Review

The importomer—A peroxisomal membrane complex involved in protein translocation into the peroxisome matrix

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Abstract

The import of proteins into the peroxisome matrix is an essential step in peroxisome biogenesis, which is critical for normal functioning of most eukaryotic cells. The translocation of proteins across the peroxisome membrane and the dynamic behavior of the import receptors during the import cycle is facilitated by several peroxisome–membrane-associated protein complexes, one of which is called the importomer complex [B. Agne, N.M. Meindl, K. Niederhoff, H. Einwachter, P. Rehling, A. Sickmann, H.E. Meyer, W. Girzalsky, W.H. Kunau, Pex8p: an intraperoxisomal organizer of the peroxisomal import machinery, Mol. Cell 11 (2003) 635–646; P.P. Hazra, I. Suriapranata, W.B. Snyder, S. Subramani, Peroxisome remnants in pex3Δ cells and the requirement of Pex3p for interactions between the peroxisomal docking and translocation subcomplexes, Traffic 3 (2002) 560–574. [1,2]]. We provide below a brief historical perspective regarding the importomer and its role in peroxisome biogenesis. We also identify areas in which further work is needed to uncover the physiological role of the importomer.
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1. Discovery of the docking and RING subcomplexes and the importomer

The receptors that bind the PTS1 and/or PTS2 sequences present in peroxisomal proteins are located predominantly in the cytosol, and are only partially associated with peroxisomes. The receptors, Pex5p [3–5] and Pex7p [6–9], and the fungal-specific co-receptors, Pex18p/Pex21p [10] or Pex20p [11], interact directly [3,12,13] or indirectly [11,14,15] with PTS-containing cargoes in the cytosol. It was therefore anticipated that these receptor/cargo complexes would need to dock with one or more peroxisome–membrane-associated protein/s during cargo translocation across the peroxisome membrane. The first constituent required for this receptor/cargo docking event at the peroxisome membrane was shown to be Pex13p, which interacts with the

PTS1 receptor, Pex5p [16–18]. Initial reports characterizing Pex13p showed that in *S. cerevisiae* it did not interact with Pex7p [16], so a peroxisomal docking factor for the PTS2 receptor still appeared to be missing (however, later studies revealed that ScPex13p could bind ScPex7p independently of ScPex14p [14]). The discovery of a new peroxin, Pex14p, that interacted with both Pex5p and Pex7p, as well as with Pex13p, solved this problem [19]. Later, other peroxins, such as Pex17p [20,21] and Pex8p [22] (in yeasts), as well as Pex3p [2] (only in *P. pastoris* and not in *S. cerevisiae*) were found to interact with Pex14p, leading to the concept of a docking subcomplex comprised of Pex13p, Pex14p, Pex17p and Pex8p (in several yeasts), as well as Pex3p [2,22] (only in *P. pastoris* and not in *S. cerevisiae*). The role of this subcomplex in receiving receptor/cargo for both PTS1 and PTS2 import pathways has now been further reinforced by the finding that the PTS2 pathway co-receptors, Pex18p and Pex21p in *S. cerevisiae* [15], or Pex20p in other fungi such as *N. crassa* [15], *Y. lipolytica* [23] and *P. pastoris* [11], also interact with Pex14p and Pex13p, either directly [11], or indirectly via Pex7p [14,15]. In mammals, there are no PEX8, PEX17 or PEX18/PEX20/PEX21-like proteins, so

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the docking subcomplex has PEX13 and PEX14, but other constituents are poorly defined.

Because Pex14p was the first peroxisomal–membrane-associated protein shown to interact with both Pex5p and Pex7p, it was suggested to be the point of convergence of the two matrix protein import pathways [19]. This concept of PEX14 as the initial docking site for receptor/cargo complexes on the peroxisomal membrane has been corroborated by the finding that mammalian PEX5 [9,24] and PEX7 [9] are completely cytosolic in *pex14*-deficient cells, and PEX7 is protease-sensitive [9]. In contrast, in wild-type mammalian cells, a fraction of both these receptors is peroxisome-associated and intraperoxisomal, as judged either by protease protection or fluorescence microscopy using permeabilization of only the plasma membrane with low concentrations of digitonin [9,24]. One notable difference between the fungal versus mammalian and plant systems is that in fungi, the receptors involved in the PTS1 and PTS2 pathways dock independently at the peroxisome membrane, whereas in the mammalian and plant systems, a long isoform of PEX5 (PEX5L) binds PEX7, and serves as a single conduit for interaction of both PTS1 and PTS2 pathway receptors with the peroxisomal PEX14 [25–27]. As a result, PEX7-dependent import requires the presence of Pex5L.

