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Structural biology of the import pathways of peroxisomal matrix proteins

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ABSTRACT

The peroxisomal proteins (peroxins) that mediate the import of peroxisomal matrix proteins have been identified. Recently, the purification of a functional peroxisomal translocon has been reported. However, the molecular details of the import pathways and the mechanisms by which the cargo is translocated into the lumen of the organelle are still poorly understood. Structural studies have begun to provide insight into molecular mechanisms of peroxisomal import pathways for cargo proteins that harbor peroxisomal targeting signals, PTS1 and PTS2, at their C- and N-termini, respectively. So far structures have been reported for binary or tertiary protein–protein interfaces, and highlight the role of intrinsically disordered regions for these interactions. Here, we provide an overview of the currently available structural biology of peroxisomal import pathways. Current challenges and future perspectives of the structural biology of peroxisomal protein translocation are discussed.
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1. Introduction

Almost one third of protein in a living cell has to be targeted to a specific compartment to function properly [1–3]. Throughout evolution cells have devised a variety of ways to tackle this problem. Typically signal peptide motifs are cotranslated with a cargo protein for targeting to a specific compartment. These motifs are then recognized by molecular machines to perform the actual translocation. The molecular pathways that target proteins to mitochondria [4,5], the ER [6] or the nucleoplasm [7] have been rather well studied for many decades. In contrast, molecular mechanisms that target proteins to peroxisomes and peroxisome biogenesis are less well understood as peroxisomes have only been discovered relatively recently [8–12]. Peroxisomes are present in all eukaryotic cells and facilitate enzymatic reactions that require oxidizing conditions, such as involving catalases or D-amino acid oxidases [13].

Although not all peroxisomal proteins (Pex, peroxins) that are involved in peroxisomal matrix import are conserved across species, the main pathways of peroxisomal import share some common features (Fig. 1) [8–11]. The import of peroxisomal matrix proteins is truly remarkable based on some unique aspects. It is now well accepted that the nature of the pore is highly dynamic, thereby rationalizing how a wide size range of cargo can be translocated, *i.e.* including folded proteins and oligomers but even gold particles of 9 nm diameter [14–21]. It has been recently shown that the size of the pore depends on that of the cargo to be imported [22]. Peroxisomal matrix enzymes are recognized by soluble receptor proteins and are then targeted to the peroxisomal membrane by binding to a docking complex of membrane-associated proteins. Although the molecular mechanisms by which the cargo is translocated into the lumen of the organelle are still poorly understood, the key peroxins involved in this process have been identified and the purification of a functional peroxisomal translocon has been reported [22]. The peroxisomal transport receptors actively participate in the import process and are recycled back *via* ubiquitination into the cytosol by another set of membrane-associated peroxins [8,23–25].

This review focuses on the currently available structural biology of molecular interactions and mechanisms involved in peroxisomal import pathways. Therefore, available structural insights into the export and recycling of peroxisomal pathways [26,27] are not addressed. Future perspectives and open questions will be discussed at the end.

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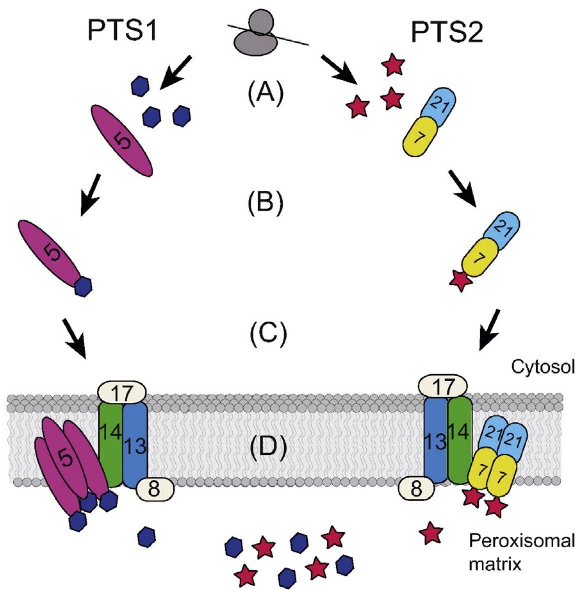

Fig. 1. Import pathways of peroxisomal matrix proteins. The import of peroxisomal matrix proteins occurs *via* two pathways, based on the presence of distinct peroxisomal targeting signal (PTS) sequences in the cargo protein. Cargo proteins with a PTS1 motif exhibit a C-terminal tripeptide (S/A/C)–(K/R/L)–(L/M) (PTS1, left) and bind to the Pex5 receptor in the PTS1 import pathway. Cargo proteins harboring an N-terminal PTS2 motif with a consensus sequence (R/K)-(L/V/I)-X5-(H/Q)-(L/A) are recognized by Pex7/Pex21 and imported *via* the PTS2 pathway (right). (A) Newly made cargo proteins and peroxins are released from polyribosomes into the cytosol. (B) In the cytosol, PTS1 cargo is recognized by the soluble Pex5 receptor, while PTS2 cargo is recognized by a complex of the Pex7/Pex21 receptors leading to the formation of receptor–cargo complexes. Note, that the coreceptor of Pex7 differs depending on the species. In human a long splice isoform of Pex5 acts as a coreceptor. In yeast species Pex21/Pex18 and Pex20, act as coreceptors (C) The cargo–receptor complexes bind to a docking subcomplex comprising Pex14 and Pex13 at the peroxisomal membrane. (D) Subsequently, the receptor–cargo complexes translocate the matrix protein into the peroxisome. Note, that — although the basic steps along the pathways are similar — the peroxisomal proteins involved and their specific roles vary between organisms.

