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Review

Peroxisome targeting signal 1: Is it really a simple tripeptide?

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Abstract

Originally, the peroxisomal targeting signal 1 (PTS1) was defined as a tripeptide at the C-terminus of proteins prone to be imported into the peroxisomal matrix. The corresponding receptor PEX5 initiates the translocation of proteins by identifying potential substrates via their C-termini and trapping PTS1s through remodeling of its TPR domain. Thorough studies on the interaction between PEX5 and PTS1 as well as sequence-analytic tools revealed the influence of amino acid residues further upstream of the ultimate tripeptide. Altogether, PTS1s should be defined as dodecamer sequences at the C-terminal ends of proteins. These sequences accommodate physical contacts with both the surface and the binding cavity of PEX5 and ensure accessibility of the extreme C-terminus. Knowledge-based approaches in applied Bioinformatics provide reliable tools to accurately predict the peroxisomal location of proteins not yet determined experimentally.

Keywords: PTS1; PEX5; Targeting; Peroxisome; Predictor

To fulfill their cellular function proteins need to be transported to their final destination. This process always involves targeting signals, receptors and docking proteins. For example, proteins targeted to the ER, the mitochondria or the chloroplasts usually comprise an N-terminal signal recognized by distinct receptors which in turn initiate a sequence of events leading to their translocation. Mitochondria and chloroplasts use the nuclear encoded gene products to complement their autonomously synthesized set of proteins. Peroxisomes neither contain DNA nor do they hold their own protein synthesis machinery and, therefore, import their complete protein content from the cytosol. Interestingly, a high number of peroxisomal proteins are oligomeric and some peroxisomal proteins do contain prosthetic groups such as heme or FAD.

Considering the biological and medical significance of sub-cellular compartmentalization, predictions of the right protein location exclusively based on the amino acid sequence constitute a major advancement in modern biology. However, such prediction is only possible with a clear conception of the signal and its interaction partners. In this review we summarize the current knowledge on the C-terminal peroxisome targeting signal 1 and we wish to put forward a new definition.

1. PTS1 is a C-terminal amino acid sequence

A major breakthrough in the elucidation of the mechanism of protein import into peroxisomes was reached about twenty years ago when Keller et al. [1] while working with recombinant luciferase of the firefly *Photinus pyralis* as a reporter protein expressed in monkey kidney cells found that the protein colocalized with catalase in peroxisomes. Following this surprising finding luciferase was localized to the peroxisomes of the lantern organ of the firefly. Moreover, using genetic engineering and immunofluorescence microscopy Gould et al. [2] identified a region at the C-terminus of luciferase that was necessary and sufficient to direct proteins to peroxisomes defining the first peroxisome targeting signal (PTS1). Today we know that the majority of peroxisomal matrix proteins contain such a PTS1. Very few proteins contain an N-terminal PTS2. Like most peroxisomal matrix proteins, luciferase is not processed after translocation into peroxisomes [3]. Import into peroxisomes therefore differs from the import mechanisms into the endoplasmic reticulum, mitochondria or chloroplasts in which the targeting signal is located at the amino-terminus of

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the proteins and in most cases is processed during or immediately after translocation. As a consequence of its carboxy-terminal topology, peroxisomal matrix proteins harboring a PTS1 have to be fully synthesized in the cytosol prior to their import. Amino acid insertions or deletions far away from the C-terminus disrupted the translocation [2] and the authors of this work already reasoned that alteration of the three-dimensional structure could be the cause of the targeting impairment. Remarkably, accessibility of the signal was already a matter in the first report on PTS1.