Cargo-loaded PEX5 in mammalian cells has a greater affinity for PEX14 than for PEX13, whereas cargo-free receptors bind more tightly to PEX13 [28,29]. In mammals, both cargo-loaded PEX5 and PEX13 bind to PEX14 homo-oligomers, but each of these proteins binds to PEX14 oligomers of different molecular masses. Cargo-unloaded PEX5 apparently disassembles PEX14 homo-oligomers [30]. These studies have led to the suggestion that cargo-loaded PTS receptors dock first with PEX14 on the docking subcomplex, and that the cargo-free forms of the receptors interact with PEX13 at a later temporal stage (e.g. just prior to the release of cargo-free receptors to the cytosol).

The study of protein–protein interactions between peroxisome–membrane-associated components required for matrix protein import using yeast two-hybrid and co-immunoprecipitation experiments revealed that several components of the docking subcomplex also interact with a set of three RING-domain proteins, Pex2p, Pex10p and Pex12p that interact with each other, and with Pex3p (in *P. pastoris*, but perhaps not in *S. cerevisiae*) [1,2]. Thus, the RING subcomplex was postulated [1,2] and it was further proposed that a supercomplex, consisting of members of both RING and docking subcomplexes existed in the peroxisome membrane [1]. Solubilization and purification of individual peroxisome–membrane-associated components required for peroxisomal matrix protein import, followed by mass spectrometric and immunoblotting analyses of the co-purified components confirmed the concept of an import complex, comprised in *S. cerevisiae* of both docking (Pex8p, Pex13p, Pex14p and Pex17p) and RING subcomplex (Pex2p, Pex10p and Pex12p) members [1]. The same components have been found to comprise the importomer in *P. pastoris*, but in addition, Pex3p is also a part of this complex [2, N. Rayapuram and S. Subramani, unpublished observations]. Because all the constituents of this import complex are necessary for peroxisomal matrix protein import by both the PTS1 and PTS2 path-

ways, this entire complex was named the importomer [1]—a complex that is responsible for protein translocation across the peroxisome membrane.

More recent work has shown that the peroxisome membrane also has a receptor-recycling machinery for the recycling of receptors/co-receptor from the peroxisomes back to the cytosol [11,31–33], in accord with the extended shuttle model for receptor dynamics [11,34,35]. Interestingly, several components of this receptor-recycling machinery (the ubiquitin-conjugating enzyme Pex4p and its peroxisome-anchoring protein Pex22p in yeasts, the AAA ATPases Pex1p and Pex6p, and the peroxisome-associated Pex6p-anchoring proteins, Pex15p [36] and PEX26 [37] in *S. cerevisiae* and mammals, respectively) also associate with the importomer. Although this receptor-recycling machinery is also involved in the matrix protein import cycle, for the purposes of this review, our view of the importomer is restricted, for two reasons, to the original structural definition that includes only components of the docking and RING subcomplexes. First, the use of the term importomer in a functional sense to refer to components involved in peroxisomal matrix protein import would require the addition of several components, such as the recycling subcomplex, that were not part of the original description of the importomer [1]. Second, there is emerging new evidence that parts of the importomer could also be involved in events such as receptor export [11].