2. Proteins involved in peroxisomal translocation

The vast majority of peroxisomal matrix proteins are targeted based on peroxisomal targeting signals (PTS1, PTS2) located at either the C-terminus (PTS1) or the N-terminus (PTS2). PTS1- and PTS2-cargo proteins reach the matrix *via* different pathways, although basic steps and some of the proteins involved are shared between these pathways. Peroxisomal cargo proteins carrying the C-terminal PTS1 signal peptide are most abundant. PTS1 cargo is transported by the Pex5 protein, which acts both as a chaperone [28] and a receptor factor for PTS1 cargo [29,30]. At the membrane the cargo-loaded Pex5 complex interacts with the membrane-associated Pex14 and Pex13 proteins [31–35] which together with Pex17 (in yeast) form the docking complex (Fig. 1). In this complex the interaction of Pex5 involves short WxxxF sequence motifs in its N-terminal region (Fig. 2), which bind to a small globular domain in the N-terminal region of Pex14. Pex14 and Pex13 play distinct roles in the import process. It has been suggested that the presence of cargo proteins influences the membrane interaction of Pex5 [36,37]. Pex14 preferentially binds to cargo-loaded Pex5 receptor, while Pex13 has higher affinity to the free receptor [38–40]. Functional assays using black lipid membranes implicate only Pex14 and Pex5 in gating of the peroxisomal pore. This suggests that Pex14 and Pex5 together are responsible for pore formation, while Pex13 may be involved in later steps, such as in coupling of translocation to receptor recycling (Fig. 1) [8]. The Pex17 protein is an additional factor associated with the Pex13/Pex14 docking complex in yeast, although its specific role is not well understood [41,42].

A smaller subgroup of peroxisomal proteins harbor an N-terminal peroxisomal targeting signal 2 (PTS2) motif and thereby utilize a second pathway to reach and import into the organelle. For PTS2 cargo recognition the Pex7 protein is required [43]. Even though Pex7 functionally resembles Pex5 with respect to cargo recognition it lacks WxxxF/Y motifs to mediate an interaction with Pex14 for docking and localization to the peroxisomal membrane. Instead, Pex14 binding depends on additional regions or coreceptors [44], depending on the organism. For example, in *Homo sapiens* a longer splice isoform of Pex5, which includes a short stretch of additional residues in the N-terminal region of Pex5(L) establishes an interaction with Pex7. Thereby, the Pex5 WxxxF motifs provide the molecular contacts with Pex14. In contrast, in *Saccharomyces cerevisiae* Pex18 and Pex21 harbor a WxxxF/Y motif and bind to Pex7 thus providing the contact with Pex14 [45,46]. All coreceptors share WxxxF-like motifs as Pex14 binding element for peroxisome targeting and a Pex7 binding domain [40,47]. Similar to PTS1-dependent import, the peroxisomal membrane-anchored proteins Pex13 and Pex14 serve as docking platform for the cargo-loaded Pex5 receptor proteins. The import complex has the capacity to adapt to the size of the cargo imported. Various distinct types of PTS cargos have been reported that exhibit a wide range of binding affinities [48–52], oligomeric state [53], import partners ("piggyback") [18,54,55], as well

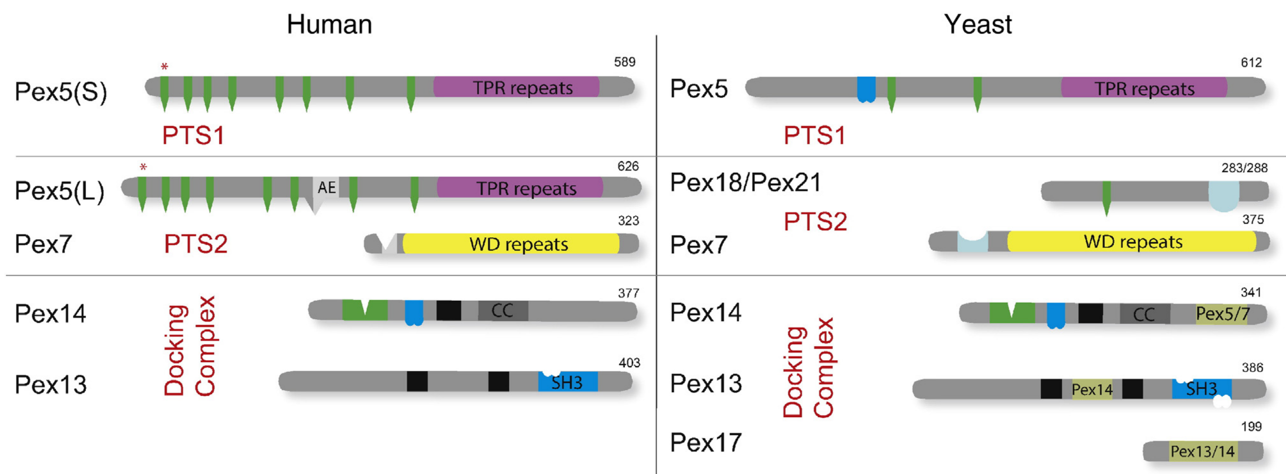

Fig. 2. Domains and interactions of key proteins involved in peroxisomal matrix protein import. Protein–protein interactions between interacting partners are indicated by complementary elements. Green arrows represent WxxxF and LVxAF (indicated by a red "*") motifs that bind to the Pex14 NTD (green). The alternative exon (AE) in the long splice isoform Pex5(L) that mediates Pex7 binding in the PTS2 pathway is colored light gray. Pex7 WD and Pex5 TPR domains are shown in yellow and purple, respectively. The Pex7 binding region of Pex18/Pex21 is shown in light blue. Black boxes in Pex14 and Pex13 indicate membrane interacting regions. The putative coiled coil domain of Pex14 is marked as CC. The Pex13 SH3 domain is shown in blue.

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as proteins showing "non-PTS import" [56]. The transient pore hypothesis [8,22] implies that the import machinery may flexibly adapt to a given cargo and thereby enable the translocation of a wide range of cargo types.

In the following, we review available structural biology of key peroxisomal protein involved in peroxisomal matrix protein import.