Site directed mutagenesis in luciferase led to the identification of the PTS1 as the tripeptide Serine–Lysine–Leucine (–SKL) at the extreme C-terminal end of the protein [4]. Additional mutagenesis experiments revealed that variants of the PTS1 may exist that conserve the property of a translocation signal and target either luciferase or the chimera chloramphenicol-acetyl-transferase (CAT)-luciferase into peroxisomes of CV-1 cells [4,5]. Finally, amino acid residue permutations soon yielded an extended definition of the PTS1-consensus sequence: amino acids at the first position contain a small uncharged side chain (S/A/C), at the penultimate position a positively charged residue (K/R/H) and at the extreme C-terminal position leucine [4,6]. Many peroxisomal proteins indeed contain a genuine PTS1 adhering to the original consensus, except the prototype of peroxisomal proteins, catalase. Besides an unusual C-terminus yeast catalase A contains an internal targeting signal [7] and the import of human catalase was demonstrated to depend on its C-terminal tetrapeptide [8]. Nevertheless, it became soon obvious that the presence of the consensus tripeptide does not suffice for peroxisomal targeting. PTS1 is not a guarantee for import of proteins across the peroxisomal membrane [9,10]. The canonical PTS1 –SKL attached to the carboxy terminus of the mouse dihydrofolate reductase [7,11] or the signal –AKI fused to the C-terminus of CAT [9] did not lead to the targeting of the reporter proteins to peroxisomes in the yeast *Saccharomyces cerevisiae* whereas the C-terminal SKL-tagging of CAT was sufficient for its import into the peroxisomal matrix. When its C-terminus was extended by 6 or 7 amino acids ending in the sequence SKL DHFR was targeted to peroxisomes clearly indicating that the extreme C-terminus should be accessible for correct transport. The opposite has also been found and proteins have been reported that do not need their PTS1 to be imported via the PTS1-receptor pathway. Deletion of the C-terminal sequences of either isocitrate lyase from castor bean or catalase A or carnitine acetyltransferase from *S. cerevisiae* did not prejudice their import into peroxisomes in this yeast [7,12,13]. It is important to point out that in most studies either heterologous expressed proteins (e.g. firefly luciferase in *Trypanosoma brucei* or CV-1 cells) or non-peroxisomal reporter proteins (e.g. CAT; DHFR) were used which may have influenced the findings leading to the definition of the PTS1.

At the time of its discovery the C-terminal location of the PTS1 was surprising but provided a good explanation for the post-translational mode of transport into peroxisomes [14]. Subsequently, it was demonstrated that proteins can enter the peroxisomal matrix in a folded or oligomerized state [15–17]. The PTS1 does not even need to be part of the primary sequence of a protein instead it could be conjugated with the protein to be imported [15,18]. McNew and Goodman [16] demonstrated that oligomerized proteins are translocated across the peroxisomal membrane using CAT as a reporter protein. The mature form of CAT is a homotrimer; its oligomerization takes place in the cytosol within a few minutes. The analysis of co-expressed CAT extended by a PTS1 at its carboxy-terminal end together with both a hemagglutinin-tagged version of CAT (HA-CAT) lacking PTS1 and a truncated version of CAT without PTS1 showed that the two PTS1-less versions of CAT were imported into peroxisomes. Stoichiometric analysis of the imported trimers confirmed, that at least one subunit of each trimer harbored a PTS1. Additional experiments demonstrated that peroxisomal proteins cross-linked or stabilized with disulfide bonds, mature folded proteins and PTS1-harboring IgG as well as colloidal gold particles (9 nm) conjugated to PTS1-containing human serum albumin (HSA) were all efficiently imported into peroxisomes [17].

These results could suggest that peroxisomal proteins have to be completely folded prior to their import into the organellar matrix, a step which could serve as quality control for entry into peroxisomes. Only mature and enzymatically active proteins would enter the organelle, and cofactor-binding or oligomerization processes occur outside prior to the translocation step. However, *in vivo* experiments with reduced albumin bearing a PTS1 indicate that proteins can be substrates for peroxisomal import despite their inability to assume their native conformation [19], suggesting that PTS1 is the major requirement for entry into the peroxisomes and that no other quality control exists at the substrate protein level. Obviously, it makes no difference for peroxisomal translocation whether proteins are folded and assembled or not. However, peroxisomes save the need for import of co-factors or assembly components when importing fully functional proteins. These observations support the benefit of C-terminal sequences as targeting signals, although such signals are disadvantageously placed very low in the hierarchy of targeting signals [20]. Decisions for the localization of proteins are made according to the flow of their synthesis from the N-terminus to the C-terminus. The N-terminal sequence leaving the ribosome is tested for its interaction with the signal recognition particle (SRP) and either bound followed by ER-translocation or not, the presence of mitochondrial or chloroplast targeting signals at the N-terminus is scanned and transmembrane regions are determined by their hydrophobic properties. Only after the tertiary structure is adopted the protein is transported either into the nucleus or into peroxisomes or becomes a cytosolic resident.