2. The importomer—A molecular machine in search of a clear function

When the importomer was first described, two possibilities were discussed for its possible role in peroxisome matrix protein import [1]. In the first model (Fig. 1A), the receptor/cargo complexes would interact with the docking subcomplex, and then with the RING subcomplex, before cargo (and perhaps the receptor) is translocated into the matrix. This model was based on earlier findings that Pex5p could interact with constituents of both docking (Pex13p and Pex14p) and RING (Pex10p and Pex12p) subcomplexes, and that its interaction with the RING peroxins occurred downstream of the binding to the docking subcomplex. The finding that in certain mammalian mutants of *pex10* and *pex12*, PEX5 accumulated inside peroxisomes was interpreted to mean that the RING subcomplex may constitute the translocon for cargo [38] (and perhaps receptors). However, this observation is also consistent with a second model (Fig. 1B) in which the interaction of receptor/cargo with the docking subcomplex is followed by translocation of cargo (and receptor) into the matrix via the docking subcomplex as the translocon [11,39]. In the matrix, Pex5p and Pex20p would interact with Pex8p, and then with the RING subcomplex during their return back to the cytosol. Thus, despite reasonable agreement on the structural constituents of the importomer, there is quite a bit of uncertainty regarding its function. In the time since the importomer was first described, more putative functions have been ascribed to the importomer. Recently, a third "transient pore" model was proposed for the action of Pex5p, in which Pex5p has the ability to act like a pore-forming toxin whose insertion and assembly into the peroxisome membrane assembles
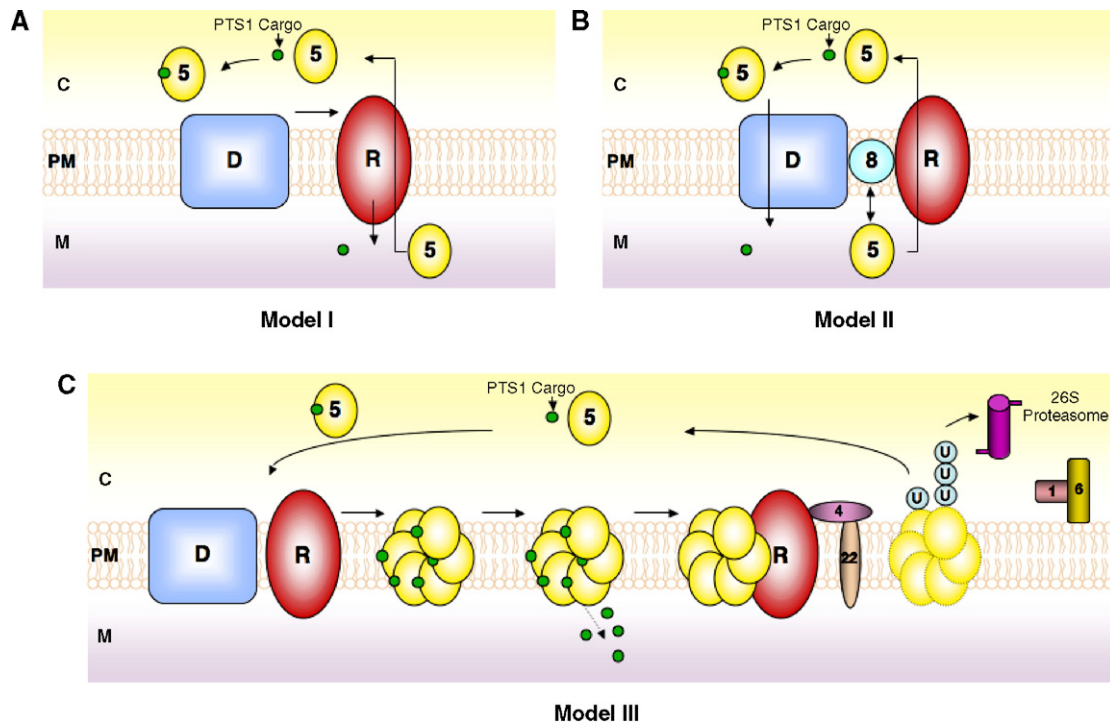
Fig. 1. Three different models proposed for the possible role of the importomer in peroxisomal matrix protein import. See text for details. D: Docking subcomplex, R: RING subcomplex, 5: Pex5p, 3: Pex3p, 8: Pex8p, 14: Pex14p, 22: Pex22p, 4: Pex4p, 1: Pex1p, 6: Pex6p, U: Ubiquitin, C: Cytosol, PM: Peroxisome membrane, M: Peroxisome matrix.

a translocon of variable size to accommodate different cargoes [40]. In this model (Fig. 1C) the role of the importomer might be to (a) assemble the translocation pore comprised of oligomeric receptors, (b) tether receptor/cargo at the peroxisome membrane for delivery to the translocation pore, or (c) have the RING subcomplex of the importomer serve as a putative E3 ligase whose action is postulated to be necessary for pore disassembly and receptor recycling to the cytosol. The receptor-recycling machinery that associates with the peroxisome membrane and the importomer would then recycle the receptors back to the cytosol for another round of matrix protein import [31,32]. In all three models, the absence of receptor recycling would impede peroxisomal matrix protein import either by sequestration of a pool of receptors at the peroxisome membrane, or by physically obstructing part of the import machinery. Under these conditions, a quality-control pathway called RADAR (receptor accumulation and degradation in the absence of recycling) [11] is involved in receptor polyubiquitylation, followed by proteolytic degradation of the receptors (shown at present only for Pex5p and Pex20p) by the proteasome (Fig. 1C) [11,31,41–43]. These multiple models make it obvious that the precise role/s of the importomer in peroxisomal matrix protein import is still far from being clear.