3. Pex5 receptor — PTS1 cargo

Pex5 is an essential factor for peroxisome biogenesis. The Pex5 protein is a soluble receptor that binds to PTS1-containing cargo in the cytoplasm and imports peroxisomal matrix proteins into the peroxisome lumen [57]. The overall architecture of the Pex5 protein is characterized by an intrinsically disordered N-terminal domain (NTD) comprising half of the protein and a globular C-terminal TPR domain that recognizes PTS1 cargo proteins [58,59]. This bipartite architecture is reflected in NMR fingerprint spectra (Fig. 3). The narrow range of chemical shifts and relative narrow linewidths of amide NMR signals of the Pex5 NTD (Fig. 3A) demonstrate the lack of a globular structure, while the well-dispersed spectrum of the C-terminal TPR domain (Fig. 3B) is consistent with the presence of a globular fold. Available structural studies have so far focused on the separate N- and C-terminal domains only.

3.1. The Pex5 N-terminal domain

The Pex5 N-terminal domain (NTD) harbors seven WxxxF/Y motifs and a LVAEF motif, which all are able to bind to a small helical fold in the N-terminal region of Pex14. This interaction is essential for docking of Pex5 to the Pex14/Pex13 complex at the peroxisomal membrane to initiate translocation of the cargo. The long isoform of human Pex5 harbors an additional sequence that mediates an interaction with Pex7 and thereby supports docking complex formation in PTS2-dependent import (Fig. 2) [34,59–61].

The binding affinities of Pex5 WxxxF/Y or LVAEF motifs to the Pex14 N-terminal domain (NTD) correspond to dissociation constants in the high nanomolar range [34,59]. The presence of seven WxxxF/Y and one LVAEF motif in human Pex5 NTD suggests that higher order complexes of 1:8 Pex5:Pex14 stoichiometry can be formed *in vitro*. This is has been confirmed by Isothermal Titration Calorimetry (ITC) [59] and Small Angle X-ray Scattering (SAXS) measurements [62]. However, it is unknown whether such higher order complexes will be relevant in a cellular context near the peroxisomal membrane. Structural details for the specific recognition of these motifs by the Pex14 NTD are presented in the next section.

SAXS, NMR, biophysical and biochemical data show that the N-terminal half of Pex5 is intrinsically disordered [58,59,62,63] (Fig. 3A). Nevertheless, NMR studies have shown that some of the WxxxF/Y motifs adopt a partial helical conformation [60]. SAXS studies with the full-length Pex5 protein indicate that the NTD remains flexible also when cargo protein is bound to the C-terminal TPR domain. This suggests that the NTD does not contribute to recognition of the SCP2 cargo protein studied [62]. SAXS and static light scattering (SLS) experiments have established that Pex5 is monomeric in solution [62]. However, recently it was proposed that cargo binding and release of Pex5 from *Pichia pastoris* may be regulated by redox-dependent intermolecular disulfide bond formation involving a cysteine residue close to the N-terminus of the Pex5 protein [64].

The main function of Pex5 NTD is thought to mediate docking of the receptor onto the Pex14/Pex13 complex at the peroxisomal membrane to initiate cargo translocation [65]. However, a contribution of the NTD Pex5 to cargo recognition has been observed by two-hybrid analysis with pumpkin catalase, cat1 [66] as well as for of acyl–CoA oxidases [67] and alcohol oxidase [56]. This suggests that additional interactions can modulate cargo binding and import.

The functional importance of the Pex5 NTD is indicated by mutations in the Pex5 gene which result in peroxisome biogenesis disorder (PBD) group A, the Zellweger spectrum disorders (ZSD) [68]. A recently identified mutation in exon 9, which is only present in the N-terminal domain of the long Pex5(L) splice isoform results in peroxisomal dysfunction. The mutation leads to a specific defect in the import of PTS2-tagged proteins, causing rhizomelic chondrodysplasia punctata (RCDP) type 1 instead of ZSD [69]. Dysfunctional peroxisomes are eliminated by lysosomal autophagy [70].

The protein degradation process is triggered by ubiquitination of a matrix protein [61,71]. For example, in *S. cerevisiae* Pex5 is monoubiquitinated at a conserved cysteine at position 4 by the ubiquitin-conjugating and ligase enzymes, Pex4 and Pex12, respectively [71,72]. It has been shown that Pex22 functions as a cofactor for Pex4, as an interaction of Pex22 enhances Pex4's ability to transfer ubiquitin to a substrate *in vitro*, and mutations in Pex4 that affect the Pex22 interaction are unable to ubiquitinate the peroxisomal import receptor Pex5

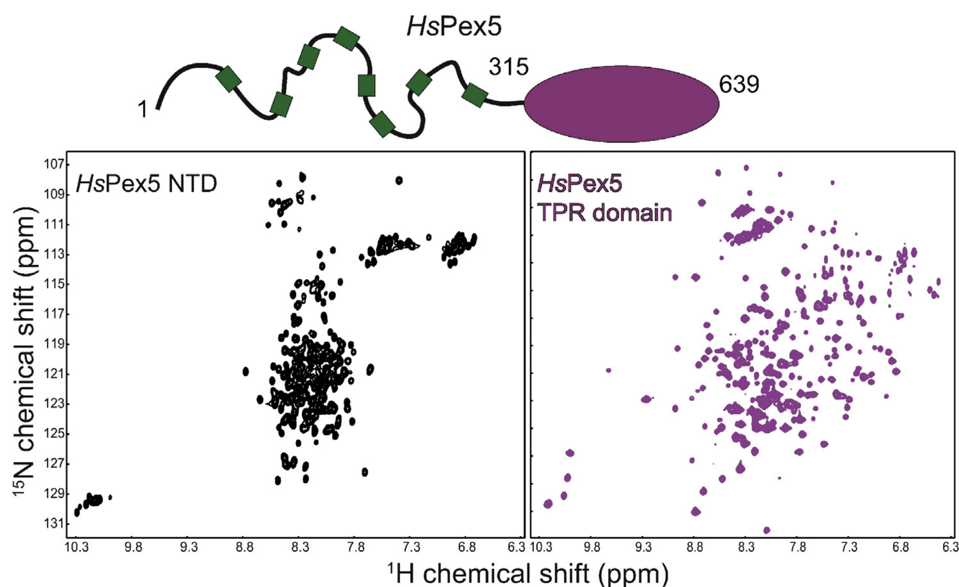

Fig. 3. Pex5 comprises an intrinsically disordered N-terminal domain. NMR ^{1}H–^{15}N correlation spectra of the NTD (left) and the C-terminal TPR domain (right) of human Pex5 are shown. The very small chemical shift range observed for amide signals of the NTD indicates that it is intrinsically disordered, while the large dispersion of chemical shifts observed for the TPR domain shows that it adopts a globular fold.