2. PTS1 is universal but species-specific

Antibodies raised against the short amino acid sequence SKL specifically recognized multiple peroxisomal proteins in various species [21]. The enzyme luciferase was used as reporter protein and was localized into peroxisomes of yeasts, plants and mammals when expressed in those cells [22] supporting the universality of peroxisomal import mechanisms and the

hypothesis that peroxisome targeting may have been conserved throughout evolution of eukaryotes. Studies on the PTS1-dependent peroxisomal matrix protein transport in yeasts [11,23], plants [24,25], mammals [18] and trypanosomes [26,27] corroborated this assumption.

A detailed analysis of the PTS1 in yeast by Aitchison et al. [9] demonstrated that the PTS1 of the multifunctional enzyme (hydratase–dehydrogenase epimerase) of *Candida tropicalis* was contained in its C-terminal sequence –AKI which was also necessary to target the ectopically expressed protein to peroxisomes in both *Candida albicans* and *S. cerevisiae*. However, substitutions within this tripeptide to GKI or AQI supported targeting to peroxisomes in *C. albicans* but no longer in *S. cerevisiae.* Additional mutational studies with luciferase in *T. brucei* indicated that the PTS1 consensus may be more relaxed for lower eukaryotic organisms than for mammalian cells [5,28,29]. These and further studies [30,31] supported a new definition for PTS1 aside from the archetype sequence –SKL with a small neutral amino acid residue at the first position of the tripeptide (A/C/G/H/W/P or T), an amino acid residue capable of hydrogen bonding at the penultimate position (H/M/N/Q/R or S) and an hydrophobic amino acid residue (I/M or Y) at the extreme carboxy terminus [26]. These studies posed for the first time the question of a species-specificity in the import mechanism of peroxisomal proteins similar to the carboxy terminal tetrapeptides KDEL, HDEL or DDEL used for retrieval of proteins to the ER in different species [32,33]. The variations observed may represent an evolutionary divergence in the peroxisomal targeting signals and their recognition by the cognate receptors.

Elgersma et al. [34] studied the PTS1 degeneracy in a homologous context using the malate dehydrogenase (MDH3) of *S. cerevisiae*. Many of the PTS1-variants found did not fit the PTS1-consensus previously defined to target heterologous proteins. Therefore, the authors assumed that similar to the context dependence of the nuclear localization signal the PTS1 may have to be specifically presented in order to be bound by the cytosolic receptor that in turn will target the protein to the peroxisomal membrane. A systematic study was performed in our laboratory applying the two-hybrid system [35] with a peptide-library [36] to screen for peptides interacting specifically with the PTS1-binding domain of either the human PTS1-receptor or the one from the yeast *S. cerevisiae*. We noted a much broader degeneracy for the human and the yeast PTS1 than the original consensus [37]. Among the novel PTS1 peptides identified with the human receptor only very few bound the yeast receptor demonstrating a species-specific recognition process. Moreover, in our screen peptides ending with the same tripeptide bound the PTS1-receptor with various efficiencies [37]. The only difference between these peptides resided in the upstream region indicating that PTS1 is more than just a C-terminal tripeptide.

An explanation for these differences may be that like in a default pathway PTS1-containing proteins would be targeted to peroxisomes unless the region upstream to the PTS1 inhibits the interaction with the receptor. Consequently, no transport across the peroxisomal membrane would occur regardless of the context of the protein. Thus the species differences observed may reside in a modulating specificity that during evolution became more subtle to guarantee that proteins are properly compartmentalized to peroxisomes.

Genetic evidence suggested the existence of a third import pathway. Zhang et al. [38] isolated an *onu*-mutant (*peb5*) in the yeast *S. cerevisiae* impaired in import of catalase (frequently used as PTS1-protein marker). Moreover, in these mutants the PTS2-containing protein thiolase and acyl CoA oxidase (AOX) were both correctly imported into the peroxisomal matrix. The authors concluded that AOX uses an import pathway different from PTS1 or PTS2. Studies in our lab by Kragler et al. [7] revealed that in the yeast *S. cerevisiae* catalase A is targeted to peroxisomes whether or not it contains a PTS1 at its extreme C-terminus. In addition, we demonstrated that the integrity of the region between amino acids 125 and 140 is necessary for its location [7], and the 126 N-terminal amino acid residues serve as targeting signal (in a PEX7-independent manner). Surprisingly, Elgersma et al. [12] described that carnitine acetyltransferase could be imported into peroxisomes of *S. cerevisiae* without any of the two defined targeting signals. Similar to catalase the latter enzyme did not need its PTS1 to be imported. Despite these findings the existence of a third import pathway remains doubtful since deletion of the *PEX5* gene encoding the PTS1-receptor totally abolished import of both catalase A and carnitine acetyltransferase [12,39]. Moreover, preliminary results in our laboratory showed that the yeast AOX interacted with *Sc*PEX5 in the two-hybrid system (Lametschwandtner, G., unpublished data). These results can be interpreted in different ways: (1) an additional receptor exists whose activity depends on the presence of PEX5. No such receptor was reported; (2) another possibility is that certain proteins do contain sequences necessary for interaction with PTS1-containing proteins with which they oligomerize and are co-imported into the peroxisomal matrix via the PTS1-import pathway [15,16]. A detailed analysis of the structure of such required regions might help to answer this question. (3) Alternatively, these proteins may interact with PEX5 avoiding the PTS1-binding cavity.

