl with one end of its elongated structure. The UbcH7p active site cysteine is indicated (**Cys**). The RING domain of c-Cbl consists of a three-stranded β sheet, an α helix and two loops. The coordinating Zinc ions that bind to the RING domain of c-Cbl are also shown (**Zn**).

Attachment of ubiquitin usually occurs via an NH_2 group present in the substrate, forming an iso-peptide bond between the substrate and ubiquitin. Lysine residues are the most common conjugation sites, although the N-terminal NH_2 group can also act as an attachment site (Breitschopf *et al.*, 1998). However, recent data have suggested that non-NH_2 groups present in a substrate are capable of conjugating ubiquitin. Cadwell *et al.* showed that the ubiquitination, resulting in the degradation of a lysine-less substrate was dependent on the presence of a cysteine residue (Cadwell and Coscoy, 2005) and recently it was reported that serine and threonine residues are also targets for ubiquitination (Wang *et al.*, 2007). Conjugation of the C-terminal glycine in ubiquitin to a cysteine residue in the substrate results in the formation of a thioester bond, similar to the bond formed between ubiquitin and the active site cysteine of E_1/E_2/HECT E_3 enzymes. Thioester bonds are susceptible to reducing agents, such as β-mercaptoethanol, whereas iso-peptide linkages (amide bonds) are resistant to these reducing agents. Interestingly, a well-conserved cysteine residue in Pex5p appears to be the conjugation site for Pex4p dependent ubiquitination (Williams *et al.*, 2007, Chapter 5).

The underlying role of ubiquitination within the cell is one of regulation. However, the type of regulation depends very much on the number of ubiquitins attached. For example, the attachment of a chain of four or more ubiquitin moieties (known as poly-ubiquitination) targets the modified substrate for degradation by the 26S proteasome. First, a single ubiquitin moiety is conjugated to the substrate. The lysine at position 48 in the initial ubiquitin then becomes the site for the attachment of another ubiquitin. The process then continues until a chain consisting of four or more ubiquitins is reached. This chain is subsequently recognised by poly-ubiquitin recognition factors present in the proteasome, "reeled in" and the substrate is destroyed (Thrower *et al.*, 2000). In contrast, the attachment of between one to three ubiquitin moieties (known as mono-ubiquitination) is usually for the non-proteolytic regulation of the substrate (reviewed in Hicke, 2001). These ubiquitin chains are often non-lysine 48 linked, with lysine 63 as a common alternative. Indeed, ubiquitin contains seven lysines in total, all of which are capable of conjugating another ubiquitin moiety. The mono-ubiquitination of histones regulates both the expression and silencing of certain genes (Osley, 2004). Mono-ubiquitination acts as an internalisation signal for plasma membrane proteins in the endocytosis pathway, as well as playing a role in the sorting of proteins to multivesicular bodies (MVB's), the regulation of DNA repair

and virus budding (Harty *et al.*, 2001; Hoege *et al.*, 2002; Katzmann *et al.*, 2001; Odorizzi *et al.*, 1998; Reggiori and Pelham, 2001; Schubert *et al.*, 2000). The mono-ubiquitination of a substrate can result in it being recognised by one of the many ubiquitin-binding domains. Currently, a broad range of domains or motifs that recognise and bind ubiquitin are known. The ubiquitin interacting motif (UIM), the ubiquitin associated domain (UBA), the Npl4p zinc-finger (NZF), the zinc-finger ubiquitin-binding domain (ZnF UBP) and the ubiquitin E_2 variant domain (UEV) are a few examples. Most of these domains bind to the region surrounding a large hydrophobic residue, isoleucine 44, in ubiquitin (reviewed in Hurley *et al.*, 2006). The mono-ubiquitination of a substrate can act as an initialising signal that results in a cascade effect. Interestingly, proteins that contain UIM and CUE domains can themselves be ubiquitinated. Their ubiquitin binding domains play crucial roles in this modification, acting as guides for the conjugation of ubiquitin by an E_3 ligase (Woelk *et al.*, 2006).

Another important step in the ubiquitin cycle is deubiquitination. Prior to their degradation by the 26S proteasome, poly-ubiquitinated proteins often have the ubiquitin chain removed by a deubiquitinating enzyme (DUB), allowing the ubiquitin to be recycled, rather that destroyed (Wilkinson, 2000). DUB's hydrolyse the amide bond between glycine 76 in ubiquitin and the NH_2 group in the substrate. Currently, five subclasses of DUB are known: the ubiquitin-specific protease (USP), the ubiquitin C-terminal hydrolase (UCH), the Otubain protease (OTU), the Machado-Joseph disease protease (MJD) and the JAMM (JAB1/MPN/Mov34) protease (Balakirev *et al.*, 2003; Doss-Pepe *et al.*, 2003; Rose and Warms, 1983; Tobias and Varshavsky, 1991; Verma *et al.*, 2002). DUB's are not only involved in the recycling of ubiquitin. Indeed, several examples of DUB enzymes regulating mono-ubiquitination events have been reported. The mono-ubiquitination of PCNA (proliferating cell nuclear antigen) is tightly regulated by the action of the DUB USP1. Mono-ubiquitinated PCNA activates error-prone translesion DNA synthesis (TLS); an event that is only desirable when the replication of UV damaged DNA is necessary. Upon exposure to UV radiation, USP1 is down regulated, allowing the build up of mono-ubiquitinated PCNA and the activation of TLS (Huang *et al.*, 2006).

As we have seen, the different functions associated with mono- and poly-ubiquitination suggests that tight regulation of the modification process is required. One important point is how to know the number of ubiquitin moieties that need to be added to a

given substrate. Several examples are known of substrates that are both mono- and poly-ubiquitinated by different E_3 ligases. However, another set of substrates can be both mono- and poly-ubiquitinated by the same E_3 ligase, raising questions about the timing and specificity of ubiquitination (Pickart, 2001). We are still a long way from understanding all the details about this process.

X. Scope of this thesis

Pex5p is responsible for the import of peroxisomal matrix proteins containing a PTS1. Pex5p achieves this by binding to the newly synthesised PTS1 proteins in the cytosol and directing them to the peroxisome. Once this destination is reached, Pex5p assists in the translocation of the cargo into the peroxisome and is itself recycled back to the cytosol, in order to partake in another round of import. During this complex multi-step process, regulation is likely to play a crucial role. This thesis describes experiments aimed at defining more clearly the role of Pex5p in PTS1 protein import, with the emphasis on how Pex5p's function is regulated.

In Chapter 2, the interaction between Pex5p and the docking protein Pex14p was studied in more detail. In humans, this interaction occurs between W-x-x-x-F/Y motifs present in the N-terminus of Pex5p and the N-terminal region of Pex14p. We discovered that *S. cerevisiae* Pex14p contains two Pex5p binding domains and that the presence of both binding sites is essential for Pex14p function. In addition, we saw that the W-x-x-x-F/Y motifs are not essential for the interaction between Pex5p and the N-terminal 94 amino acids of Pex14p but that a region of Pex5p containing a reverse W-x-x-x-F/Y motif can interact directly with Pex14 1-94.

The docking theme is continued in Chapter 3, in which the SH3 domain containing protein Pex13p is reviewed in detail. Pex13p is a peroxisomal membrane protein essential for the import of both PTS1 and PTS2 proteins. In this chapter, we present an overview of the current knowledge concerning Pex13p, including its interaction with Pex7p, Pex14p and Pex5p and speculate as to what the function of Pex13p in PTS1/2 protein import.

Post-translational modification plays an important role in protein function. Modification of a protein often influences the way it interacts with other proteins. Ubiquitination of a protein, for example, may allow it to bind to an ubiquitin interacting

domain of another protein. In Chapter 4, we identified a putative ubiquitin interacting motif (UIM) present in Pex5p. Although no interaction between Pex5p and ubiqutin could be found, mutation of two conserved residues in this putative UIM resulted in a protein unable to complement the *pex5*Δ phenotype. However, further analysis of this mutant indicated that its inability to complement the *pex5*Δ strain was likely to be caused by a general loss of Pex5p's interacting capabilities, rather than a specific effect, arguing against a role for these residues in ubiquitin binding.

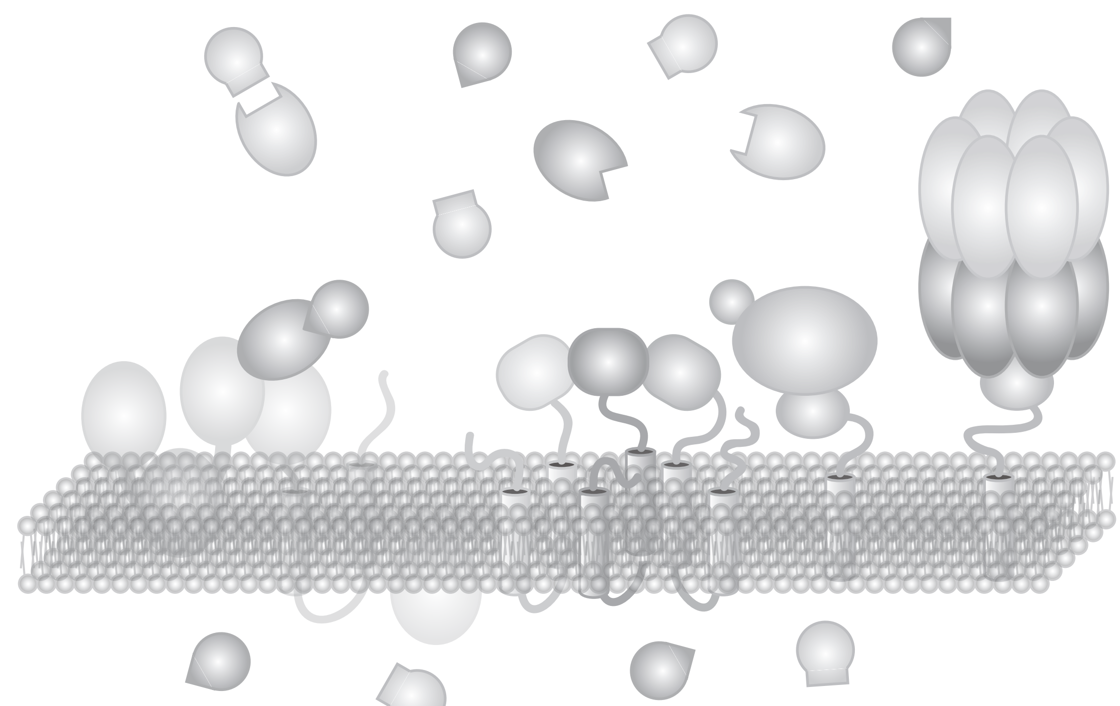
Previous reports suggest an important role for ubiquitin in Pex5p's life cycle. Pex5p is ubiquitinated in both an Ubc4p-dependent and -independent manner. In Chapter 5, we show that these two modifications occur at different sites in Pex5p. While the Ubc4p-dependent ubiquitination of Pex5p occurs on two lysine residues present in the N-terminus, Ubc4p-independent ubiquitination is likely to a target a well-conserved cysteine residue present at position 6 in Pex5p. We also show that the Ubc4p-independent ubiquitination of Pex5p requires the presence of Pex4p and that this form of ubiquitination is essential for Pex5p function, while Ubc4p-dependent ubiquitination is not.

The ubiquitination of Pex5p is again the underlying theme for Chapter 6. Here we continue the analysis of the Pex4p-dependent ubiquitination of Pex5p. The conserved cysteine residue at position 6 in Pex5p was mutated to a lysine (C_6K), resulting in a protein that could partially rescue the *pex5Δ* phenotype. In addition, this mutant showed increased levels in ubiquitination, very likely present on the introduced lysine residue. Also in this chapter, we show that the RING domain of Pex10p and not those of either Pex2p or Pex12p has E_3 ligase activity in *in vitro* ubiquitination assays in combination with UbcH5a, a homologue of *S. cerevisiae* Ubc4p. These observations suggest that Pex10p RING may be the E_3 ligase for Ubc4p-dependent ubiquitination of Pex5p *in vivo*.

Protein quality control in peroxisomes: Ubiquitination of the Peroxisomal targeting signal receptors

Chris Williams and Ben Distel

Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands



Contents

I. Introduction
II. PTS (co-) receptor ubiquitination: conundrum and confusion
III. *S. cerevisiae* Pex18p is degraded in an ubiquitin dependent manner
IV. *S. cerevisiae* Pex5p, two distinct ubiquitination events, two distinct functions
V. The ubiquitination of *P. pastoris* Pex20p: two independent ubiquitination events?
VI. Involvement of the RING proteins in PTS (co-) receptor ubiquitination
VII. AAA protein mediated (co-) receptor recycling: an ubiquitin dependent event?
VIII. Conclusions

I. Introduction

The ability to import folded, co-factor bound and even oligomeric proteins makes peroxisomes unique when compared to subcellular organelles such as mitochondria and chloroplasts (Leon *et al.*, 2006a). Proteins destined for the peroxisomal matrix begin their journey in the cytosol, where they are synthesized on free polyribosomes. Peroxisomal sorting, like the sorting into other sub-cellular components, relies on targeting signals, in this case a peroxisomal targeting signal (PTS). To date, two signals have been identified: the PTS1 and the PTS2. Of these two signals, the PTS1 is by far the most common and is a C-terminal tripeptide related to the canonical S-K-L sequence of firefly luciferase (Gould *et al.*, 1989). The PTS2 however, is less common and is an N-terminal nona-peptide with the consensus (R/K)(L/V/I)X_5(H/Q)(L/A) (Gietl *et al.*, 1994). Proteins equipped with a PTS are recognised and bound in the cytosol by a cycling receptor. The cycling receptor for PTS1 proteins, peroxin 5 (Pex5p) is a bi-domain protein. The N-terminal region is involved in the docking and recycling of the receptor (Costa-Rodrigues *et al.*, 2004; Schafer *et al.*, 2004), while the C-terminal region contains seven tetratricopeptide repeats (TPR's), which specifically interact with the PTS1 sequence (Klein *et al.*, 2001; Van der Leij *et al.*, 1993). Peroxin 7 (Pex7p) on the other hand, is a WD-40 repeat containing protein and the cycling receptor for PTS2 proteins (Marzioch *et al.*, 1994; Rehling *et al.*, 1996). A number of "helper proteins" have been identified for the PTS2 pathway. These co-receptors, known as the Pex20p family and consisting of the yeast proteins Pex18p, Pex20p and Pex21p, assist Pex7p in the import of PTS2 proteins (Einwächter *et al.*, 2001; Purdue *et al.*, 1998). The members of the Pex20p family have a similar domain structure to that of the N-terminal region of Pex5p. Indeed, expression of a chimeric protein consisting of Pex18p fused to the TPR domains of Pex5p can rescue the PTS1 protein import defect of *pex5Δ* cells, indicating that Pex18p fulfils the same function as the N-terminal region of Pex5p (Schafer *et al.*, 2004).

During a typical cycle (Fig. 1), the receptor recognises and binds the PTS protein in the cytosol (I), transports it to the peroxisomal membrane (II), aids in the translocation of the cargo protein into the peroxisomal matrix (III) and recycles to the cytosol for another round of import (IV) (for review see Purdue and Lazarow, 2001a). Around twelve peroxins (the precise number depending on the organism) play important roles in the receptor cycle. A complex, consisting of the peroxisomal membrane proteins (PMP's) Pex13p and Pex14p

(with Pex17p in yeast) is responsible for receptor docking (Albertini *et al.*, 1997; Elgersma *et al.*, 1996a; Erdmann and Blobel, 1996; Gould *et al.*, 1996; Huhse *et al.*, 1998). A separate complex, consisting of the really interesting new gene (RING) domain containing proteins Pex2p, Pex10p and Pex12p as well as the intra-peroxisomal protein Pex8p are involved in the translocation process, but the individual role of each component is not fully understood (Agne *et al.*, 2003). In addition, the ubiquitin conjugating enzyme Pex4p (alternate name Ubc10p), together with its membrane anchor PMP Pex22p may also play a role in this step or, alternatively, may be involved, together with the AAA (ATPase associated with various cellular activities) proteins Pex1p and Pex6p and the PMP Pex15p, in receptor recycling (Collins *et al.*, 2000; Platta *et al.*, 2007; Platta *et al.*, 2005).

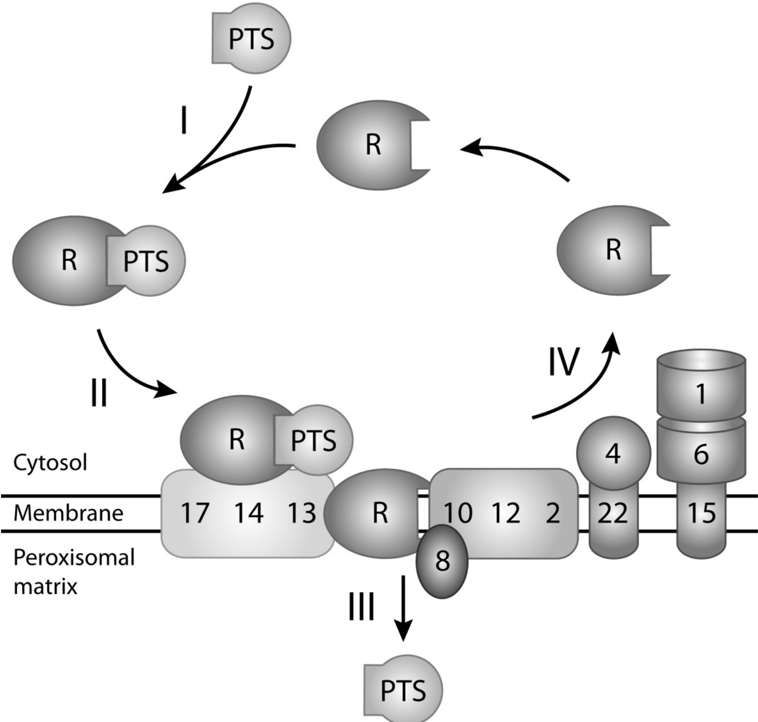


Fig. 1 Model for the import of peroxisomal matrix proteins and receptor recycling
Peroxisomal matrix proteins containing a peroxisomal targeting signal (PTS) are synthesised in the cytosol and recognised by an associated cycling receptor (**I**). The receptor-cargo complex then docks on the peroxisomal membrane (**II**). Next, the PTS cargo is dissociated from the receptor and translocated into the peroxisome (**III**) and the receptor is recycled to the cytosol for another round of import (**IV**). R represents the cycling receptors and numbers indicate specific peroxins. See text for details.

Recent efforts in the peroxisome field are aimed at understanding how such a complex import cycle, involving many different steps, may be regulated. Remarkably, the import of PTS proteins does not require a membrane potential or an energy source, such as ATP. The recycling of the receptors, however, does require ATP hydrolysis and there is compelling evidence that the AAA proteins Pex1p and Pex6p are involved in this ATP-dependent step (Imanaka *et al.*, 1987; Platta *et al.*, 2007; Platta *et al.*, 2005). Are their other potential regulators of the import process? Over the last few years, it has become clear that ubiquitination of peroxins plays a key role in the regulation of peroxisomal protein import.

Ubiquitination is the attachment of ubiquitin to a substrate protein. Ubiquitination plays a key role in a wide range of cellular events, including protein degradation, DNA repair, cell cycle control, multivesicular body sorting and endocytosis, amongst others (Mukhopadhyay and Riezman, 2007). The attachment of ubiquitin is an ATP-dependent process and requires the action of three distinct enzymes. Firstly, the ubiquitin-activating enzyme (E_1) activates ubiquitin in an ATP dependent manner. Next, the activated ubiquitin is transferred to the active site cysteine residue of an ubiquitin-conjugating enzyme (UBC or E_2). Finally, with the aid of an ubiquitin ligase (E_3), ubiquitin is conjugated to the substrate protein, usually to an NH_2-group of a lysine residue. In turn, the ubiquitin itself can become a substrate for ubiquitination, resulting in the formation of an ubiquitin chain. The attachment of a chain consisting of at least four ubiquitin moieties, linked through lysine 48 (K_{48}) of ubiquitin, is often referred to as poly-ubiquitination and targets the modified substrate for 26S proteasome-mediated degradation (Thrower *et al.*, 2000). The attachment of less than four ubiquitin moieties, usually linked via other lysine residues in ubiquitin can be referred to as mono-ubiquitination and is for the non-proteolytic regulation of the modified substrates (Hicke, 2001).

This section discusses the ubiquitination of the PTS (co-) receptors and addresses the role of each of the proteins involved in this process together with the implications of receptor ubiquitination on peroxisomal matrix protein import.

II. PTS (co-) receptor ubiquitination: conundrum and confusion

More than a decade ago, the E_2 enzyme Pex4p (Ubc10p) was identified as an essential factor in PTS-mediated peroxisomal protein import (Wiebel and Kunau, 1992). However, it took many years before the first ubiquitinated peroxin, the PTS2 co-receptor Pex18p from

the yeast *Saccharomyces cerevisiae*, was identified (Purdue and Lazarow, 2001b). Since then, other ubiquitinated peroxins have been identified, for example the PTS1 receptor Pex5p, as well as other members of the Pex18p/Pex20p/Pex21p family of PTS2 co-receptors (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Leon *et al.*, 2006b; Platta *et al.*, 2004). Nevertheless, the situation is far more complex than would, at first, appear. For example, in the absence of the presumed E_2 enzyme Pex4p, the PTS (co-) receptors are still ubiquitinated. Also, the fate of the ubiquitinated peroxins can vary between rapid degradation and accumulation, depending on the type of ubiquitination and the organism under study.

III. *S. cerevisiae* Pex18p is degraded in an ubiquitin dependent manner

The yeast *S. cerevisiae* is unique in that, unlike the other yeast species that only possess one PTS2 co-receptor, Pex20p, it contains two, partially redundant proteins, Pex18p and Pex21p that are required for PTS2 import (Einwächter *et al.*, 2001; Purdue *et al.*, 1998). The expression of Pex18p is dramatically upregulated when cells are grown on oleic acid, a carbon source requiring active peroxisomes for its metabolism. This regulation at the transcriptional level is combined with a rapid turnover of the mature protein that is dependent on ubiquitin and the E_2 enzymes Ubc4p and Ubc5p. It is therefore not surprising that Pex18p is ubiquitinated, via the conjugation of one or two ubiquitin moieties. A direct involvement for Ubc4/5p in this process, however, was not shown (Purdue and Lazarow, 2001b). Interestingly, Pex18p is stabilised in certain peroxin deletion strains, including *pex14Δ*, *pex1Δ* and, significantly, *pex4Δ*. The observation that both Pex4p and Ubc4/5p are required for Pex18p turnover led the authors to propose a model where Pex18p is sequentially ubiquitinated by Pex4p and Ubc4/5p, respectively, ultimately leading to proteasomal degradation of the protein (Lazarow, 2003). Additional data concerning the ubiquitination of Pex18p have, however, not been forthcoming. We are therefore left with several unanswered questions, such as is the turnover of Pex18p essential for its function and crucially, if so why? Further developments in this field are eagerly awaited.

IV. *S. cerevisiae* Pex5p, two distinct ubiquitination events, two distinct functions

Pex5p is poly-ubiquitinated by Ubc4p on two lysine residues

In contrast to Pex18p, rapid turnover of Pex5p is only observed in cells that lack certain late acting peroxins. In the yeast *Pichia pastoris*, cells deleted for either Pex4p or Pex22p show a strong reduction in the levels of Pex5p. This effect is also observed in a *pex1*Δ and *pex6*Δ deletion strain, although to a lesser extent (Collins *et al.*, 2000). Similarly, in mammalian cells where either Pex1p or Pex6p are absent, Pex5p levels are again reduced (Gould *et al.*, 1996). The reasons for the reduction remained unclear until data on *S. cerevisiae* Pex5p showed that the levels of this protein were not severely reduced in cells lacking one of these same peroxins but instead, ubiquitinated forms of the protein accumulated in the cell (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). Later, it was also shown that certain Pex5p mutants are poly-ubiquitinated, in much the same way as in a *pex4Δ* strain (Williams *et al.*, 2007). The ubiquitination pattern in these strains varies and can be divided into two sub-groups. In the first group, consisting of *pex4*Δ, *pex22*Δ and certain Pex5p mutants, two ubiquitinated species were observed, corresponding to the attachment of one or two ubiquitin moieties. In the second group, containing *pex1*Δ, *pex6*Δ and *pex15*Δ three and sometimes four ubiquitinated species were present, consistent with the attachment of 3-4 ubiquitin moieties (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004; Williams *et al.*, 2007). The presence of such an ubiquitin "ladder" is reminiscent of poly-ubiquitination, which as stated above, targets modified substrates for degradation via the 26S proteasome (Thrower *et al.*, 2000). The appearance of a ladder can be explained by the fact that each ubiquitin molecule in the chain is attached separately and not transferred as a chain of ubiquitins in bulk (Deffenbaugh *et al.*, 2003). Ubiquitin chain elongation can be efficiently blocked by mutation of the lysine residue present at position 48 in ubiquitin to an arginine (Ub-K_{48}R). Indeed, the expression of Ub-K_{48}R in *pex4*Δ or *pex1*Δ cells results in a significant decrease in the larger ubiquitinated Pex5p species, indicating that Pex5p is poly-ubiquitinated at a single site, rather than at a number of different sites (Platta *et al.*, 2004). Nevertheless, poly-ubiquitination for 26S proteasome mediated degradation usually requires the attachment of a chain of more than four ubiquitin moieties, which is not often observed in the *S. cerevisiae pex* deletion strains. Does this then mean that the purpose of

poly-ubiquitination of Pex5p in *S. cerevisiae* is not its degradation, or alternatively, that the ubiquitination machinery in this organism is not efficient, leading to relatively short ubiquitin chains and failure to degrade the protein? Conclusions in this area are difficult as most of the data concerning the poly-ubiquitination of Pex5p come from *S. cerevisiae*. One exception, however, is the methylotrophic yeast *Hansenula polymorpha*, where Pex5p abundance is severely reduced in a *pex4*Δ strain, much the same as in *P. pastoris*. Expression of the ubiquitin $K_{48}R$ mutant stabilises Pex5p levels and results in the formation of a higher molecular weight species of Pex5p, corresponding to the addition of a single ubiquitin moiety, suggesting that Pex5p is modified but, due to the inability of the Ub-$K_{48}R$ to form a ubiquitin chain, is no longer degraded (Kiel *et al.*, 2005b). It remains to be seen whether the poly-ubiquitination of Pex5p is also responsible for the apparent degradation observed in mammalian and *P. pastoris* cells lacking one of the late acting peroxins. Data concerning the ubiquitination of the PTS2 co-receptor Pex20p in *P. pastoris* suggests that, at least for this organism, this is indeed the case (see below). Although reduction in Pex5p levels is not observed in *S. cerevisiae*, mutants deficient in proteasome function cause an apparent build up of poly-ubiquitinated Pex5p (Kiel *et al.*, 2005a; Platta *et al.*, 2004). These results, however, must be treated with caution as they were obtained with temperature sensitive mutants grown at the restrictive temperature of 37˚C, conditions where others have observed that in wild type cells, poly-ubiquitination of Pex5p already occurs (Kragt *et al.*, 2006).

It seems rather contradictory that in the absence of the E_2 enzyme Pex4p (Ubc10p), Pex5p is ubiquitinated (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). *S. cerevisiae* contains 13 E_2 enzymes, of which Ubc9p and Ubc12p are specific for the ubiquitin like proteins SUMO (small ubiquitin-related modifier) and Nedd8p (neural precursor cell-expressed developmentally down-regulated), respectively. The question then became which one of these E_2 enzymes was responsible for Pex5p poly-ubiquitination? Deletion of Ubc4p, in combination with either Pex1p or Pex4p results in a reduction in the amount of ubiquitinated Pex5p, when compared to the *pex1*Δ *or pex6Δ* strain alone (Kiel *et al.*, 2005a; Platta *et al.*, 2004). This reduction becomes more severe when Ubc1p, a homologue of Ubc4p, was also deleted in an *ubc4*Δ*/pex4*Δ strain, suggesting that some redundancy exists between Ubc4p and Ubc1p (Kragt *et al.*, 2005b). Ubc4p is an E_2 enzyme involved in the degradation of short-lived and abnormal proteins (Seufert and Jentsch,

1990), and has already been shown to have a hand in peroxisome biogenesis, being involved in the ubiquitination and turnover of Pex18p (Purdue and Lazarow, 2001b). These observations, coupled with the fact that, at least in the *S. cerevisiae pex* deletion strains, ubiquitin chains rarely consist of more than three ubiquitin moieties, led Williams *et al*. (2007) to refer to this form of ubiquitination as Ubc4p dependent-ubiquitination, rather than poly-ubiquitination.

The current data suggest that poly-ubiquitination of Pex5p represents an attempt by the cell to degrade Pex5p. Assuming that, like many other Ubc4p substrates, Pex5p is non-functional under conditions that induce poly-ubiquitination of the protein, the question arises as to why Pex5p has to be removed? Poly-ubiquitination/degradation of Pex5p is observed in the absence of a number of peroxins implicated in Pex5p recycling. This, coupled with the fact that poly-ubiquitinated Pex5p predominantly associates with peroxisomes (Kiel *et al.*, 2005a; Platta *et al.*, 2004) suggests that this modification may be a way to clear the peroxisomal membrane of unwanted Pex5p caused by inefficient recycling. Further supporting evidence is provided by the stability of Pex5p in certain double mutants. As already mentioned, Pex5p levels are severely reduced in a *P. pastoris pex4Δ* or *pex1Δ* strain (Collins *et al.*, 2000; Dodt and Gould, 1996). However, Pex5p becomes stable when elements of the docking complex are also deleted (Collins *et al.*, 2000), indicating that docking at the peroxisomal membrane is a prerequisite for poly-ubiquitination.

The conjugation of ubiquitin usually occurs onto a lysine residue present in the substrate, although the α-NH_2 group can also be used (Ciechanover and Ben-Saadon, 2004; Hershko *et al.*, 1984). Mutation of the lysine residue present at position 21 (K_{21}) in *H. polymorpha* Pex5p stabilises the protein in a *pex4Δ* strain, suggesting that this residue has an important role in the ubiquitin-dependent turn over of the protein (Kiel *et al.*, 2005b). A role for lysine residues in the N-terminal domain of Pex5p was confirmed in *S. cerevisiae*. Replacing the lysines at positions 18 and 24 with arginines ($K_{18/24}R$) blocked Ubc4p-dependent modification of Pex5p (Platta *et al.*, 2007; Williams *et al.*, 2007). This effect was specific for lysine 18 and 24, as mutation of the other 13 lysine residues present in the N-terminal region of Pex5p had no effect on poly-ubiquitination (Williams *et al.*, 2007). Sequence alignments of the N-terminal ~40 amino acids of a number of Pex5p's shows the presence of at least one lysine residue (Fig. 2). Interestingly, a similar analysis of the N-terminal region of the Pex20p family of PTS2 co-receptors also shows the presence of at

least one lysine residue (Fig. 2). In the yeast *P. pastoris*, the poly-ubiquitination of Pex20p depends on lysine 19 (Leon *et al.*, 2006b and see below), confirming the role of N-terminal lysines in PTS (co-) receptor ubiquitination.

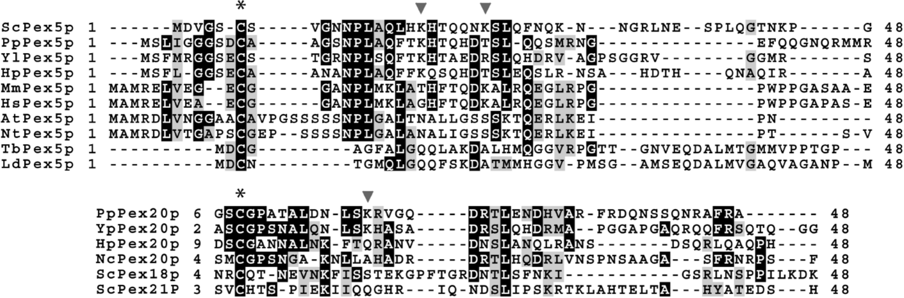


Fig. 2 Sequence alignment showing the N-terminal 48 amino acids of a number of Pex5 (*upper panel*) and Pex18/20 (*lower panel*) proteins from different species
* Indicates the conserved cysteine residue. Arrowheads indicate lysine residues shown to be involved in poly-ubiquitination (for Pex5p, lysines 18 and 24 in *S. cerevisiae* and 21 in *H. polymorpha* and for Pex20p lysine 19 in *P. pastoris*). Sc; *Saccharomyces cerevisiae*, Pp; *Pichia pastoris*, Yl; *Yarrowia lipolytica*, Hp; *Hansenula polymorpha*, Mm; *Mus musculus*, Hs; *Homo sapiens*, At; *Arabidopsis thaliana*, Nt; *Nicotiana tabacum*, Tb; *Trypanosoma brucei*, Ld; *Leishmania donovani*, Nc; *Neurospora crassa*.

It appears likely that ubiquitin-mediated degradation of Pex18p is required for its function (see above). Is this the same for the poly-ubiquitination of Pex5p, since poly-ubiquitinated Pex5p is only observed in mutants? Opinions are somewhat divided on this point. Platta and co-workers (2004) claimed that deletion of Ubc4p together with its homologue Ubc5p causes a growth defect on oleic acid media, suggesting that their presence is important for peroxisome function. However, two other groups reported the exact opposite result, that deletion of these E_2 enzymes has no effect of peroxisome function (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b). It is also known that *ubc4Δubc5Δ* cells are temperature sensitive and grow quite slowly under most conditions (Seufert and Jentsch, 1990), which may account for the observed growth defect on oleic acid. Other evidence against a vital role for Pex5p poly-ubiquitination in PTS import comes from work with Pex5p lysine mutants. In both *H. polymorpha* and *S. cerevisiae*, Pex5p mutants blocked in poly-ubiquitination through mutation of the target lysines can rescue the growth phenotype of a *pex5Δ* strain (Kiel *et al.*, 2005b; Williams *et al.*, 2007). Although these results are quite convincing, they do not completely rule out an important role for poly-ubiquitination in Pex5p function. It is conceivable that, due to the small amount of Pex5p that needs to be

degraded at any one time, no effect on growth on oleic acid is observed when poly-ubiquitination is blocked. However, this mechanism may become important under stress conditions such as heat shock. Indeed, *pex5Δ* cells expressing Pex5p mutants blocked in poly-ubiquitination exhibit growth retardation when grown on oleic acid for long periods of time (our unpublished results). The conserved nature of the N-terminal lysine residues support the notion that poly-ubiquitination is important as some stage of cellular life.

Pex5p is mono-ubiquitinated by Pex4p on a cysteine residue

Interestingly, the poly-ubiquitination of Pex5p is not the only ubiquitin related event associated with this protein. Kragt *et al*. (2005) demonstrated that Pex5p is post-translationally modified in wild-type cells. Immunoprecipitation analysis with cells overexpressing a myc-tagged form of ubiquitin revealed the presence of a single, discreet, Pex5p band around 20 kDa heavier than unmodified Pex5p that specifically reacted with myc tag antibodies. A molecular weight increase of around 20 kDa is consistent with the attachment of two ubiquitin moieties (inclusive two myc tags), indicating that Pex5p is mono-ubiquitinated. Expression of a lysine-less form of myc-tagged ubiquitin (Ub-K_0), which in a similar way to the Ub-K_{48}R mutant cannot undergo chain elongation, did not reduce the levels of mono-ubiquitinated Pex5p or result in the formation of a faster migrating band, leading the authors to suggest that Pex5p is mono-ubiquitinated at two different sites (Kragt *et al.*, 2005b). However, these data were obtained with cells where wild-type ubiquitin was still present, which if conjugated to Pex5p first, would be able to take part in chain elongation. In addition, the myc tag itself contains a lysine residue, which may also act as a conjugation site.