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in vivo [73,74]. A crystal structure of the Pex4–Pex22 complex and biochemical data demonstrate that Pex4 and Pex22 together function as the E2 enzyme required for Pex5 ubiquitination [73]. The ubiquitinated Pex5 is subsequently extracted from the peroxisomal membrane in an ATP-dependent manner by the receptor export machinery for receptor recycling [61]. When the receptor recycling pathway is blocked, Pex5 is polyubiquitinated by the ubiquitin-conjugating and ligase enzymes, Ubc4 and Pex2, respectively, and directed to the proteasome for degradation [71,75]. How the receptor recycling and degradation pathways are regulated is not known [64].

3.2. The Pex5 C-terminal TPR domain mediates cargo binding

The C-terminal cargo-binding region of Pex5 is a globular domain consisting of seven tetratricopeptide repeats (TPRs), followed by a helix bundle [48]. Two TPR triplets (TPR1-3, TPR5-7) are arranged in a ring-like array forming the PTS1 binding groove for the matrix enzymes destined to peroxisomes. Both triplets are linked by the non-canonical TPR4 that is partially folded in available structures [48,57] (Fig. 4A).

The PTS1 peptide sequence establishes the most crucial contacts in cargo binding as it determines the strength of interaction with binding affinities ranging from low nanomolar to low millimolar dissociation constants [76]. First described as a serine–lysine–leucine (SKL) tripeptide in firefly luciferase [51] several other variations — [S/A/C]–[K/H/R]–[L/M] — have been found [49,50]. Some exceptions exist where only two out of three positions do not match this consensus [52,77] Four conserved asparagine residues in the TPR array facilitate binding to the PTS1 backbone *via* hydrogen bonds [48,57] (Fig. 4B). These residues are critical for the function of Pex5, as mutations lead to isolated PTS1 protein import defects *in vivo* [78]. Multiple sequence alignments of Pex5 TPR domains show that these residues are highly conserved [40].

The PTS1 side-chain interaction varies depending on the PTS1 signal sequence and can be direct or mediated *via* water molecules. An acidic pocket within the TPR domain accommodates interaction with basic side-chains on position −2 (counting from the C-terminus of the cargo PTS1) [48,57] (Fig. 4B). The PTS1 position-3, which favors small side chains, plays a vital role in receptor-cargo recognition. Steric clashes at this position can prevent efficient binding by causing a 1000-fold reduction in binding affinity, as observed in mutant PTS1 studies of the full-length alanine-glyoxylate aminotransferase (AGT) Pex5 complex [79,80]. Based on this observation and comparisons of cargo-loaded and *apo* TPR domain structures [80], an induced fit model was proposed to explain the adaptation of the TPR domains to accommodate different PTS1 peptides of peroxisomal matrix proteins. The TPR 1–3 and TPR 5–7 triplets that both contribute to cargo PTS1 binding, adapt their position by reducing the size of the binding pocket volume, thus ensuring a snug fit. This in turn increases the binding affinity [80] (Fig. 4A). The TPR4 in this scenario operates as a conformational mobile linker [81,82].

Additional residues beyond the C-terminal PTS1 tripeptide were described to be crucial for binding and successful peroxisomal import, which led to an expanded definition of the PTS1 taking all positions of the C-terminal dodecamer peptide into consideration [83]. The complex structures of full-length cargo protein AGT bound to Pex5 for example, revealed that an eight-residue helix preceding the PTS1 tripeptide additionally interacts with the cargo receptor [79]. PTS1-independent interactions are also observed within the two currently existing crystal structures of Pex5 with sterol carrier protein (SCP2) (Fig. 4C,D) and AGT (Fig. 4E,F). In both cases, additional contacts are detected with the C-terminal helical bundle of Pex5 beyond the TPR array [48,79] (Fig. 4D,E).

Interactions of the unstructured N-terminal region with the globular C-terminal cargo binding domain of Pex5, as well as conformational changes that may occur upon cargo binding to facilitate docking complex binding have been suggested [58,84]. However, low-resolution