3. Interactions between the docking and RING components of the importomer

Several lines of evidence suggest that the docking and the RING sub-complexes are associated in yeast, and perhaps also in mammalian cells. In immuno-isolation experiments, ScPex12p

co-purified with Pex10p, Pex5p, Pex13p and Pex14p, indicating that the RING and docking subcomplexes might be associated *in vivo* [44]. In *P. pastoris*, protein complexes were cross-linked using a thio-cleavable agent, DSP, and immunoprecipitation of any of the RING proteins brought down Pex13p, while Pex12p also brought down Pex17p [2]. In immunoprecipitates of Pex3p, the authors showed the presence of components of both the docking and the RING subcomplexes. In the absence of Pex3p, the docking subcomplex was still found, but the stability of the RING proteins, and the interactions between them, were drastically reduced. Consequently, it was concluded that Pex3p is the protein vital for the association of both the subcomplexes [2].

Purification of proteinA-fusions of either Pex2p or Pex14p of *S. cerevisiae* showed that the docking and RING subcomplexes are associated [1]. While all the docking subcomplex proteins, RING subcomplex proteins, Pex5p and Pex8p co-purified with Pex2p, Pex14p was co-purified with all the above proteins except Pex2p. The authors further carried out similar experiments in *pex8Δ* cells and demonstrated that Pex2p co-purified with other RING, but not the docking, peroxins. Conversely, when Pex14p was purified from *pex8Δ* cells, the other docking proteins, but not the RING peroxins, were co-purified. Pex5p was found associated with the docking complex and not with the RING complex in the absence of Pex8p suggesting that the association of the two sub-complexes is essential for the transfer of Pex5p from the docking to the RING subcomplex.

The RING domain of mammalian PEX12 interacts with PEX10 and with PEX5 [38,45]. PEX10 also interacts with PEX2 and PEX5 *in vitro* [45]. These studies support the existence of a RING subcomplex in mammalian cells, although the evidence

for the intact subcomplex comes primarily from pairwise interactions and not from the isolation and characterization of the constituents of the whole subcomplex.

Mammalian cells lack PEX8 and PEX17, so the docking subcomplex in mammals must differ from that in yeasts. Cargo-free PEX5 binds to PEX13 and PEX14 independently. However, cargo-loaded PEX5 is in a subcomplex with PEX13 and PEX14 [29], which might be the mammalian equivalent of the docking subcomplex bound to PEX5. This subcomplex dissociates in the presence of cargo-free PEX5, and this result is true even when a mutant form of PEX5L (Pex5L–Mut234) incapable of interacting with PEX13 is used. This fact, and the finding that cargo-free PEX5 binds PEX13, suggest that PEX5 docking with PEX14 occurs upstream of its interaction with PEX13. Also, as mentioned earlier, mammalian PEX7 appears to dock to peroxisomes via PEX5L [25,26], rather than doing so independently of PEX5, as is the case in fungi.

Evidence for the existence of a supercomplex in mammalian cells comprised of docking and RING subcomplex components is either not available [32] or only indirect [46]. Using an *in vitro* mammalian system that imports and exports ^{35}S-PEX5 to and from peroxisomes, organelle fractions were solubilized, and analyzed by BN-PAGE and autoradiography [32]. Two subcomplexes containing ^{35}S-PEX5 were detected, with molecular masses of ~800 kDa and ~500 kDa. The former contained PEX14 and the latter had PEX2, but notably neither subcomplex had both PEX14 and PEX2, indicative of the components of the intact importomer. Neither subcomplex formed in *pex14*-deficient cells, suggesting the necessity of PEX14 for the formation of both complexes. In the absence of PEX2 or PEX12, only the 800 kDa subcomplex, but not the 500 kDa subcomplex, was detected suggesting that the 500 kDa subcomplex is not required for the formation of the 800 kDa subcomplex and that the 500 kDa subcomplex acts downstream of the 800 kDa subcomplex.

Using solubilized rat liver peroxisomes, co-migration of PEX5, PEX14 and a fraction of PEX12 was observed following native gel electrophoresis and sucrose gradient sedimentation. Immunoprecipitates of PEX14 from such fractions revealed the presence of PEX2, PEX5, PEX12 and PEX14 in a complex. Small, non-stoichiometric amounts of PEX13 were also detected in this immunoprecipitate that might represent the importomer [46]. However, it was unclear if the immunoprecipitate contained a single or multiple PEX14-containing subcomplexes.