Experiments using mutants deficient in proteasomal and vacuolar degradation indicated that mono-ubiquitinated Pex5p is not a breakdown intermediate of either system. In addition, this modification does not require the E_2 enzymes Ubc1p or Ubc4p (Kragt *et al.*, 2005b). At the time, it was suggested that Pex4p is responsible for the mono-ubiquitination of Pex5p but, due to the observed Ubc4p-dependent poly-ubiquitination in *pex4Δ* cells, this was difficult to prove (Kragt *et al.*, 2005b). However, the involvement of Pex4p in mono-ubiquitination was confirmed using lysine mutant versions of Pex5p that can not be poly-ubiquitinated by Ubc4p. Such a mutant is still mono-ubiquitinated when introduced into a Pex5p wild type or *pex6Δ* strain, but the mono-ubiquitination is lost in a

pex4Δ strain (Williams *et al.*, 2007). In addition, mono-ubiquitination of Pex5p was seen in a cell free ubiquitination assay using Pex4p as E_2 (Platta *et al.*, 2007).

As discussed above, poly-ubiquitination of Pex5p is Ubc4p-dependent and targets conserved lysines present in the N-terminus (Kiel *et al.*, 2005b; Platta *et al.*, 2007; Williams *et al.*, 2007). These two lysine residues however, are not involved in the mono-ubiquitination of Pex5p (Platta *et al.*, 2007; Williams *et al.*, 2007), nor are other lysine residues present in the N-terminal half of Pex5p (Williams *et al.*, 2007). Furthermore, conjugation of ubiquitin to the α-NH2 group of Pex5p was also ruled out, as the α-NH_2 group is blocked by acetylation (Williams *et al.*, 2007). The ubiquitination of non-NH_2 groups by viral E_3 ligases, where cysteine, serine and threonine residues are potential conjugation sites has recently become a hot topic (Cadwell and Coscoy, 2005; Wang *et al.*, 2007). Sequence alignments of Pex5p show the presence of a well-conserved cysteine residue in the N-terminal ~40 amino acids (Williams *et al.*, 2007 and Fig. 2). Such a residue is also present in the N-terminal domain of the Pex20p family of proteins (Leon *et al.*, 2006b and Fig. 2). Mutation of this cysteine residue renders Pex5p non-functional and causes the protein to be poly-ubiquitinated. Furthermore, when the cysteine mutant is combined with the poly-ubiquitination disturbing lysine mutations, Pex5p is no longer mono-ubiquitinated in wild type cells. Further evidence for the cysteine as conjugation site came from experiments using reducing agents. Cysteine residues form thioester bonds with the C-terminus of ubiquitin, whereas lysine residues form amide (iso-peptide) bonds. Thioester bonds exhibit different chemical properties to amide bonds, one of which is their susceptibility to the reducing agent β-mercaptoethanol (β-me). Mono-ubiquitinated Pex5p is susceptible to β-me, whereas poly-ubiquitinated Pex5p is not (Williams *et al.*, 2007). Final proof of the role of the cysteine residue in mono-ubiquitination, such as mass spectrometry, so far is lacking. However, recent data showing the importance of the cysteine residue in mammalian Pex5p, as well as *P. pastoris* Pex20p recycling indicate the crucial nature of this residue in receptor function (Carvalho *et al.*, 2007; Leon and Subramani, 2007).

V. The ubiquitination of *P. pastoris* Pex20p: two independent ubiquitination events?

As with the ubiquitination of Pex5p, many of the data concerning Pex20p ubiquitination comes from a single organism, in this case *P. pastoris*. Many similarities can be drawn between the behaviour of Pex20p and that already mentioned for Pex5p from the same organism (see above). In the absence of Pex1p, Pex4p or Pex6p, levels of Pex20p are severely reduced when cells are grown overnight on oleic acid medium (Leon *et al.*, 2006b). Mutation of the lysine at position 19 in Pex20p to an arginine ($K_{19}R$) stabilises the proteins in these deletion strains, analogous to the $K_{21}R$ mutant in *H. polymorpha* Pex5p (Kiel *et al.*, 2005b). If cells lacking Pex1p, Pex4p or Pex6p are exposed to oleic acid medium for a 6 h period, Pex20p levels are not reduced. Instead, the formation of a ladder of higher molecular weight species is observed. Significantly, these higher molecular weight species are not observed in the $K_{19}R$ mutant, suggesting poly-ubiquitination of the protein takes place via lysine 19 (Leon *et al.*, 2006b). A conserved lysine residue is present in the N-terminal ~40 amino acids of all proteins from the Pex20p family and, as already discussed, the N-terminal region of Pex5p (Leon *et al.*, 2006b and Fig. 2). The same authors also expressed the Ub-$K_{48}R$ mutant in *pex4Δ* cells and, again like the HpPex5p situation, observed a build up of ubiquitinated Pex20p. Poly-ubiquitinated Pex20p is also predominantly membrane associated, like its Pex5p counterparts. In addition, expression of the Pex20p $K_{19}R$ mutant in one of the above mentioned deletion strains not only blocks degradation, it also results in a build up of the protein on the peroxisomal membrane, confirming the role already suggested for poly-ubiquitination, *i.e.* the removal of non-functional/unwanted PTS receptors from the peroxisomal membrane.

The story of PpPex20p does not end here. The sequence alignments of the Pex20p family of proteins show that besides the conserved lysine residue, also a conserved cysteine residue at position 8 (Fig. 2). Although mono-ubiquitination of Pex20p has never been shown, a number of interesting observations concerning this cysteine residue in Pex20p are worth mentioning. Unlike the ScPex5p situation, mutation of this cysteine in Pex20p does not render the protein non-functional, although the protein is degraded over time. However, when this mutation is combined with the $K_{19}R$ mutant, cells can no longer grow on oleic acid medium. Co-expression of Pex20p C_8S with the Ub-$K_{48}R$ mutant, allowing ubiquitin

conjugation to a substrate but interfering with ubiquitin chain formation, results in ubiquitinated Pex20p, suggesting that, in the absence of a cysteine residue, Pex20p is poly-ubiquitinated. Mutation of both Cys_8 and Lys_{19} in Pex20p also results in a non-recycling phenotype (Leon and Subramani, 2007).

Although direct proof for mono-ubiquitination is still lacking, it seems quite clear that the PTS2 co-receptor Pex20p, much like its PTS1 counterpart Pex5p, can undergo two distinct ubiquitination events, one involved in the recycling of the protein and the other in degradation of non-functional proteins. An obvious difference between Pex20p and Pex5p is that mutation of the cysteine in Pex5p results in a growth defect on oleic acid, whereas the same mutation in Pex20p can partially rescue a *pex20Δ* strain. This may stem from the number of cargo proteins that are handled by the PTS2 co-receptors. So far, only one PTS2 protein has been identified in the yeasts *P. pastoris* and *S. cerevisiae*, thiolase. The number of PTS1 proteins, on the other hand, is considerable. Therefore, sufficient thiolase may be imported if Pex20p only undergoes one round of import and then, due to a lack of recycling, is degraded, the subsequent import being performed by newly synthesised Pex20p. It is conceivable that, due to the large number of PTS1 proteins, each Pex5p molecule must perform multiple rounds of import and that a block in recycling limits the amount of PTS1 proteins imported. On the other hand, a lack of data concerning the ubiquitination of *P. pastoris* Pex5p makes it difficult to predict if such a cysteine mutation would result a non-functional protein, as in *S. cerevisiae*.

VI. Involvement of the RING proteins in PTS (co-) receptor ubiquitination

The third and final step of the ubiquitination pathway is the attachment of ubiquitin to the substrate. This task is performed by an E_3 ligase enzyme. Currently, two main groups of E_3 ligases have been identified: the HECT (homologous to E6-AP C-terminus) E_3's and the RING (really interesting new gene) E_3's (Pickart, 2001). RING E_3's contain a zinc-binding RING domain that acts as a bridge between the conjugating E_2 enzyme and the substrate, allowing transfer of ubiquitin from the E_2 to the substrate to occur (Jackson *et al.*, 2000; Joazeiro *et al.*, 1999). Three RING domain containing proteins are important in PTS import: Pex2p, Pex10p and Pex12p (Albertini *et al.*, 2001; Chang *et al.*, 1999; Fujiki *et al.*,

2000). However, zinc binding has only been shown for Pex10p and not for the other RING proteins (Kalish *et al.*, 1995). In addition, Pex2p and Pex12p lack a complete set of cysteine/histidine residues necessary for zinc coordination. Analysis using SMART (http://smart.embl-heidelberg.de) predicts that both Pex2p and Pex12p contain a U-box, instead of a RING domain. U-box domain-containing proteins represent a subgroup of the RING E_3 ligase family. Although they lack zinc-coordinating residues, their overall fold is very similar to that of the RING domain (Hatakeyama and Nakayama, 2003).

The RING proteins are present as a complex at the peroxisomal membrane and RING domain of Pex10p can interact with the RING/U-box domains of both Pex2p and Pex12p (Agne *et al.*, 2003). In mammals, both Pex10p and Pex12p RING domains can interact with Pex5p (Albertini *et al.*, 2001; Chang *et al.*, 1999; Okumoto *et al.*, 2000). Epistasis analysis places all three RING proteins downstream of the docking complex, suggesting that Pex5p is handed over from the docking complex to the RING complex (Chang *et al.*, 1999; Collins *et al.*, 2000).

The presence of three potential E_3 ligase enzymes on the peroxisomal membrane raises many questions concerning their individual roles in the ubiquitination of the PTS (co-) receptors. Indeed, all three proteins are required for both the poly- and mono-ubiquitination of Pex5p (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). The RING domain of Pex10p shares considerable homology with that of c-Cbl, a well-characterised RING E_3 ligase (Joazeiro *et al.*, 1999) and can act as an E_3 ligase with UbcH5a, a homologue of *S. cerevisiae* Ubc4p (Chapter 6 of this thesis). Interestingly, Pex10p can interact with Pex4p in the split-ubiquitin system (Eckert and Johnsson, 2003), which may suggest that Pex10p acts as the E_3 for Pex4p *and* Ubc4p. This theory does not explain the role of the other RING proteins in mono-ubiquitination, but does explain the need for Pex10p in Pex5p poly-ubiquitination (Kiel *et al.*, 2005a; Platta *et al.*, 2004). A number of RING E_3's cannot attach ubiquitin to a substrate alone and act in collaboration with other proteins. These complexes are known as multisubunit E_3's and include the SCF (skip1-Cul-F-box) and the CBC/VCB (elongin C-elongin B-Cul2/Von Hippel-Lindau-elongin C/B) ligases (Fang and Weissman, 2004). A similar mechanism could be envisaged for the RING complex on the peroxisomal membrane, where Pex10p would function as E_3 ligase while the other two RING proteins, Pex2p and Pex12p, would have a role in binding of the substrate.

VII. AAA protein mediated (co-) receptor recycling: an ubiquitin dependent event?

The recycling of the receptors from the peroxisomal membrane requires ATP as an energy source (Gouveia *et al.*, 2003). So far, only two proteins essential for PTS import with the ability to bind and hydrolyse ATP have been identified: Pex1p and Pex6p. These AAA (ATPase associated with various cellular activities) proteins are capable of forming a high molecular weight complex that can cycle between the cytosol and the peroxisomal membrane (Birschmann *et al.*, 2005; Faber *et al.*, 1998; Kiel *et al.*, 1999). Membrane association is achieved through Pex6p's interaction with Pex15p in yeast (Birschmann *et al.*, 2003) and Pex26p is mammals (Matsumoto *et al.*, 2003). ATP plays a role in the Pex15p-Pex6p interaction, its hydrolysis being required for dissociation of the two proteins (Birschmann *et al.*, 2003).

AAA-proteins are involved in a variety of cellular processes. They are often employed as protein complex dissociation factors. Indeed, the AAA protein Cdc48p (p97 or VCP in mammals) functions in, amongst other things, the retrotranslocation step in ER-associated degradation (ERAD), where misfolded proteins are targeted for degradation. Several reports suggest an important role for ubiquitin in this process, possibly acting as the signal for Cdc48p-mediated removal (Bays and Hampton, 2002; Richly *et al.*, 2005; Tsai *et al.*, 2002). Could Pex1p and Pex6p be involved in a similar process in peroxisomes? A role for the AAA-proteins in recycling has been proposed on many occasions. This was largely based on genetic analysis, showing their involvement in a late step in the import process (Collins *et al.*, 2000). Recent data however, gives us a clear indication that, at least for Pex5p, the AAA-proteins perform such a function. *In vitro* export assays using membrane fractions have shown that the addition of a purified complex consisting of Pex1p and Pex6p is sufficient to remove Pex5p from the peroxisomal membrane (Platta *et al.*, 2005). These observations were expanded upon to include a role for ubiquitin in the recycling process. Results from Platta *et al*. (2007) indicate that either mono- or poly ubiquitination of Pex5p are required to remove the protein from the peroxisomal membrane. In the absence of one of these pathways, the other is capable of taking over. For example, AAA-protein dependent Pex5p recycling is observed with the Pex5p $K_{18/24}R$ mutant and in a *pex4Δ* strain. Only when both modifications are blocked, by combining the Pex5p $K_{18/24}R$ mutant

with the *pex4Δ* strain, does Pex5p fail to recycle (Platta *et al.*, 2007). Also, the addition of recombinant Pex4p, but not the active site cysteine mutant Pex4p $C_{115}S$, to the Pex5p $K_{18/24}R$/*pex4Δ* system can stimulate Pex5p recycling. Therefore, it seems likely that the AAA-proteins play a major role in the ubiquitin-dependent recycling of Pex5p. The mechanistic details of such a process however are not yet known. An interaction between ubiquitinated Pex5p and one or both of the AAA-proteins may be expected. Further developments in this field are eagerly awaited.

VIII. Conclusions

It is clear that two independent ubiquitination events can occur in the Pex5p cycle. One, the mono-ubiquitination of Pex5p targets a cysteine residue, is dependent on the E_2 enzyme Pex4p and is likely to regulate the function of Pex5p, possibly the recycling of the protein to the cytosol. On the other hand, the poly-ubiquitination of Pex5p on lysine residues by the E_2 enzyme Ubc4p is implicated in quality control, resulting in the degradation of non-functional protein stuck at the peroxisomal membrane. Although only one such event, ubiquitin mediated degradation has been shown for the PTS2 co-receptors, the evidence heavily suggests the presence of the other. It is noteworthy that these two distinct events are both regulated by the same molecule, ubiquitin and that such diversity can be achieved through the conjugation site as well the action of different enzymes. Based on the results presented, we can draw up a hypothetical model concerning the role of ubiquitin in receptor function (Fig. 3). After completion of the docking and PTS translocation steps, the membrane associated PTS (co-) receptor is mono-ubiquitinated by Pex4p, allowing recognition by the AAA-proteins Pex1p and Pex6p. The PTS (co-) receptor is then pulled out of the membrane and the ubiquitin is removed, possibly by the action of a deubiquitinating enzyme. The PTS (co-) receptor is then free to partake in another round of PTS protein import. In the situation that no efficient recycling is possible, due to the absence of one of the peroxins involved in recycling or in certain PTS (co-) receptor mutants, PTS (co-) receptor poly-ubiquitination, mediated by Ubc4p is observed. This modified form is then removed from the membrane in an as yet unknown way and destroyed by the 26S proteasome, effectively removing the blockage.

Questions that still need answering concerning the role ubiquitin plays in PTS protein import include the identification of the true E_3 enzyme for poly- and mono-

ubiquitination. The possibility that they are one and the same is very real and that the regulation comes from other factors, the E_2 enzyme for example. Alternatively, one RING protein may be the E_3 for Pex4p and another for Ubc4p. Analysis of the different E_2 and E_3 enzymes in *in vitro* ubiquitination assays should resolve this issue. The confirmation that mono-ubiquitination on a cysteine residue also occurs in the other members of the Pex5p and Pex20p family would add further weight to the results obtained with *S. cerevisiae* Pex5p. We are clearly still a long way from fully understanding the important role ubiquitin plays in the import of peroxisomal matrix proteins.

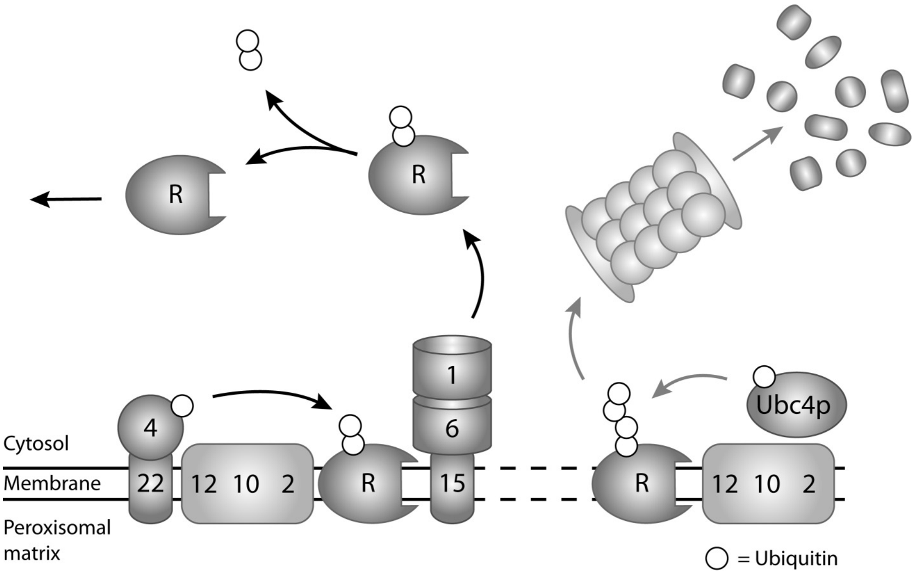


Fig. 3 Hypothetical model for PTS (co-) receptor ubiquitination showing ubiquitin-dependent recycling and degradation

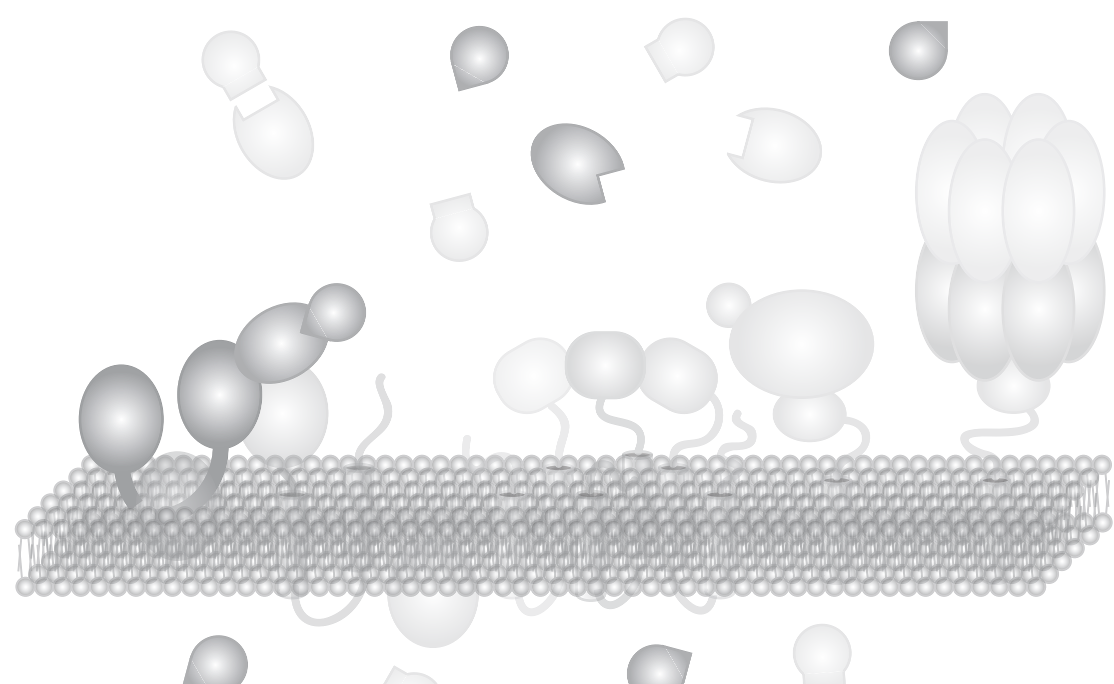
Once the PTS (co-) receptor has released its cargo into the peroxisomal matrix, it is recycled to the cytosol. The E_2 enzyme Pex4p, with the aid of Pex2p, Pex10p and Pex12p acting as RING E_3 ligases, mono-ubiquitinates the PTS (co-) receptor on the conserved cysteine residue. The modified PTS (co-) receptor then becomes a substrate for the AAA-protein complex consisting of Pex1p and Pex6p and is pulled out of the peroxisomal membrane, a step that requires ATP hydrolysis. Ubiquitin is then removed from the PTS (co-) receptor, allowing it to partake in another round of import. In the absence of functional recycling machinery, the PTS (co-) receptor is poly-ubiquitinated by the E_2 enzyme Ubc4p, with Pex10p possibly acting as an E_3 ligase. This modification targets the PTS (co-) receptor for degradation by the 26S proteasome. R represents the cycling (co-) receptors and numbers indicate specific peroxins. See text for details.

2

Saccharomyces cerevisiae Pex14p contains two independent Pex5p binding sites, which are both essential for PTS1 protein import

Chris Williams, Marlene van den Berg and Ben Distel

Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands

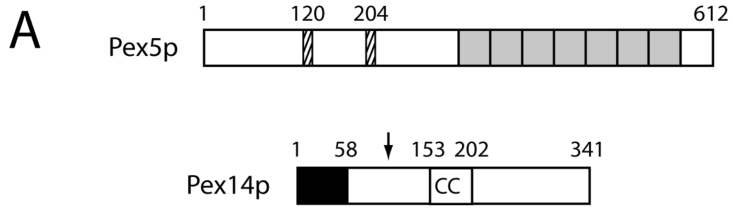


Abstract

Pex14p is a peroxisomal membrane-associated protein involved in docking of both Pex5p and Pex7p to the peroxisomal membrane. Previous studies have shown that, in humans, the N-terminal region of Pex14p interacts with W-x-x-x-F/Y motifs in Pex5p. Here we report that *Saccharomyces cerevisia*e Pex14p contains two independent Pex5p binding sites, one in the N- and one in the C-terminus. Using deletion analysis we show that, *in vivo*, both of these interactions are needed for PTS1 import. Furthermore, we show that the characterised W-x-x-x-F/Y motifs of Pex5p are not essential for binding to the N-terminus of Pex14p but do play a role in the interaction with the Pex14 C-terminus. Thus, the data suggest that the mechanism of the Pex14p-Pex5p interaction in yeast is different from that previously reported for humans.

Introduction

Peroxisomes are single membrane bound organelles that are ubiquitous in eukaryotic cells. Their function can vary between different organisms and different cell types, but two well-conserved functions are the β-oxidation of fatty acids and hydrogen peroxide detoxification (Purdue and Lazarow, 2001). Proteins destined for the peroxisomal matrix are synthesised on free ribosomes in the cytosol (Lazarow and Fujiki, 1985) and post translationally imported via the use of one of the characterised peroxisomal targeting signals; type1 (PTS1) or type2 (PTS2). The PTS1, consisting of the extreme C-terminal tri-peptide sequence (S/C/A)(K/R/H)(L/M) is by far the most common signal (Gould *et al.*, 1989; Swinkels *et al.*, 1992). The less abundant PTS2 is an N-terminal nona-peptide with the consensus (R/K)(L/V/I)X_5(H/Q)(L/A) (Gietl *et al.*, 1994; Glover *et al.*, 1994). Both PTS1 and PTS2 proteins are recognised in the cytosol by their associated cycling receptors, Pex5p (Dodt and Gould, 1996; Van der Leij *et al.*, 1993; Wiemer *et al.*, 1995) and Pex7p (Marzioch *et al.*, 1994; Zhang and Lazarow, 1995), respectively. Arguably the most studied Peroxin, Pex5p, is a bi-domain protein (Fig. 1 A). The highly conserved C-terminal domain contains 7 tetratricopeptide repeats (TPR's) shown to be sufficient and essential for PTS1 protein binding (Gatto *et al.*, 2000; Klein *et al.*, 2001). The N-terminal domain shows little conservation, with the notable exception of multiple W-x-x-x-F/Y motifs that have been identified in all Pex5p orthologs and are thought to be involved in binding to the peroxisomal membrane-associated protein Pex14p (Choe *et al.*, 2003; Saidowsky *et al.*, 2001). This protein is believed to be the point of convergence for both PTS1 and PTS2 import as it can interact with proteins associated with both pathways (Albertini *et al.*, 1997; Stein *et al.*, 2002). Pex14p has been identified in numerous organisms and contains a conserved N-terminus (Choe *et al.*, 2003), shown to bind the W-x-x-x-F/Y motifs of Pex5p in humans (Saidowsky *et al.*, 2001), a P-x-x-P motif, responsible for the interaction with Pex13p (Girzalsky *et al.*, 1999) and a coiled coil region, thought to be involved in dimerisation (Oliveira *et al.*, 2002) (Fig. 1 A). Pex14p also contains a poorly conserved C-terminal region, whose function has not yet been defined. It is believed that Pex14p, together with Pex13p (and Pex17p in yeast) constitutes the initial docking complex for both Pex5p and Pex7p (Agne *et al.*, 2003).



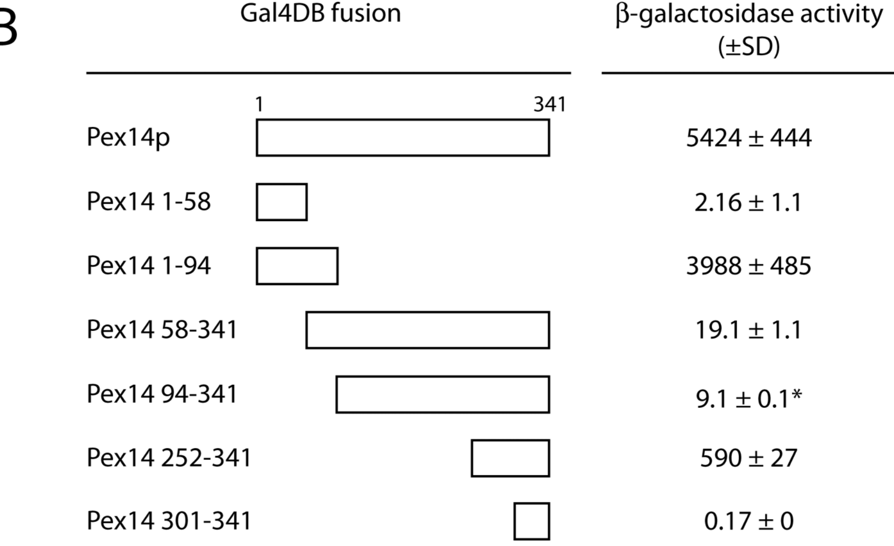


Fig. 1 Two-hybrid analysis of the ScPex14p-ScPex5p interaction
A. Domain structures of *S. cerevisiae* Pex14p and Pex5p, showing for Pex5p, the 7 TPR repeats (*grey boxes*) and the two W-x-x-x-F/Y motifs (*hatched boxes*). For Pex14p, the conserved N-terminus (*black box*) and the P-x-x-P motif (*arrow*) are indicated. A coiled coil region (**CC**), as predicted by SMART (http://smart.embl-heidelberg.de/) is also shown.
B. Constructs expressing Gal4DB fused to WT and truncated forms of Pex14p were co-transformed with Gal4AD Pex5p to the yeast two-hybrid strain PCY2. Activity of the reporter β-galactosidase (defined as absorbance at 420 nm per mg of protein per min) was used to determine the strength of interactions. Values correspond to the mean ± SD of four independent measurements, except * which was taken from two measurements. All Gal4DB Pex14 constructs were tested against the Gal4AD domain alone and showed no activity. Likewise, Gal4AD Pex5p showed no interaction with Gal4DB (not shown).

In earlier work from our group, we reported that mutation of the two W-x-x-x-F/Y motifs in *S. cerevisia*e Pex5p (ScPex5p) did not abolish the interaction with full length ScPex14p (Bottger *et al.*, 2000). This would suggest that the mechanism by which ScPex14p binds to ScPex5p is different from that previously seen in other species (Choe *et al.*, 2003; Saidowsky *et al.*, 2001). In this study, we present an in depth analysis of the

Pex14p-Pex5p interaction in *S. cerevisia*e. We demonstrate that ScPex14p contains two independent ScPex5p binding sites and that both of these sites are essential for PTS1 import. In addition, we show that the W-x-x-x-F/Y motifs are not essential for binding to the N-terminus of Pex14p but that a region of Pex5p containing a reverse W-x-x-x-F/Y motif may be involved.

Materials and Methods

Strains and culture conditions

The yeast strains used in this study are as follows: *S. cerevisiae* BJ1991 *pex14Δ (MATα, pex14::LEU2, leu2, trp1, ura3-251, prb1-1122, pep4-3, gal2)* and PCY2 *(MATα, Δgal4, Δgal80, URA3::GAL1-lacZ, lys2-801, his3-Δ200, trp1-Δ63, leu2, ade2-101)*. Yeast transformations were performed as described in (Van der Leij *et al.*, 1993). Transformants were grown on minimal media containing 0.67% yeast nitrogen base (Difco), 2% glucose and amino acids (20 µg/ml) as required. The *E. coli* strain DH5α *(recA, hsdR, supE, endA, gyrA96, thi-a, relA1, lacZ)* was used for all plasmid isolations. The *E. coli* strains BL21 DE3 (B, F^-, *dcm, ompT, hsdS* ($r_B^- m_B^-$), *gal* λ(DE3)) and SG13009 (F^-, *his, pyrD, Δlon-100, rpsL)* were used for the expression of His_6-GST and His_6 fusion proteins, respectively. Cells transformed with bacterial expression constructs were grown at 37°C to an OD_{600} of 0.4 in 1 litre LB medium supplemented with 2% glucose, 50 mM Tris pH 7.4 and antibiotics as required. Cells were then transferred to 21°C and grown to an OD_{600} of 0.7 and induced with 0.25 mM IPTG (Invitrogen) for 6 h. Cells were harvested by centrifugation for 20 min at 9,000 x g, washed with water and stored at -20°C.

Cloning procedures

Details of primers can be seen in Table I. The following plasmids have been described previously: pAN92, a fusion of the Gal4 trans-activating domain (Gal4AD) and Pex5p 239-300 (Klein *et al.*, 2002); pAN4, a fusion of Gal4AD and Pex5p (Klein *et al.*, 2001); pGB47, a fusion of the Gal4 DNA binding domain (Gal4DB) and Pex14p (Bottger *et al.*, 2000); Gal4AD Pex5 W_{204}A, a fusion of Gal4AD and Pex5 W_{204}A (Bottger *et al.*, 2000). Plasmids expressing fusions of Gal4DB and Pex14p were made as follows; polymerase chain reaction (PCR) was performed on pGB47 using the primer combinations PR100 and PR104

(1-58, pCW33), PR122 and PR127 (1-94, pCW56), PR101 and PR105 (94-341, pCW34), PR102 and PR105 (252-341, pCW35) and PR103 and PR105 (301-341, pCW36), the products were digested with *Eco*RI and *Spe*I and ligated into the *Eco*RI-*Spe*I sites of pPC86 (Chevray and Nathans, 1992). The resulting vectors were digested with *Sma*I and *Spe*I and ligated into the *Sma*I-*Spe*I sites of pPC97 (Chevray and Nathans, 1992). To produce the fusion of Gal4DB and Pex14 59-341 (pGB36) the *Eco*RI-*Spe*I fragment from pGB29 (see below) was ligated into *Eco*RI-*Spe*I digested pPC86. The resulting product was digested with *Sal*I and *Not*I and the fragment was ligated into *Sal*I-*Not*I digested pPC97. To produce the fusion of Gal4AD and Pex5p 239-612 (pCW60), PCR was performed on pAN4 using the primers PR66 and PR184 and the resulting product was digested with *EcoR*I and *Spe*I and ligated into *Eco*RI-*Spe*I digested pPC86. Gal4AD fusions of Pex5 $W_{120}A$ and $W_{120/204}A$ were produced using the QuikChange^{TM} site directed mutagenesis kit (Stratagene) and primers $W_{120}A$ and $W_{120}A$ R with, respectively, pAN4 and Gal4AD Pex5 $W_{204}A$ as templates. His_6-GST tagged Pex5 239-300 containing a tobacco etch virus (Tev) protease cleavage site (pCW68) was constructed by ligating the *Nco*I-*Hind*III fragment of pAN94 (Klein *et al.*, 2002) into the *Nco*I-*Hind*III sites of pETM-30 (a kind gift from Dr. Stier). His_6 Pex14 1-94 (pCW76) was made by ligating the *Bam*HI-*Pst*I fragment from Gal4AD Pex14 1-94 (pCW52) into *Bam*HI-*Pst*I digested pQE9 (Qiagen).

Plasmids for *in vivo* expression of Pex14p constructs under control of the Pex14 promoter (P_{Pex14}) were made as follows: The Pex14 promoter, representing the region -617bp to -1bp upstream of PEX14, was amplified by PCR on genomic DNA using primers P14 PRO 5 and P14 PRO 3, the product was digested with *Eco*RI and *Sac*I and ligated into *Eco*RI-*Sac*I digested pUC19 producing pGB27. The *Bam*HI-*Pst*I fragment from pGB4 (Bottger *et al.*, 2000) was ligated into the *Bam*HI-*Pst*I sites of pEL43 (Elgersma *et al.*, 1993) and the *Nar*I-*Sac*I fragment was removed and replaced by the *Nar*I-*Sac*I fragment of pGB27 to produce P_{Pex14} Pex14 (pGB30). P_{Pex14} Pex14 1-252 (pCW51) was constructed by PCR on pGB47 using primers PR122 and PR123. The product was digested with *Bam*HI and *Pst*I and ligated into *Bam*HI-*Pst*I digested pEL43. The *Nar*I-*Sac*I fragment was replaced by the *Nar*I-*Sac*I fragment from pGB27. PCR was performed on pGB4 using primers PstI P14 and Δ52 P14 or Δ58 P14. The products were digested with *Bam*HI and *Cla*I and ligated into the *Bam*HI-*Cla*I sites of pGB4 producing pUC19 Pex14 53-341 and 59-341 (pGB28 and 29) respectively. The resulting vectors were then digested with *Bam*HI

and *Pst*I and the fragments were ligated into pGB30 partially digested with *Bam*HI and *Pst*I, producing P_{Pex14} Pex14 53-341 (pGB31) and 59-341 (pGB32). All constructs made using PCR were confirmed by sequencing.