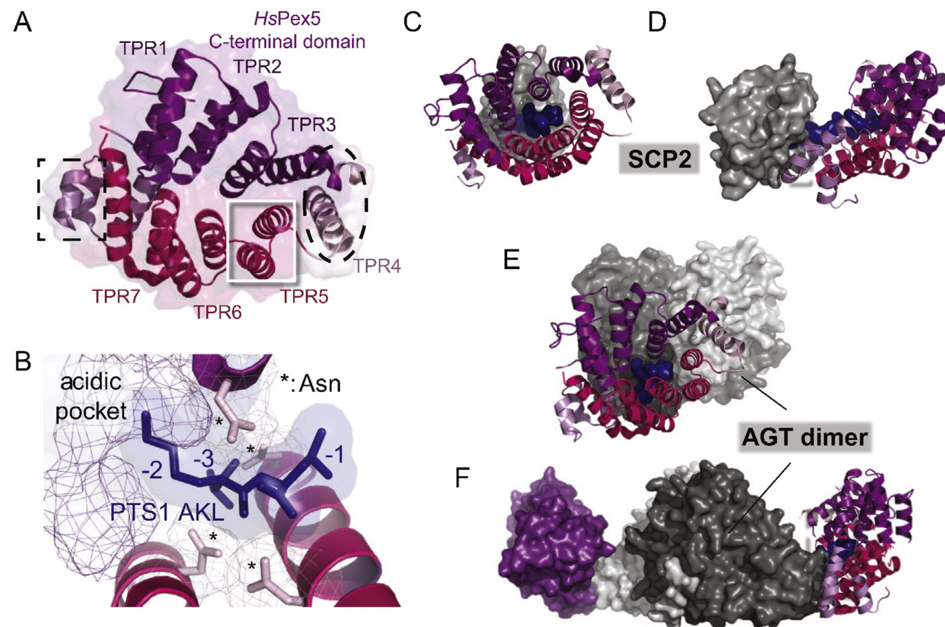

Fig. 4. Structural biology of Pex5 PTS1–cargo complexes. Colors: PTS1 C-terminal residue triplet, blue; Pex5 TPR domain, pink/purple; cargo proteins, gray surface representation. (A) Structure of the Pex5 cargo binding domain. The C-terminal domain of Pex5 harbors seven TPR domains followed by a C-terminal helical bundle (dotted box). Two TPR triplets, TPR1-3 (purple) and TPR 5–7 (pink) form a ring-like PTS1 binding groove linked by a non-canonical TPR4 that is only partially folded (dotted cycle). The solid box indicates an exemplary TPR domain (PDB accession 4KYO). (B) PTS1 binding groove. Conserved asparagine residues (indicated by "*") interact with the AKL amino acid sequence in the PTS1 motif. The Pex5 surface is shown in mesh presentation. The acidic pocket recognizes the residue at position −2, small side chains are preferred at position −3 counting from the C-terminus of the cargo PTS1 AKL in the structure (PDB accession code 4KYO). (C) Complex of the cargo protein SCP2 (gray, surface representation) with the Pex5 TPR domain (cartoon representation, front view, PDB: 2COL). (D) Structure of the SCP2-Pex5 TPR domain complex (side view). The white dotted box highlights an area with PTS1-independent contacts involving the C-terminal helical bundle. (E) Complex of the cargo protein AGT dimer (two different gray shades for each of the protomer, surface representation) with the Pex5 TPR domain (cartoon representation, front view, PDB: 4KYO). The PTS1 is shown in blue. (F) Structure of the AGT-Pex5 TPR domain complex (side view). The white dotted box highlights an area with PTS1-independent contacts involving the C-terminal helical bundle.

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SAXS data of the full length Pex5 cargo complex by itself, as well as bound to Pex14 did not indicate a significant rearrangement of Pex5 complex in complex with a SCP2 cargo protein [62].

4. Pex7 receptor/Pex21 coreceptor — PTS2 cargo

Structural mechanisms linked to PTS2 import are less well characterized. While in mammals, a small number of PTS2-cargo proteins exist, in *Arabidopsis thaliana* 30% of peroxisomal matrix proteins are imported based on a PTS2 motif [50]. The proteins involved in the PTS2 pathway resemble those of the PTS1 import [85] and both pathways converge at the membrane upon interaction with the docking complex (Pex13, Pex14 and Pex17, Fig. 1). Binding to PTS2 cargo is mediated by the Pex7 protein, which harbors a WD domain responsible for recognition of the PTS2 peptide (Fig. 2). Thus, the Pex7 WD/PTS2 interaction functionally corresponds to the Pex5 TPR/PTS1 complex in the PTS2 import pathway. However, an important aspect of the PTS2 pathway is that Pex7 requires coreceptors for the import of PTS2 cargo. Since Pex7 lacks characteristic WxxxF motifs to mediate binding to Pex14 at the peroxisomal membrane, coreceptors provide such Pex14 binding motifs. Interestingly, different solutions are employed depending on the organism. For example, Pex7 binds to the paralog proteins Pex18 or Pex21 in *S. cerevisiae*, to Pex20 in *P. pastoris* and to the longer splice isoform of Pex5(L) in *H. sapiens* and thereby acquires the ability to bind to Pex14 [45,86] (Fig. 2).

Recently, the crystal structure of a heterotrimeric *S. cerevisiae* Pex7–Pex21–PTS2 complex was reported, providing first high-resolution structural insight into PTS2 cargo recognition [87] (Fig. 5). In the crystal structure the C-terminal region of Pex21 (Pex21C), which mediates the interaction with Pex7, was also required to form a stable complex with the PTS2 ligand. The PTS2 peptide derived from the Fox3 cargo protein was fused to maltose-binding protein (MBP), creating an artificial cargo protein to enhance crystallization. The PTS2 peptide folds into an amphipathic α-helix, which is sandwiched between Pex7 and Pex21 (Fig. 5A). Thus, PTS2 recognition requires cooperative binding by the Pex7 receptor and the Pex21 coreceptor involving contacts of the hydrophobic face of the amphipathic PTS2 helix with both Pex7 and Pex21C (Fig. 5B). In contrast, the charged face of the PTS2 helix is mainly solvent-exposed, except two important residues, Arg4 and His11 which form electrostatic contacts with resides in Pex7 (Fig. 5B). These findings demonstrate that cooperation of Pex7 and its coreceptor Pex21 is required for PTS2 recognition, in addition to docking complex formation, which further depends on Pex14 binding motifs provided by the coreceptor.

5. Pex14

Subsequent to recognition of cargo proteins in the cytoplasm the PTS1- and PTS2 cargo/receptor complexes are targeted to the peroxisomal membrane. This step is mediated by the docking complex, which includes the membrane-anchored proteins Pex14 and Pex13 as key components [31,33]. Our structural knowledge about domain organization of these two key components of peroxisome biogenesis is rather limited. Sequence analysis and experimental data of known Pex14 homologs reveal three key features of the protein, an N-terminal helical domain, a hydrophobic patch that is expected to mediate membrane interactions and a C-terminal region.