Much more work is necessary for the characterization of the importomer in mammalian cells.

4. What is/are the importomer bridging proteins? Evidence and evolutionary implications

Two different proteins have been proposed to hold the docking and the RING subcomplexes together in the importomer (Fig. 2). In *P. pastoris*, components of both the docking and RING subcomplexes interact with Pex3p, and this protein has been found in purified preparations of the importomer, using either TAP-tagged Pex10p or Pex12p (N. Rayapuram and S. Subramani, unpublished observations). In cells lacking Pex3p, the RING subcomplex constituents were found to be less stable and immunoprecipitates of the RING subcomplex components did not reveal the presence of the other constituents of the RING subcomplex. However, in the absence of Pex3p, the docking subcomplex constituents were stable and found to interact with each other [2]. In *S. cerevisiae*, no Pex3p was detected in the purified importomer. Instead, Pex8p was proposed to be the organizer of the importomer because in its absence, the RING subcomplex was formed, but the docking subcomplex constituents were not associated with the RING subcomplex [1]. Whether this difference is due to organism-specific or methodological differences is unclear.

Interesting questions are raised by the nature of the bridging proteins necessary for importomer assembly. If Pex8p is critical for the integrity and function of the importomer, then because it is not conserved beyond fungi, does the importomer exist in plants and mammals, and if so, how is the importomer held together in these organisms? Additionally, if Pex8p and an intact importomer are needed for PTS1 and PTS2 import, how could Pex8p itself (with PTS1 and/or PTS2) be imported into the matrix initially? In contrast, Pex3p is conserved in all organisms analyzed to date and is a better candidate for a bridging protein.

Recent evidence suggests the use of two redundant pathways for import of PpPex8p into the peroxisome matrix [39]. One pathway depends on the TPR motifs in Pex5p, the C-terminal PTS1 sequence (AKL) in PpPex8p, and the intra-peroxisomal presence of this peroxin. The alternative pathway uses the PTS2 receptor, Pex7p, its accessory protein, Pex20p, and a putative PTS2 motif in PpPex8p, but does not require intra-peroxisomal PpPex8p. In this second pathway, clearly Pex8p is not necessary for peroxisomal import of itself, raising questions about the role
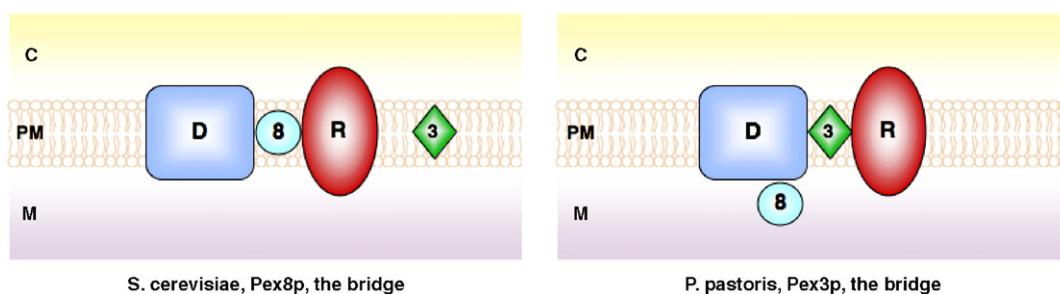

Fig. 2. Two different proteins, Pex8p in *S. cerevisiae* and Pex3p in *P. pastoris*, are reported to bridge the docking and RING subcomplexes [1,2]. D: Docking subcomplex, R: RING subcomplex, 3: Pex3p, 8: Pex8p, C: Cytosol, PM: Peroxisome membrane, M: Peroxisome matrix.

of Pex8p and the entire importomer in Pex8p translocation into peroxisomes. We have proposed that the dual targeting pathways for Pex8p import into the peroxisome matrix may have aided the evolution of a system in which its import became dependent on the prior presence of Pex8p in the matrix. During evolution, Pex8p may have needed Pex20p and Pex7p to first enter peroxisomes without requiring Pex8p to be present in the organelle. It may have then evolved a redundant pathway, either as a backup or for an increased efficiency of Pex8p import [39].