Table I. Primers used in this study

Primer	Sequence (5'-3')	Comments
PR184	cggactagtaagcttgcatgcctgcag	Pex5 612 SpeI (R)
PR66	cggaattcttggctcgggatcaccagaaactgttgagaaggaag	Pex5 239 EcoRI (F)
PR100	ggaattctaggaagcggaagcatgagtgacgtggtcagtaaag	Pex14 1 EcoRI (F)
PR101	ggaattctaggaagcggaagctggaaggactattttgtgatggc	Pex14 94 EcoRI (F)
PR102	ggaattctaggaagcggaagctccatatctcctaatggtatacc	Pex14 252 EcoRI (F)
PR103	ggaattctaggaagcggaagcaaaaaagcaagagaacaaactattg	Pex14 301 EcoRI (F)
PR104	ccactagtactattcaatctccttttctgttaaccc	Pex14 58 SpeI (R)
PR105	ccactagtactatgggatggagtcttcgac	Pex14 341 SpeI (R)
PR122	ggaattctaggatccatgagtgacgtggtcagtaaag	Pex14 1 EcoRI/BamHI (F)
PR123	ccactagtactgcagctaggaaaataatctgttgtcctgc	Pex14 252 PstI/SpeI (R)
PR127	ccactagtactgcagctaccaatccctgtggggcagc	Pex14 94 PstI/SpeI (R)
Δ52 P14	cgggatccatggagcccaagaaagacggtatc	Pex14 53 BamHI (F)
Δ58 P14	cgggatccatgatcgtaggcgatgaagtatcg	Pex14 59 BamHI (F)
PstI P14	aactgcagctatgggatggagtcttcgac	Pex14 341 PstI (R)
P14 PRO 5	cgaattccctcccgccataattg	P_{pex14} EcoRI (F)
P14 PRO 3	cgagctccttattcaccttacaacttc	P_{pex14} SacI (R)
$pW_{120}A$	gagtgaacgatatatctcatgcgtcacaggaatttcaagg	Pex5 $W_{120}A$ (F)
$pW_{120}A$ R	ccttgaaattcctgtgacgcatgagatatatcgttcactc	Pex5 $W_{120}A$ (R)

Purification of fusion proteins expressed in E. coli

Cell pellets were thawed in buffer 1 (100 mM potassium phosphate pH 7.4, 5 mM β-mercaptoethanol, 0.1% triton X-100 and 2 mM PMSF), treated with 1mg/ml lysozyme at 21°C for 15 min and pulse sonicated on ice for 8 x 30 sec. Cell debris was removed by centrifugation at 4°C for 30 min at 12,500 x g. Lysates were loaded onto either Ni-NTA (Qiagen) or glutathione sepharose 4B (GE Healthcare) resin equilibrated with buffer 1. The resin was washed with buffer 1 and the fusion proteins were eluted with buffer 2 (100 mM potassium phosphate pH 7.4, 5 mM β-mercaptoethanol and 2 mM PMSF) containing either 330 mM imidazole or 20 mM reduced glutathione. His_6-GST tags were removed by addition of 100 µg recombinant His_6 Tev protease (a kind gift from Dr. Stier) followed by

incubation at 4°C overnight and at 30°C for 3 h. Both His_6-GST and His_6 Tev were removed by loading lysates onto Ni-NTA resin and collecting the run through. Purified proteins were concentrated and equilibrated in buffer 2 using Amicon™ Ultra centrifugal filters (Millipore) and stored at -80°C. Purity was monitored by SDS-PAGE analysis. Protein concentrations were measured using the Bradford method with BSA as standard (Bradford, 1976).

In vitro pull down assay

150 µg of Pex5 239-300 was incubated with either 150 µg His_6 Pex14 1-94, 150 µg His_6 Tev or alone in a total volume of 1 ml buffer 2 for 30 min at 21°C and loaded onto 0.5 ml Ni-NTA resin equilibrated with buffer 2. The resin was washed with 6 x 0.5 ml buffer 2 and proteins were eluted with 0.5 ml buffer 2 containing 330 mM imidazole. Samples of the elution fractions were subjected to 8-20% SDS-PAGE followed by western blotting and staining with antibodies specific for Pex14p, His_6 (Sigma) or Pex5p.

Miscellaneous

Published procedures were used for subcellular fractionation (Bottger *et al.*, 2000), β-galactosidase enzyme activity determination (Klein *et al.*, 2002) and immuno electron microscopy (Van der Leij *et al.*, 1993).

Results

ScPex14p contains two separate binding sites for ScPex5p

In the yeast *S. cerevisiae*, Pex14p has been shown to interact with Pex5p (Albertini *et al.*, 1997) and it is believed that the first 58 amino acids are responsible for this interaction (Schliebs *et al.*, 1999). Previously, we observed that deletion of the first 58 amino acids of Pex14p resulted in an oleate non-utilising phenotype when expressed in a *pex14Δ* strain but the truncated protein retained the ability to interact weakly with Pex5p in the two-hybrid system (our unpublished data). This suggested that Pex14p might contain multiple Pex5p binding sites. To address this, we constructed systematic deletions from both the N- and C-terminus of Pex14p and determined their interaction with full-length Pex5p using the two-hybrid system (Fig. 1 B). In addition to wild-type Pex14p, two separate fragments of

Pex14p strongly interacted with Pex5p, Pex14 1-94 and Pex14 252-341. Further deletion of these fragments resulted in an almost complete loss of the interaction.

Both ScPex5p binding sites in ScPex14p are essential for PTS1 protein import

To test whether these interactions were important *in vivo*, constructs were made in which one of the potential binding sites was deleted. *Pex14Δ* cells expressing either wild type (WT) Pex14p, Pex14 1-252, Pex14 53-341 (Pex14 59-341 was either degraded or not expressed when tested) or an empty vector (Ycplac33) were homogenised and the 600 x g post nuclear supernatant was subjected to centrifugation at 17,500 x g. Equivalent volumes of the organellar pellet and supernatant fractions were analysed by western blotting with antibodies specific for catalase A (a PTS1 protein) and 3-ketoacyl-CoA thiolase (a PTS2 protein). In *pex14Δ* cells expressing WT Pex14p, both catalase and thiolase were located predominantly in the pellet (Fig. 2), indicating that both proteins were imported. In cells transformed with an empty vector, both catalase and thiolase were mislocalised to the cytosol. Similarly, cells expressing Pex14 53-341 also miss-targeted catalase and thiolase to the cytosol. Expression of Pex14 1-252 resulted in a catalase import deficiency but had only a very limited effect on thiolase import. Antibodies against Pex14p confirmed that in all cases, Pex14p was expressed and targeted to peroxisomes (not shown).

Fig. 2 Localisation of catalase and thiolase in *pex14Δ* cells expressing truncated forms of Pex14p
Pex14Δ cells expressing wild type Pex14p, Pex14 1-252, Pex14 53-341 or an empty vector were subjected to subcellular fractionation. Equivalent volumes of the 600 x g post-nuclear supernatant (**H**), 17,500 x g organellar pellet (**P**) and 17,500 x g supernatant (**S**) were subjected to western blotting and staining with antibodies specific for catalase A (a PTS1 protein) and 3-ketoacyl-CoA thiolase (a PTS2 protein).

To verify these results using an independent method, immuno electron microscopy was performed. Cells expressing WT Pex14p targeted catalase efficiently to peroxisomes, as seen by the labeling of peroxisomal profiles (Fig. 3 A), whereas in cells expressing the deletion constructs or empty vector, gold particles were only found in the cytosol (Fig. 3 B-

D). Thiolase localisation was similar to that observed in the fractionation, with peroxisomal staining in the case of WT Pex14p (not shown) and Pex14 1-252 (Fig. 3 F) and cytosolic with the empty vector (not shown) and Pex14 53-341 (Fig. 3 E). Fractionation studies were also performed on a strain expressing Pex14 1-301, which showed wild-type distribution of both catalase and thiolase (not shown), indicating that the region important for catalase import is located between residues 252 and 301.

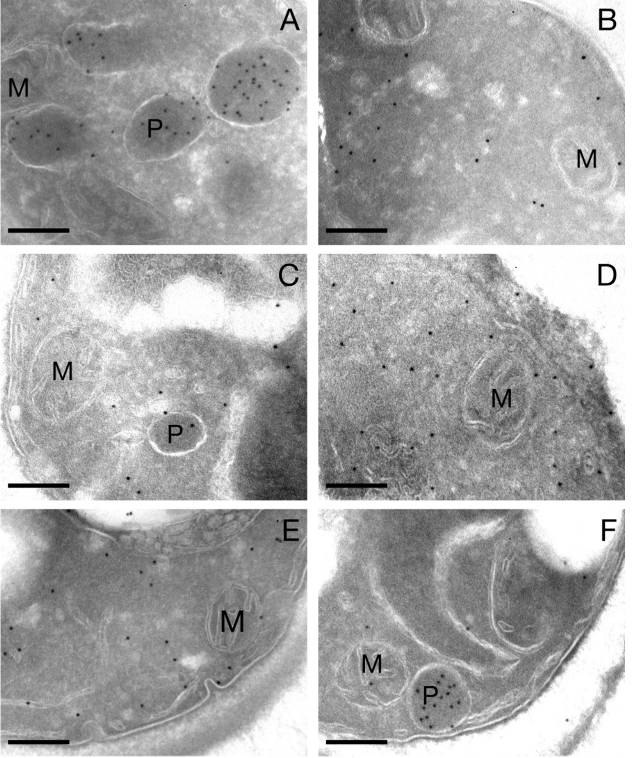


Fig. 3 Electron microscopy analysis of *pex14Δ* cells expressing truncated forms of Pex14p
Cryosections of oleic acid-grown *pex14Δ* cells expressing wild type Pex14p (**A**), Pex14 53-341 (**B** and **E**), Pex14 1-252 (**C** and **F**) or an empty vector (**D**) were labeled with primary antibodies specific for catalase (**A**-**D**) or thiolase (**E** and **F**) and immuno gold particles conjugated to ProteinA. M, mitochondrion, P, peroxisome. Bar, 0.2μm.

The two W-x-x-x-F/Y motifs in ScPex5p are not essential for binding to ScPex14 1-94

Previously, we observed that mutation of the two W-x-x-x-F/Y motifs at positions 120 and 204 in ScPex5p did not abolish the interaction with full length ScPex14p in the two-hybrid (Bottger *et al.*, 2000). This raised the question as to the location of the Pex14p binding

site(s) in Pex5p. Two-hybrid analysis was performed using the two Pex14p fragments shown to bind to Pex5p in combination with several Pex5p constructs where the W-x-x-x-F/Y motifs were either mutated or deleted (Fig. 4). The interaction between Pex14 252-341 and Pex5p was unaffected by mutation of tryptophane 120 to alanine ($W_{120}A$) but a severe decrease was observed when a $W_{204}A$ mutation was introduced. Pex14 1-94 retained the ability to strongly interact with Pex5p where one or both of the tryptophane residues at positions 120 and 204 were mutated to alanines ($W_{120}A$, $W_{204}A$ and $W_{120/204}A$). An interaction, although reduced, could also be detected even when the region containing both of these motifs was deleted (239-612). This interacting region could be further reduced to residues 239-300.

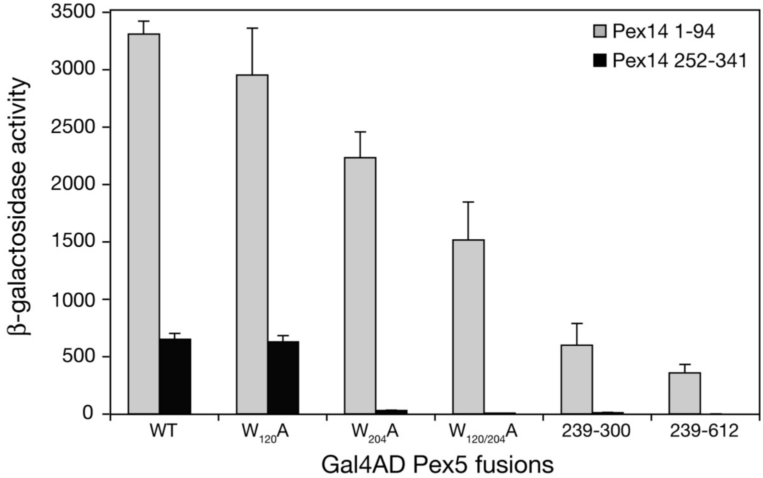


Fig. 4 Pex14p binding sites in Pex5p
Gal4DB Pex14 1-94 and 252-341 were tested in a two-hybrid assay against fusions of Gal4AD and wild type Pex5p (**WT**), Pex5 $W_{120}A$ (**$W_{120}A$**), Pex5 $W_{204}A$ (**$W_{204}A$**), Pex5 $W_{120/204}A$ (**$W_{120/204}A$**), Pex5 239-612 (**239-612**) and Pex5 239-300 (**239-300**). The strength of the interactions was measured as β-galactosidase activity (see legend to Fig. 1). The values given are the mean ± SD of four independent measurements. All Gal4AD fusions showed no interaction with the Gal4DB domain alone (not shown).

ScPex14 1-94 can interact with ScPex5 239-300 in vitro

To determine if the interaction between Pex14 1-94 and Pex5 239-300 was direct, an *in vitro* binding assay was performed. Purified Pex5 239-300 alone, or pre-incubated with either His_6 Pex14 1-94 or His_6 Tev (a negative control), was loaded onto Ni-NTA resin, washed and the proteins were eluted. Equivalent volumes of the elution fractions were

analysed by western blotting using antibodies specific for His_6, Pex14p (His_6 Pex14 1-94 could not be visualised using His_6 antibodies) and Pex5p (Fig. 5). Pex5 239-300 was only found in the elution fraction when His_6 Pex14 1-94 was present.

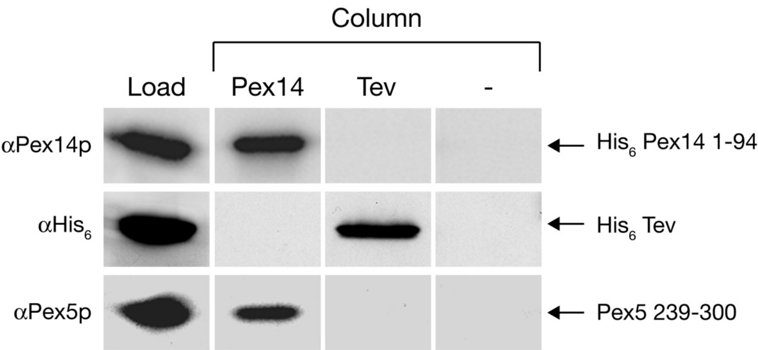


Fig. 5 *In vitro* pull down assay
Purified Pex5 239-300 was incubated in the presence of His_6 Pex14 1-94 (**Pex14**), His_6 Tev (**Tev**) or buffer alone (-) and loaded onto Ni-NTA resin. After thorough washing, proteins were eluted with imidazole. Equivalent volumes of the His_6 Pex14 1-94, His_6 Tev and Pex5 239-300 samples were loaded onto the Ni-NTA resin (**Load**) and the elution fractions were analysed by western blotting using antibodies directed against Pex14p (*upper panel*), His_6 (*middle panel*) or Pex5p (*lower panel*).

Discussion

Pex14p is believed to function as a docking factor for both PTS1- and PTS2-mediated peroxisomal matrix protein import. In this study we have explored the interaction between the PTS1 receptor Pex5p and Pex14p in *S. cerevisiae*.

Using two-hybrid analysis with truncated forms of Pex14p, two independent Pex5p binding sites could be identified. The N-terminal region of ScPex14p has already been postulated as a Pex5p binding site. In our study, a fragment consisting of the first 94 amino acids showed binding to Pex5p. Removal of even part of this region resulted in a severe reduction in the interaction. The C-terminal interaction has not been previously reported and was most efficient when only residues 252-341 were present. As to why the larger C-terminal constructs showed only a weak interaction, even when these residues were present, remains unclear but may be due to a masking of this binding site by, for example, the coiled coil region.

Both of the interactions appear to be essential for PTS1 import, as truncated forms of Pex14p containing only one binding site misdirected catalase to the cytosol. This may

imply that the two binding sites act in collaboration, as the presence of only one is not sufficient to fully restore the function of Pex14p. Removal of the N-terminal binding site also resulted in a PTS2 import deficiency. It is known that Pex7p, Pex18p and Pex21p (proteins specifically involved in PTS2 import) can interact with Pex14p (Stein *et al.*, 2002), but the regions responsible for these interactions have, so far, not been identified. Our data suggest that the N-terminus of Pex14p may play a role in both PTS1 and PTS2 import.

Two-hybrid analysis using Pex5p constructs lacking the characterised W-x-x-x-F/Y motifs showed that they are not essential for binding to Pex14 1-94, although a 2-5 fold reduction in the interaction was observed when both motifs were mutated or deleted. Therefore, the participation of these motifs in the interaction cannot be ruled out and would require further investigation. In our study, a positive interaction in the two-hybrid as well as *in vitro* was observed between Pex14 1-94 and Pex5 239-300. This fragment of Pex5p has been previously identified as the Pox1p binding region (Klein *et al.*, 2002) and contains a reverse W-x-x-x-F/Y motif. Whether this motif is responsible for the interaction remains to be determined.

The binding of Pex14 252-341 to Pex5p was unaffected by the mutation $W_{120}A$, but was severely disturbed, although not completely abolished, when tryptophane 204 was mutated or deleted, suggesting that it may play a role in the interaction. We were unable to reproduce the two-hybrid interaction in direct *in vitro* experiments, suggesting that it is either too weak to detect using the technique employed or that other factors are needed. A potential candidate for another factor could be Pex13p, as the interaction between Pex5p and Pex14 252-341 was disturbed when tryptophane 204, a residue shown to be involved in the Pex13p-Pex5p interaction (Bottger *et al.*, 2000), was absent.

As to why *S. cerevisiae* Pex14p contains two separate Pex5p binding sites, whereas only one has been identified in other organisms, remains to be seen. The N-terminal region of Pex14p might represent the initial docking site for Pex5p on the peroxisomal membrane, as the interaction between Pex14 1-94 and Pex5p is direct. The C-terminal region of Pex14p, acting alone or in combination with another factor, such as Pex13p, could therefore be involved in a later step. Alternatively, this interaction might be involved in the actual translocation of the Pex5p-PTS1 complex across the peroxisomal membrane, as Pex14p has been suggested to play a role in this process (Agne *et al.*, 2003).

The nature of the Pex14p-Pex5p interaction needs to be investigated further, if only to determine the function of these multiple binding sites.

Acknowledgements

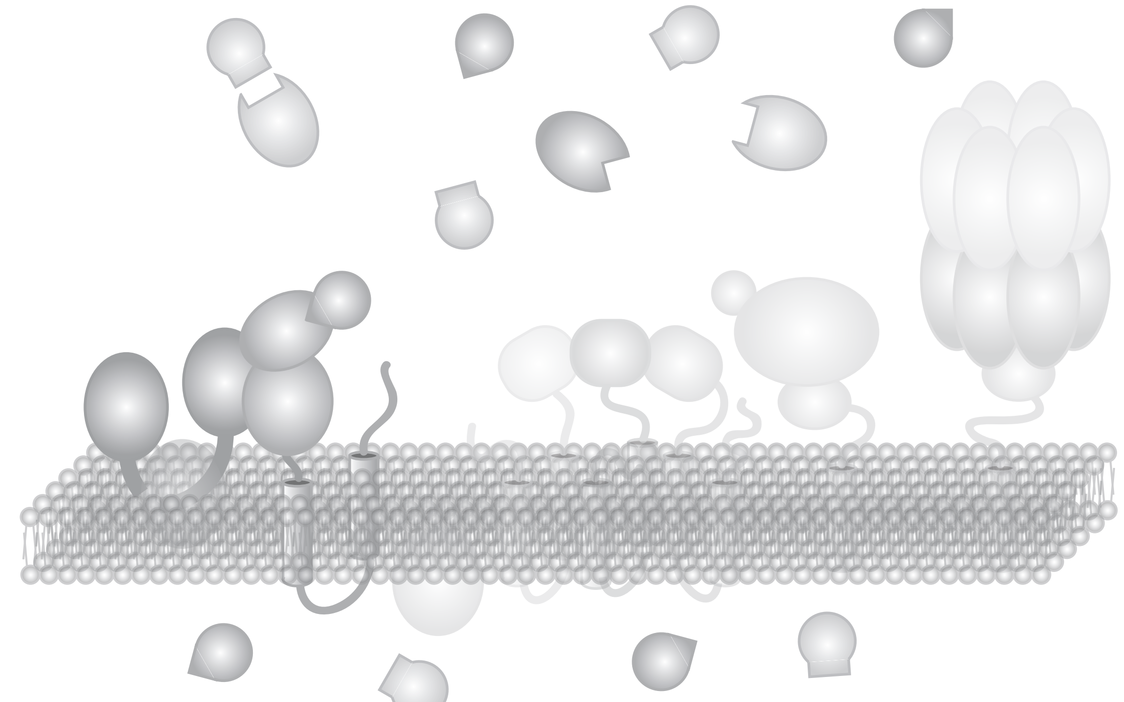
We would like to thank Dave Speijer, Will Stanley and Christian Neufeld for useful advice and discussions, Gunter Stier for the pETM-30 plasmid and His_6 Tev and Rob Benne for critically reading the manuscript.

3

Pex13p: Docking or cargo handling protein?

Chris Williams and Ben Distel

Department of Medical Biochemistry, Academic Medical Center,
Amsterdam, The Netherlands



Abstract

The Src homology 3 (SH3) domain-containing peroxisomal membrane protein Pex13p is an essential component of the import machinery for matrix proteins and forms a binding site for the peroxisomal targeting type I (PTS1) receptor Pex5p. The interaction between these two proteins can be described as novel in several ways. In the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, the SH3 domain itself is responsible for the interaction but not via the typical P-x-x-P motifs that are common to SH3 ligands as Pex5p lacks such a motif. Instead, a region of Pex5p containing a W-x-x-x-F/Y motif is crucial for this binding. In mammals, again W-x-x-x-F/Y motifs appear to be important for the interaction but the SH3 domain seems not to be the site for Pex5p binding, this being located in the N-terminus of Pex13p. Despite these differences in the details of the Pex13p-Pex5p interaction, the association of the two proteins is a crucial step in Pex5p-mediated protein import into peroxisomes in both yeasts and mammals.

Introduction

After Pex5p has recognised and bound its PTS1-containing cargo protein in the cytosol, there is still a long journey ahead before this cargo sees its final destination, the peroxisomal matrix. One of the initial steps on this journey and, arguably, one of the most essential, is that of association with the peroxisomal membrane. For this, a docking platform situated on the cytosolic side of the peroxisomal membrane is required. So far, three proteins have been identified as potential components of this platform: Pex13p, Pex14p and Pex17p (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Elgersma *et al.*, 1996; Erdmann and Blobel, 1996; Fransen *et al.*, 1998; Gould *et al.*, 1996; Huhse *et al.*, 1998; Komori *et al.*, 1997; Shimizu *et al.*, 1999; Will *et al.*, 1999). Of these three proteins, Pex17p has, up until this point, only been identified in yeast and its actual function in the docking process still remains enigmatic (Huhse *et al.*, 1998). The other two components, Pex13p and Pex14p, have been identified in all organisms where peroxisomes (or peroxisome like organelles) are found and in each case they were shown to be essential for both PTS1- and PTS2-mediated protein import (reviewed in Purdue and Lazarow, 2001). Pex13p and Pex14p are capable of interacting with each other, as well as with the cycling receptors Pex7p and Pex5p (Albertini *et al.*, 1997; Barnett *et al.*, 2000; Bottger *et al.*, 2000; Brocard *et al.*, 1997; Elgersma *et al.*, 1996; Erdmann and Blobel, 1996; Fransen *et al.*, 1998; Girzalsky *et al.*, 1999; Gould *et al.*, 1996; Otera *et al.*, 2000; Schliebs *et al.*, 1999; Urquhart *et al.*, 2000). These interactions are thought to play a role in the early stages of the import of both PTS1 and PTS2 proteins, namely, the association of the cargo-bound receptor with the peroxisomal membrane. However, the actual details of these interactions are far from clear and can be highly variable depending on the species under observation. The interaction between Pex5p and Pex13p is a much-studied process, both in yeasts and mammals and with the aid of a variety of techniques.

Here we will discuss different aspects of this interaction and its role in Pex5p-mediated protein import into peroxisomes.

Pex13p: the original docking protein

PEX13 was originally isolated as a *p*eroxisome *as*sembly (*pas*) mutant in yeast using random mutagenesis screening and given the names *pas20* in *S. cerevisiae* (Elgersma *et al.*, 1993) and *pas6-1* in *P. pastoris* (Gould *et al.*, 1992). Initial characterisation revealed that

these mutants were disturbed in the import into peroxisomes of the PTS1-containing protein catalase and that cells with these mutations could not grow on media containing methanol/oleic acid as sole carbon source, conditions where functional peroxisomes are critical. Since its initial isolation in yeast, Pex13p has been identified in a variety of other eukaryotes. Sequence alignment revealed that the overall amino acid conservation throughout evolution is not high, but that the domain structure *is* well conserved; all Pex13p's contain two transmembrane regions and they all contain an Src homology 3 (SH3) domain in the extreme C-terminal region (Fig. 1). An obvious role for a protein containing two transmembrane domains that is essential for PTS1 protein import would be in the docking of a cargo-laden receptor onto the peroxisomal membrane or in the translocation of PTS1-containing proteins into the peroxisomal matrix or both. Indeed, localisation studies confirmed that Pex13p is peroxisome-associated and biochemical assays showed that it behaves as an integral membrane protein, being resistant to both high salt and high pH treatment (Elgersma *et al.*, 1996; Erdmann and Blobel, 1996; Gould *et al.*, 1996). It was also shown that the SH3 domain is cytoplasmically oriented and essential for Pex13p function, its absence leading to a similar phenotype as that of a complete *PEX13* deletion *i.e.* the inability to grow on methanol containing media (Gould *et al.*, 1996). The observation that in the absence of Pex13p, the amount of Pex5p associated with peroxisomes is 40 times less than in the wild type situation, again strengthened the claim that Pex13p might be a docking factor for the PTS1 receptor Pex5p (Gould *et al.*, 1996). However this, apparently, is not its only function as *pex13*Δ cells also mislocalise the PTS2 protein 3-ketoacyl-CoA thiolase to the cytosol, giving us the first indication that the PTS1 and PTS2 pathways may converge at the peroxisomal membrane, and that Pex13p might represent this point of convergence. Later studies, however, have assigned this role to Pex13p’s fellow docking factor Pex14p (Albertini *et al.*, 1997), posing the question as to what the function of Pex13p is in the process of peroxisomal matrix protein import. Before addressing this issue we will first discuss the multiple interactions Pex13p is involved in, focussing on Pex13p’s most distinctive feature, the SH3 domain.

The Pex13p SH3 domain: a protein-protein interaction module

The function of most proteins is, for a large part, defined by their ability to interact with other cellular components and the identification of interacting partners can give

considerable information about the function of the protein in question. Any docking protein involved in PTS1 and PTS2 protein import would be expected to have multiple interactions with other proteins involved in the import process. Yeast two-hybrid analysis and, later on, *in vitro* binding studies gave us the first ideas about a protein-linkage map for Pex13p, which consists of the docking factor Pex14p, the PTS1 import receptor Pex5p and the PTS2 import receptor Pex7p. Of the latter interaction, little is known other than that it is a direct interaction and that the N-terminal region of Pex13p would appear to be the binding site in both yeast and mammals (Girzalsky *et al.*, 1999; Otera *et al.*, 2002; Stein *et al.*, 2002). Removal of the Pex7p binding site in *S. cerevisiae* Pex13p resulted in a PTS2 but not a PTS1 import defect, indicating that the contact sites for Pex5p and Pex7p are separate (Stein *et al.*, 2002).

Early in the Pex13p story, it was clear that the SH3 domain would have a pivotal role in these interactions, as it is a well-known protein-protein interaction module and its presence is essential for Pex13p function. Indeed, two SH3 domain ligands have been identified: Pex14p and Pex5p. To understand the molecular details of the Pex13p SH3-Pex5p/Pex14p interaction some general knowledge about SH3 domain structure and ligand binding is needed. SH3 domains have been found in a huge range of proteins across the evolutionary board. Approximately 60 amino acids in length and retaining a high level of sequence similarity, especially among residues involved in ligand recognition, SH3 domains are known to mediate protein-protein interactions involved in several cellular processes including cytoskeletal organisation, signal transduction and protein localisation (reviewed in Kay *et al.*, 2000; Mayer, 2001). Not only is their primary structure well conserved, the topology is also very similar throughout the family, consisting of a tightly packed five stranded ß-barrel fold surrounded by three variable loops, known as the RT, n-Src and the distal-loop (Figs. 1 and 2). The ligand-binding surface of the SH3 domain consists of an elongated patch of aromatic residues, that form a hydrophobic cleft well-suited to accommodate the typical SH3 ligand, consisting of a proline-rich or poly-L-proline (PP) region in the binding partner. Upon binding, these PP motifs adopt a so-called polyproline type II helix. PP motifs come in two main types, type I (PP-I) and type II (PP-II) and contain a core P-x-x-P motif (where x represents any amino acid) and ancillary, often positively-charged, residues present in front or behind this core module (Feng *et al.*, 1994; Lim *et al.*, 1994). These positive charges adjacent to the P-x-x-P motif contribute to

the affinity and specificity of the SH3-ligand interaction by contacting negatively charged residues in the RT loop of the SH3 domain. The PP-I class conform to the consensus R-x-x-P-x-x-P while the PP-II class possesses a P-x-x-P-x-R motif. Other sequences are also possible, with P-x-x-D-Y and R-K-x-x-Y-x-x-Y both capable of contacting the canonical hydrophobic binding pocket on SH3 domains (Kang *et al.*, 2000; Mongiovi *et al.*, 1999).

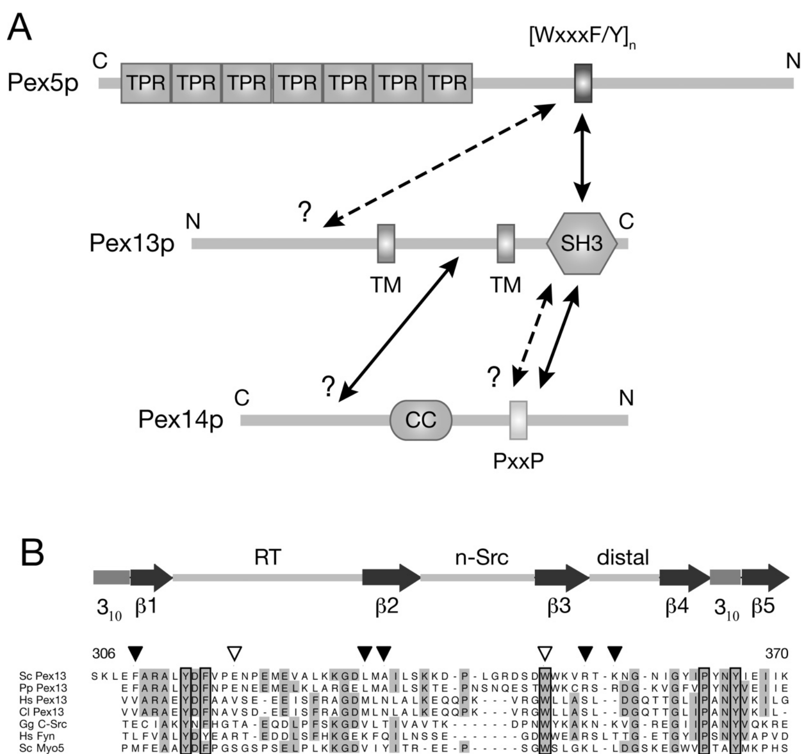


Fig. 1 A. Domain structure of the PTS1 receptor Pex5p and the docking factors Pex13p and Pex14p
Arrows indicate interactions among the three proteins in yeasts (*solid*) and mammals (*dashed*). Binding sites that are not precisely mapped are indicated by question marks. For clarity the interactions between Pex5p and Pex14p are not shown. Only one W-x-x-x-F/Y motif in the N-terminal half of Pex5p is shown, but the number (n) can vary between 2 (yeasts) and 7 (mammals). TPR, tetratricopeptide repeat; TM, transmembrane domain; CC, coiled-coil domain; P-x-x-P, proline-rich SH3 binding motif; SH3, Src homology 3 domain.
B. Secondary structure and multi-sequence alignment of Pex13p SH3 domains
The positions of the secondary structural elements (β strands and 3_{10} helixes) and the RT, n-Src and distal loops, connecting these elements, are indicated above the alignment. Also shown is the additional short 3_{10} helix present in the N-terminus of the *S. cerevisiae* Pex13p SH3 domain. The SH3 domain sequence of four different Pex13 proteins is compared with the amino acid sequences of several representative SH3 domains. Conserved residues are highlighted in grey and residues involved binding the canonical P-x-x-P motif are boxed. Arrowheads indicate residues in *S. cerevisiae* Pex13p SH3 that when mutated specifically affect the interaction with Pex14p (*open*) and Pex5p (*closed*), respectively. *Sc, Saccharomyces cerevisiae; Pp, Pichia pastoris; Cl, Cricetulus longicaudatus; Gg, Gallus gallus; Hs, Homo sapiens*. See text for details

The Janus face of the Pex13p SH3 domain

The interaction with Pex14p

X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have shown that the SH3 domain of *S. cerevisiae* Pex13p is very similar to its family-members, consisting of the five stranded β-barrel and three loop regions, including the typical hydrophobic cleft, involved in P-x-x-P recognition (Douangamath *et al.*, 2002; Pires *et al.*, 2003). However, an extra 3_{10} helix at the N-terminus as well as several additional residues present in the RT and n-Src loops that are not found in other SH3 domains were also observed (Fig. 1). These extra residues in the loops have been suggested to play a role in separating the binding sites for Pex5p and Pex14p (Douangamath *et al.*, 2002) (see below). Looking at the SH3 binding partners in detail, sequence comparisons show that Pex14p contains a well-conserved PP-II motif. Mutation of the two conserved proline residues in yeast Pex14p confirmed that this motif was responsible for the interaction between Pex14p and the SH3 domain (Girzalsky *et al.*, 1999). Also, mutations of a well-conserved tryptophane residue ($W_{349}A$) found in the hydrophobic cleft of the SH3 domain and a glutamic acid residue ($E_{320}K$) in the RT loop abrogate the Pex14p-Pex13p SH3 interaction (Bottger *et al.*, 2000; Girzalsky *et al.*, 1999). Co-crystal structures of the SH3 domain with a dodeca-peptide of Pex14p containing the P-x-x-P motif as well as NMR chemical shift experiments with a similar but larger Pex14p peptide again verified that the interaction between Pex14p and the SH3 domain of Pex13p is typical of that between SH3 domains and proline-rich ligands, with residues in the RT and n-Src loops playing an important role (Douangamath *et al.*, 2002; Pires *et al.*, 2003). Taken the above, it is amazing that, *in vivo*, mutation of the P-x-x-P motif (into A-x-x-A) in *S. cerevisiae* Pex14p does not result in a growth defect on oleic acid medium (Girzalsky *et al.*, 1999). In particular since the Pex13p SH3 mutant $E_{320}K$, which is specifically disturbed in its interaction with Pex14p but not with other proteins, does give rise to a growth defect on oleic acid, although the mislocalisation of PTS1 and PTS2 proteins is not as severe as that observed in a *pex13* knockout (Elgersma *et al.*, 1996). Recent observations however have shed new light on the details of the Pex13p-Pex14p interaction, namely that Pex13p contains a second binding site for Pex14p in-between the two transmembrane regions (Schell-Steven *et al.*, 2005). This interaction, when disturbed, appears to have little effect on the import of matrix proteins and does not result in a growth defect on oleic acid. However, when the two

independent Pex14p binding sites in Pex13p are mutated, the import of both PTS1 and PTS2 proteins is considerably less efficient than in cells with either of the single mutants, suggesting that the Pex13p-Pex14p interaction may be important but not essential for import. A possible explanation for this observation is that Pex5p, the other protein shown to interact with the SH3 domain of Pex13p, might play a role in the interaction between Pex14p and Pex13p by acting as a bridge. Indeed Schell-Steven and co-workers (Schell-Steven *et al.*, 2005) report that the import of PTS2 proteins, a process that is independent of Pex5p, is disturbed when the Pex14p binding sites in Pex13p are mutated and Pex5p is no longer present. Collectively, these data indicate that the association of Pex13p with Pex14p is mediated by multiple direct and indirect interactions and that blocking all these interactions completely abrogates peroxisomal matrix protein import.