The best characterized of Pex14 is the conserved N-terminal domain (NTD) (Fig. 6). The small, 60-residue Pex14 NTD domain (Fig. 6A) has been shown to mediate binding to various interaction partners, ranging from peroxisomal proteins involved in PTS-mediated and peroxisomal membrane protein (PMP) import pathways, to tubulin [59,60,88]. A crystal structure of the rat Pex14 NTD revealed a three helical bundle fold [63] (Fig. 6B,C). Stacking of the aromatic side chains of Phe52 and Phe35, located in helices α2 and α3, respectively, stabilize the fold of the domain and contribute to a hydrophobic surface cavity of the protein. A dimeric arrangement of the NTD observed in the crystal (Fig. 6B,C) mimics interactions with WxxxF-like helical ligands (see below), while NMR analysis indicates that even at high micromolar concentrations the Pex14 is predominantly monomeric [60].

The structural basis for the interactions of Pex14 with WxxxF-like ligands was determined using solution NMR with motifs of Pex5, the transport receptors for peroxisomal matrix protein import, as well as of Pex19, the import receptor for peroxisomal membrane proteins [59,60]. Structures of the human Pex14 NTD bound to a prototypic WxxxF [60] and a non-canonical LVxAF motif [59] found in the N-terminal domain of Pex5, and of a FFxxxF motif found in the intrinsically disordered N-terminal region of Pex19 [60] were reported (Fig. 6D,F). The structures reveal how two hydrophobic binding cavities in the Pex14 NTD accommodate conserved aromatic (and aliphatic) side chains of the peptide ligands. An additional salt bridge (not shown) stabilizes the interaction of Pex14 with the Pex5 WxxxF and LVxAF motifs. A corresponding salt bridge is not present in the complex with Pex19 FFxxxF peptide consistent with the reduced binding affinity (K_D values of 0.5 μM for Pex5 WxxxF, 0.16 μM for Pex5 LVxAF, and 9 μM for Pex19 FFxxxF, respectively). Interestingly, while all three peptides employ aromatic or aliphatic side chains to bind into the two hydrophobic cavities in the Pex14 NTD the Pex19 FFxxxF helix binds in an opposite orientation [60]. The functional role of the overlapping (and thus competitive) binding site in Pex14 for peptide motifs found in Pex5 and Pex19 remains to be clarified.

Structural information for Pex14 beyond the N-terminal region is scarce. Sequence analysis reveals the presence of a PxxP motif, which has been shown to interact directly with SH3 domain of Pex13 [60,89]. However, in the context of the Pex14 NTD this motif is not fully accessible so that binding would require a conformation change in the Pex14

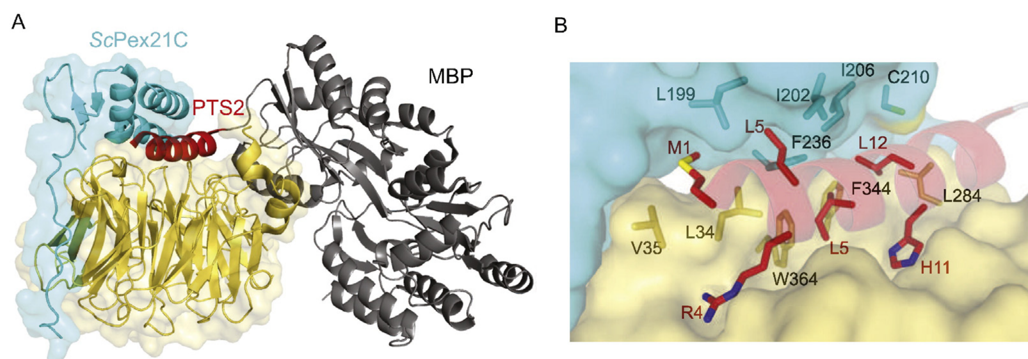

Fig. 5. PTS2 cargo recognition by the Pex7–Pex21 receptor. (A) Cartoon and surface representation of the PTS2 cargo–receptor complex. The Pex7 WD domain (yellow) and the C-terminal domain of the coreceptor Pex21 (cyan) together recognize the helical PTS2 signal peptide of Fox3N (red) fused to MBP (gray). (B) Zoomed view of the PTS2 helix with side chains depicted as sticks.

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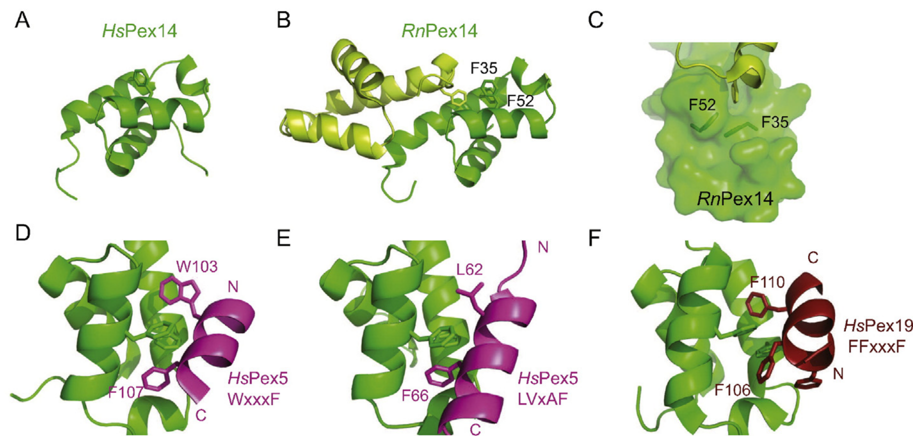

Fig. 6. Structures of N-terminal domain of Pex14. (A) Cartoon representation of the NMR structure of the human Pex14 NTD (green) when bound to the WxxxF motif (PDB accession 2W84) and (B) of the crystallographic Pex14 dimer from Rattus norvegicus (PDB 3FF5). The helical bundle is stabilized by stacking of two phenylalanines, which also contribute to the formation of a hydrophobic cavity on the surface of the structure. (C) In the dimer a phenylalanine derived from the expression vector binds to this cavity of the other monomer. (D–F) Cartoon representation of Pex14 NTD complexes with (D) Pex5 WxxxF (magenta, PDB 2W84), and (E) LVxEF motifs (magenta, PDB 4BXU) as well as (F) with an FFxxxF motif found in the N-terminal region of Pex19 (brown, PDB 2W85). Conserved phenylalanines of Pex14 and aromatic/hydrophobic side chains of the ligands are depicted as sticks.