5. Role of the importomer in import of PTS1 and PTS2 cargo

Because mutations in any of the components of the importomer affect both PTS1 and PTS2 import, it has been assumed that the importomer plays a role in the import of all proteins into the peroxisome matrix [1]. However, in the light of new knowledge of receptor dynamics and Pex8p import, this implied function of the importomer may not be applicable to all peroxisomal proteins. Although there are parts of the importomer that play a role in protein import, there is also evidence for a new role of the importomer in Pex20p export that does not fully fit its name.

5.1. All components of the importomer are not necessary for Pex8p, Pex5p and Pex20p import into peroxisomes

There are three proteins whose entry into the peroxisome matrix complicates the simple notion that the importomer is needed only for protein import. In *P. pastoris*, the import of Pex8p, the only intraperoxisomal peroxin, is Pex14p-dependent but Pex2p-independent, suggesting that targeting of Pex8p to the peroxisome matrix (by the PTS2 pathway at least) requires an intact docking, but not the RING, subcomplex [39]. PpPex8p is the first peroxisomal cargo for which only a subset of the importomer proteins is necessary for import function. This naturally raises the question regarding whether PpPex8p behaves like other matrix cargo, or whether it is a special cargo with less stringent requirements than generic cargo. The entry of PpPex8p into peroxisomes by the PTS1 pathway appears to be just like other PTS1-containing cargoes because it is dependent on its PTS1-, Pex5p- and intraperoxisomal Pex8p. In contrast, the entry of PpPex8p into peroxisomes via the PTS2 pathway is unlike that of other PTS2 cargo in two respects. First, even though PpPex8p has a PTS2-like sequence, it is not bound directly by the PTS2-receptor, Pex7p, but it interacts instead with Pex20p, the co-receptor for the PTS2 pathway. This point alone is not sufficient to label PpPex8p as a special cargo because of a recent report that in some cases Pex20p may bind the PTS2 sequence in cargo proteins [13]. Second, the peroxisomal entry of PpPex8p via the PTS2 pathway is independent of intraperoxisomal Pex8p, which is different from the behavior of generic PTS2-containing cargoes [39].

Like PpPex8p, PpPex5p is also peroxisomal and protease protected in cells lacking Pex2p [39]. In view of previous work that in *pex2Δ* cells Pex10p and Pex12p are unstable [2,11], it seems likely that Pex5p import into peroxisomes also requires only the docking subcomplex and not the entire importomer. This conclusion is reinforced by the additional finding that Pex20p–GFP accumulation in peroxisomes also requires Pex14p of the docking subcomplex, but not Pex2p, Pex10p or Pex12p [11]. It should be noted that Pex5p and Pex20p are import receptors, so it is possible that the requirements for their import may be different from that of generic cargo. These caveats not withstanding, one can no longer envision the *entire* importomer as being necessary for protein import into the peroxisome matrix.

5.2. What is the translocon for PTS cargoes and for import receptors?

As stated above, Pex5p, Pex8p and Pex20p can be peroxisomal and protease protected, even in the absence of RING components, but their entry into peroxisomes requires Pex14p, a key component of the docking complex [11,39]. In *Yarrowia lipolytica pex8Δ* cells, Pex20p was found to be peroxisomal and protease-protected [47]. If Pex8p is indeed required to assemble the importomer in this organism, then Pex20p import must not require the entire importomer. Further work is needed to determine whether these are special cargoes, or whether the docking subcomplex is the translocon for most or all cargoes.

5.3. A role for the RING subcomplex in receptor export?

If the RING subcomplex is not the translocon (at least for Pex5p, Pex8p and Pex20p), what is its function? It should be noted, in this context, that the RING subcomplex appears to be needed for the recycling of certain receptors, such as Pex5p and Pex20p, from the peroxisome matrix to the cytosol [11,48]. Thus the RING subcomplex plays a role in receptor exit or export from the peroxisome matrix. What we do not know, of course, is whether a separate RING subcomplex, or the RING subcomplex of the importomer, performs this receptor export function. Components of the RING subcomplex could function directly or indirectly (e.g., by associating with and modifying the directionality of the translocon used for protein entry into peroxisomes, or by acting as E3 ligases to ubiquitylate a target protein on the peroxisome membrane) as retrotranslocon subunits. In such a model, it is easy to understand why mutations in the RING subcomplex components would affect all matrix protein import.

6. Future directions

Future efforts will have to focus on the determination of the subunit stoichiometry and structure of the importomer, which will provide valuable insights into the dynamic functioning of the importomer and help to elucidate its precise function in the import cycle. Additionally, the reconstitution of the importomer complex into liposomes would serve as an important tool to elucidate key events in the import process.

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