The interaction with Pex5p

The binding of Pex5p to Pex13p, however, is a very different story from that of Pex14p. Being a well-known protein interaction module, the SH3 domain of yeast Pex13p was used as bait in two-hybrid screens, either against a limited library consisting of known Pex proteins (Erdmann and Blobel, 1996) or a full-genome library (Elgersma *et al.*, 1996). In both screens Pex5p turned-up as a ligand of the Pex13p SH3 domain and further two-hybrid and *in vitro* binding studies confirmed that the Pex5p-SH3 interaction is specific and direct. Surprisingly, yeast Pex5p appeared to lack the classical SH3 binding motif, P-x-x-P, and such a sequence was also not present in Pex5p from other species. At first sight, this may not be cause for concern as variations in the P-x-x-P motif have been observed (for example P-x-x-D-Y, see above) but still with the ability to interact with SH3 domains. Deletions of *P. pastoris* Pex5p gave a peptide containing residues 100-213 as the minimum Pex13p SH3 binding domain, a region lacking any recognisable P-x-x-P-like motifs (Urquhart *et al.*, 2000). The specific residues involved in the interaction were found by random mutagenesis of *S. cerevisiae* Pex5p, followed by screening of the mutants for loss of interaction with Pex13p SH3 in a two-hybrid assay (Bottger *et al.*, 2000). This resulted in 5 mutants that had lost the ability to interact with Pex13p SH3 but retained binding to other Pex and PTS1 proteins. Sequencing the constructs showed that, although all mutants contained multiple substitutions, the glutamic acid at position 212 was found mutated in three independent clones (either to a valine or a glycine) while the other two clones contained substitutions in

the same region namely, phenylalanine 208 to leucine ($F_{208}L$) and glutamic acid 214 to glycine ($E_{214}G$). Site directed mutagenesis to make the individual mutants $E_{212}V$ and $F_{208}L$ confirmed that this region of Pex5p is important for the binding with the Pex13p SH3 domain. Closer examination of this region of Pex5p shows that, although lacking a typical P-x-x-P motif, the sequence $P_{203}WTDQ_{207}$ is present, which shares some homology with P-x-x-D-Y, a P-x-x-P motif variant that can contact SH3 domains through the classical hydrophobic binding cleft (see above). However, alanine scanning of the region of Pex5p between residues 203 and 214 followed by two-hybrid analysis indicated that proline 203 was not involved, suggesting that the Pex5p-Pex13 SH3 interaction is a novel, non P-x-x-P motif-mediated process (Barnett *et al.*, 2000). The same alanine scanning experiment resulted in several mutants that could no longer interact with the SH3 domain, namely $W_{204}A$ and again, $F_{208}A$ and $E_{212}A$. The ability of two other mutants, $L_{211}A$ and $E_{214}A$, to interact with the SH3 domain was reduced but not abolished. All of these mutants retained the ability to interact with Pex14p and MDH3, a PTS1 protein, indicating that the effect was specific for the Pex13p interaction. This leaves us the potential recognition sequence W-x-x-x-F-x-x-L-E-x-E, quite different from the typical P-x-x-P motif found in Pex14p.

The residues W_{204} and F_{208} make up another well-known protein motif, the W-x-x-x-F/Y motif. These motifs are very well conserved throughout the Pex5p family and are often present in multiple copies, ranging from 2 in *S. cerevisiae* to 7 in humans. They have been shown to play a role in the interaction between Pex14p and Pex5p although this depends very much upon the specific motif and the species under observation, with some W-x-x-x-F/Y motifs being essential for binding and others, apparently, not being involved at all (Otera *et al.*, 2002; Saidowsky *et al.*, 2001; Schliebs *et al.*, 1999). This would appear to be the case with the motif $W_{204}TDQF_{208}$ in *S. cerevisiae* Pex5p, as mutation of either the tryptophane or phenlyalanine does not abolish the interaction with Pex14p but does effect the Pex13p SH3 interaction (Barnett *et al.*, 2000; Bottger *et al.*, 2000). A close look at the peptide of *P. pastoris* that interacts with the SH3 domain (amino acid 100-213) reveals three separate W-x-x-x-F/Y motifs all of which are followed within 4-5 amino acids by a glutamic or aspartic acid, suggesting that these motifs may play a role in the Pex5p-SH3 interaction in this organism as well. Does this then mean that W-x-x-x-F/Y motifs can be added to the list of potential SH3 ligands along with P-x-x-P motifs? Yes, but there is an interesting twist to the story. The *S. cerevisiae* Pex13p SH3 mutant $E_{320}K$ has already been

mentioned as it lacks the ability to interact with Pex14p as this residue is involved in P-x-x-P motif recognition. If the binding between Pex13p SH3 and Pex5p would resemble that of the binding between Pex14p and Pex13p, then it might be expected that this mutant would also be disturbed in the Pex5p interaction, which is not the case (Bottger *et al.*, 2000; Girzalsky *et al.*, 1999). The same is seen with the $W_{349}A$ mutation in the hydrophobic binding pocket of Pex13p SH3. This mutant cannot interact with Pex14p but retains, albeit reduced, the ability to interact with Pex5p (Bottger *et al.*, 2000). These observations led away from the typical P-x-x-P motif recognition site being involved in Pex5p binding and pointed towards the possibility that another, novel binding site may be present in the Pex13p SH3 domain. In line with this suggestion it was found that in an *in vitro* competition assay, a peptide of Pex5p and Pex14p can interact with Pex13p SH3 simultaneously and do not compete with each other for binding (Barnett *et al.*, 2000). The first indication as to the position of the Pex5p binding site in the *S. cerevisiae* Pex13p SH3 domain came from suppressor mutation analysis (Barnett *et al.*, 2000). Random mutagenesis of the SH3 domain resulted in several mutants that were able to rescue the interaction with the mutant forms of Pex5p no longer capable of binding to the wild type SH3 domain in the two-hybrid. These mutants ($N_{321}Y/I$, $R_{353}G$, $E_{323}V$ and $K_{355}R$), are not clustered over a small region, but are found in both the RT and the distal loops and, significantly, have no effect on the capability of the SH3 domain to interact with Pex14p. The RT loop has already been implicated in the binding of SH3 domains to P-x-x-P ligands but the distal loop appears to play no direct role in this binding. The presence of two suppressor mutations in this region indicated that Pex5p binding might take place here.

NMR analysis of the binding between Pex13p SH3 and both Pex5p and Pex14p did indeed confirm, as expected, that these two binding sites could be found in different parts of the SH3 domain, although some overlap was also observed (Douangamath *et al.*, 2002; Kami *et al.*, 2002; Pires *et al.*, 2003) (Fig. 2). The binding of Pex14p was typical for that of the characterised SH3-P-x-x-P motif interaction (see above). The binding of Pex5p to the SH3 domain was considerably different, with large chemical shifts observed in the first, second and third β-sheet elements as well as part of the distal and RT loops. These results also confirmed that the two residues from the distal loop (R_{353} and K_{355}) identified in the suppressor screen were specifically involved in the Pex5p interaction, as they underwent strong chemical shifts in the presence of the Pex5 peptide but not the Pex14

peptide. The other residues that are part of the Pex5p binding site are F_{310} (β1), L_{333} and A_{335} (both β2). Most of these residues are not, as might be expected, conserved in the SH3 domain family but, rather surprisingly, they are also not well conserved in the mammalian Pex13p SH3 domains (see Fig. 1).

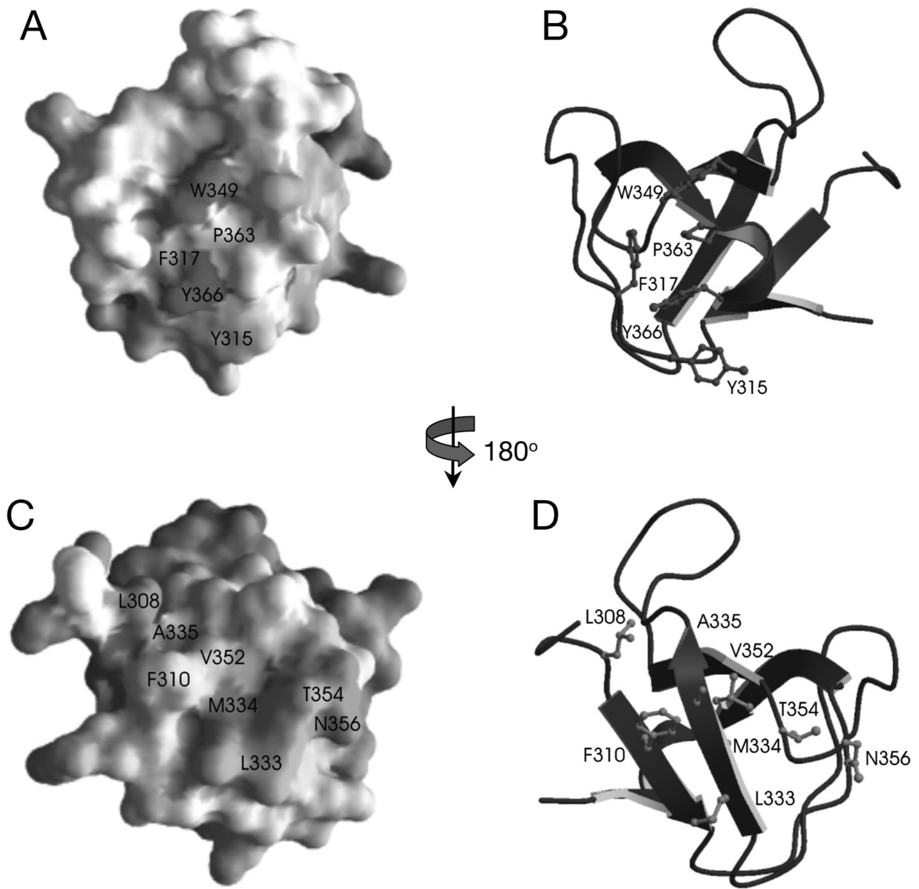


Fig. 2 The Pex14p and Pex5p binding sites are located at opposite surfaces of the *S. cerevisiae* Pex13p SH3 domain
Side chains of residues involved in ligand to Pex14p (**A** and **B**) and Pex5p (**C** and **D**) are shown in surface (**A** and **C**) and ribbon (**B** and **D**) representations, respectively. Modified from (Douangamath *et al.*, 2002). See text for details.

This may indicate that this novel-binding site for Pex5p on the Pex13p SH3 domain is specific for yeasts and is not present in mammals. Indeed, the Chinese hamster Pex5p (and, therefore very likely other mammalian Pex5p's as well) does not interact with

the SH3 domain of the corresponding Pex13p. Instead, a region of the Pex13p N-terminus, corresponding to the Pex7p binding site appears to be the location for the interaction (Otera *et al.*, 2002). The SH3 domain, however, functions much like its yeast counterparts in binding to Pex14p, presumably via the P-x-x-P motif present in Pex14p although this remains to be seen (Otera *et al.*, 2002). Amazingly, the interaction between Pex5p and Pex13p appears to be mediated by the presence of W-x-x-x-F/Y motifs in Pex5p, just like the situation in *S. cerevisiae*. And again, these W-x-x-x-F/Y motifs are highly variable in their function, with some being essential for Pex13p binding, others for Pex14p binding and some for both. Although the *P. pastoris* Pex13p SH3 domain reveals a strict conservation of the residues that form the Pex5p binding site (see Fig. 1), several mutants in this SH3 domain show a rather unexpected behaviour. The mutant $E_{291}K$ is considerably reduced in its ability to interact with Pex5p but not Pex14p (Urquhart *et al.*, 2000). This position corresponds to glutamic acid 320 in the *S. cerevisiae* SH3 domain, where a mutation to a lysine ($E_{320}K$) has the opposite effect, specifically disturbing the interaction with Pex14p (Bottger *et al.*, 2000). Also, a *P. pastoris* Pex5 peptide is capable of competing with Pex14p for binding to the SH3 domain, indicating that the binding sites for both proteins may, at least partially, overlap (Urquhart *et al.*, 2000). The reasons for such diversity between the different species are not clear and, until further more in depth study is performed on the interaction between Pex13p and Pex5p in *P. pastoris* and mammals will remain so.

So many interactions, but what is the actual function of Pex13p?

Pex13p's location on the peroxisomal membrane, its interaction with both PTS receptors and the fact that it is essential for both PTS1 and PTS2 protein import make it an extremely interesting protein and suggested that the function might be in docking. This fact was compounded by its ability to interact with multiple partners, which include Pex14p, Pex5p and Pex7p, all proteins involved in the import of peroxisomal matrix proteins. But, to call it just a docking factor is to over simplify the matter. Several lines of evidence suggest that Pex14p is the initial docking factor responsible for the first contact of the cargo-bound receptor with the peroxisomal membrane. This would mean that Pex13p is essential for a process that happens directly after this initial association. Three observations are in support of this: i) in *in vitro* binding experiments, Otera *et al.* and Urqhuart *et al.* found that PTS1-loaded Pex5p interacts more strongly with Pex14p than with Pex13p (Otera *et al.*, 2002;

Urquhart *et al.*, 2000), ii) the absence of Pex14p in CHO cells results in a reduction in the amount of Pex5p associated with the peroxisomal membrane, whereas overexpression of Pex14p results in an increased amount of peroxisomal Pex5p (Otera *et al.*, 2000) and iii) in biochemical isolation experiments the majority of Pex13p does not co-purify with the presumed docking proteins Pex14p and Pex17p (Agne *et al.*, 2003; Reguenga *et al.*, 2001). However, the stochiometric relationship between Pex13p and Pex14p is crucial for functional protein import as overexpression of either protein results in a lack of growth on oleic acid but co-overexpression of both does not (Bottger *et al.*, 2000). What the post-docking function of Pex13p would entail is still a matter of speculation, but in all scenarios it is assumed that the cargo-laden Pex5p is transferred from Pex14p to Pex13p. This handing over of the receptor may be either coupled to dissociation of the receptor-cargo complex and subsequent translocation of the cargo protein or may provide a means by which the receptor is able to cycle back to the cytoplasm, or possibly both. The only experimental evidence supporting the view that Pex14p may not be the initial docking site for Pex5p comes from work with the methylotrophic yeast *Hansenula polymorpha*. It was shown that in a *H. polymorpha pex14*Δ strain the PTS1 import defect could be partially suppressed by overexpressing Pex5p suggesting that under conditions of Pex5p excess Pex14p can be bypassed and the receptor can reach the peroxisome (Salomons *et al.*, 2000). Whether Pex13p plays an important role in Pex5p docking in the *pex14*Δ strain was not addressed in this study. Moreover, in *S. cerevisiae* overexpression of Pex5p using the oleic acid inducible catalase promoter and a multi-copy vector in a *pex14*Δ strain does not rescue PTS1 import, either partially or completely, indicating that Pex14p is not dispensable for Pex5p docking in this organism (our unpublished observations). In conclusion, there seem to be (at least) two binding sites for Pex5p at the peroxisome membrane: Pex13p and Pex14p. The association of Pex5p with Pex13p is mediated by W-x-x-x-F/Y motifs present in the N-terminal half of Pex5p that contact either a novel binding cleft on the SH3 domain of yeast Pex13p or the N-terminus in the case of mammalian Pex13p. Whether Pex13p functions in the initial receptor docking event or plays a role at a later stage in peroxisomal matrix protein import is subject for future studies, which most likely require the reconstitution of the import process *in vitro*. Finally, it should be noted that Pex13p is not only present in mature peroxisomes but also has been detected in a specialised sub-domain of the endoplasmic reticulum in mouse dendritic cells (Geuze *et al.*, 2003), structures that

are thought to be the origin of newly synthesised peroxisomes in a cell (Tabak *et al.*, 2006). It cannot be excluded therefore that Pex13p has a dual function, and that in addition to its role in peroxisomal matrix protein import, Pex13p is also involved in the early steps of peroxisome biogenesis by contributing to the formation of the specialised ER.

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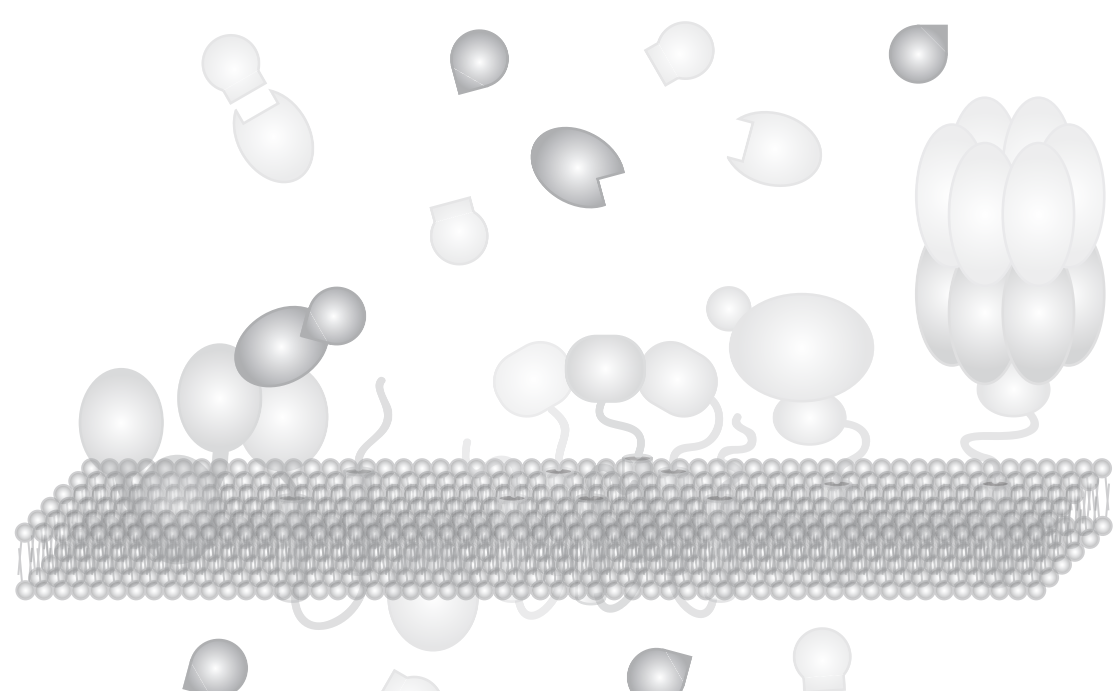
4

Studies on a putative ubiquitin interacting motif present in the fourth TPR motif of *Saccharomyces cerevisiae* Pex5p

Chris Williams, Simon van Haren, Sarah Meeuws and Ben Distel

Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands



Abstract

Recent reports have suggested that ubiquitination of the cycling receptor Pex5p may play a role in the import of PTS1 proteins into peroxisomes. We observed, using sequence alignments, that *Saccharomyces cerevisiae* Pex5p contains a putative ubiquitin interacting motif (UIM) in its fourth TPR motif. Binding studies performed between Pex5p and ubiquitin failed to show any interaction between these two proteins. However, mutation of residues within the UIM consensus caused a limited growth defect on media containing oleic acid as sole carbon source and resulted in a build up of Pex5p on the peroxisomal membrane.

Introduction

Peroxisomes are extremely diverse organelles that play a crucial role in eukaryotic cells (for review, see (Purdue and Lazarow, 2001). Their importance is shown by the occurrence of several human disorders resulting from a defect in peroxisomal function (Weller *et al.*, 2003). The post-translational import of proteins into these organelles is a well-studied multi-step process. Proteins destined for the peroxisome are equipped with a peroxisomal targeting signal (PTS) type-1, 2 or 3, which enables them to be recognised in the cytosol by their associated cycling receptors and subsequently transported to the peroxisomal membrane and be imported. The receptors are then recycled back to the cytosol for another round of import. So far, around 32 proteins involved in peroxisome biogenesis, called peroxins, or Pex proteins (Distel *et al.*, 1996) have been identified. The cycling receptor for PTS1 and -3 containing proteins, Pex5p, can be post-translationally modified by ubiquitin (Kiel *et al.*, 2005; Kragt *et al.*, 2005; Platta *et al.*, 2004). Ubiquitination is the attachment of the 7 kDa protein ubiquitin to a substrate and has been shown to regulate numerous processes in the cell, such as 26S proteasome mediated degradation, endocytosis, DNA repair and ER retrotranslocation (for review, see Pickart, 2001).

Ubiquitination of a substrate can often lead to structural changes in the substrate, as well as altering the way the substrate interacts with other proteins. Recent evidence suggests that those interactions often involve the recognition of ubiquitinated substrate by another protein by virtue of an ubiquitin-binding domain. Several ubiquitin binding domains have been identified, such as the UIM (ubiquitin interacting motif), UBA (ubiquitin associated) domain, CUE (coupling of ubiquitin conjugating to ER degradation) domain, the GAT (Gga and TOM1) domain, the PAZ (poly-ubiquitin-associated zinc finger) domain, the NZF (Npl4 zinc finger) motif, as well as the VHS (Vps27, HRS, STAM) and the GLUE (GRAM like ubiquitin-binding in Eap45) domains (Fisher *et al.*, 2003; Hicke *et al.*, 2005; Hofmann and Bucher, 1996; Shih *et al.*, 2003). The first ubiquitin-binding domain discovered was the UIM, present in the proteasomal protein S5a (Young *et al.*, 1998). Bioinformatics searches recorded that similar sequences are present in a wide range of proteins but appear to be prevalent in proteins involved in endocytosis and vacuolar protein sorting. This motif was shown to be able to both bind ubiquitin and to direct mono-ubiquitination of substrates (Polo *et al.*, 2002). The consensus for UIM's consists of Φ-x-x-alanine-x-x-x-serine-x-x-Ac, where Φ represents a large hydrophobic

residue, Ac an acidic residue and x any amino acid (Klapisz *et al.*, 2002). Although not included in the consensus, a region of around 4 acidic residues directly upstream of the large hydrophobic residue is present in most UIM's and appears to play an important role in ubiquitin recognition (Fisher *et al.*, 2003).

Using sequence alignments, we found that the fourth tetratricopeptide repeat (TPR) motif of *Saccharomyces cerevisiae* Pex5p (ScPex5p) contains a region that bears resemblance to an UIM, although it lacked an acidic region upstream of the consensus. Binding studies using ubiquitin and Pex5p were performed, but without success. However, we show that mutation of conserved residues in this consensus caused a limited growth on oleic acid phenotype and resulted in a build up of Pex5p on the peroxisomal membrane. We further characterised the effect these of mutations on the interactions between Pex5p and other proteins.

Materials and methods

Strains and culture conditions

Yeast strains used in this study are as follows: *S. cerevisiae* BJ1991 *(MATα, leu2, trp1, ura3-251, prb1-1122, pep4-3, gal2)* and PCY2 *(MATα, Δgal4, Δgal80, URA3::GAL1-lacZ, lys2-801, his3-Δ200, trp1-Δ63, leu2, ade2-101)*. Yeast transformations were performed as described in (Van der Leij *et al.*, 1993). Transformants were grown on minimal media containing 0.67% yeast nitrogen base (YNB), 2% glucose, 2% agar and amino acids (20 μg/ml) as required. Native lysates and sub-cellular fractionations were performed on cells grown at 28°C overnight in rich oleic acid media containing 0.1% oleic acid, 0.5% potassium phosphate buffer pH 6.0, 0.3% yeast extract, 0.5% peptone and 0.2% tween 40. For growth analysis, cells were grown on 0.67% YNB containing 0.3% glucose for at least 24 h and then shifted to 0.67% YNB media containing 0.5% oleic acid, 0.1% yeast extract and amino acids as required.

The *E. coli* strain DH5α *(recA, hsdR, supE, endA, gyrA96, thi-a, relA1, lacZ)* was used for all plasmid isolations. The *E. coli* strain BL21 RIL was used for the expression of MBP and GST fusion proteins. Cells transformed with bacterial expression constructs were grown at 37°C to an OD_{600} of 0.5 in LB medium supplemented with 1% glucose and antibiotics as required. Cells were then transferred to 28°C and induced with 1 mM IPTG

(Invitrogen) for 3-6 h. Cells were harvested by centrifugation for 20 min at 9,000 x g, washed with water and stored at -20°C.

Cloning procedures

The following plasmids have been described previously; pAN4, a fusion of the Gal4 activating domain (Gal4AD) and Pex5p (Klein *et al.*, 2001); pGB47, a fusion of the Gal4 DNA binding domain (Gal4DB) and Pex14p (Bottger *et al.*, 2000); pGB15, a fusion of Gal4DB and Pex13 SH3 domain (Bottger *et al.*, 2000); pDBMDH3, a fusion of Gal4DB and Mdh3p (Klein *et al.*, 2001); pEL128, a fusion of Gal4DB and ΔN-CAT2-ΔC (Elgersma *et al.*, 1995); pAN81, a fusion of Gal4DB and Pox1p (Klein *et al.*, 2002); pGST-Pex5p, encoding a fusion of glutathione-S-transferase (GST) and Pex5p (Bottger *et al.*, 2000); pTI98, a yeast plasmid for the expression of Pex5p from the PEX5 promoter (Klein *et al.*, 2002). The plasmid p1779, expressing VPS27 with a C-terminal HA tag was a generous gift from Dr. Piper (Bilodeau *et al.*, 2002). Details of primers used in this study can be found in Table I. Gal4AD fusion's of Pex5 $A_{449}L$ (pSI1), $L_{452}A$ (pSI2), $S_{453}D$ (pSI3) and $FL_{445}AA$ (pSI4) were produced using the QuikChange^{TM} site directed mutagenesis kit (Stratagene) with pAN4 as a template and the primer combinations PR82 and PR83, PR84 and PR85, PR86 and PR87 and PR80 and PR81, respectively. Plasmids for *in vivo* expressing of Pex5 UIM mutants under the control of the PEX5 promoter were constructed as follows: the plasmids pSI1, pSI2, pSI3 and pSI4 were digested with *BamHI* and *PstI* and the resulting fragments were ligated into BamHI-PstI digested pEL91 (Bottger *et al.*, 2000), creating pSA2, pSA3, pSA4 and pSA5, respectively. The individual $F_{445}A$ and $L_{446}A$ mutants were made in a similar way as the $FL_{445}AA$ double mutant and using the primer combinations PR90 and PR91 and PR92 and PR93, respectively. For the overexpression of the Pex5 $FL_{445}AA$ mutant, pSI4 was digested with *BamHI* and *PstI* and ligated into *BamHI-PstI* digested pEL43 (Elgersma *et al.*, 1993), creating Pex5 $FL_{445}AA$ under the control of the catalase A promoter (pSI9). Maltose binding protein (MBP) tagged ubiquitin (pAN106) was constructed by PCR on plasmid LHP276 (a generous gift from Dr. Hicke) using the primer combination PR71 and PR72 and the resulting fragment was digested with *EcoRI* and *HindIII* and cloned into *EcoRI-HindIII* digested pMalC2 (New England Biolabs). All constructs made using PCR were confirmed by sequencing.

Table I. Primers used in this study

Primer	Sequence (5'-3')	Comments
PR80	gtgcttaaattgtttgctagttgcgcagcttgctttgtgatatgagcg	Pex5 $FL_{445}AA$ (F)
PR81	cgctcatatcacaaagcaagctgcgcaactagcaaacaatttaagcac	Pex5 $FL_{445}AA$ (R)
PR82	caggatctattgtgcttaaattgtttagtagttgcaaaaattgctttgtg	Pex5 $A_{449}L$ (F)
PR83	cacaaagcaatttttgcaactactaaacaatttaagcacaatagatcctg	Pex5 $A_{449}L$ (R)
PR84	catagttgtatttcaggatctattgtgcttgcattgtttgctagttgc	Pex5 $L_{452}A$ (F)
PR85	gcaactagcaaacaatgcaagcacaatagatcctgaaatacaactatg	Pex5 $L_{452}A$ (R)
PR86	caagcatagttgtatttcaggatctattgtgtctaaattgtttgctagttgc	Pex5 $S_{453}D$ (F)
PR87	gcaactagcaaacaatttagacacaatagatcctgaaatacaactatgcttg	Pex5 $S_{453}D$ (R)
PR90	gtgcttaaattgtttgctagttgcaaagcttgctttgtgatatgagcg	Pex5 $F_{445}A$ (R)
PR91	cgctcatatcacaaagcaagctttgcaactagcaaacaatttaagcac	Pex5 $F_{445}A$ (F)
PR92	gtgcttaaattgtttgctagttgcgcaaattgctttgtgatatgagcg	Pex5 $L_{446}A$ (R)
PR93	cgctcatatcacaaagcaatttgcgcaactagcaaacaatttaagcac	Pex5 $L_{446}A$ (F)

Native lysates

Oleic acid grown cells expressing HA tagged VPS27 were washed and resuspended in column buffer (20 mM tris pH 7.4, 200 mM NaCl and 1 mM EDTA) containing 1 mM dithiothreitol (DTT), 1 mM PMSF and a yeast protease inhibitor cocktail (Sigma). Cells were pulse vortexed for 10 x 1 min using glass beads with intermediate cooling on ice for 1 min. Triton X-100 was added to a final concentration of 0.5% and lysates were incubated at 4°C for 20 min, followed by 2 x centrifugation at 17,500 x g. Protein concentrations were measured using the Bradford method with BSA as standard (Bradford, 1976).

Purification of E. coli expressed fusion proteins and binding studies

Pellets of cells expressing MBP, MBP-ubiquitin, GST or GST-Pex5p were thawed in column buffer, treated with 1 mg/ml lysozyme at 21°C for 30 min and pulse-sonicated on ice for 8 x 30 sec. Cell debris was removed by centrifugation at 4°C for 30 min at 9,000 x g. Lysates were loaded onto either amylose (New England Biolabs) or glutathione sepharose 4B (GE Healthcare) resin equilibrated with column buffer and incubated at 4°C for 1 h with agitation followed by washing with column buffer. 500 µl of amylose resin loaded with 500 µg of MBP/MBP-ubiquitin was mixed with 1 ml (7 mg/ml total protein concentration) of native yeast lysate, followed by incubation for 3 h at 4°C with agitation. The resin was washed with column buffer containing 0.5% triton X-100 and elution was

carried out using column buffer containing 10 mM maltose. Elution fractions were precipitated with 10% trichloroacetic acid (TCA) and the pellet was resuspended in laemmli sample buffer (LSB) and subjected to SDS-PAGE and immunoblotting using anti-Pex5p and the HA tag (12CA5). 70 µl of Glutathione sepharose 4B resin loaded with 10 µg GST, GST-Pex5p or GST-S5a (Affiniti) was incubated with 4 µg of ubiquitin chains (Affiniti) in column buffer containing 0.1% tween 20 for 1 h at 4°C with agitation. The resin was washed with column buffer containing 0.1% tween 20 and elution was carried out by heating the resin in 50 µl of LSB at 95ºC for 5 min. Samples were analysed by SDS-PAGE and immunoblotting using anti-GST (Sigma) and anti-ubiquitin (Biotrend).

Growth curves

Glucose grown cells were inoculated into minimal oleic acid medium to an OD_{600} of 0.05 and grown for 6 days at 28ºC. Samples of 1 ml were taken at 6, 24, 30, 48, 54, 72, 78 and 144 h. Samples were centrifuged at 10,000 rpm for 5 min and washed with 1 ml H_2O. The washing was repeated twice and the cells were resuspended in 1 ml H_2O and the OD_{600} value was measured.

Miscellaneous

Protocols for subcellular fractionation (Bottger *et al.*, 2000) and β-galactosidase enzyme activity determination (Klein *et al.*, 2001) have been described previously.

Results

Using sequence alignments, we identified a putative ubiquitin interacting motif (UIM) in the fourth TPR motif of *S. cerevisiae* Pex5p. Further analysis showed that certain residues in this motif are conserved in other Pex5p's, such as the large hydrophobic residue and the alanine, whereas others, such as the serine and the acidic residue at the end of the motif, are not well conserved (Fig. 1 A). We also observed other residues that are well conserved in this region of Pex5p, namely a leucine or phenylalanine residue adjacent to the large hydrophobic residue of the UIM consensus and a leucine adjacent to the serine of the UIM consensus (Fig. 1 A). In addition, secondary structure predictions using the program PredictProtein (http://cubic.bioc.columbia.edu/predictprotein) suggest that this region of Pex5p is largely α-helical, a common feature of UIM's.

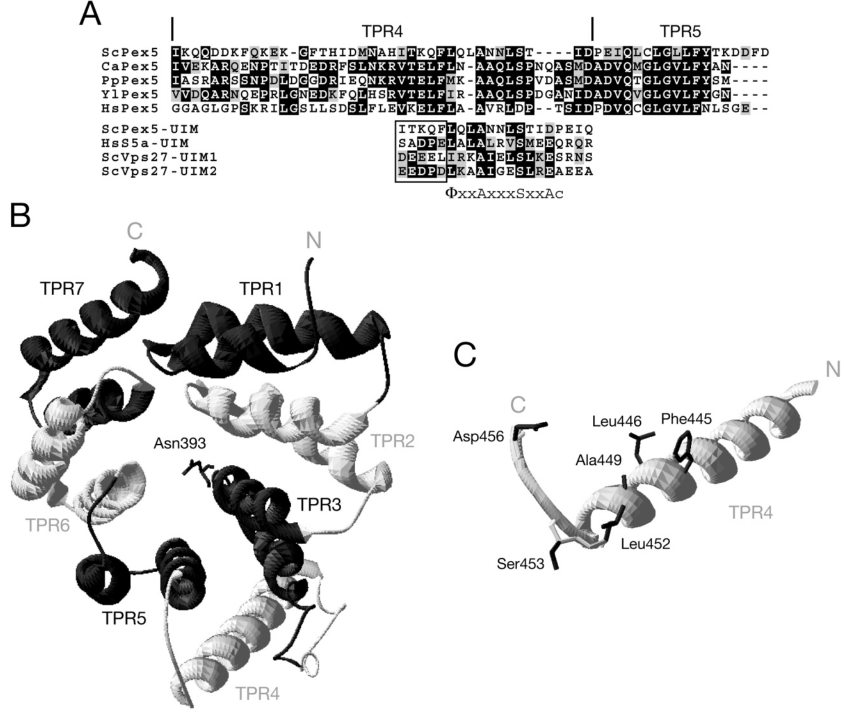


Fig. 1 The fourth TPR motif of ScPex5p contains a putative ubiquitin interacting motif (UIM)
A. Sequence alignment showing the fourth tetratricopeptide repeat (TPR) of Pex5p proteins from different species (*upper panel*) and the putative UIM present in ScPex5p aligned with the UIM's found in S5a and VPS27 (*lower panel*). The general UIM consensus is indicated, where Φ represents a large hydrophobic residue, x represents any residue and Ac represents an acidic residue. The acidic region present upstream in most UIM's is boxed. Sc; *Saccharomyces cerevisiae*, Ca; *Candida albicans*, Pp; *Pichia pastoris*, Hp; *Hansenula polymorpha*, Hs; *Homo sapiens*.
B. Predicted tertiary structure of the 7 TPR domains of ScPex5p, using the 3D-JIGSAW program (http://www.bmm.icnet.uk/servers/3djigsaw/html/form_v2.0.html). The asparagine at position 393 (**Asn393**), an important residue involved in the interaction with the PTS1 protein, is indicated.
C. Predicted tertiary structure of the fourth TPR domain of ScPex5p. The residues that make up the putative UIM are indicated.