NTD [60]. A structure of the Pex13 SH3 domain with the Pex14 PxxP motif [90] will be discussed below in the chapter on Pex13.

In the primary sequence of Pex14 the PxxP motif is followed by a hydrophobic patch of residues responsible for membrane association of the protein. It is not known how these residues interact with the membrane and the topological arrangement of the full-length Pex14 protein with respect to the membrane is unclear. While the C-terminal part of Pex14 is thought to face the cytoplasm [91] the localization of the N-terminal helical domain is unclear and inconsistent results have been reported [92–96]. In addition, it has been shown that a loop facing the peroxisomal lumen in the *S cerevisiae* Pex13 protein can interact with Pex14 [97]. This suggests that a region of Pex14 at some stage may be localized in the peroxisomal lumen to mediate such an interaction.

6. Pex13

Pex13 is the second factor of the docking complex [98]. It exhibits two membrane spanning domains (TMD) that are connected by a loop in the peroxisomal lumen [97], while flanking N- and C-terminal parts of the protein are cytosolic [99]. The N-terminal cytosolic part is not well characterized and no structural data are available so far. Nevertheless some interactions have been reported. In yeast, the N-terminal 100 residues of Pex13 mediate binding to Pex7 and deletion of these residues impairs PTS2 import [44]. Structural details have been reported for the SH3 domain in the C-terminal region, downstream of the second TMD [33,90,95,99–101]. Interestingly, a mutation in the Pex14 binding interface of the Pex13 SH3 domain has been reported for a Zellweger syndrome patient [102]. Two independent studies demonstrated that the yeast Pex13 SH3 domain exhibits two distinct and independent binding sites for Pex14 and Pex5 [90,101] (Fig. 7). A crystal structure of SH3 domain bound to a PxxP motif from Pex14 showed that the PxxP motif is recognized in a poly-proline type II (PPII) helical conformation on the canonical PPII binding site of the SH3 domain [90] (Fig. 7B,C). NMR studies showed that a WxxxF peptide derived from the N-terminal domain of Pex5 was found to bind to the opposite site of the SH3 domain in an α-helical conformation [90,101]. The binding of Pex14 PxxP and Pex5 WxxxF motifs is non-competitive and independent [90]. Thus, the Pex13 SH3 domain appears to be an important adaptor that establishes a molecular link between Pex5, Pex13 and Pex14 in the docking complex. So far the interaction between the SH3 domain and a Pex5 WxxxF motif has only been found in yeast and appears not to be conserved in human Pex13. Nevertheless, the importance of the Pex13–Pex5 interaction for PTS1 import has been confirmed by *in vivo* experiments [90].

7. Conclusions

Despite the fact that peroxisomes have been studied for decades by traditional biochemical means, within the last 15 years structural studies have begun to help elucidating molecular mechanisms in peroxisomal import. Here, we have presented an overview of currently available structural biology linked to PTS1- and PTS2-mediated import into peroxisomes. A number of interesting features and principles are emerging from these studies.

- The key proteins involved in PTS-mediated import cooperate by a network of protein–protein interactions that in most cases involve the

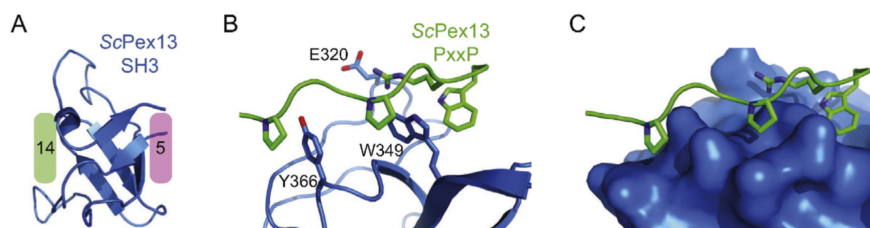

Fig. 7. Structure and interactions of the Pex13 SH3 domain. (A) Cartoon representation of crystal structure of the Pex13 SH3 domain (blue, PDB 1NM7). Green and magenta boxes indicate the interaction sites with Pex14 PxxP and Pex5 WxxxF peptide motifs, respectively. (B,C) Crystal structure of the Pex13 SH3 domain bound to a Pex14 derived PxxP peptide (green) (PDB 1N5Z). Important residues, which contribute to the interaction are shown as sticks.

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recognition of short helical motifs, such as the characteristic biaromatic/aliphatic motifs found in the Pex5 and Pex18/Pex21 receptors.

- These short helical motifs are typically found in intrinsically disordered regions of peroxisomal proteins, which represent significant parts of the full-length sequence. It is expected that unique features associated with the intrinsic flexibility of these regions play important roles. For example, the flexible connection of short helical motifs in an unstructured poly-peptide chain supports malleable interactions with multiple distinct binding partners, as for example seen for the recognition of WxxxF-like motifs by Pex14, Pex13 and Pex19. The extended conformation also greatly increases the search space for the exploration of binding partners.
- The Receptor proteins for peroxisomal matrix (Pex5) and peroxisomal membrane proteins (Pex19) share a common bipartite architecture: The N-terminal half of the receptor is intrinsically disordered and harbors short peptide motifs to mediate protein–protein interactions with other peroxins, *i.e.* in the docking complex at the peroxisomal membrane. The C-terminal domain is dedicated for the recognition of cargo proteins depending on targeting signals (PTS by Pex5 or mPTS by Pex19). In the PTS2 pathway, this bipartite architecture is provided by separate proteins, Pex7 and Pex21/Pex18.
- The PTS1 and PTS2 pathways use distinct molecular mechanisms to recognize their respective PTS motifs: The PTS1 motif is recognized in an extended conformation by the Pex5 TPR domain, while the PTS2 motif is sandwiched in a α-helical conformation between a WD domain of the Pex7 receptor and a helical domain of the Pex21 coreceptor.
- Structural studies of a number of different PTS1 cargo proteins bound to Pex5 have identified that recognition of additional regions outside the PTS1 motif contribute to cargo recognition, thereby presumably providing additional specificity.