3. PEX5 recognizes PTS1 via its TPR domain

With the signal for translocation into the peroxisomal matrix at hand the next challenge was to identify the corresponding receptor(s). Which factor(s) recognize the C-terminus of peroxisomal matrix proteins? In the yeast *S. cerevisiae* mutants have been described that are impaired in either PTS-dependent transport [40]. The gene able to complement the defect in the PTS1-protein transport was cloned from various species and later commonly named *PEX5* [39,41–43]. A common feature in the mutant analysis was that cells were defective in growth but not in division of peroxisomes, as demonstrated in the *pex5*-mutants of *Pichia pastoris* [41] or *Hansenula polymorpha* [42]. Since PTS1-containing proteins were properly expressed and induced, these studies led to the conclusions that the PTS1-import pathway is impaired in *pex5*-mutant cells and that PEX5 represents the PTS1-receptor. *In vitro*-translated *Pp*PEX5 protein could be bound to immobilized peptides terminating

with the tripeptide –SKL [41] providing the first biochemical hints for the function of PEX5 as PTS1-receptor.

An alternative strategy exploited the two-hybrid system using PTS1 as bait. This approach resulted in the identification of *Sc*PEX5 as interaction partner [44]. With a meticulous characterization of the interaction between bait and prey-fragments we demonstrated that the PTS1-peptides physically interact with the C-terminal half of PEX5 containing the tetratricopeptide repeat (TPR)-domain, which was splendidly corroborated by the crystal structure 6 years later [45]. According to this 3D-structure, the PTS1 appears to be locked in a groove with various interactions towards both sets of 3 TPR-motifs linked together by a helical hinge (Fig. 1).

TPR-containing proteins are involved in numerous protein–protein interactions. Although the sequence conservation among the TPRs is very low, these motifs fold into a very similar compact conformation of two short antiparallel alpha-helices termed A and B separated by a turn [46]. At least three of these repeats are stacked upon each other forming a particularly shaped surface resembling a bent half-pipe, with the A-helices of the individual repeats building the inner concave surface. The conserved amino acids responsible for the structural shape are buried, and the variable ones in the helices or in the turn between the two short α-helices in each of the repeats are exposed at the surface. The structure formed by staggering the repeats provides a distinct surface for protein–protein interactions, the specificity of which is determined by amino acid residues not involved in structure formation.

Besides the recognition of PTS1 by PEX5 another well-characterized protein–protein interaction involving a TPR domain is the one between the adaptor protein Hop and the C-termini of the two molecular chaperones Hsp70 and Hsp90 consisting of the amino acid sequence –EEVD [47]. Hop contains two TPR domains, both recognize identical amino acid sequences and each one is able to form a "two-carboxylate clamp" tightly interacting with the ultimate aspartate of the Hsps. Among the 11 proteins forming the mammalian ana-phase-promoting complex (APC), 4 contain TPR domains: APC3, 6, 7 and 8. Two of these, namely APC3 and APC7 [48],

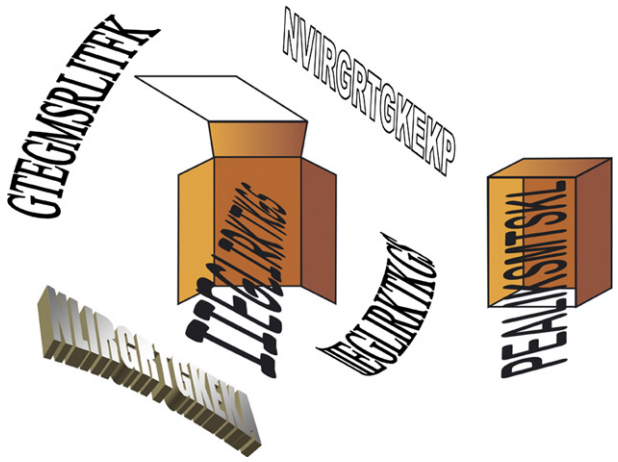

Fig. 1. PEX5 recognizes C-terminal ends and successful interaction closes the PEX5 clamp.