Testing the binding of ubiquitin to Pex5p

To investigate if the putative UIM in ScPex5p was capable of interacting with ubiquitin, we tried to pull down Pex5p from a native yeast lysate using MBP-tagged ubiquitin (Fig. 2 A). As a positive control, lysates were made from cells expressing the well-characterised UIM containing protein VPS27, tagged at its C-terminal with a HA tag (Bilodeau *et al.*, 2002). This protein was shown to interact directly with ubiquitin through its UIM (Shih *et al.*, 2002). Fractions were analysed by coomassie brilliant blue (upper panels) and immunoblotting using anti-HA (middle panels) or anti-Pex5p (lower panels) antibodies.

Fig. 2 A shows that only VPS27-HA, but not Pex5p, could bind to MBP-ubiquitin while neither of these proteins bound to MBP alone. These results suggest that, under these specific conditions, Pex5p is not able to bind ubiquitin.

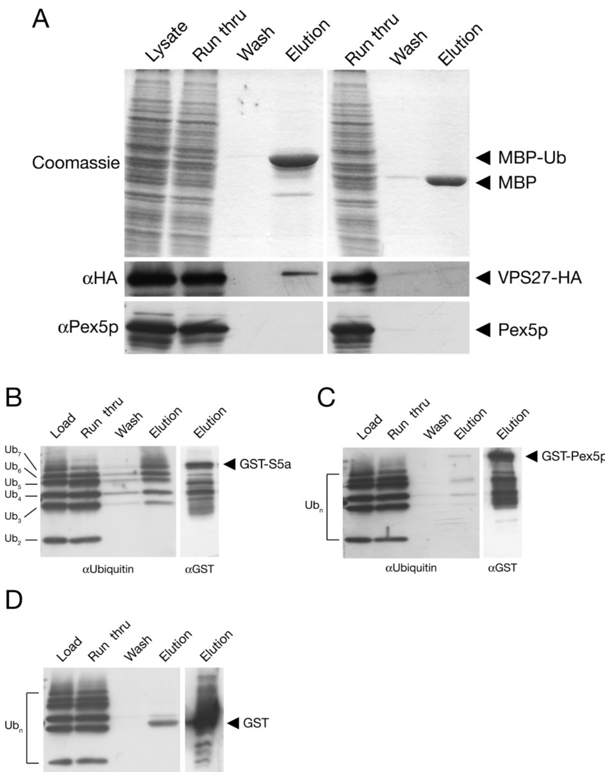


Fig. 2 Testing the putative ubiquitin interacting motif *in vitro*
A. Amylose beads loaded with either MBP-Ubiquitin (*left panels*) or MBP alone (*right panels*) were incubated with a native yeast lysate, thoroughly washed and bound proteins were eluted with maltose. Equivalent amounts of the run through, wash and elution fractions were analysed by coomassie brilliant blue staining (*upper panels*) and immunoblotting using antibodies against the HA tag (*middle panels*) or Pex5p (*lower panels*).
Glutathione beads loaded with GST-S5a (**B**), GST-Pex5p (**C**) or GST alone (**D**) were incubated with a mix of poly-ubiquitin chains, thoroughly washed and proteins were eluted with glutathione. Equivalent amounts of the run through, wash and elution fractions were analysed by immunoblotting with antibodies directed against ubiquitin (*left panels*) or GST (*right panels*).

It has been reported that certain ubiquitin interacting domains have preferences for ubiquitin chains rather than single ubiquitin moieties. The UIM containing protein S5a, an essential component of the 19S regulator of the 26S proteasome, has a preference for chains of more than 4 ubiquitin moieties (Deveraux *et al.*, 1994). Therefore, we performed a similar binding experiment, except that purified GST-Pex5p or GST-S5a was mixed with ubiquitin chains, consisting of between 2 and 7 ubiquitin moieties, linked via lysine 48. While we found significant binding of the ubiquitin chains to GST-S5a (Fig. 2 B) only a small amount of ubiquitin bound to GST-Pex5p (Fig. 2 C). However, similar amounts of ubiqutin bound to GST alone (Fig. 2 D). Furthermore, the anti-ubiquitin antibodies also recognised the GST tag (Fig. 2 C and D), which resulted in the signals seen in the GST and

GST-Pex5p elutions. Also, two-hybrid analysis of ubiquitin with full length and N-terminal deletion constructs of Pex5p failed to show an interaction between the two proteins (not shown).

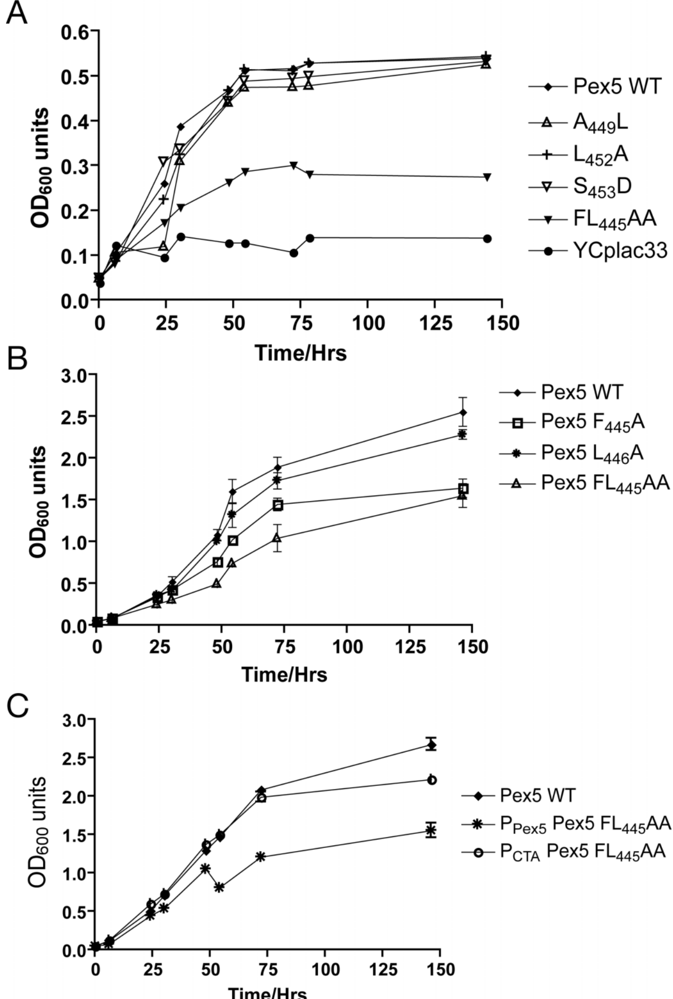


Fig. 3 Pex5p $FL_{445}AA$ shows reduced growth on oleic acid

A. *Pex5Δ* cells expressing wild type (**WT**) or mutant forms of Pex5p were grown overnight on medium containing 0.3% glucose and shifted the next morning to minimal oleic acid medium and grown for 6 days at 28°C. Samples of 1 ml were taken, the cells were washed and the OD_{600} was measured.

B. *Pex5Δ* cells expressing either wild type (**WT**) or mutant forms of Pex5p were grown as described in A.

C. *Pex5Δ* cells expressing wild type Pex5p (**WT**) or Pex5 $FL_{445}AA$ under control of either the Pex5 promoter (**P_{pex5}**) or the catalase A promoter (**P_{CTA}**) were grown as described in A.

Mutation of the conserved residues in the putative UIM in Pex5p

As no interaction could be detected between Pex5p and ubiquitin, we mutated the putative UIM residues in Pex5p, as well as the conserved leucine and phenylalanine residues (Fig. 1 A) and expressed these mutants in a *pex5Δ* strain, to determine their effect *in vivo*. Secondary and tertiary (Fig. 1 B and C) structure predictions suggest that this region of Pex5p is helical. We therefore made mutations that were predicted to retain the helical structure, but that would result in a considerable change to the residue in question.

Expression of the mutants Pex5 $A_{449}L$, $L_{452}A$ and $S_{453}D$ could rescue the growth of a *pex5Δ* strain on oleic acid, a carbon source requiring functional peroxisomes for growth (Fig. 3 A). However, the mutant Pex5 $FL_{445}AA$ could only partially complement the *pex5Δ* phenotype (Fig. 3 A). The individual mutants ($F_{445}A$ and $L_{446}A$) were constructed and tested in a similar way. Growth analysis revealed that the single $L_{446}A$ mutation had little effect on Pex5p function, while Pex5 $F_{445}A$ showed a similar phenotype to the $FL_{445}AA$ double mutant (Fig. 3 B), indicating that the phenotype of the double mutant is caused by the mutation of the phenylalanine residue. Overexpression of Pex5 $FL_{445}AA$, under the control of the strong catalase A promoter, almost completely restored the growth phenotype of a *pex5Δ* strain (Fig. 3 C).

Pex5 $FL_{445}AA$ localises to peroxisomes to a larger degree than wild type Pex5p

Further characterisation of the Pex5 $FL_{445}AA$ mutant was performed by organellar fractionation. *Pex5Δ* cells expressing either wild type (WT) Pex5p, Pex5 $FL_{445}AA$ or an empty vector (Vector) were homogenised and the 600 x g post nuclear supernatant was subjected to centrifugation at 17,500 x g. Equivalent volumes of the organellar pellet and supernatant fractions were analysed by immuno blotting with antibodies specific for catalase A (a PTS1 protein), 3-ketoacyl-CoA thiolase (a PTS2 protein) and Pex5p (Fig. 4). In both the wild type and $FL_{445}AA$ mutant, catalase A was predominantly present in the organellar pellet fraction. *Pex5Δ* cells containing an empty vector only (Vector) mislocalised a large portion of catalase A to the cytosol (Fig. 4, middle panel). 3-ketoacyl-CoA thiolase, a PTS2 protein and therefore, not dependent on Pex5p for its import into peroxisomes, was targeted to peroxisomes in all cases (Fig. 4, lower panel). Interestingly, unlike wild type Pex5p, the majority of the Pex5 $FL_{445}AA$ mutant was localised to the

peroxisomal fraction, rather than the cytosolic fraction (Fig. 4, upper panel) indicating that the mutation affects the distribution of Pex5p between the cytosol and peroxisome.

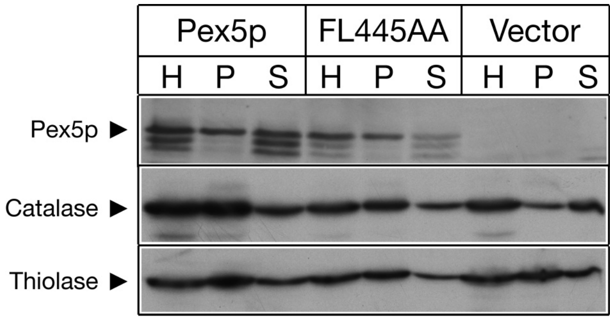


Fig. 4 Pex5 $FL_{445}AA$ associates more with the peroxisomal membrane than wild type Pex5p
Pex5Δ cells expressing wild type (**Pex5p**), Pex5 $FL_{445}AA$ (**$FL_{445}AA$**) or an empty vector (**Vector**) were subjected to subcellular fractionation. Equivalent volumes of the 600 x g post-nuclear supernatant (**H**), 17,500 x g organellar pellet (**P**) and 17,500 x g supernatant (**S**) were subjected to western blotting and staining with antibodies specific for Pex5p, catalase A (a PTS1 protein) or 3-ketoacyl-CoA thiolase (a PTS2 protein).

Pex5 $FL_{445}AA$ shows reduced binding with a range of Pex5p interacting proteins

To test the ability of the $FL_{445}AA$ to interact with several known Pex5p interacting partners, we performed two-hybrid analysis of the mutant and wild type forms of Pex5p against the docking factors Pex13p and Pex14p, the PTS1 containing protein malate dehydrogenase (MDH3) and the PTS3 containing proteins acyl-CoA oxidase (Pox1p) and carnitine acetyltransferase (CAT2). The ability of the $FL_{445}AA$ mutant to interact with all the proteins tested in this assay was significantly reduced (Fig. 5).

Discussion

We identified a putative ubiquitin interacting motif (UIM) in the fourth TPR motif of S*accharomyces cerevisiae* Pex5p. In this study, we tested this putative UIM in its ability to interact with ubiquitin as well as the effect of the mutations in the conserved residues on the overall functionality of Pex5p.

Using both purified proteins and native lysates we were unable to observe an interaction between Pex5p and ubiquitin (Fig. 2). We were also unable to show an interaction between these two proteins using two-hybrid analysis (not shown). This does not mean *per se* that there is no interaction between Pex5p and ubiquitin. Previous reports

have suggested that certain ubiquitin binding domains, such as UIM's and CUE domains, have very low binding affinity for ubiquitin but still play a role in ubiquitin recognition (Hurley *et al.*, 2006; Shih *et al.*, 2003). The interactions between these low affinity domains and ubiquitin are likely to be very transient.

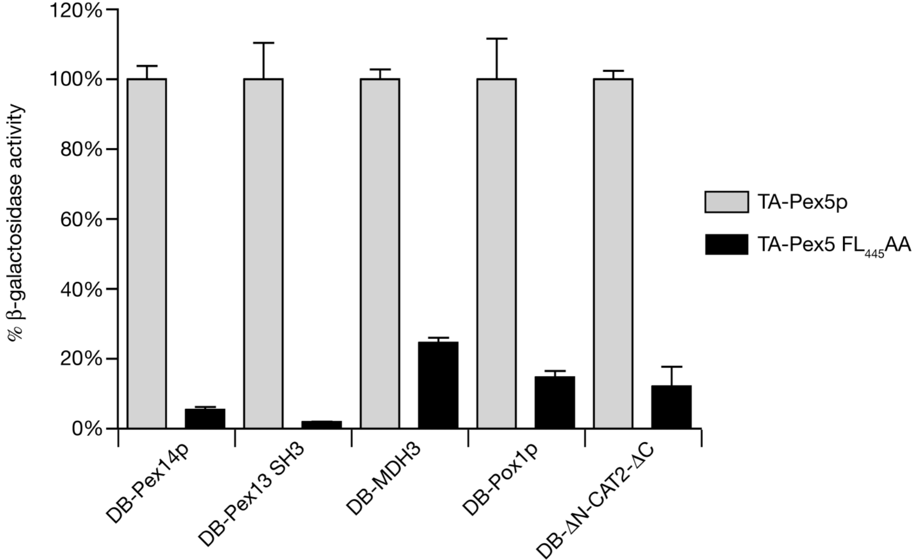


Fig. 5 Pex5 FL$_{445}$AA shows reduced interactions with Pex5p binding partners
Gal4AD (TA) Pex5p and Pex5 FL$_{445}$AA were tested in a two-hybrid assay against fusions of Gal4DB and Pex14p, the SH3 domain of Pex13p, MDH3 (a PTS1 protein), and the PTS3 proteins Pox1p and CAT2 (lacking both the N- and C-terminal regions). Activity of the reporter β-galactosidase (defined as absorbance at 420 nm per mg of protein per min) was used to determine the strength of interactions. Values correspond to the mean ± SD of four independent measurements. The strength of interaction of the wild type Pex5p with each binding partner was set to 100%. All Gal4AD fusions showed no interaction with the Gal4DB domain alone and visa versa (not shown).

Therefore, it is conceivable that the putative UIM of Pex5p has a very low affinity for ubiquitin, which is below the level of detection using the methods described here. However, the acidic region that is present upstream of nearly all UIM's, and which plays an important role in ubiquitin binding, is absent in the putative UIM of Pex5p. This single observation is rather suggestive of the fact that the putative UIM in Pex5p is either not functional or not a real UIM at all, and only bears some resemblance to UIMs.

To tackle this issue from the other end, we mutated the conserved residues in the putative UIM consensus as well as the conserved phenylalanine and leucine residues and analysed their effect *in vivo*. Only the double mutant FL$_{445}$AA showed a reduced growth on

oleic acid (Fig. 3 A), a phenotype that could be partially rescued when the protein was overexpressed (Fig. 3 C). This mutant was also observed to associate more with the peroxisomal fraction when analysed using fractionation (Fig. 4). Further analysis of Pex5 $FL_{445}AA$ using the two-hybrid system showed that this mutant's ability to interact with Pex5p binding partners was seriously disturbed (Fig. 5). We observed an effect on the mutant's ability to interact with proteins known to bind in both the N- (Pex14p, Pex13p, Pox1p and CAT2) and C-terminal (MDH3) of Pex5p. Such an effect across the board of interactions is indicative that the Pex5 $FL_{445}AA$ mutant may be unstable or incorrectly folded, as interactions in the N-terminal region of Pex5p are severely disturbed by mutations in the C-terminal. The fourth TPR motif of Pex5p, where these residues are found, does not adopt a typical TPR domain "helix-turn-helix" fold (D'Andrea and Regan, 2003) but is in a distorted arrangement (Stanley *et al.*, 2006) and is believed to act as a "hinge" between the other two clusters of TPR domains (Fig. 1 B). Mutation of the phenylalanine at position 445 may result in an incorrectly folded TPR4 domain, which in turn, could destabilise the rest of the TPR region and/or protein and cause a severe reduction in the ability to interact with other proteins. If this is indeed the case, it is curious that *in vivo* expression of the $FL_{445}AA$ mutant does not give a stronger phenotype, similar to a complete *pex5* knockout. Further questions that arise include why the $FL_{445}AA$ mutant localises more to peroxisomes that wild type Pex5p. If anything, a more cytosolic localisation would be expected, as the interactions with Pex14p and the SH3 domain of Pex13p, two docking proteins, were reduced to 5% and 2% of the wild type Pex5p, respectively. This change in localisation may represent a build up of Pex5p on the peroxisomal membrane that is not efficiently recycled back to the cytosol. We also observed that catalase A associated predominantly with the peroxisome fraction in the fractionation analysis, suggesting that PTS1 protein import was functional, although is conceivable that the catalase A associated with this fractionation was only present on the surface of the peroxisomes, still bound to Pex5p and, therefore not imported. Protease protection experiments would answer this question.

The recent observation, made by several groups, that Pex5p is both poly- and mono-ubiquitinated indicates that ubiquitin may play an important role in the function of Pex5p (Kiel *et al.*, 2005; Kragt *et al.*, 2005; Platta *et al.*, 2004). Poly-ubiquitination of Pex5p appears to be for the proteasomal breakdown of the protein. The function of Pex5p

mono-ubiquitination, on the other hand, appears to be involved in the recycling of Pex5p from the peroxisomal membrane (Platta *et al.*, 2007). A functional ubiquitin binding domain in Pex5p may function as a guide for the ubiquitination of the protein, as is seen with the UIM of Eps15p (Woelk *et al.*, 2006). This explanation would seen unlikely, as a C-terminal truncated version of Pex5p, lacking the entire TPR domain, can still be both poly- and mono-ubiquitinated (Williams *et al.*, 2007), indicating that the putative UIM is not essential for these modifications. We also analysed the ubiquitination status of the Pex5p mutants, and saw that all were both poly- and mono-ubiquitinated at levels comparable with the wild type protein (data not shown).

In conclusion, it seems unlikely that the putative UIM that we identified in ScPex5p indeed functions as an ubiquitin-binding domain, although the possibility cannot be completely ruled out. It is, however, a likely hypothesis that the TPR4 domain of Pex5p is structurally very important for the overall folding of the TPR region and/or the whole protein. Further analysis into this region is needed before further conclusions can be made.

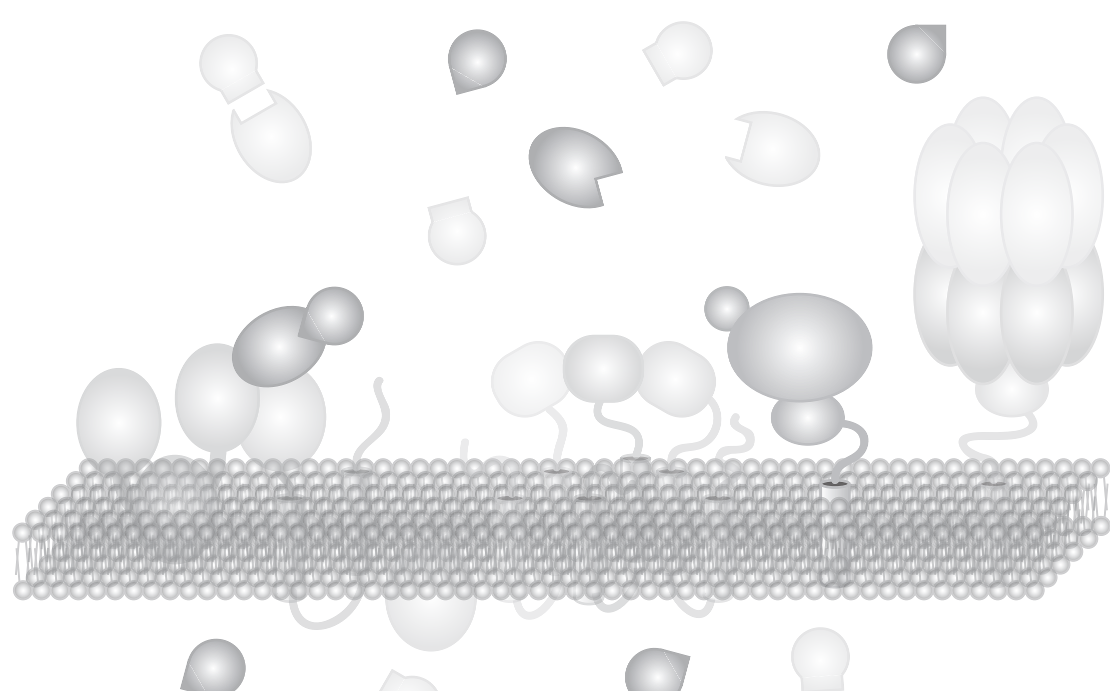
Acknowledgements

We are grateful to Dr. Piper for the p1779 plasmid and Dr. Hicke for the plasmid LHP276.

A conserved cysteine is essential for Pex4p-dependent ubiquitination of the peroxisomal import receptor Pex5p

Chris Williams, Marlene van den Berg, Richard Sprenger and Ben Distel

Department of Medical Biochemistry, Academic Medical Center,
Amsterdam, The Netherlands



Abstract

The peroxisomal protein import receptor Pex5p is modified by ubiquitin, both in an Ubc4p-dependent and -independent manner. Here we show that the two types of ubiquitination target different residues in the N-terminal region of Pex5p and we identify Pex4p (Ubc10p) as the ubiquitin-conjugating enzyme required for Ubc4p-independent ubiquitination. While Ubc4p-dependent ubiquitination occurs on two lysine residues, Pex4p-dependent ubiquitination requires neither lysine residues nor the N-terminal α-NH_2 group. Instead, a conserved cysteine residue appears to be essential for both the Pex4p-dependent ubiquitination and the overall function of Pex5p. In addition, we show that this form of ubiquitinated Pex5p is susceptible to the reducing agent β-mercaptoethanol, a compound that is unable to break ubiquitin-NH_2 group linkages. Together, our results strongly suggest that Pex4p-dependent ubiquitination of Pex5p occurs on a cysteine residue.

Introduction

Conjugation of ubiquitin to a substrate protein is a well-conserved process in eukaryotic cells, sequentially involving an ubiquitin-activating enzyme (E_1), an ubiquitin conjugating enzyme (E_2) and an ubiquitin ligase (E_3) (Hershko and Ciechanover, 1998), while ubiquitin chain elongation sometimes requires the action of an additional conjugation factor called E_4 (Koegl *et al.*, 1999). The effect of ubiquitination on a particular protein is, in part, determined by the length of the ubiquitin chain. 'Poly-ubiquitination', i.e. the attachment of 4 or more ubiquitin moieties, typically results in degradation of the substrate by the 26S proteasome (Thrower *et al.*, 2000), whereas 'mono-ubiquitination', comprising the linkage of 1-3 ubiquitins, usually has a non-proteolytic function, e.g. inducing a change in activity or cellular location (or both) (Hicke, 2001). Many important cellular processes, such as DNA repair, ER-retrotranslocation, endocytosis, cell division and apoptosis are regulated by the poly- or mono-ubiquitination of participating proteins (for review, see Mukhopadhyay and Riezman, 2007). Not surprisingly, defective ubiquitination has been implicated in the etiology of important human diseases, such as neurodegenerative disorders and cancer.

In the large majority of cases, ubiquitin appears to be conjugated to the ε-NH_2 group of a lysine residue in the substrate protein, while in a limited number of proteins the N-terminal α-NH_2 group is used as a conjugation site (Ben-Saadon *et al.*, 2004; Bloom *et al.*, 2003; Breitschopf *et al.*, 1998). Recently, however, it was reported that the ubiquitination of a lysine-less C-terminal tail of the MHC class I heavy chain was dependent on the presence of a cysteine residue, suggesting that ubiquitin conjugation is not restricted to NH_2 groups and that the SH-group of a cysteine may also serve as a target (Cadwell and Coscoy, 2005). The frequency of this novel mode of ubiquitination and the functional and mechanistic differences (if any) with ubiquitination on NH_2 groups remains to be established.

A recent addition to the list of processes potentially regulated by ubiquitination is that of the import of proteins into peroxisomes. Peroxisomes are eukaryotic organelles with a wide range of functions, two of which, β-oxidation of long chain fatty acids and H_2O_2 detoxification, are very well conserved throughout evolution (for review, see Van den Bosch *et al.*, 1992). Peroxisomes post-translationally import all their matrix enzymes with the aid of a peroxisomal targeting signal (PTS) type one, two or three. Proteins that contain

a PTS signal are recognised in the cytosol by their corresponding cycling receptor (Pex5p for PTS1/3 proteins and Pex7p for PTS2 proteins) and transported to the peroxisomal membrane, where docking takes place. The PTS protein is then released into the peroxisomal matrix and the receptor is recycled to the cytosol for another round of import (reviewed in Purdue and Lazarow, 2001a). So far, 32 Pex proteins (called peroxins) have been identified, with around 12 being directly involved in protein import (Distel *et al.*, 1996). Characteristic features of some of these twelve proteins seem to point at a role for ubiquitin in the import process. Pex4p, one of the first peroxins characterised (Wiebel and Kunau, 1992), belongs to the E_2 family of ubiquitin conjugating enzymes. Pex4p is associated with the peroxisomal membrane (Crane *et al.*, 1994; Koller *et al.*, 1999) and genetic experiments have suggested that it functions in the late steps of protein import (Collins *et al.*, 2000). Additionally, there are three membrane-localised peroxins, Pex2p, Pex10p and Pex12p each possessing a RING finger domain (Albertini *et al.*, 2001; Chang *et al.*, 1999), the hallmark of a specific class of E_3 ligases (Joazeiro and Weissman, 2000).

Indeed, two ubiquitinated peroxins have recently been identified: Pex5p, the PTS1 receptor (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004) and Pex18p/Pex20p which act as co-receptors in the PTS2 pathway (Leon *et al.*, 2006b; Purdue and Lazarow, 2001b). Two distinct forms of Pex5p ubiquitination have been reported, one of which is dependent on the E_2 enzyme Ubc4p, while the other one is not. Ubc4p-dependent ubiquitination is only observed in certain pex deletion strains, namely *pex4*, *pex22*, *pex1*, *pex6* and *pex15* (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). These mutants are blocked at a stage where Pex5p is normally recycled from the peroxisome membrane to the cytosol (Collins *et al.*, 2000). Such a situation seems to trigger the ubiquitination of Pex5p, which accumulates at the peroxisomal membrane (Kiel *et al.*, 2005a; Platta *et al.*, 2004). The available evidence suggests that Ubc4p-dependent ubiquitination serves a quality control function, priming Pex5p that is unable to recycle for proteasomal degradation (Kiel *et al.*, 2005a; Platta *et al.*, 2004). For this reason, Ubc4-dependent Pex5p ubiquitination has been referred to as ‘poly-ubiquitination’, although in this particular case only one to four ubiquitin residues are attached. As mentioned above, this type of ubiquitination is also observed in a *pex4* deletion strain, demonstrating that Pex4p (Ubc10p) is not involved. In wild type cells, on the other hand, Pex5p is transiently ubiquitinated at the peroxisome membrane, a process that has been shown to be independent of Ubc4p

(Kragt *et al.*, 2005b). In view of the fact that only two ubiquitin molecules seemed to be attached, which did not affect the stability of Pex5p, this type of ubiquitination was called 'mono-ubiquitination'. The function of this second form of ubiquitination and the E_2 enzyme responsible however, remained enigmatic (Kragt *et al.*, 2005b).

Here we report that the two forms of Pex5p ubiquitination target different amino acid residues within the N-terminal region of the protein. While Ubc4p-dependent ubiquitination occurs on two lysines, Ubc4p-independent ubiquitination does not require lysine residues or the N-terminal α-NH_2 group. Instead, a conserved cysteine residue in the N-terminal domain is absolutely essential for this modification. Mutation of this cysteine not only blocks Ubc4p-independent ubiquitination, but also results in a non-functional Pex5p. In addition, we show that the E_2 enzyme Pex4p is involved in the Ubc4p-independent ubiquitination of Pex5p.

Materials and methods

Strains, media and culture conditions

The *E. coli* strain DH5α (*recA, hsdR, supE, endA, gyrA96, thi-a, relA1, lacZ*) was used for all plasmid isolations. The following yeast strains were used in this study: *S. cerevisiae* BJ1991 *pex5Δ* (*MATα; pex5::KanMX4, leu2, ura3-251, trp1, prb1-1122, pep4-3, gal2*), BJ1991 *pex4Δpex5Δ* (*MATα, pex4::KanMX4, pex5::LEU2, leu2, trp1, ura3-251, prb1-1122, pep4-3, gal2*) and BJ1991 *pex5Δpex6Δ* (*MATα, pex5::KanMX4, pex6::LEU2, leu2, trp1, ura3-251, prb1-1122, pep4-3, gal2*). Yeast transformations were performed as described in (Van der Leij et al., 1993). Transformants were grown on minimal medium containing 0.67% yeast nitrogen base (YNB, Difco), 2% glucose, 2% agar and amino acids (20 μg/ml) as required. For immunoprecipitations, trichloroacetic acid (TCA) lysates and sub cellular fractionations, cells were grown on 0.67% YNB containing 0.3% glucose for at least 24 h and then shifted to 0.1% oleic acid medium containing 0.5% potassium phosphate buffer pH 6.0, 0.3% yeast extract, 0.5% peptone and 0.2% tween 40 and grown for 7-16 h. TCA lysates for Pex5 1-308 and controls (Fig. 1 A) were prepared from cells grown overnight on 0.67% YNB containing 0.3% glucose. $CuSO_4$ (100 μM final concentration) was added to cultures for expression of CUP1 promoter-controlled myc-

ubiquitin. Oleate plates contained 0.67% YNB, 0.1% oleic acid, 0.5% tween 40, 2% agar, 0.1% yeast extract and amino acids (20 μg/ml) as required.

Plasmids

Plasmids used in this study are listed in Table I. All plasmids, except the myc-Ub expressing plasmids, are low-copy shuttle vectors that are maintained in 1-2 copies per cell. Further details of plasmids are available on request. Pex5p site directed mutants were constructed using either the QuikChange^{TM} site-directed or multi site-directed mutagenesis kits (Stratagene) and confirmed by sequencing. The plasmid YEP105, expressing myc-tagged ubiquitin was a generous gift from Dr. Ellison (Ellison and Hochstrasser, 1991).

Table I. Plasmids used in this study

Name	Promoter	Comments	Reference
pTi98	PEX5	wild type Pex5	(Klein *et al.*, 2002)
YEP105	CUP1	myc tagged Ubiquitin	(Ellison and Hochstrasser, 1991)
pCW122	PEX5	Pex5 C_6R	This study
pCW127	CUP1	6 x myc tagged Ubiquitin	This study
pCW131	CUP1	myc tagged Ubiquitin	This study
pCW138	PEX5	fusion of HsPex5p 1-41 and ScPex5 43-612	This study
pCW145	PEX5	Pex5 C_6R K_0N	This study
pCW80	Catalase	Pex5 1-308-His_6	This study
pMB23	Catalase	Pex5 1-308	This study
pMB35	PEX5	Pex5 $K_{18/24}R$	This study
pMB36	PEX5	Pex5 $K_{18/24/31}R$	This study
pMB37	PEX5	Pex5 $K_{31/46/81}R$	This study
pMB38	PEX5	Pex5 $K_{81/112/142}R$	This study
pMB39	PEX5	Pex5 $K_{210/213/238/244}R$	This study
pMB40	PEX5	Pex5 $K_{238/244/266/289}R$	This study
pMB41	PEX5	Pex5 $K_{142/193/210/213}R$	This study
pMB42	PEX5	Pex5 $K_{193/210/213/227}R$	This study
pMB74	PEX5	Pex5 K_0N	This study
pMB94	PEX5	Pex5 1-308	This study
pMB95	PEX5	Pex5 1-308 K_0	This study
pMB112	PEX5	HsPex5 $C_{11}R$/Sc	This study
pMB113	PEX5	HsPex5 K_0N/Sc	This study
pMB114	PEX5	HsPex5 $C_{11}R$ K_0N/Sc	This study

Immunoprecipitation

Oleate-grown cells (20 A_{600} units) co-expressing myc tagged ubiquitin and wild type or mutant forms of Pex5p were lysed with glass beads in 5% TCA and precipitates were resuspended in 175 µl 50 mM tris pH 7.5, 6 M urea and 1% SDS and heated to 65°C for 10 min. Undissolved material was pelleted and 1.75 ml of IP-tween buffer (50 mM tris pH 7.5, 150 mM NaCl, 0.5% tween 20 and 0.1 mM EDTA) was added, containing 0.1% bovine serum albumin (BSA), 20 mM N-ethylmaleimide (NEM), 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Sigma). Lysates were first pre-cleared with 20 µl of Protein-A sepharose (GE Healthcare) and then incubated with 5 µl rabbit polyclonal Pex5p antiserum and 50 µl of Protein-A sepharose beads for 2 h at 4°C. Precipitates were washed 3 times with IP-tween buffer, twice with IP-urea buffer (100 mM tris pH 7.5, 2 M urea, 200 mM NaCl and 0.5% tween-20) and twice with TBS buffer (50 mM tris pH 7.5 and 150 mM NaCl) and elution was carried out by heating the beads in 25 µl IP-elution buffer (125 mM tris pH 6.8, 1.5% SDS, 6 M urea and 20% glycerol) for 10 mins at 65°C. Samples were analysed by SDS-PAGE and immunoblotting. The antibodies used for immunoprecipitation or immunoblotting were anti-Pex5p (raised in our own laboratory, rabbit polyclonal) and anti-myc (Cell Signalling technology, Inc., mouse monoclonal). For anti-myc immunoblotting analysis, 10 µl of the elution fraction was used. For anti-Pex5p analysis, 1 µl of the elution fraction was diluted in 20 µl IP-elution buffer containing 50 mM dithiothreitol (DTT) and heated at 37°C for 5 mins. Subsequently, 5 µl of this sample was used for SDS-PAGE and immunoblotting.

Purification of Pex5 1-308 His_6

Oleate-grown cells (300 A_{600} units) expressing Pex5 1-308 with a C-terminal His_6 tag were lysed with glass beads in lysis buffer (75 mM tris pH 7.4, 200 mM NaCl, 15 mM NaF, 1.5 mM Na_3VO_4, 5 mM β-mercaptoethanol, 20 mM NEM, 1% triton X-100, 0.5% octyl β-D-glucopyranoside (OGP, Sigma), 1 mM PMSF and protease inhibitor cocktail). Guanidine-HCl was added to a final concentration of 6 M and undissolved material was removed by centrifugation at 10,000 x g. The lysate was passed over Ni- NTA resin (Qiagen) and sequentially washed with buffers W1 (lysis buffer containing 6 M guanidine-HCl), W2 (lysis buffer containing 2 M guanidine-HCl) and W3 (75 mM tris pH 7.4, 200 mM NaCl, 5

mM β-mercaptoethanol, 1% triton X-100, 0.5% OGP and 1 mM PMSF). Elution from the resin was carried out using buffer W3 containing 330 mM imidazole and the sample was concentrated using an Amicon™ Ultra centrifugal filter (Millipore) and analysed by SDS-PAGE and coomassie brilliant blue staining (Serva).