The structural and molecular mechanisms of docking complex formation and protein translocation are still poorly understood. For example, multiple WxxxF-like motifs are presented in the Pex5 N-terminal domain (*i.e.* up to eight in *H. sapiens* Pex5). Most of these motifs can individually bind to the Pex14 NTD, but not all motifs are required for functional peroxisomal matrix protein import [91].

What is the role for this redundancy? Some non-mutually exclusive considerations are:

1) The unstructured extended N-terminal domain of Pex5 can employ a fly-casting mechanism to find and bind Pex14 at the peroxisomal membrane [59] (Fig. 8A). The presence of multiple motifs thereby increases the probability of interaction and thus provides avidity for the Pex5–Pex14 interaction due to increased local concentrations of WxxxF-like motifs.
2) Considering different binding kinetics of the various motifs, the Pex14 NTD could make initial contact with the N-terminal LVxEF motif and then “slide” along the Pex5 N-terminal domain towards the cargo binding domain. Thereby the unstructured Pex5 N-terminal domain could act like a rope and pull the cargo-bound TPR domain towards the membrane [59].
3) The presence of multiple motifs also may reflect multifunctional interactions and can contribute to the formation of higher order complexes, where the individual motifs can form distinct interactions with other peroxisomal proteins. For example, in yeast, a ternary complex can be formed involving two WxxxF motifs in Pex5, which bind to the Pex14 NTD and the Pex13 SH3 domain, respectively.
4) Multiple binding motifs enable the formation of higher order complexes as has been observed *in vitro* [59,62]. It is, however, unknown whether complexes with higher Pex5–Pex14 stoichiometry exist in the cellular context. The molecular weight of the purified PTS1 pore [22] can only be explained by the presence of multiple Pex14 and/or Pex5 components. As an interesting possibility each WxxxF-like motif could bind to one Pex14 NTD at the peroxisomal membrane (Fig. 8B). For the human protein this could lead to a 1:8 Pex5:Pex14 complex at the membrane, in other organisms with fewer WxxxF motifs perhaps multiple Pex5 proteins could be involved. In any case, this assembly could contribute to the formation of a pore that enables cargo translocation and is consistent with the so-called “transient” pore hypothesis. Clearly, experimental evidence and eventually structural details are required to probe such a model in the future.

8. Outlook

Structural biology has become an important aspect of peroxisome research and continues to provide unique insight into detailed molecular mechanisms of the peroxisomal translocation machinery. The peroxisomal translocon exhibits unique features, based on multiple protein–protein interaction that connect the various peroxisomal proteins, and by the presence of large, functionally important intrinsically disordered regions.

To unravel the molecular details of the peroxisomal tranlocon at a structural level integrated structural biology approaches are required. High-resolution crystal structures of globular domains are complemented by solution methods such as small angle scattering

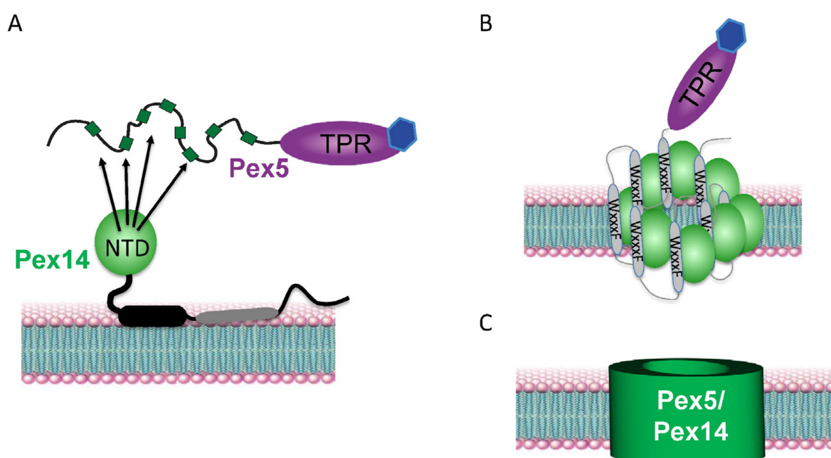

Fig. 8. Possible models for Pex5–Pex14 interactions. (A) The presence of multiple WxxxF-like motifs in the N-terminal half of the soluble Pex5 receptor may enable fly-casting for binding to Pex14 at the peroxisomal membrane. (B) The peroxisomal translocon could involve the formation of Pex5–Pex14 complexes, where each WxxxF-like motif binds to one Pex14 molecule near or within the peroxisomal membrane, (C) thereby forming a transient and dynamic pore. The blue hexagon represents a PTS1-cargo protein bound to the C-terminal TPR domain of Pex5.

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and NMR spectroscopy to capture conformational dynamics of disordered regions. While SAXS can characterize overall conformational changes and dynamics, NMR provides unique opportunities to study molecular interactions as well as the intrinsic flexibility, local conformation and dynamics of protein complexes including intrinsically disordered regions [103,104]. Especially given the recent exciting technical advances [105], cryo-electron microscopy will be an important tool for studying the peroxisomal translocon. However, this will require that pure and homogenous biochemical preparations of functional peroxisomal pores become available, preferably, based on reconstitution *in vitro*.

Future efforts need to focus on studying peroxisomal proteins in a membrane mimicking environment, where additional interactions with the membrane are expected to strongly affect molecular interactions and to shape the structure of the peroxisomal translocon. While taking a divide-and-conquer approach will remain useful to obtain structural insight and study molecular interfaces, it will also be important to study full-length proteins and complexes where intrinsically disordered regions could potentially interact with globular domains in the context of a larger complex. As is being applied to other translocation machineries, it will be crucial to establish mutants that trap the translocation at specific steps to provide homogeneous complexes suitable for structural studies.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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