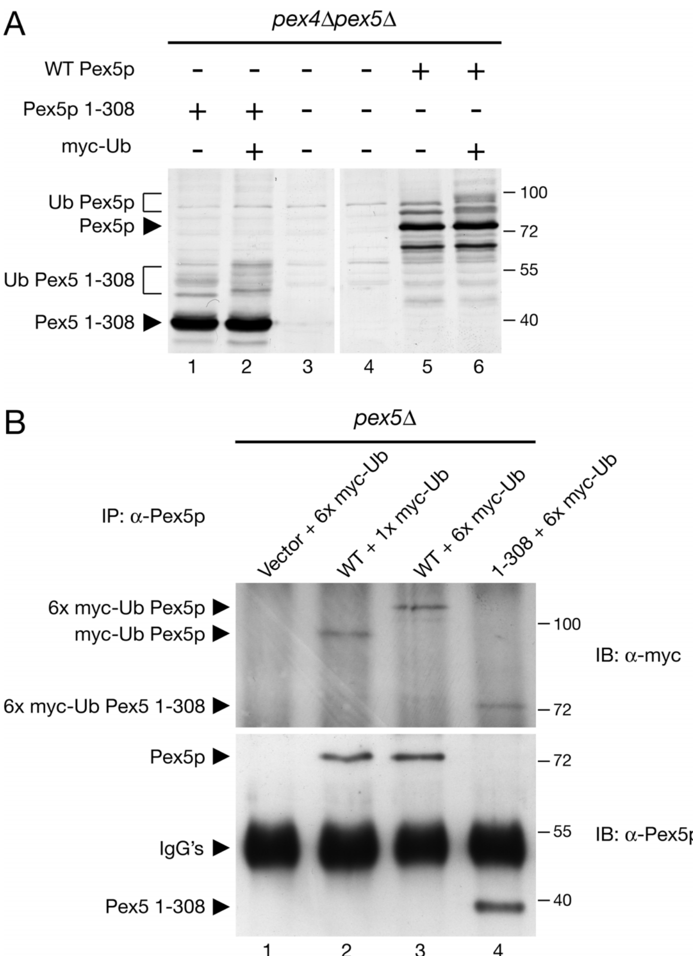


Fig. 1 The first 308 amino acids of Pex5p are sufficient for both Ubc4-dependent and -independent ubiquitination

A. *Pex4Δpex5Δ* cells bearing a plasmid expressing myc-tagged ubiquitin (**+**) or a control vector (**-**) and co-expressing either wild type (**WT**) Pex5p or a deletion construct consisting of the first 308 amino acids of Pex5p (**Pex5 1-308**), were lysed and cell extracts were analysed by SDS-PAGE and anti-Pex5p immunoblotting.

B. *Pex5Δ* cells co-expressing myc-tagged or 6x myc-tagged ubiquitin and either wild type Pex5p (**WT**), Pex5p 1-308 or a control vector (**Vector**) were lysed and Pex5p was precipitated using anti-Pex5p anti-serum (**IP**). Immunoprecipitates were analysed with SDS-PAGE and immunoblotting (**IB**) using antibodies raised against the myc-tag and Pex5p.

Mass spectrometry

Coomassie stained bands were excised from gel, treated with DTT and iodoacetamide in order to block the thiol groups on cysteine residues by carbamidomethylation, and digested overnight with sequence grade trypsin (Roche Molecular Biochemicals). Peptides were extracted as described in (Sprenger *et al.*, 2004) and analysed by peptide mass fingerprinting and peptide sequencing, using a QSTAR-XL equipped with an oMALDI interface (Applied Biosystems/MDS Sciex, Toronto, Canada). The resulting peptide spectra were used to search the MASCOT search engine (http://www.matrixscience.com).

Treatment of immunoprecipitates with reducing agent

Immunoprecipitation was performed as described above except that 150 µl of CNBr activated sepharose beads (GE Healthcare) conjugated with polyclonal Pex5p antiserum was used. Beads were treated for 10 mins at 65°C with 25 µl IP-elution buffer without urea and either lacked reducing agent (control) or contained 10% β-mercaptoethanol. For anti-myc immunoblotting, 10 µl of the elution was used. For anti-Pex5p immunoblotting, 1 µl of the elution was diluted in 20 µl IP-elution buffer without urea and 5 µl of this dilution was used.

Miscellaneous

The preparation of TCA lysates, SDS-PAGE and immunoblotting has been described previously (Kragt *et al.*, 2005b).

Results

The N-terminal 308 amino acids of Pex5p are sufficient for ubiquitination

The N-terminal half of Pex5p contains the binding sites for the docking proteins Pex13p and Pex14p and, therefore, is required for the association of the protein with the peroxisomal membrane (Bottger *et al.*, 2000; Saidowsky *et al.*, 2001; Schäfer *et al.*, 2004). As membrane association is also essential for the ubiquitination of Pex5p (Kragt *et al.*, 2005b; Platta *et al.*, 2004), we examined whether a C-terminal truncated version of Pex5p, consisting of the first 308 amino acids (Pex5 1-308) could still be ubiquitinated. To assess Ubc4p-dependent ubiquitination, wild type (WT) Pex5p or Pex5 1-308 constructs were expressed in a *pex4Δpex5Δ* strain containing either a vector expressing myc-tagged

ubiquitin or a control vector. Next, total protein lysates were prepared and analysed by immunoblotting with anti-Pex5p antibodies (Fig. 1 A). In cells expressing a control vector, two slower migrating bands could be observed in addition to the major Pex5p and Pex5p 1-308 species (lanes 1 and 5), patterns that are characteristic for Ubc4p-dependent ubiquitination in a *pex4Δ* strain (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). Indeed, these slower migrating bands shifted upon overexpression of myc-ubiquitin in the cell (lanes 2 and 6), confirming that the Pex5 1-308, like wild type Pex5p, is ubiquitinated.

Previously, we have shown that in wild type cells Pex5p is transiently ubiquitinated in an Ubc4p-independent manner (Kragt *et al.*, 2005b). To detect this low abundance ubiquitinated form of Pex5p, an immunoprecipitation assay was developed using cells expressing myc-tagged ubiquitin (Kragt *et al.*, 2005b). *Pex5Δ* cells expressing either wild type Pex5p, Pex5 1-308 or a control vector and myc-tagged ubiquitin were subjected to immunoprecipitation with anti-Pex5p antibodies and analysed by anti-Pex5p and anti-myc immunoblotting (Fig. 1 B). In the blot probed with anti-myc antibodies, a single band typical for Ubc4p-independent ubiquitination was detected in cells expressing either wild type Pex5p (lanes 2 and 3) or Pex5 1-308 (lane 4), but not in the empty vector control (lane 1). Together, these results show that the first 308 amino acids of Pex5p contain the target residues of both Ubc4p-dependent and -independent ubiquitination.

Ubc4p-dependent ubiquitination of Pex5p requires lysines 18 and 24 but Ubc4p-independent ubiquitination is not reliant on lysine residues

Having identified the likely region involved in the ubiquitination of Pex5p, we reverted to using the full-length protein for further analysis, because the 1-308 construct does not complement the *pex5Δ* strain as it lacks the essential PTS1 binding region (Klein *et al.*, 2001; Terlecky *et al.*, 1995). Since the conjugation of ubiquitin to a substrate is usually via a lysine residue, we mutagenized all 15 lysines present in the N-terminal 308 amino acids of Pex5p to arginines, in combinations of two, three or four lysine residues at a time. Next, the constructs were tested in their ability to undergo both forms of ubiquitination. Mutation of lysines 18 and 24 resulted in a severe reduction in the slower migrating Pex5p bands when expressed in a *pex4Δ/pex5Δ* (Fig. 2 A) or *pex5Δ/pex6Δ* (not shown) strain, indicating that Ubc4p-dependent ubiquitination was inhibited. No other combinations of lysine mutations resulted in a loss of ubiquitination (Fig. 2 A), suggesting that these two residues

are the main targets. Construction of the individual mutants Pex5p $K_{18}R$ and $K_{24}R$ revealed that lysine 24 is the main target for Ubc4p-dependent ubiquitination, but that lysine 18 to a certain extent can also act as a target (not shown). This is in line with other recent work, in which the ubiquitination of *H. polymorpha* Pex5p and *P. pastoris* Pex20p in a *pex4Δ* strain was shown to be dependent on lysine residues present in the N-terminal region of the proteins (Kiel *et al.*, 2005b; Leon *et al.*, 2006b).

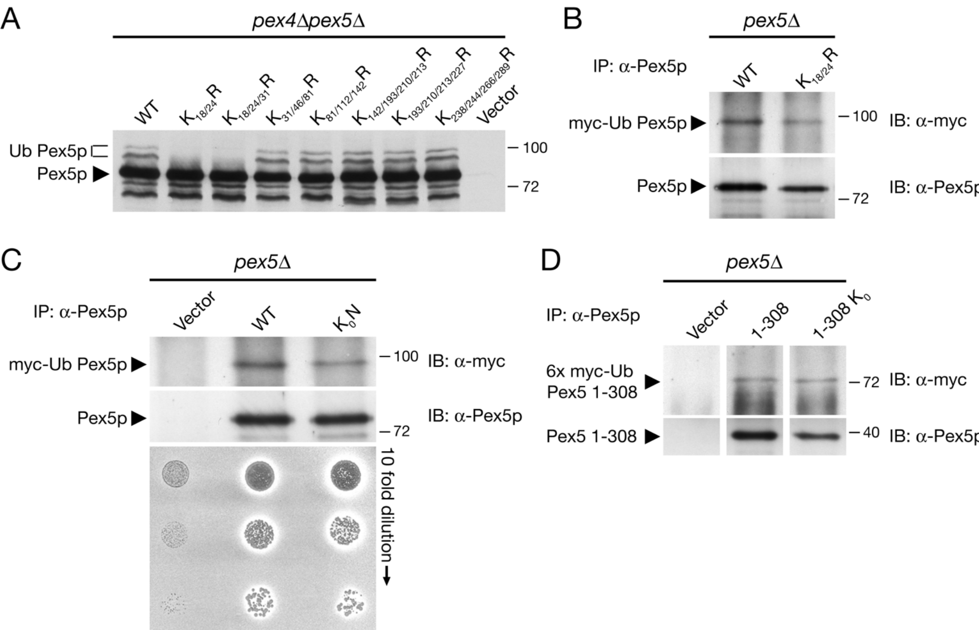


Fig. 2 Ubc4p-dependent ubiquitination of Pex5p requires lysines 18 and 24 but Ubc4p-independent ubiquitination is not reliant on lysine residues

A. Equal amounts of *pex4Δpex5Δ* cells expressing either wild type (**WT**) or lysine mutant forms of Pex5p were lysed and cell extracts were separated by SDS-PAGE and analysed by anti-Pex5p immunoblotting.

B. Total lysates of *pex5Δ* cells co-expressing myc-tagged ubiquitin and either wild type or the lysine 18/24 to arginine mutant form of Pex5p were subjected to immunoprecipitation (**IP**) and immunoblotting (**IB**) as described in Fig. 1.

C. *Pex5Δ* cells co-expressing myc-tagged ubiquitin and either wild type Pex5p (**WT**), a mutant form of Pex5p lacking N-terminal lysine residues (**K_0N**) or an empty vector (**Vector**) were lysed and subjected to immunoprecipitation (**IP**) and immunoblotting (**IB**) as described in Fig. 1 (*upper panels*) or spotted onto plates containing oleic acid as sole carbon source and grown for 7 days at 28°C (*lower panel*).

D. *Pex5Δ* cells co-expressing 6x myc-tagged ubiquitin and either the first 308 amino acids of Pex5p (**1-308**), Pex5 1-308 without lysine residues (**1-308 K_0**) or an empty vector (**Vector**) were lysed and subjected to immunoprecipitation (**IP**) and immunoblotting (**IB**) as described in Fig. 1.

Remarkably, Ubc4p-independent ubiquitination was not blocked in the $K_{18/24}R$ mutant form of Pex5p (Fig. 2 B) or in all the other lysine mutants tested (not shown). All of these Pex5p lysine mutants were functional, as they could rescue the growth of a *pex5Δ* strain on oleic acid, a carbon source requiring peroxisomes for its metabolism (not shown). To test whether any of the remaining lysine residues in the N-terminal region could act as a target for Ubc4p-independent ubiquitination, we made a version of Pex5p in which all the lysines in this domain were mutated to arginines (K_0N). Surprisingly, this mutant was still ubiquitinated, although at a lower level than the wild type Pex5p (Fig. 2 C, upper panels) and was able to complement the *pex5Δ* phenotype, indicating that the K_0N Pex5p is functional (Fig. 2 C, lower panel). Because it cannot be ruled out that the lysines still present in the C-terminal region of the Pex5p construct that was used in Fig. 2 C may have become targets for ubiquitin conjugation, we checked the extent of ubiquitination of a lysine-less N-terminal fragment (1-308 K_0). The 1-308 K_0 fragment was ubiquitinated to a level comparable to that of the lysine-containing 1-308 control construct (Fig. 2 D), indicating that the Ubc4p-independent ubiquitination of Pex5p does not occur on lysine residues.

A conserved cysteine residue near the N-terminus is essential for Ubc4p-independent ubiquitination and function of Pex5p

Conjugation of ubiquitin to the α-NH_2 group on the N-terminus of the polypeptide backbone has been observed for a number of proteins, providing a clear example of a non-lysine linkage (Ciechanover and Ben-Saadon, 2004). In principle, Ubc4p-independent ubiquitination of Pex5p could also be targeted to the N-terminal NH_2 group. However, analysis of Pex5p using the TermiNator program (http://www.isv.cnrs-gif.fr/Terminator) predicts the N-terminal NH_2 group to be acetylated, a modification that would prevent ubiquitin conjugation on the α-NH_2 group. In order to analyze its acetylation status, we purified Pex5 1-308 using a C-terminal hexa-histidine tag and excised the most prominent band from gel for trypsin digestion followed by mass spectrometry analysis (Fig. 3 A). Database searches and peptide sequencing revealed that the majority of peptides recovered corresponded to Pex5 1-308-His_6 with a total coverage of 80%. Nevertheless, an N-terminal peptide with an unmodified α-NH_2 group was not detected. Instead, a peptide was found that corresponded to a carbamidomethylated N-terminal peptide, but with an extra 42 Da

(Fig. 3 A, 1968.9 MH^+). Carbamidomethylation (CAM, see Fig. 3 B) is the result of treatment of the peptides with iodoacetamide, while the 42 Da increase in mass is consistent with an extra acetyl group being attached to the peptide. Peptide sequencing analysis revealed that this additional mass is present on the first methionine residue, as the b-ion series (N-terminal containing fragments), but not the y-ion series (C-terminal containing fragments) show the 42 Da increase (Fig. 3 B). These data indicate that the α-NH_2 group on the first methionine residue of Pex5p is acetylated, effectively blocking it for ubiquitin conjugation.

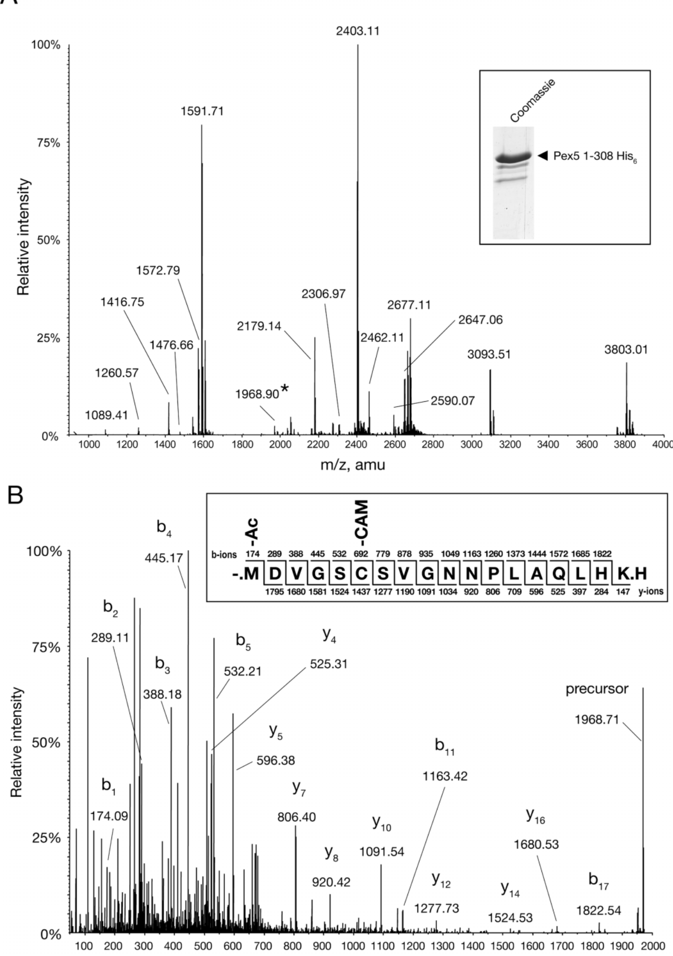
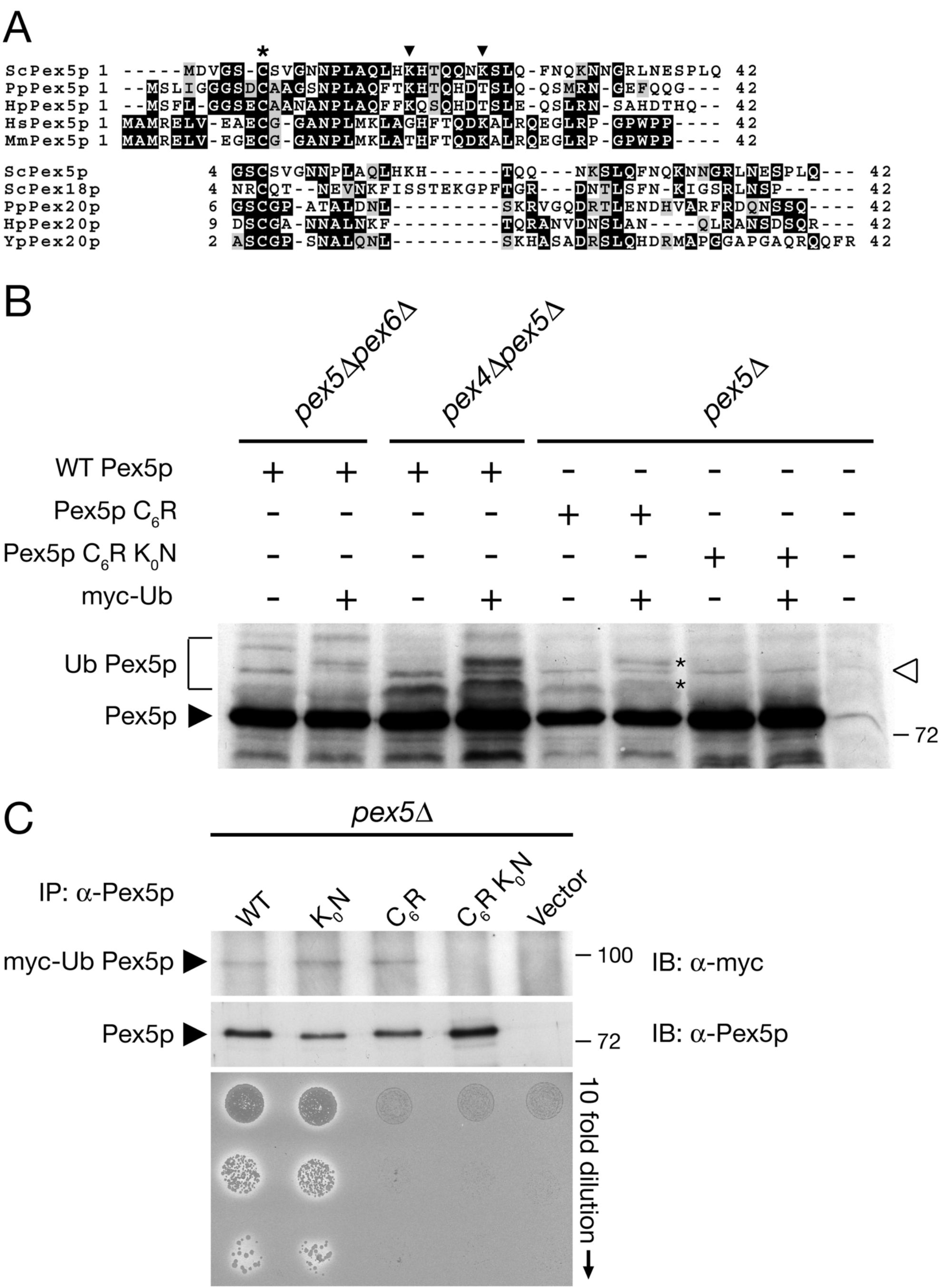


Fig. 3 The N-terminus of Pex5p is acetylated
A. MALDI-TOF spectrum of a tryptic digest of purified Pex5 1-308-His_6 (*inset*). The molecular masses (monoisotopic MH^+) of abundant Pex5p peaks are indicated. The acetylated, carbamidomethylated N-terminal fragment is labelled with an asterisk.
B. Peptide sequence of the 1968.9 MH^+ fragment indicated in A. Fragments representing b and y ions are shown. Ac – acetyl group. CAM – Carbamidomethyl group.



A
ScPex5p 1 -----MDVGS-CSVGNNPLAQLHKHTQQNKSLQ-FNQKNNGRLNESPLQ 42
PpPex5p 1 --MSLIGGGSDCAAGSNPLAQFTKHTQHDTSLQ-QSMRN-GEFQQG--- 42
HpPex5p 1 --MSFL-GGSECAANANPLAQFFKQSQHDTSLE-QSLRN-SAHDTHQ-- 42
HsPex5p 1 MAMRELV-EAECG-GANPLMKLAGHFTQDKALRQEGLRP-GPWPP---- 42
MmPex5p 1 MAMRELV-EGECG-GANPLMKLATHFTQDKALRQEGLRP-GPWPP---- 42
ScPex5p 4 GSCSVGNNPLAQLHKH------TQQ---NKSLQFNQKNNGRLNESPLQ--- 42
ScPex18p 4 NRCQT--NEVNKFISSTEKGPFTGR---DNTLSFN-KIGSRLNSP------ 42
PpPex20p 6 GSCGP-ATALDNL---------SKRVGQDRTLENDHVARFRDQNSSQ---- 42
HpPex20p 9 DSCGA-NNALNKF---------TQRANVDNSLAN----QLRANSDSQR--- 42
YpPex20p 2 ASCGP-SNALQNL---------SKHASADRSLQHDRMAPGGAPGAQRQQFR 42
B
pex5Δpex6Δ
pex4Δpex5Δ
pex5Δ
WT Pex5p
Pex5p C6R
Pex5p C6R K0N
myc-Ub
Ub Pex5p
Pex5p
72
C
pex5Δ
IP: α-Pex5p
WT
K0N
C6R
C6R K0N
Vector
myc-Ub Pex5p
Pex5p
100
72
IB: α-myc
IB: α-Pex5p
10 fold dilution

Fig. 4 A conserved cysteine residue in the N-terminal region of Pex5p is crucial for Ubc4p-independent ubiquitination and function
A. Sequence alignment showing the N-terminal 42 amino acids of a number of Pex5p (*upper panel*) and Pex18/20p (*lower panel*) proteins from different species. * Indicates the conserved cysteine residue. Arrowheads indicate lysine residues 18 and 24 in *S. cerevisiae* Pex5p. Sc; *Saccharomyces cerevisiae*, Pp; *Pichia pastoris*, Hp; *Hansenula polymorpha*, Hs; *Homo sapiens*, Mm; *Mus musculus*, Yp; *Yarrowia lipolytica*.
B. *Pex5Δ*, *pex4Δpex5Δ* or *pex5Δpex6Δ* cells bearing a plasmid expressing myc-tagged ubiquitin (+) or a control vector (-) and co-expressing either wild type Pex5p (**WT**), Pex5p cysteine mutant (**C_6R**) or Pex5p C_6R without N-terminal lysines (**C_6R K_0N**) were lysed and cell extracts were analysed by SDS-PAGE and anti-Pex5p immunoblotting. The open arrowhead indicates a band that cross-reacts with the anti-Pex5p antibody. * Indicates the myc-ubiquitin conjugated Pex5p species in the C_6R mutant protein.
C. *Pex5Δ* cells co-expressing myc-tagged ubiquitin and either wild type Pex5p (**WT**), Pex5p lacking N-terminal lysines (**K_0N**), Pex5p with the conserved cysteine residue mutated to an arginine (**C_6R**) or a similar construct without N-terminal lysines (**C_6R K_0N**) were lysed and subjected to immunoprecipitation (**IP**) and immunoblotting (**IB**), as described in Fig.1 (*upper panels*) or spotted onto plates containing oleic acid as the sole carbon source and grown for 7 days at 28°C (*lower panel*).

Recently, ubiquitination on a non-NH_2 group of a protein was reported, which appeared to target the SH group of a cysteine residue (Cadwell and Coscoy, 2005). Interestingly, sequence alignment analysis of Pex5p from various species shows a well-conserved cysteine residue in the N-terminal region of the protein, while the N-terminus of all members of the Pex20p family also harbours a conserved cysteine (Fig. 4 A). To assess the importance of this cysteine (C_6 in *S. cerevisiae* Pex5p) in Ubc4p-independent ubiquitination and Pex5p function, we replaced it with an arginine (Fig. 4 B and C), alanine, tryptophane or a serine (not shown). None of the cysteine point mutants were able to complement the growth of a *pex5Δ* strain on oleic acid (Fig. 4 C, lower panel and data not shown), a phenotype that was caused by the inability of the mutant proteins to import PTS1 proteins (Fig. 5). Immunoblot analysis of total lysates showed that the pattern of ubiquitination of the C_6R mutant was similar to that of wild type Pex5p in a *pex4Δ* mutant (Fig. 4 B). These data suggest that although the C_6R mutant does not import PTS1 proteins, it still associates with the peroxisomal membrane and is ubiquitinated in an Ubc4p-dependent manner. The introduction of the cysteine mutation into Pex5p lacking lysines 18 and 24 (C_6R $K_{18/24}$R, not shown) or all N-terminal lysines (C_6R K_0N) resulted in a significant reduction of the ubiquitinated Pex5p bands (Fig. 4 B, compare C_6R with C_6R K_0N). However, these mutants were unable to rescue the *pex5Δ* phenotype (Fig. 4 C, lower panel and Fig. 5) indicating that the presence of the cysteine residue is essential for the function of Pex5p and that the growth phenotype of the C_6R mutant was not caused by Ubc4p-dependent ubiquitination of the protein.

To directly assess the role of the conserved cysteine residue in Ubc4p-independent ubiquitination, we immunoprecipitated different Pex5p mutants from cells expressing myc-ubiquitin and analysed ubiquitination by anti-myc immunoblotting (Fig. 4 C). While the lysine-less Pex5p mutant (K_0N) was still modified, this modification was no longer visible when the conserved cysteine was mutated to arginine (C_6R K_0N). The ubiquitinated species seen in the C_6R mutant most likely represents Ubc4p-dependent modification on lysines. Together, the results suggest that the conserved cysteine residue plays a crucial role in the Ubc4p-independent ubiquitination of Pex5p.

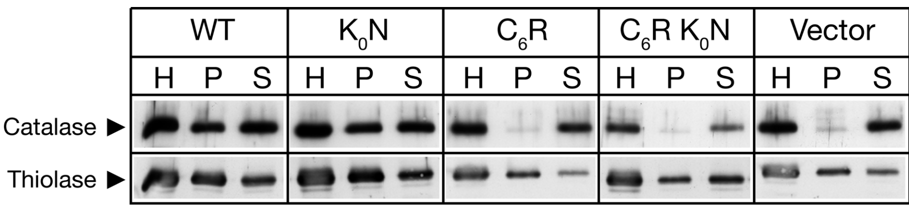


Fig. 5 PTS1 import requires the presence of the conserved cysteine residue in Pex5p
Pex5Δ cells expressing wild type Pex5p (**WT**), Pex5p lacking N-terminal lysines (**K_0N**), Pex5p with the conserved cysteine residue mutated to an arginine (**C_6R**) or a similar construct without N-terminal lysines (**C_6R K_0N**) or an empty vector (**Vector**) were subjected to subcellular fractionation. Equivalent volumes of the 600 x g post-nuclear supernatant (**H**), 100,000 x g organellar pellet (P) and 100,000 x g supernatant (**S**) were subjected to western blotting and staining with antibodies specific for the PTS1 protein catalase A (*upper panels*) and the PTS2 protein 3-ketoacyl-CoA thiolase (*lower panels*).

The N-terminal domain of human Pex5p can functionally replace that of S. cerevisiae Pex5p and ubiquitination of the chimeric protein requires the conserved cysteine

The sequence conservation of the N-terminal 35-40 amino acids in the Pex5p proteins, including the strictly conserved cysteine residue (Fig. 4 A), suggests that this part of the protein serves an important function and may be sufficient for Ubc4p-independent ubiquitination of Pex5p. We therefore replaced the first 42 amino acids of *S. cerevisiae* (Sc) Pex5p with the first 41 amino acids from human Pex5p (Hs/Sc), in which the conserved cysteine residue is present at position 11, and analysed both the ubiquitination status and the functionality of the chimeric protein. Remarkably, the chimeric Pex5p was modified at a level comparable to that of ScPex5p, and could fully complement the *pex5Δ* phenotype (Fig. 6). Again, mutation of all lysines in the Pex5p chimera (Hs K_0N/Sc) did not block ubiquitination and the mutant protein could restore growth of the *pex5Δ* strain on oleic acid, albeit incompletely. The reduced amounts of the mutant protein present in the cell may

account for this phenotype (Fig. 6). Mutation of the cysteine residue either in the Pex5p chimera (Hs C_{11}R/Sc) or in the lysine less chimera (Hs C_{11}R K_0N/Sc) resulted in an almost complete inhibition of ubiquitination and rendered the protein non-functional. These data re-emphasise the fact that the conserved cysteine residue is critical for both Ubc4p-independent ubiquitination and function of Pex5p.

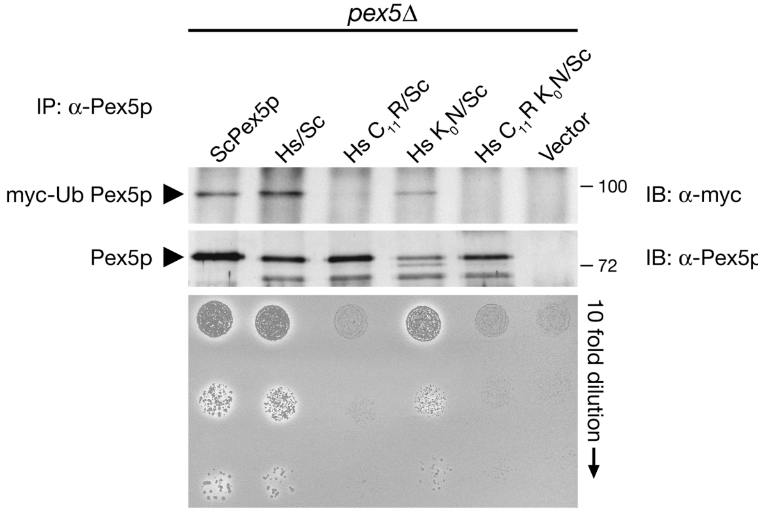


Fig. 6 The N-terminal domain of human Pex5p can functionally replace that of *S. cerevisiae* Pex5p and is ubiquitinated in a cysteine-dependent manner
Pex5Δ cells co-expressing myc-tagged ubiquitin and either wild type *S. cerevisiae* Pex5p (**ScPex5p**), a chimeric Pex5p constructs containing the first 41 amino acids from human Pex5p (**Hs/Sc**) or the same chimera with cysteine 11 mutated to arginine (**HsC_{11}R/Sc**), the chimera lacking the N-terminal lysines (**HsK_0N/Sc**) or lacking both the N-terminal lysines and cysteine 11 (**HsC_{11}R K_0N/Sc**) were lysed and subjected to immunoprecipitation (**IP**) and immunoblotting (**IB**) as described in Fig. 1 (*upper panels*), or spotted onto plates containing oleic acid as the sole carbon source and grown 7 days at 28°C (*lower panel*).

The ubiquitinated K_0N form of Pex5p is susceptible to β-mercaptoethanol

The results obtained so far imply that the conserved cysteine residue in the N-terminal domain of Pex5p can function as a site for ubiquitin attachment. Conjugation of ubiquitin to a cysteine residue would result in the formation of a thioester bond between the COOH group of the terminal glycine residue in ubiquitin and the SH group of the cysteine residue. This bond, which is also the type of linkage E_1 and E_2 enzymes form with ubiquitin, can be broken by the reducing agent β-mercaptoethanol (β-me), while a ubiquitin-lysine linkage (iso-peptide or amide bond) is not susceptible to β-me (Cadwell and Coscoy, 2005). We

compared the effect of β-me on the two different forms of ubiquitinated Pex5p. Treatment of immunoprecipitates of the K_0N form of Pex5p with β-me drastically reduced the amount of ubiquitinated Pex5p (Fig. 7 A). In contrast, the levels of lysine-linked ubiquitinated Pex5p, isolated from the *pex4Δ* strain, were unaffected by this treatment (Fig. 7 B). The data clearly show that the ubiquitin linkage in the K_0N form of Pex5p behaves as a thioester bond and not as an amide bond, adding strong support for the conserved cysteine being the conjugation site for Ubc4p-independent ubiquitination.

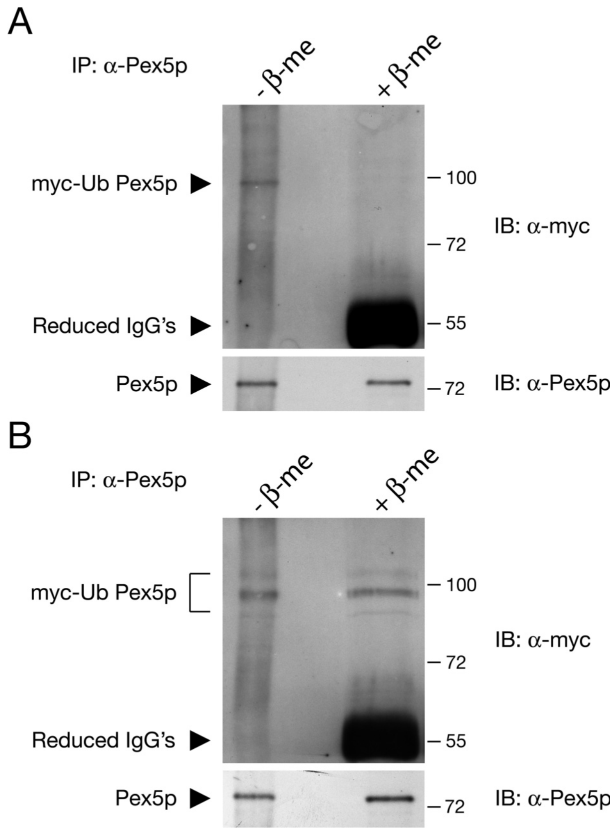


Fig. 7 The ubiquitinated K_0N form of Pex5p is susceptible to ß-mercaptoethanol
Immunoprecipitation analysis (**IP**) was performed on either *pex5Δ* cells expressing Pex5p lacking N-terminal lysine residues (K_0N) (**A**) or *pex4Δpex5Δ* cells expressing wild type Pex5p (**B**). Immunoprecipitates were subjected to treatment with buffer without reducing agent (**- β-me**) or containing 10% β-mercaptoethanol (**+ β-me**). Samples were analysed by SDS-PAGE and immunoblotting (**IB**) as described in Fig. 1. IgG's are only observed in the presence of β-me, due to the reduction of the disulphide bonds present between the heavy and light chains. In the absence of β-me, IgG's remain conjugated to the CNBr beads.

Ubc4p-independent ubiquitination of Pex5p requires Pex4p

Previously, it has been shown that ubiquitination of Pex5p in the deletion strains *pex4Δ*, *pex22Δ*, *pex1Δ*, *pex6Δ* and *pex15Δ* is dependent on the E_2 enzyme Ubc4p (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). The E_2 responsible for the ubiquitination of Pex5p in wild type cells, however, has not yet been identified although Pex4p (Ubc10p) is a

very likely candidate (Crane *et al*., 1994; Wiebel and Kunau, 1992; Van der Klei *et al*., 1998). Our observation that Ubc4p-dependent ubiquitination of Pex5p could be efficiently blocked by mutation of the lysine residues present in the N-terminal region (Fig. 2), allows a direct test of the involvement of Pex4p in Ubc4p-independent ubiquitination. To this end, the ubiquitination patterns of the Pex5p K_0N mutant were compared to those of wild type Pex5p (WT), both in a *pex4Δpex5Δ* and a *pex5Δpex6Δ* strain, using immunoprecipitation analysis (Fig. 8). The results show that the lysine-less Pex5p mutant is only ubiquitinated in the *pex5Δpex6Δ* strain and not in the *pex4Δpex5Δ* strain (Fig. 8, K_0N), conditions in which wild type Pex5p displayed the expected ubiquitination patterns (Fig. 8, WT). These data demonstrate that Ubc4p-independent ubiquitination of Pex5p requires the presence of Pex4p, and that Pex6p is not involved. We also checked the ubiquitination status of the Pex5p cysteine mutants in the *pex4Δpex5Δ* strain. The absence of the cysteine residue did not significantly affect Ubc4p-dependent ubiquitination (Fig. 8, C_6R), ubiquitinated Pex5p species being present even at a somewhat higher level than in the experiment with wild type Pex5p (Fig. 8, WT). Ubc4p-dependent ubiquitination appeared to be completely blocked, however, by the absence of lysines in the N-terminal region of this cysteine mutant (Fig. 8, C_6R K_0N). From these results, we conclude that the conserved cysteine residue in Pex5p is essential for Pex4p-dependent ubiquitination, but plays no role in Ubc4p-dependent ubiquitination.

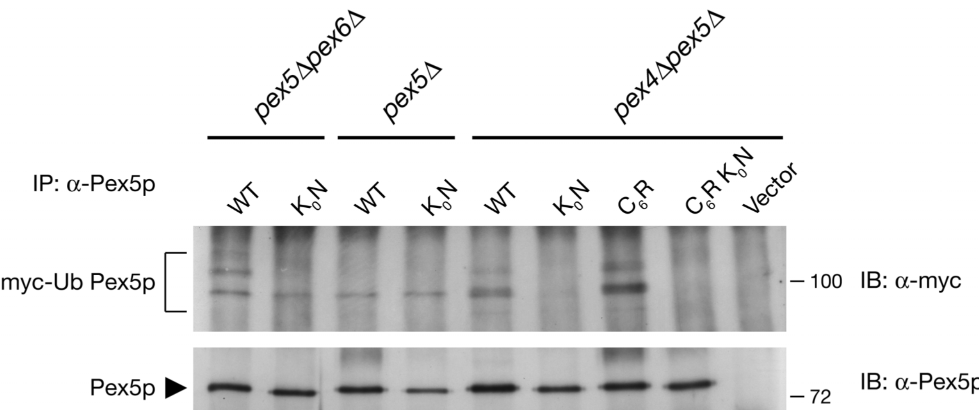


Fig. 8 Ubc4p-independent ubiquitination of Pex5p requires Pex4p
Pex5Δ, *pex4Δpex5Δ* and *pex5Δpex6Δ* cells co-expressing myc-tagged ubiquitin and either wild type (**WT**) or mutant forms of Pex5p were subjected to immunoprecipitation (**IP**) and immunoblotting (**IB**) as described in Fig. 1.

Discussion

The PTS1 import receptor *S. cerevisiae* Pex5p is modified by ubiquitin in two clearly distinct ways, only one of which is dependent on the E_2 enzyme Ubc4p. In this study, we have shown that these different ubiquitination processes target different residues in the conserved N-terminal domain of Pex5p. While Ubc4p-dependent ubiquitination occurs on two lysines present at positions 18 and 24, Ubc4p-independent ubiquitination targets a well-conserved and essential cysteine residue at position 6. In addition, we show that only the cysteine-targeted ubiquitination requires the E_2 enzyme Pex4p. Together, these findings support the idea that these two different ubiquitination processes represent two distinct mechanisms involved in the regulation of Pex5p.

Ubc4p-dependent ubiquitination

Ubc4p-dependent ubiquitination of *S. cerevisiae* Pex5p is observed in certain *pex* deletion strains, namely *pex4Δ*, *pex22Δ*, *pex1Δ*, *pex6Δ and pex15Δ*. This form of ubiquitination has previously been described as “poly-ubiquitination” (see introduction) and is seen as a ladder of 1-4 slower migrating Pex5p bands in SDS-PAGE (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). The proteins Pex1p, Pex4p, Pex6p, Pex15p and Pex22p are believed to have functions late in the import cycle, possibly in the recycling of Pex5p from the peroxisomal membrane to the cytosol (Collins *et al.*, 2000; Platta *et al.*, 2005). Therefore, Ubc4p-dependent ubiquitination in these deletion strains might represent an attempt to overcome a block in recycling by removing Pex5p from the membrane (Kiel *et al.*, 2005a; Kragt *et al.*, 2006; Platta *et al.*, 2004). However, removal and degradation of ubiquitinated Pex5p appears to be inefficient as it builds up at the membrane (Kragt *et al.*, 2005b; Platta *et al.*, 2004). Studies performed in other organisms have shown that in the absence of the same peroxins, levels of Pex5p, as well as the PTS2 import protein Pex20p are severely diminished (Dodt and Gould, 1996; Koller *et al.*, 1999; Leon *et al.*, 2006b), suggesting degradation of the proteins via the 26S proteasome. Whether degradation is observed or not, a question that remains is whether this form of ubiquitination is essential for the function of these proteins in wild type cells. Platta and co-workers suggested an important role for Ubc4p-mediated ubiquitination in Pex5p function, as a deletion of Ubc4p and Ubc5p, two redundant E_2 enzymes, resulted in slow growth and a PTS1 import defect (Platta *et al.*, 2004). The identification of the two lysines (Lys18 and 24) in Pex5p that are

used as targets of Ubc4p-dependent ubiquitination (Fig. 2) allowed us to address the function of this modification in wild type cells. Our data show that the replacement of these lysines by arginine residues does not affect the function of the protein (Fig. 2), which suggests that Ubc4p-dependent ubiquitination of Pex5p does not play an essential role in the import of PTS1 proteins. This is in line with previous work in other organisms, where lysine residues present in the N-terminal region of *H. polymorpha* Pex5p (Kiel *et al.*, 2005b) and *P. pastoris* Pex20p (Leon *et al.*, 2006b) at positions 21 and 19, respectively, were shown to be essential for Ubc4p-dependent ubiquitination, but their mutation had no effect on protein function. Interestingly, sequence alignments of the N-terminal region of different Pex5p and Pex20p family members (Fig. 4) shows that, in all cases, a lysine residue is present in the first 25 amino acids. It appears, therefore, that Ubc4p-dependent ubiquitination of the (co-) receptors Pex5p and Pex20p on lysines is a conserved, but non-essential, process that is activated in certain mutants blocked in a step at which these receptors are recycled. Whether the protein is degraded by the proteasome seems to depend on the organism.

Ubc4p-independent ubiquitination

We have shown that Ubc4p-independent ubiquitination of Pex5p also occurs in the N-terminal 308 amino acids of the protein but does not require lysine residues (Fig. 2). This raised the question as to which other residue(s) could potentially act as an attachment site for ubiquitin. Our data point towards a well-conserved cysteine residue that is essential for both Ubc4p-independent ubiquitination and function of Pex5p. First, mutation of the cysteine (Cys_6) in the lysine-less K_0N mutant blocked Ubc4p-independent ubiquitination and resulted in a protein no longer able to rescue the *pex5Δ* phenotype (Fig. 4). Second, by swapping the N-terminal region of ScPex5p with that of HsPex5p, we again showed that the presence of a cysteine residue, cysteine 11 in human Pex5p, is essential for both the Ubc4p-independent ubiquitination and receptor function (Fig. 6). Finally, and most significantly, biochemical studies showed that the ubiquitin linkage in the lysine-less form of Pex5p is susceptible to the reducing agent β-mercaptoethanol, while ubiquitin-lysine bonds are not (Fig. 7). The theoretical possibility remains that the cysteine does not act as the final conjugation site but is involved in the transfer of ubiquitin to a side-chain of another residue in Pex5p, analogous to the transfer of ubiquitin from the active site cysteine

of an E_2 enzyme to the substrate. Transfer to a lysine seems unlikely because none of the lysine mutants had a phenotype, whereas mutation of the cysteine alone resulted in a non-functional protein. If the residue involved in ubiquitin transfer is essential, one might expect the residue(s) that receive the ubiquitin to be, likewise, essential. In the absence of any lysines, in Pex5p K_0N, other potential targets would include the α-NH_2 group or serine, threonine and tyrosine residues that could form ester bonds with ubiquitin through their hydroxyl groups. The α-NH_2 group is a highly unlikely conjugation site, as we have shown that it is blocked by acetylation (Fig. 3), which is a co-translational modification (Polevoda and Sherman, 2003). Even if a small portion of Pex5p remains un-acetylated, the susceptibility of the ubiquitinated Pex5p K_0N to β-me indicates that the linkage between Pex5p and ubiquitin is not an amide bond, the type of bond formed between NH_2 groups and ubiquitin. The same argument applies to an ester bond that would be formed between ubiquitin and a serine, threonine or tyrosine residue as this type of linkage would also not be susceptible to β-me. We attempted to isolate the ubiquitinated forms of Pex5p for mass spectrometric analysis to confirm the role of the cysteine residue as the conjugation site, but we were unable to isolate sufficient amounts of modified Pex5p. This may be due to the weak nature of the thioester bond between ubiquitin and Pex5p.

Our data strongly suggest that the conserved cysteine residue in Pex5p represents the conjugation site for ubiquitin. So far, ubiquitination on a cysteine has only been suggested for the MHC class I heavy chain, a reaction that is catalysed by a viral E_3 ligase (Cadwell and Coscoy, 2005). Our data would represent the first example of cysteine ubiquitination performed by the cellular ubiquitination machinery. Why a cysteine, rather than a more standard lysine residue, is the preferred conjugation site remains to be investigated. We speculate that the timely removal of the ubiquitin from Pex5p may represent an important step in the import cycle, a process that may occur at a faster rate when ubiquitin is linked to a cysteine, as thioester bonds are more labile than iso-peptide linkages. The small amounts of ubiquitinated Pex5p that are present at any one time in the cell are in line with this suggestion, although it cannot be ruled out that the liability of the thioester bond to reduction may also hamper the detection.

Which step in the import cycle is regulated by Ubc4p-independent ubiquitination of Pex5p remains to be addressed, although two recent observations suggest a role in the recycling of Pex5p from the peroxisomal membrane to the cytosol. First, Costa-Rodrigues

and co-workers (Costa-Rodrigues *et al.*, 2004) showed that the extreme N-terminal 17 amino acids of human Pex5p containing the conserved cysteine, are essential for the release of the receptor from the peroxisome membrane. Second, the group of Subramani (Leon and Subramani, 2007) suggested that the conserved cysteine residue near the N-terminus of *P. pastoris* Pex20p (cysteine 8) is required for cytosolic relocation of peroxisomal Pex20p. Although modification of the cysteine residue in Pex20p was not addressed in this article, it is conceivable that this cysteine is also a target for ubiquitination.

Using subcellular fractionation, we have, thus far, been unable to show an accumulation of the C_6R K_0N or the C_6R mutants at the peroxisomal membrane, suggesting that cysteine ubiquitination of Pex5p may be required for another important step in the import cycle, for example PTS1 cargo release/delivery. The severe PTS1 protein import defect observed in the C_6R and C_6R K_0N mutants (Fig. 5) is in line with this suggestion, while a deficiency in Pex5p recycling would be expected to result in a milder PTS1 protein import defect (Collins *et al.*, 2000). We are currently investigating these possibilities further. Irrespective of its possible function, the highly conserved nature of the cysteine residue in both Pex5p and the Pex20p families implies that cysteine ubiquitination is not restricted to yeast's but may also occur in other organisms.

Pex4p: the E_2 required for Ubc4p-independent ubiquitination

Our finding that the E_2 enzyme Pex4p (Ubc10p) is required for the Ubc4p-independent ubiquitination of Pex5p resolves a long-standing debate about the possible substrates of this ubiquitin-conjugating enzyme. Following its identification in the early 1990's as a genuine E_2 enzyme, ubiquitinated peroxins were not identified until nearly 10 years later (Platta *et al.*, 2004; Purdue and Lazarow, 2001b). All potential Pex4p substrates belong to the two families of cycling (co)-receptors involved in either the PTS1 (Pex5p) or PTS2 (Pex20/Pex18p) protein import pathways. However, the notion that in the absence of Pex4p, Pex5p and Pex20p are still ubiquitinated by another E_2 (Ubc4p) has cast serious doubts on the role of Pex4p in ubiquitination of these proteins (Kiel *et al.*, 2005b; Leon *et al.*, 2006b; Platta *et al.*, 2004). By blocking ubiquitination on lysines, through mutation of the target residues, we now show that Pex4p is required for the Ubc4p-independent ubiquitination of Pex5p (Fig. 8).

In a *pex6*Δ strain, Pex4p-dependent ubiquitination of Pex5p is undisturbed (Fig. 8), an observation that explains why in this strain and, by inference, in the *pex1*Δ and *pex15*Δ strains, larger ubiquitinated species (3-4 ubiquitins) are seen than those found in a *pex4*Δ cell (1-2 ubiquitins). In the absence of Pex6p, Pex1p or Pex15p, both Pex4p and Ubc4p modify Pex5p, while in the absence of Pex4p, only Ubc4p-mediated ubiquitination occurs. The similarity in ubiquitination pattern observed with the C_6R mutant of Pex5p in wild type cells and wild type Pex5p in *pex4*Δ cells (Fig. 4) supports the notion that both mutants have a deficiency in the same process, i.e. Pex4p-dependent ubiquitination of Pex5p.

Perspectives

Until recently, it was believed that the attachment of the first ubiquitin moiety to a substrate protein invariably occurs on an NH_2 group present on either an internal lysine or the N-terminal residue. However, the recent finding that ubiquitin may also be conjugated to a cysteine residue has added a further level of complexity to ubiquitin-regulated processes (Cadwell and Coscoy, 2005). Our observation that ubiquitination on a cysteine is likely to occur on proteins involved in peroxisome biogenesis indicates that this alternate form of ubiquitination may be more widespread in nature then previously thought. Its recent discovery may be explained by the relative instability of a thioester bond (cysteine-ubiquitin) compared to an amide linkage (lysine/α-NH_2-ubiquitin). The identification of more proteins that are ubiquitinated on a cysteine will help to unravel the potential function(s) of this novel form of ubiquitination.

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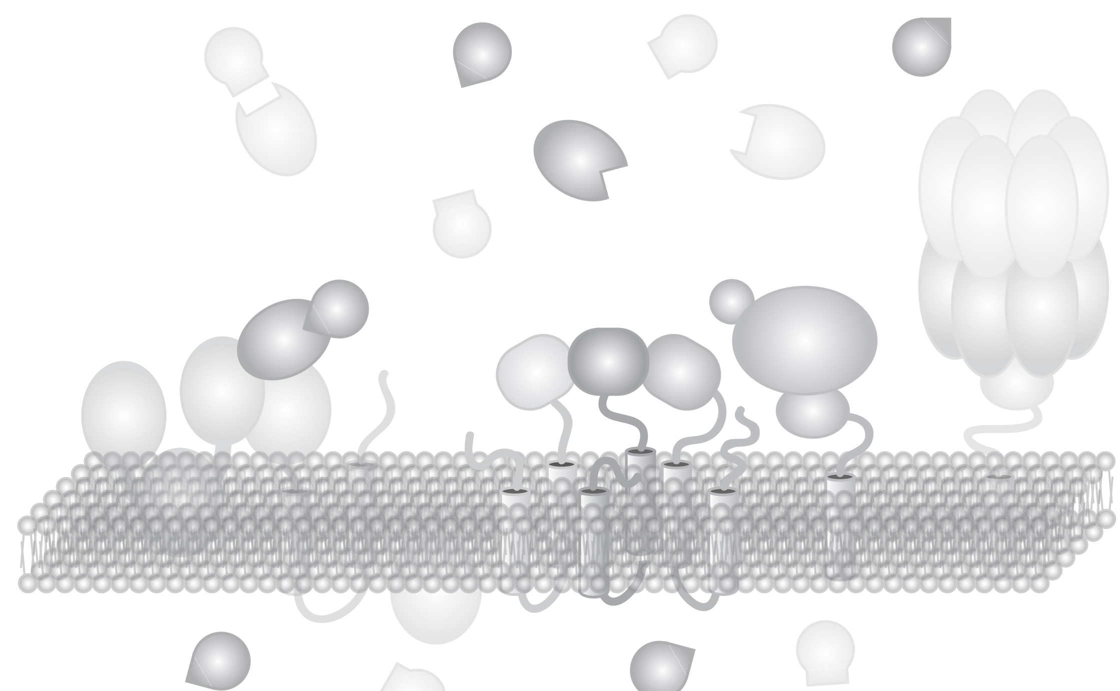
6

Further analysis on the ubiquitination of the PTS1 receptor Pex5p

Chris Williams, Marlene van den Berg, Erica Geers and Ben Distel

Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands



Abstract

Pex5p, the peroxisomal protein import receptor for PTS1 proteins is modified by ubiquitin, both in an Ubc4p-dependent and a Pex4p-dependent manner. While Ubc4p-dependent ubiquitination targets two lysines in Pex5p, Pex4p-dependent ubiquitination is likely to target a cysteine residue (Cys_6) in the N-terminal region of Pex5p. Here, we report that mutation of this cysteine residue to a lysine results in a partially functional protein that shows increased levels of ubiquitination. This increase does not depend on other N-terminal lysine residues and is not observed in cells lacking Pex4p, indicating that the introduced lysine residue at position 6 can conjugate ubiquitin in a Pex4p-dependent manner. The increased amounts of ubiquitinated Pex5p allows its detection using Pex5p antibodies and opens up the possibility for future studies on Pex4p-dependent ubiquitination. In addition, we show that the RING domain of Pex10p exhibits E_3 ligase activity in combination with the Ubc4p orthologue UbcH5a.

Introduction

Peroxisomes are diverse single membrane bound organelles that can be found in all eukaryotic cells. Peroxisomal enzymes are synthesised in the cytosol of free polyribosomes and imported with the aid of a peroxisomal targeting signal (PTS). The most common signal is the PTS1, located at the extreme C-terminus and consisting of variants of the canonical S-K-L sequence of firefly luciferase (Gould *et al.*, 1989). The less common PTS2 is an N-terminal nona-peptide, with the consensus (R/K)(L/V/I)X_5(H/Q)(L/A) (Gietl *et al.*, 1994). The import of PTS containing proteins requires the action of a cycling import receptor: peroxin 5 (Pex5p) for PTS1 proteins (Van der Leij *et al.*, 1993; Wiemer *et al.*, 1995) and peroxin 7 (Pex7p) for PTS2 proteins (Marzioch *et al.*, 1994). A number of co-receptors for the PTS2 pathway, the Pex20p family, have also been identified (Einwächter *et al.*, 2001; Purdue *et al.*, 1998).

During its receptor cycle, Pex5p recognises and binds the PTS1 protein in the cytosol, transports it to the peroxisomal membrane, aids in the translocation into the peroxisomal matrix and recycles to the cytosol for another round of import (Purdue and Lazarow, 2001a). A number of other proteins play important roles in this cycle. The initial docking of cargo-laden Pex5p on the peroxisomal membrane is thought to occur via interactions with the peroxisomal membrane proteins Pex13p and Pex14p (and Pex17p in certain yeast species) (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Elgersma *et al.*, 1996; Gould *et al.*, 1996; Huhse *et al.*, 1998). The actual translocation of the cargo over the peroxisomal membrane is poorly understood. However, several proteins have been implicated in this process, including the docking factors Pex13p and Pex14p as well as three really interesting new gene (RING) domain containing proteins, Pex2p, Pex10p and Pex12p, and the intra-peroxisomal peroxin Pex8p. These three RING proteins form a complex on the peroxisomal membrane and both Pex10p and Pex12p can interact with Pex5p in mammals (Chang *et al.*, 1999; Okumoto *et al.*, 2000). Pex8p, the only intra-peroxisomal peroxin identified so far, functions in the dissociation of the receptor-cargo complex as well as connecting the docking complex with the RING protein complex (Agne *et al.*, 2003; Rehling *et al.*, 2000). Finally, the AAA proteins Pex1p and Pex6p, along with their peroxisomal membrane anchor Pex15p (Pex26p in mammals) remove Pex5p from the peroxisomal membrane (Birschmann *et al.*, 2003; Collins *et al.*, 2000; Platta *et al.*, 2005). In addition, two interacting peroxins, the peroxisomal membrane protein Pex22p and the

ubiquitin-conjugating (UBC or E_2) enzyme Pex4p (Ubc10p) are also involved at a late stage in the Pex5p cycle (Collins *et al.*, 2000; Koller *et al.*, 1999). Recently, it has been shown that both Pex5p and certain members of the Pex20p family are post-translationally modified by ubiquitin (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Leon *et al.*, 2006b; Platta *et al.*, 2004; Purdue and Lazarow, 2001b). Ubiquitination is the conjugation of the 7-kDa protein ubiquitin to a substrate. This ATP-requiring process can be divided into three distinct steps i) ubiquitin activating by the ubiquitin-activation enzyme (E_1), ii) transfer of ubiquitin to an ubiquitin conjugating enzyme (E_2) and iii) the ligation of ubiquitin to a substrate with the aid of an ubiquitin ligase enzyme (E_3) (Hershko and Ciechanover, 1998). Ubiquitination plays a role in numerous cellular processes, such as DNA repair, protein degradation, stress response and cell cycle control (Pickart, 2001).

Two distinct forms of ubiquitination are observed with *Saccharomyces cerevisiae* Pex5p (ScPex5p) (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). These two forms of Pex5p ubiquitination, referred to here as Pex4p-dependent and Ubc4p-dependent ubiquitination respectively (Williams *et al.*, 2007), are very distinct from each other. Ubc4p-dependent ubiquitination is the attachment of between one to four ubiquitin moieties to lysine residues in the N-terminal region of Pex5p (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2007; Platta *et al.*, 2004; Williams *et al.*, 2007). In contrast, Pex4p dependent ubiquitination is the attachment of two ubiquitin moieties and is likely to occur on a well conserved cysteine residue present in Pex5p (Williams *et al.*, 2007). The functions of these two modifications also appear to be quite distinct from each other. Ubc4p-dependent ubiquitination is observed in a number of *pex* deletion strains, namely *pex1Δ*, *pex4Δ*, *pex6Δ*, *pex15Δ* and *pex22Δ*, as well as certain Pex5p mutants and is thought to be a quality control mechanism for the degradation of non-functional Pex5p (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004; Williams *et al.*, 2007). Pex4p-dependent ubiquitination, on the other hand occurs in wild-type cells and current data suggest that it acts as a signal for the removal of Pex5p from the peroxisomal membrane, allowing the receptor to participate in another round of import (Platta *et al.*, 2007).

Several unanswered questions concerning these modifications remain. For example, two E_2 enzymes essential for Pex5p ubiquitination have been identified, but the identity of the E_3 (‘s) remain(s) unknown. E_3 ligase domains come in two main types: RING and HECT (homologous to E6-AP carboxyl terminus) E_3’s. HECT E_3’s are able to directly

conjugate ubiquitin to themselves from the E_2 before passing it on to a substrate, whereas RING E_3's act as a bridge between the E_2 and the substrate, allowing direct transfer from the E_2 to the substrate (Pickart, 2001). The three RING domain-containing proteins Pex2p, Pex10p and Pex12p all appear to be essential for both forms of Pex5p ubiquitination *in vivo* (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2004). However, direct evidence for E_3 ligase activity for any of the RING proteins is lacking.

Here we report that mutation of the conserved cysteine residue in Pex5p to a lysine residue results in a partially functional protein, whereas all other mutations at this position are not functional (Williams *et al.*, 2007). In addition, this mutant form of Pex5p is heavily ubiquitinated in a Pex4p-dependent manner and this modification does not require other lysine residues present in the N-terminal region of Pex5p. Furthermore, the ubiquitinated form of Pex5p C_6K localises predominantly to membrane fractions, in much the same way as wild-type ubiquitinated Pex5p (Kragt *et al.*, 2005b). Due to the increase in Pex4p-dependent ubiquitination, this mutant may allow us to study the Pex5p ubiquitination process in more detail. We also show that the Pex10 RING domain, and not those of either Pex2p or Pex12p can act as an E_3 ligase with UbcH5a, indicating the presence of at least one functional E_3 ligase at the peroxisomal membrane.

Materials and methods

Strains, media and culture conditions

The *E. coli* strain DH5α (*recA*, *hsdR*, *supE*, *endA*, *gyrA96*, *thi-a*, *relA1*, *lacZ*) was used for all plasmid isolations. The *E. coli* strain BL21 DE3 (*B*, *F-*, *dcm*, *ompT*, *hsdS* ($r_B^- m_B^-$), *gal* *λ*(*DE3*)) was used for the expression of His_6-HA Ubiquitin and His_6-GST RING domain fusions. Cells transformed with bacterial expression constructs were grown at 37°C to an OD_{600} of 0.4 in 1 litre LB medium supplemented with 1% glucose, 50 mM tris (pH 7 for His_6-HA ubiquitin and 8.5 for RING constructs) and antibiotics as required. Cells were then transferred to 21°C and grown to an OD_{600} of 0.7 and induced with 0.25 mM IPTG (Invitrogen) for 2-6 h. Cells were harvested by centrifugation for 20 min at 9,000 x g, washed with water and stored at -20°C. The following yeast strains were used in this study: *S. cerevisiae* BJ1991 *pex5Δ* (*MATα*; *pex5*::*KanMX4*, *leu2*, *ura3-251*, *trp1*, *prb1-1122*, *pep4-3*, *gal2*), BJ1991 *pex4Δ pex5Δ* (*MATα*, *pex4*::*KanMX4*, *pex5*::*LEU2*, *leu2*, *trp1*, *ura3-251*, *prb1-1122*, *pep4-3*, *gal2*). Yeast transformations were performed as described in (Van

der Leij *et al.*, 1993). Transformants were grown on minimal medium containing 0.67% yeast nitrogen base (YNB, Difco), 2% glucose, 2% agar and amino acids (20 μg/ml) as required. For immunoprecipitations and membrane fractionations, cells were grown on 0.67% YNB containing 0.3% glucose for at least 24 h and then shifted to 0.1% oleic acid medium containing 0.5% potassium phosphate buffer pH 6.0, 0.3% yeast extract, 0.5% peptone and 0.2% tween 40 and grown for 7-16 h. $CuSO_4$ (100 μM final concentration) was added to cultures for expression of CUP1 promoter-controlled myc-ubiquitin.

Plasmids, primers and cloning procedures

The following plasmids are described elsewhere; pTI98, a yeast plasmid for the expression of Pex5p from the PEX5 promoter (Klein *et al.*, 2001); pMB74, a yeast plasmid for the expression of Pex5p lacking N-terminal lysine residues from the PEX5 promoter (Williams *et al.*, 2007); pCW122, a yeast plasmid for the expression of the Pex5p cysteine to arginine mutant (Pex5p C_6R) from the PEX5 promoter (Williams *et al.*, 2007); pCW145, similar to pCW122 but lacking N-terminal lysine residues (Williams *et al.*, 2007); YEP105, a yeast plasmid for the CUP1 promoter controlled expression of myc-ubiquitin (Ellison and Hochstrasser, 1991); pET28b HA ubiquitin, for the *E. coli* expression of His_6-HA ubiquitin (Davies *et al.*, 2003). The constructs expressing Pex5p C_6K (pCW123) and Pex5p C_6K K_0N (pCW147) were made using the QuikChange^{TM} site directed mutagenesis kit (Stratagene) with the primer combination Pex5 C_6K *Bam*HI (cgggatccatggacgtaggaag taaatcagtggg) and Pex5 360 (ctattactaccttgaaattcctgtg) and either pTI98 or pMB74 as templates, respectively. *E. coli* expressing vectors of His_6-GST fusions with the RING domains of Pex2p, Pex10p and Pex12p (pCW116, pCW114 and pCW115, respectively) were made as follows: polymerase chain reaction (PCR) was performed on genomic DNA using the primer combinations Pex2 214 *Eco*RI/*Bam*HI (ggaattctaggatcctctacgactactta taagacag) and Pex2 271 *Pst*I/*Spe*I (ccactagtactgcagttagtacactggtgaggcgg), Pex10 244 *Eco*RI/*Bam*HI (ggaattctaggatcctctgttggtctacaggagcg) and Pex10 337 *Pst*I/*Spe*I (ccactagtactgcagctattgccgcaggaccagaa) and Pex12 297 *Eco*RI/*Bam*HI (ggaattctaggatccat gactactaaattgcagaaaagg) Pex12 399 *Pst*I/*Spe*I (ccactagtactgcagtcagattagtagcttcctaatac). The products were digested with *Eco*RI and *Spe*I and ligated into *Eco*RI-*Spe*I digested pRP265nb (Barnett *et al.*, 2000). The resulting vectors were digested with *Nco*I and *Hind*III and ligated into *Nco*I-*Hind*III digested pETM-30 (a generous gift from Dr. Stier), creating

His_6-GST Pex2 RING (pCW116), His_6-GST Pex10 RING (pCW114) and His_6-GST Pex12 RING (pCW115).

Growth curves

Glucose grown cells were inoculated into minimal oleic acid medium to an OD_{600} of 0.05 and grown for 6 days at 28ºC. Samples of 1 ml were taken at 6, 24, 30, 48, 54, 72, 78 and 144 h, centrifuged at 10,000 rpm for 5 min and washed with 1 ml H_2O. The washing was repeated twice and the cells were resuspended in 1 ml H_2O and the OD_{600} value was measured with a spectrophotometer.

Membrane fractionations

Oleate-grown cells (~200 A_{600} units) expressing wild type or mutant forms of Pex5p were pulse vortexed with glass beads in 300 µl fractionation buffer (50 mM HEPES pH 7.5, 50 mM MgAc, 100 mM NaCl, 5 mM NaF, 1 mM β-mercaptoethanol and 2 mM PMSF) and a protease cocktail inhibitor (Sigma) for 7x 1 min plus 7x 1 min on ice. Lysates were then centrifuged for 2 min at 2,000 rpm, the supernatant was removed and again centrifuged for 10 min at 4,000 rpm at 4°C. Homogenates were corrected for protein concentration and centrifuged at 48,000 rpm for 30 mins at 4°C. Pellet fractions were resuspended in fractionation buffer. Equal amount of the homogenate, pellet and supernatant fractions were analysed by SDS-PAGE and western blotting with antibodies directed against Pex5p, Pex13p and hexokinase.

Purification of fusion proteins expressed in E. coli

For purification of His_6-HA ubiquitin, cell pellets were thawed in buffer 1 (100 mM potassium phosphate pH 7.4, 30 mM imidazole, 5 mM β-mercaptoethanol, 0.1% triton X-100 and 2 mM PMSF), treated with 1 mg/ml lysozyme at 21°C for 15 min and pulse sonicated on ice until cells were broken. Debris was removed by centrifugation at 4°C for 15 min at 9,000 x g. Lysates were loaded onto Ni-NTA (Qiagen) resin equilibrated with buffer 1. The resin was sequentially washed with 10 bed volumes of buffer 1, 10 bed volumes of wash buffer 1 (100 mM potassium phosphate pH 7.4, 1 M NaCl, 30 mM imidazole, 5 mM β-mercaptoethanol and 2 mM PMSF and 10 bed volumes of wash buffer 2 (100 mM potassium phosphate pH 7.4, 30 mM imidazole, 5 mM β-mercaptoethanol and 2

mM PMSF). Fusion proteins were eluted with buffer 2 (100 mM potassium phosphate pH 7.4, 5 mM β-mercaptoethanol and 2 mM PMSF) containing 330 mM imidazole. For His_6-GST RING domain fusion protein purification, cell pellets were thawed in buffer A (100 mM tris pH 8.5, 150 mM NaCl, 1 M urea, 5 mM β-mercaptoethanol, 1% triton X-100 and 2 mM PMSF), treated with 1 mg/ml lysozyme at 21°C for 15 min and pulse sonicated on ice until cells were broken. Debris was removed by centrifugation at 4°C for 15 min at 9,000 x g. Lysates were loaded onto glutathione sepharose 4B (GE Healthcare) resin equilibrated with buffer A. The resin was sequentially washed with 10 bed volumes of buffer A, 10 bed volumes of buffer W1 (100 mM tris pH 8.5, 1 M NaCl, 2 M urea, 5 mM β-mercaptoethanol, 0.1% triton X-100 and 2 mM PMSF) and 10 bed volumes of buffer W2 (100 mM tris pH 8.5, 150 mM NaCl, 5 mM β-mercaptoethanol and 2 mM PMSF). Fusion proteins were eluted with elution buffer (100 mM tris pH 8.5, 5 mM β-mercaptoethanol, 2 mM PMSF and 20 mM reduced glutathione). Purified proteins were concentrated and equilibrated in 100 mM tris pH 8.1, 100 mM NaCl, 1 mM β-mercaptoethanol using Amicon® Ultra centrifugal filters (Millipore) and stored at -80°C. Purity was monitored by SDS-PAGE analysis. Protein concentrations were measured using the Bradford method with BSA as standard (Bradford, 1976).

In vitro ubiquitination assays

In vitro ubiquitination assays were carried out in 40 µl reactions containing 25 mM tris pH 8.1, 75 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and some or all of the following: 5 mM ATP, 0.5 µg E_1 enzyme (a generous gift from Dr. Höhfeld), 0.5 µg UbcH5a (Biomol), 2 µg His_6-HA Ubiquitin and 2 µg His_6-GST Pex2/10/12 RING domain. The mixture was incubated at 28°C for 1 h with gentle shaking. Reactions were stopped by addition of SDS-PAGE loading buffer and samples were then subjected to either 12% (anti-GST) or 8-20% SDS-PAGE and western blotting using antibodies directed against the HA tag (12CA5), Pex10p and Pex12p (both generous gifts from Dr. Kunau) and GST (Sigma).

Miscellaneous

Immunoprecipitation analysis was performed as described in (Williams *et al.*, 2007).

Results

Pex5p C_6K is partially functional

Previously, we have shown that a well-conserved cysteine residue present at position 6 in the N-terminal region of *S. cerevisiae* Pex5p is essential for Pex4p-dependent-ubiquitination of the protein and is very likely the conjugation site for this modification (Williams *et al.*, 2007). Mutation of this residue to a serine, alanine, tryptophane or arginine resulted in a non-functional protein unable to complement the oleate non-utilising phenotype of a *pex5Δ* strain (Williams *et al.*, 2007 and data not shown). Therefore, we investigated whether a lysine residue, a more standard ubiquitin conjugation site, could functionally replace this cysteine residue. Expression of the Pex5p C_6K mutant in a *pex5Δ* strain resulted in growth on oleic acid, a carbon source that requires functional peroxisomes for its metabolism (our unpublished results).

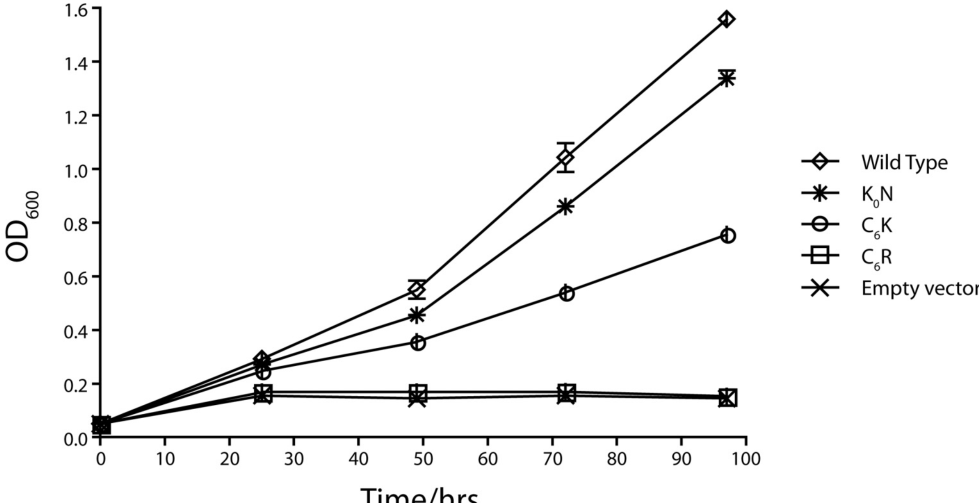


Fig. 1 Pex5p C_6K can partially complement the *pex5Δ* phenotype
Pex5Δ cells expressing wild type or mutant forms of Pex5p were grown overnight on medium containing 0.3% glucose and shifted the next morning to minimal oleic acid medium and grown for 6 days at 28°C. Samples of 1 ml were taken, the cells were washed and the OD_{600} was measured. Values correspond to the mean ± SD of two measurements each taken from two separate cultures.

To gain insight into how efficient Pex5p C_6K could complement the *pex5Δ* phenotype, growth curves were performed. As can be seen in Fig. 1, Pex5p C_6K cannot

fully complement the *pex5Δ* phenotype, but the growth of this strain was considerably better than a *pex5Δ* strain containing either an empty vector or the non-functional Pex5p C_6R mutant, indicating that Pex5p C_6K, unlike other Pex5p cysteine mutants, is partially functional.

Levels of ubiquitinated Pex5p C_6K are enhanced and this increase does not depend on other N-terminal lysine residues

In order to check the ubiquitination status of the Pex5p C_6K mutant, *pex5Δ* cells expressing either wild type or mutant forms of Pex5p and myc-tagged ubiquitin were subjected to immunoprecipitation with anti-Pex5p antibodies and analysed by anti-Pex5p and anti-myc immunoblotting (Fig. 2 A). The levels of ubiquitinated Pex5p were dramatically increased in the C_6K mutant when compared to the wild-type protein (Fig. 2 A, compare lanes WT and Pex5p C_6K), allowing its detection with Pex5p antibodies (Fig. 2 B). To determine whether ubiquitination only occurs on lysine 6 or whether other lysine residues in the N-terminal region are also targets, we made a version of Pex5p C_6K in which all the other lysine residues in the N-terminal region were mutated to arginines (Pex5p C_6K K_0N). Again, we saw considerably more ubiquitinated Pex5p than with the wild type protein (Fig. 2 A, C_6K K_0N). These data suggest that the introduced lysine residue at position six is the main target for ubiquitin conjugation. Next, we addressed the question as to whether the ubiquitination of the Pex5p C_6K mutant was dependent on Pex4p. In the absence of Pex4p, Ubc4p-dependent ubiquitination of Pex5p is observed on lysine residues in the N-terminal region of Pex5p (Kiel *et al.*, 2005a; Kiel *et al.*, 2005b; Kragt *et al.*, 2005b; Platta *et al.*, 2004; Williams *et al.*, 2007). This modification is distinct from Pex4p-dependent ubiquitination in that, instead of the presence of a single higher molecular weight species, a ladder of one to four ubiquitinated species are observed (Williams *et al.*, 2007). Immunoprecipitates of *pex4Δpex5Δ* cells expressing Pex5p C_6K and myc-ubiquitin exhibited a similar ubiquitination pattern and levels as wild type Pex5p and the C_6R mutant (Fig. 2 C). These data suggest that the ubiquitination of Pex5p C_6K is largely dependent on Pex4p. However, when the other lysine residues were mutated (C_6K K_0N) the protein was still ubiquitinated, whereas both Pex5p K_0N and Pex5p C_6R K_0N were no longer modified (Fig. 2 C), suggesting that Ubc4p targets the introduced lysine at position 6 in the absence of other lysine residues.

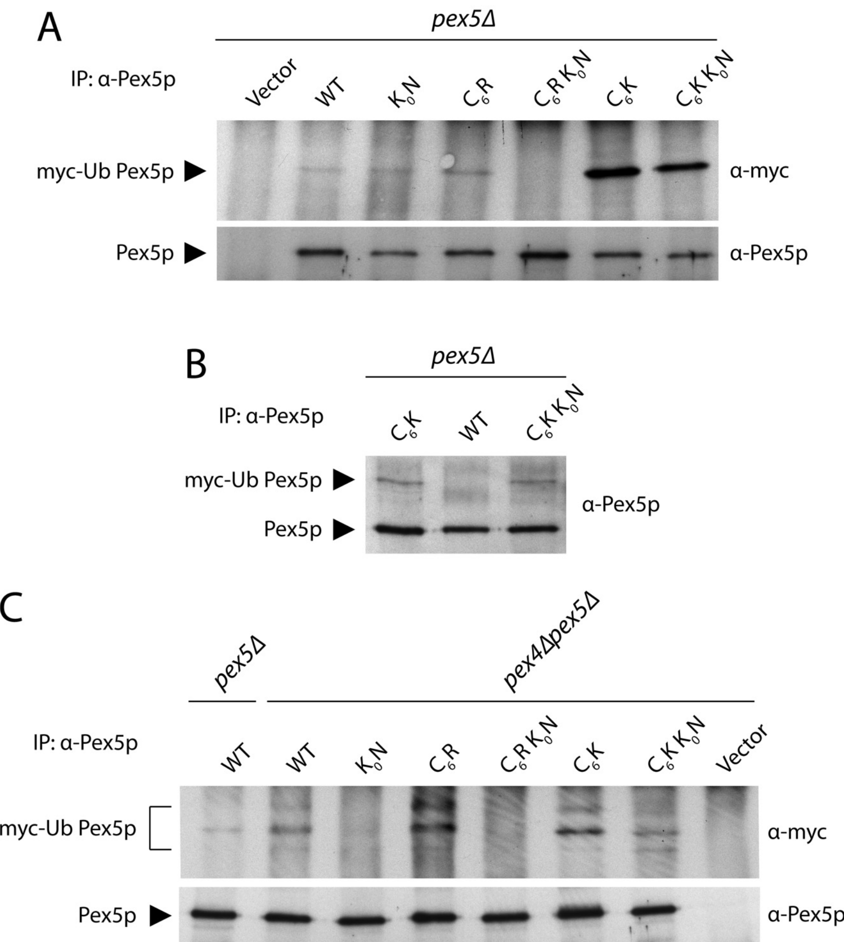


Fig. 2 Levels of ubiquitinated Pex5p C_6K are dramatically increased

A. *Pex5Δ* cells co-expressing myc-tagged ubiquitin and either wild type Pex5p (**WT**), Pex5p lacking N-terminal lysines (**K_0N**), Pex5p with the conserved cysteine residue mutated to an arginine (**C_6R**) or a similar construct without N-terminal lysines (**C_6R K_0N**), Pex5p with the conserved cysteine residue mutated to a lysine (**C_6K**) or a similar construct without N-terminal lysines (**C_6K K_0N**) or an empty vector (**Vector**) were lysed and Pex5p was precipitated using anti-Pex5p anti-serum (**IP**). Immunoprecipitates were analysed with SDS-PAGE and immunoblotting (**IB**) using antibodies raised against the myc-tag and Pex5p.

B. Long exposure of immunoprecipitates analysed with SDS-PAGE and immunoblotting (**IB**) using antibodies against Pex5p. WT, wild type Pex5p, C_6K, cysteine to lysine mutant version of Pex5p, C_6K K_0N, cysteine to lysine mutant version of Pex5p but lacking other N-terminal lysine residues.

C. *Pex5Δ* cells co-expressing myc-tagged ubiquitin and wild type Pex5p and *pex4Δpex5Δ* cells co-expressing myc-tagged ubiquitin and either wild type Pex5p (**WT**), Pex5p lacking N-terminal lysines (**K_0N**), Pex5p with the conserved cysteine residue mutated to an arginine (**C_6R**) or a similar construct without N-terminal lysines (**C_6R K_0N**), Pex5p with the conserved cysteine residue mutated to a lysine (**C_6K**) or a similar construct without N-terminal lysines (**C_6K K_0N**) or an empty vector (**Vector**) were subjected to immunoprecipitation (**IP**) and immunoblotting (**IB**) as described in Fig. 2 A.

Ubiquitinated Pex5p C_6K localises predominantly to the peroxisomal membrane

Previous reports have indicated that ubiquitinated forms of Pex5p localise predominantly to the peroxisomal membrane (Kragt *et al.*, 2005b; Platta *et al.*, 2004). Therefore, we performed membrane fractionations on the Pex5p C_6K mutant in order to determine the subcellular distribution of the ubiquitinated form of Pex5p. Since these modified forms of Pex5p C_6K were visible with Pex5p immunoblotting alone the immunoprecipitation step was omitted. We observed that the ubiquitinated forms of both the Pex5p C_6K and Pex5p C_6K K_0N mutants were predominantly present in the membrane fraction (Fig. 3), indicating that the modified mutants behave in a similar way as the modified wild-type protein (Kragt *et al.*, 2005b).

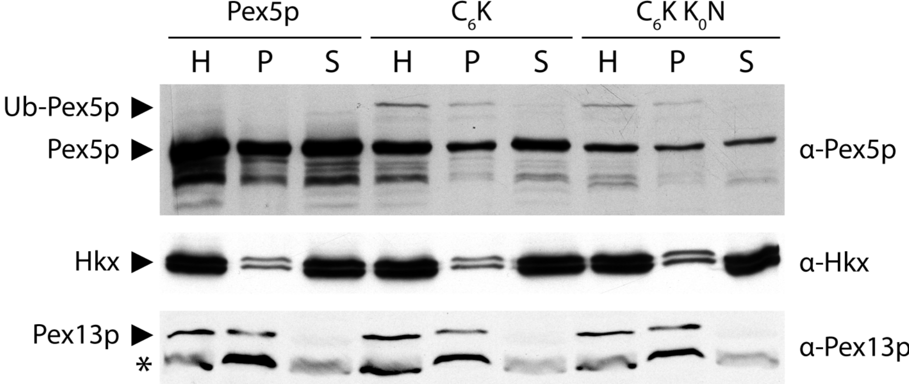


Fig. 3 Ubiquitinated Pex5p C_6K localised predominantly to membranes
Pex5Δ cells expressing wild type Pex5p (**WT**), Pex5p with the conserved cysteine residue mutated to a lysine (**C_6K**) or a similar construct without N-terminal lysines (**C_6K K_0N**) were lysed with glass beads and subjected to membrane fractionation. Equivalent volumes of the 600 x g supernatant (**H**), 100,000 x g membrane pellet (**P**) and 100,000 x g supernatant (**S**) were subjected to western blotting and staining with antibodies specific for Pex5p (*upper panel*), the cytosolic protein hexokinase (Hkx, *middle panel*) and the peroxisomal membrane protein Pex13p (*lower panel*). * Indicates a cross reacting band recognised by the Pex13p antibody.

The Pex10 RING domain but not the RING domains of Pex2p or Pex12p can act as an E_3 ligase with UbcH5a

Several groups have reported the involvement of the E_2 enzymes Pex4p and Ubc4p in the ubiquitination of Pex5p (Kiel *et al.*, 2005a; Kragt *et al.*, 2005b; Platta *et al.*, 2007; Platta *et al.*, 2004; Williams *et al.*, 2007), but the E_3 ('s) ligase responsible for these conjugations

has not been identified. There are, however, several good candidates, namely the RING domain containing peroxins Pex2p, Pex10p and Pex12p.

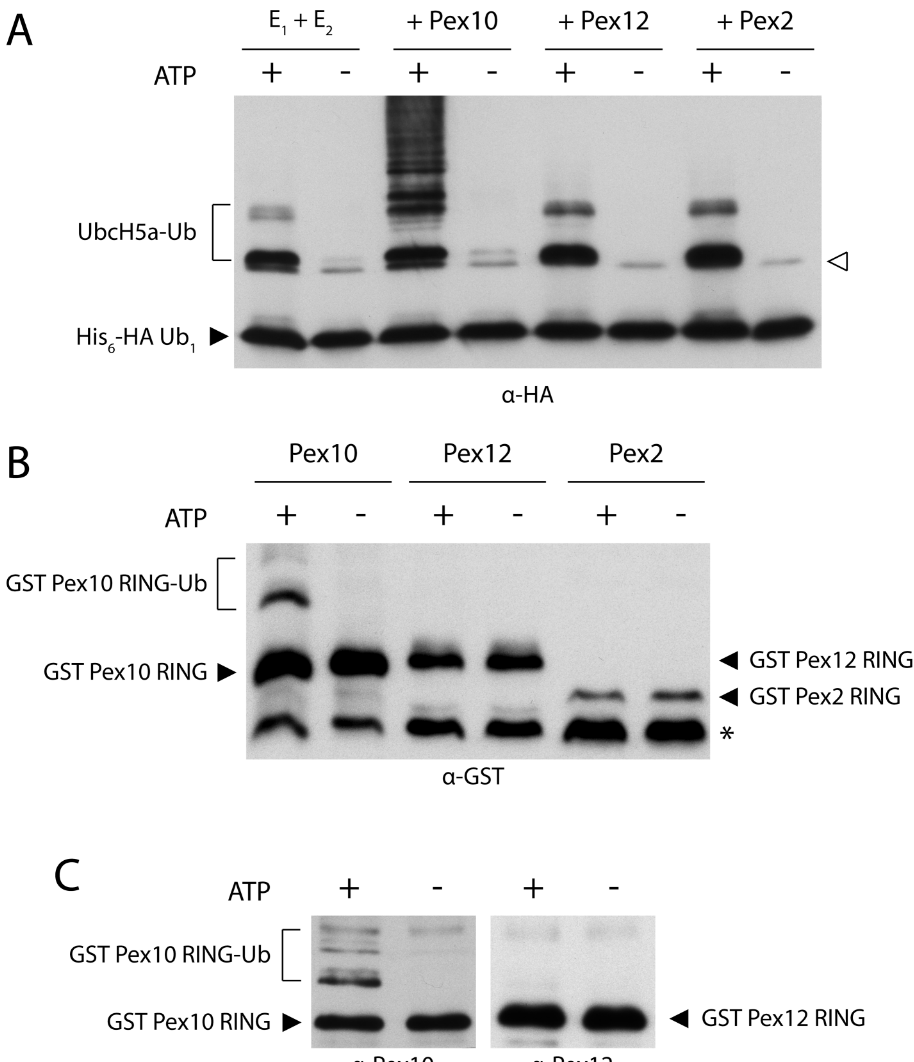


Fig. 4 Pex10 RING shows E_3 ligase activity with Ubc5Ha
In vitro ubiquitination reactions were performed using recombinant E_1 enzyme, UbcH5a (E_2), GST-RING domains and His_6-HA ubiquitin in the presence (+) or absence (-) of ATP. Samples were incubated at 28°C for 1 h with gentle shaking, subjected to SDS-PAGE and immunoblotting with antibodies against HA (**A**), GST (**B**), Pex10p and Pex12p (**C**). The open arrow indicates di-ubiquitin. * Indicates a breakdown product of GST-RING constructs, recognised by the GST antibody.

To test whether any of these RING proteins exhibited E_3 ligase activity, we expressed and purified all three RING domains as His_6-GST fusions and checked them for self-ubiquitination activity in an *in vitro* ubiquitination reaction, a phenomenon that can be observed with RING E_3's. In the absence of a substrate protein, the E_2 enzyme can transfer its ubiquitin to an accessible lysine residue in the RING domain, a reaction that is catalysed by the E_3 itself. Since we were unable to show loading of purified Pex4p with ubiquitin in the presence of recombinant E_1 and His_6-HA ubiquitin, we used human UbcH5a, a commercially available homologue of *S. cerevisiae* Ubc4p (Scheffner *et al.*, 1994) in the self-ubiquitination assay. As shown in Fig. 4 A, UbcH5a could be charged with ubiquitin in an ATP and E_1 dependent manner (Fig. 4 A, $E_1 + E_2$). When the Pex10 RING domain was added to the reaction, higher molecular weight forms of His_6-HA ubiquitin were observed, suggesting that ubiquitin conjugation had occurred (Fig. 4 A, + Pex10). To confirm that these forms were indeed ubiquitinated His_6-GST Pex10 RING, reactions were analysed with anti-GST and anti-Pex10 antibodies. Higher molecular weight forms of His_6-GST Pex10 RING were observed with both antibodies (Fig. 4 B, αGST and C, αPex10), indicating that Pex10 RING is modified by ubiquitin and can, therefore, act as an E_3 ligase. No slower migrating ubiquitin species were observed when either Pex2 RING or Pex12 RING was included in the reaction (Fig. 4), indicating that these RING domains do not exhibit E_3 ligase activity under the experimental conditions applied.

Discussion

The PTS1 cycling receptor Pex5p is post-translationally modified by ubiquitin in two distinct ways: in a Pex4p-dependent and an Ubc4p-dependent manner. Here we have studied both forms of Pex5p ubiquitination in more detail using *in vivo* and *in vitro* assays.

The Pex5p C_6K mutant: a method to study Pex4p-dependent ubiquitination?

Previously, we identified a well-conserved cysteine residue present at position 6 in Pex5p that is likely to be the conjugation site for the Pex4p-dependent ubiquitination of the protein (Williams *et al.*, 2007). Replacement of this cysteine with an alanine, arginine, tryptophane or serine both abolishes Pex4p-dependent ubiquitination and renders the protein non-functional (Williams *et al.*, 2007 and Fig. 1). However, when a lysine residue was introduced at this position, the more common target residue for ubiquitin conjugation,

partial restoration of the *pex5Δ* phenotype was observed (Fig. 1). Biochemical analysis showed a dramatic increase in the levels of ubiquitinated Pex5p in this mutant and this increase was not dependent on other N-terminal lysine residues (Fig. 2). These data suggest that the introduced lysine at position 6 acts as the conjugation site for Pex4p-dependent ubiquitination of Pex5p. Nevertheless, we must be careful in interpreting the results obtained with this mutant. For example, in the absence of Pex4p, Ubc4p-dependent ubiquitination of Pex5p occurs on the lysine residues present at positions 18 and 24 (Platta *et al.*, 2007; Platta *et al.*, 2004; Williams *et al.*, 2007). Mutation of the target lysines blocks this modification and the cysteine residue plays no part in it (Williams *et al.*, 2007). The Pex5p C_6K K_0N mutant in *pex4Δ* cells however, is still modified in a way that resembles Ubc4p-dependent ubiquitination (Fig. 2 C). This modification is very likely to be present on the introduced lysine residue at position 6, meaning that we cannot be sure that the ubiquitination of the Pex5p C_6K mutant in *pex5Δ* cells solely depends on Pex4p. Nonetheless, the fact that the Pex5p C_6K mutant is partially functional supports the notion that ubiquitination at position 6 is essential for Pex5p function as all other residues introduced at this position are unable to conjugate ubiquitin and are, therefore, unable to complement the *pex5Δ* strain (Williams *et al.*, 2007). Due to its partial functionality as well as the considerable increase in ubiquitination signal, the C_6K mutant may be useful for further characterising the function of Pex5p ubiquitination, although some caution has to be taken.

Further questions that arise concerning the Pex5p C_6K mutant include why such a large increase in the levels of ubiquitinated Pex5p is observed? This may be due to the more stable nature of the linkage formed between ubiquitin and Pex5p. In wild-type cells, ubiquitin is linked to Pex5p via a thioester bond, which is more easily broken by reducing agents, whereas ubiquitin linked via an amide bond (lysine linkage) is resistant to such agents (Williams *et al.*, 2007). Although great care was taken to avoid reducing conditions during the immunoprecipitation procedure, we cannot exclude that the recovery of thioester-linked ubiquitinated Pex5p is less than that of amide-linked ubiquitinated Pex5p.

A more likely explanation is that the higher amounts of ubiquitinated Pex5p recovered from the C_6K mutant directly reflect the *in vivo* levels. The increased levels can be caused by a more efficient ubiquitination or a less efficient deubiquitination of the mutant protein. Unfortunately, the current data do not allow us to distinguish between these

two eventualities, although we favour the latter. Previously, we have suggested that the timely removal of ubiquitin may be crucial for its function and, therefore a cysteine residue acts as the conjugation site (Williams *et al.*, 2007). As we have been unable to find evidence for the involvement of any of the 17 deubiquitinating enzymes (DUB's) present in *S. cerevisiae* in removal of cysteine linked ubiquitin from Pex5p (data not shown), this process may be accomplished by Pex1p and/or Pex6p or even occur spontaneously, due to the unstable nature of the thioester bond. The presence of an amide bond between ubiquitin and Pex5p may prevent efficient deubiquitination and cause incomplete complementation. Further study is needed in order to answer these questions.

Pex10, the E_3 ligase for Ubc4p-dependent ubiquitination of Pex5p?

The observation that Pex10p harbours E_3 ligase activity comes as no surprise. Indeed, the RING domain of Pex10p is highly homologous to that of c-Cbl, a well-characterised RING E_3 ligase (Joazeiro *et al.*, 1999) and the presence of Pex10p is essential for Ubc4p-dependent ubiquitination of Pex5p, although this evidence is indirect (Kiel *et al.*, 2005a). Since two distinct forms of ubiquitination occur on Pex5p, both performed by a separate E_2 enzyme, the question arose as to which E_3 ('s) serve(s) which E_2? Our *in vitro* ubiquitination data show that Pex10p, and not Pex2p or Pex12p, acts as an E_3 in combination with UbcH5a, the human orthologue of yeast Ubc4p. It is therefore conceivable that *in vivo* Pex10p and Ubc4p are responsible for the Ubc4p-dependent ubiquitination of Pex5p, although direct evidence for this, *i.e. in vitro* ubiquitination of Pex5p with this E_2/E_3 pair is lacking. Also, the *in vivo* data of Kiel and co-workers suggest that Ubc4p-dependent ubiquitination of Pex5p requires all three RING proteins (Kiel *et al.*, 2005a), but again the evidence is indirect. To complicate matters, the Pex4p-dependent ubiquitination of Pex5p *in vivo* also requires all three RING proteins (Kragt *et al.*, 2005b). We have, thus far been unable to see *in vitro* loading of Pex4p with ubiquitin (data not shown), preventing further testing of the RING domains for E_3 ligase activity with Pex4p as E_2. Such an experiment will give more information concerning the function and specificity of the RING domains. Maybe all three domains are needed for the conjugation of ubiquitin to Pex5p, be it Ubc4p-dependent or Pex4p-dependent. As all three RING proteins form a complex at the peroxisomal membrane, it would seem likely that their functions are somehow linked (Agne *et al.*, 2003). The possibility that they represent a multi-subunit E_3

ligase complex is rather appealing, however, more data from both *in vivo* and *in vitro* experiments are needed before we have a clear picture.

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Summary

Peroxisomes are ubiquitous organelles, bound by a single lipid bilayer. Functionally they are extremely diverse, housing enzymes that are involved in numerous metabolic pathways. Their importance is compounded by the existence of several human disorders, caused by deficiencies in peroxisome function. Matrix enzymes are imported into the peroxisome post-translationally. This is achieved with the aid of a peroxisomal targeting signal (PTS) present in the protein. A set of proteins, called peroxins or Pex proteins play an important role in the import of PTS proteins. The current model for the import of PTS proteins proposes that newly synthesised PTS proteins are recognised and bound in the cytosol by a cycling receptor. Next, the cargo-laden receptor docks on the peroxisomal membrane and aids in the translocation of the PTS protein across the peroxisomal membrane. Finally, the receptor recycles back to the cytosol for another round of import. The main areas of research covered here concerns the type 1 PTS receptor Pex5p and how its function may be regulated through protein-protein interactions and post-translational modification.

In Chapter 2, we studied the interaction between Pex5p and the docking factor Pex14p in the yeast *S. cerevisiae*. We showed that Pex14p contains two Pex5p binding sites, one in the N-terminus and one in the C-terminus. This is different from the mammalian situation where only one binding site, in the N-terminal region, has been identified. Further analysis revealed that both of these binding sites are essential for the import of PTS1-containing proteins. We also observed that, again unlike the mammalian system, the conserved W-x-x-x-F/Y motifs present in Pex5p are not essential for the interaction with Pex14p. Instead, a region containing a reverse W-x-x-x-F/Y motif binds to Pex14p *in vitro*. These results indicate that there are differences between the mammalian and yeast systems.

Docking on the peroxisomal membrane was again addressed in Chapter 3. Pex13p, an SH3 domain-containing peroxisomal membrane protein is involved in the docking of Pex5p on the peroxisomal membrane. In this chapter we presented an overview of Pex13p, and discussed the known information concerning its interaction with the PTS2 receptor Pex7p, its fellow docking protein Pex14p and the PTS1 receptor Pex5p, as well as speculating on a post-docking role for Pex13p in PTS protein import.

In Chapter 4, research on a putative ubiquitin interacting motif (UIM) present in C-terminal region of Pex5p was carried out. The presence of a UIM in a protein can fulfil

one of two functions: directing the ubiquitination of the protein itself or allowing the protein to interact with ubiquitin and/or ubiquitinated substrates. Binding studies with Pex5p and ubiquitin failed to detect any interaction, but mutation of two of the residues in the putative UIM resulted in a protein unable to efficiently complement the *pex5Δ* strain. However, two-hybrid analysis showed that this mutant could no longer bind efficiently to a number of known Pex5p-binding partners, suggesting that a general destabilisation of the protein, rather than a specific effect due to mutation of the putative UIM was the cause of its phenotype.

A relationship between Pex5p and ubiquitin was again the topic of Chapter 5. Pex5p is ubiquitinated in both an Ubc4p-dependent and -independent manner. While Ubc4p-dependent ubiquitination is observed in certain *pex* deletion strains and is likely to be involved in quality control of Pex5p, Ubc4p-independent ubiquitination is observed in wild type cells and is believed to have a role in the non-proteolytic regulation of the PTS1 receptor. In this chapter, the sites at which these two forms of ubiquitination occur in Pex5p were described. Ubc4p-dependent ubiquitination targets two conserved lysines in the N-terminal region of Pex5p. Ubc4p-independent ubiquitination, however, requires neither lysine residues nor the N-terminal α-NH_2 group. Instead, a well-conserved cysteine residue present at position 6 appears to be the target. This residue is also essential for Pex5p function, as PTS1 proteins are mislocalised to the cytosol in its absence. In addition, we showed that the E_2 enzyme Pex4p (Ubc10p) is essential for Ubc4p-independent ubiquitination of Pex5p. Not only do these data represent the first example of cysteine ubiquitination of a target protein performed by the cellular ubiquitination machinery but also answer the long-standing question as to what the substrate of Pex4p may be.

In Chapter 6, further characterisation on Pex4p-dependent ubiquitination of Pex5p was performed. Mutation of the conserved cysteine residue to a lysine resulted in a partially functional protein that was ubiquitinated at higher levels than the wild-type protein. This increase was not due to other N-terminal lysine residues and was greatly reduced in the absence of Pex4p. Due to this increase in the ubiquitination signal, we could use direct western blotting with Pex5p antibodies to observe the behaviour of this mutant. In addition, we were able to show *in vitro* that the RING domain of Pex10p can act as an E_3 ligase with the Ubc4p homologue UbcH5a, suggesting that Pex10p may function as the E_3 ligase for Ubc4p-dependent ubiquitination of Pex5p *in vivo*.

Samenvatting

Een peroxisoom is een organel dat in veel eukaryote cellen voorkomt en is omgeven door een lipidenmembraan. Ze vertonen een enorme diversiteit aan functies door enzymen te bevatten die betrokken zijn bij een groot aantal metabolische processen. Deficiënties in deze functies zijn gerelateerd aan verschillende humane aandoeningen wat het belang van deze organellen benadrukt. De matrixenzymen worden na synthese in the cytosol geïmporteerd in de peroxisomen door middel van een signaal voor peroxisomale import (PTS: peroxisomal targeting signal) aanwezig in het eiwit. Een set van eiwitten, genoemd peroxines of Pex eiwitten, spelen een belangrijke rol in de import van deze PTS eiwitten. Het huidige model van de import van PTS eiwitten is dat nieuw gesynthetiseerde PTS eiwitten worden herkend door en gebonden aan een receptor in de cytosol. Vervolgens begeeft de geladen receptor zich naar het peroxisomale membraan waar deze assisteert bij de translocatie van het PTS eiwit door het membraan heen. Uiteindelijk transloceert de receptor terug naar het cytosol voor een volgende ronde van import. Het onderzoek beschreven in dit proefschrift behandelt grotendeels de type 1 PTS receptor Pex5p en de mogelijke regulatie van zijn functie door eiwit-eiwit interacties en post-translationele modificatie.

In Hoofdstuk 2 wordt de interactie bestudeerd tussen Pex5p en de docking factor Pex14p in de bakkersgist *S. cerevisiae*. We tonen aan dat Pex14p twee bindingsplaatsen voor Pex5p bevat, de ene in de N-terminus en de andere in de C-terminus van het eiwit. Deze situatie verschilt van die in zoogdieren waar enkel een N-terminale bindingsplaats is geïdentificeerd. Verdere analyse toonde aan dat beide bindingsplaatsen essentieel zijn voor de import van PTS1 eiwitten. Ook werd waargenomen dat de geconserveerde W-x-x-x-F/Y motieven aanwezig in Pex5p, die essentieel zijn voor de functie in zoogdieren, in bakkersgist niet essentieel zijn voor de interactie met Pex14p,. In plaats daarvan is er een domein met een omgekeerde W-x-x-x-F/Y motief, dat een interactie aangaat met Pex14p *in vitro*. Deze resultaten geven aan dat het gehele proces in zoogdieren en gist niet identiek verloopt.

Het proces van de docking op het peroxisomale membraan wordt ook bestudeerd in Hoofdstuk 3. Pex13p, een peroxisomaal membraaneiwit met een SH3 domein is betrokken bij de docking van Pex5p op het peroxisomale membraan. In dit hoofdstuk wordt een overzicht gegeven van Pex13p en de interactie van dit eiwit met de PTS2 receptor

Pex7p, het docking eiwit Pex14p en de PTS1 receptor Pex5p wordt bediscussieerd. Ook wordt gespeculeerd over een additionele functie van Pex13p in PTS eiwit import na docking.

In Hoofdstuk 4 wordt een mogelijk ubiquitine-interacting motief (UIM) dat aanwezig is in het C-terminale gebied van Pex5p bestudeerd. Een UIM in een eiwit kan één van de twee volgende functies vervullen: het dirigeren van de ubiquinatie van het eiwit zelf of de stimulatie van een interactie tussen het eiwit en ubiquitine en/of met een geubiquitineerd substraat. Interactiestudies met Pex5p en ubiquitine waren negatief, maar wanneer een mutatie werd aangebracht in twee belangrijke aminozuren die deel uitmaken van de potentiële UIM , herstelde dit gemuteerde eiwit een *pex5Δ* stam niet volledig tot wildtype. Daarnaast toonde two-hybrid analyse dat deze mutant niet langer efficiënt kon binden aan een aantal bekende/gekarakteriseerde Pex5p-bindingspartners. Deze resultaten suggereren dat dit fenotype hoogstwaarschijnlijk wordt veroorzaakt door een algemene instabiliteit van het eiwit en niet door een specifiek effect door de aangebrachte mutatie in de potentiële UIM.

Een relatie tussen Pex5p en ubiquitine is wederom het onderwerp van discussie in Hoofdstuk 5. Pex5p wordt geubiquitineerd in zowel een Ubc4p-afhankelijke als onafhankelijke manier. Ubc4p-afhankelijke ubiquitinatie wordt waargenomen in bepaalde *pex* deletiestammen en deze vorm is waarschijnlijk betrokken in de kwaliteitscontrole van Pex5p. Daartegenover is Ubc4p-onafhankelijke ubiquitinatie waargenomen in wildtype cellen en aangenomen wordt dat dit een rol speelt in de non-proteolitische regulatie van de PTS1 receptor. In dit hoofdstuk wordt beschreven op welke plaatsen in Pex5p deze twee vormen van ubiquitinatie zich voordoen. Ubc4p-afhankelijke ubiquitinatie vindt plaats op twee geconserveerde lysines in het N-terminale gebied van Pex5p. Ubc4p-onafhankelijke ubiquitinatie vindt niet plaats op de terminale α-NH_2 of op andere lysines maar vereist hoogstwaarschijnlijk een sterk geconserveerd cysteine residu aanwezig op positie 6 van de receptor. In afwezigheid van deze cysteine blijven PTS1 eiwitten in het cytosol waaruit blijkt dat dit residu essentieel is voor de functie van Pex5p. Bovendien konden we aantonen dat het E_2 enzym Pex4p (Ubc10p) essentieel is voor Ubc4-onafhankelijke ubiquitinatie van Pex5p. Deze data geven niet alleen een eerste voorbeeld van cysteine ubiquitinatie uitgevoerd door het cellulaire ubiquitinatie proces maar tonen ook het mogelijke substraat van Pex4p aan.

Een verdere karakterisatie van Pex4p-afhankelijke ubiquitinatie van Pex5p wordt besproken in Hoofdstuk 6. Wanneer de geconserveerde cysteine werd gemuteerd in een lysine resulteerde dit in een gedeeltelijk functioneel eiwit dat veel meer geubiquitineerd was dan het wildtype eiwit. Deze verhoging was niet te wijten aan een ander N-terminaal lysine residu en werd grotendeels gereduceerd in de afwezigheid van Pex4p. Deze verhoging maakte het mogelijk om gebruik te maken van direct western blotting met Pex5p antilichamen om het gedrag van deze mutant te observeren. Bovendien toonden we aan dat de RING domein van Pex10p *in vitro* kan fungeren als een E_3 ligase met de Ubc4p homoloog UbcH5a. Deze resultaten suggereren dat Pex10p kan functioneren als een E_3 ligase voor Ubc4p-afhankelijke ubiquitinatie van Pex5p *in vivo*.

Dankwoord

Well, that's that then. Now that's its all done and dusted and we are left to clear away the pink party hats and half eaten cold pizza, I would like to the take this time to thank a few people who's contributions over the last five years have allowed me to be where I am today.

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Finally, this is the end. Amid cries of about %@$#*&! time, there remains but one thing left to say, and that is……………….