were shown to interact with C-terminal dipeptide motifs (–IR). Altogether, these examples suggest that TPR domains recognize short C-terminal stretches of amino acids to identify proteins for interaction.

The TPR-domain of the *Sc*PEX5 is necessary to bind the C-terminal sequence –SKL in a two-hybrid system [44]. Moreover, the amino acid residues beyond the TPR-domain do not provide binding specificity, illustrated by the finding that the two-hybrid interaction between *Sc*PEX5 harboring a modified C-terminus and an SKL-containing protein was not impaired (Brocard, C., unpublished data). The TPR-domain provides the specificity to discern PTS1 and should therefore be responsible for species-specific differences. The N-terminal half of *Sc*PEX5 fused with the C-terminal half of a plant receptor *Nt*Pex5 restored peroxisomal import in *S. cerevisiae pex5*-mutant cells [49]. Similarly, a fusion between the N-terminal part of the *Pp*PEX5 and the C-terminal half of the *Hs*PEX5 containing the TPR domain was able to rescue import of some PTS1-proteins in the *pex5*-mutant of the yeast *P. pastoris* [50]. These results do not only prove the functional homology of these factors but they also suggest that the peroxisomal import machinery is conserved from yeast to plant and human. However, two groups observed that the fusion *Pp*PEX5p–*Hs*PEX5 failed to complement the methanol growth defect of the *P. pastoris pex5*-mutant cells [50,51]. Therefore, Dodt et al. [51] proposed that the differences in peroxisomal import observed between organisms may reside in the aptitude of the TPR-domain to recognize the PTS1. The sequence selectivity in human and monkey cells excludes a phenylalanine at the extreme C-terminus of the PTS1, but the *P. pastoris* alcohol oxidase terminates with the sequence –ARF. It was shown that in the *P. pastoris* mutant strain complemented by the expression of a chimera *Pp*PEX5p–*Hs*PEX5 protein alcohol oxidase was indeed not imported clearly illustrating that the TPR domain of PEX5 determines the PTS1 sequence specificity of the organism.

Unlike the mitochondrial receptors Tom20 and Tom70 none of the PEX5 proteins identified so far contain a hydrophobic domain that could lead to their anchoring in the peroxisomal membrane. Although there is no doubt about the function of PEX5 as PTS1-receptor the exact mechanism of import of PTS1-proteins remains elusive. The receptor has been localized in different parts of the cells depending on the organism studied. Common assumption would assign this interaction to the peroxisomal membrane, and indeed many experimental results pointed into this direction. Isolated peroxisomes could be bound to immobilized peptides [52], labeled PTS1 was bound to glyoxysomal membranes [53], and human PEX5 protein was found to be associated with peroxisomes [54]. In summary, all evidence suggested a membrane-bound protein to be responsible for binding PTS1. A contrasting view was provided by Dodt et al. [51] showing that more than 90% of human PEX5 protein is cytosolic, culminating in the commonly accepted view that PEX5 shuttles between the cytosol and the peroxisomes [55]. The daring hypothesis that the whole PEX5 protein transverses the peroxisomal membrane is still a matter of debate [56]. The discrepancies observed in the PEX5 subcellular localization

could be explained by different means, but most likely the observation that PEX5 is mainly associated with the membrane [41,52] was an experimental artefact. Gould et al. [57] have shown that in the cytosol PEX5 exhibited a very pronounced protease sensitivity which was inhibited only by the addition of NaF in the buffers used for extractions. Nevertheless, *Hs*PEX5 and *Sc*PEX5 were found mainly in the cytosolic fraction and only a small fraction associated with peroxisomes [34,58]. Among all organisms so far only in *H. polymorpha* and *Yarrowia lipolytica* PEX5 was localized to the peroxisomal matrix [42,43]. It is unlikely that these two yeasts use a different mechanism of import, since it was shown that the import of peroxisomal matrix proteins is highly conserved throughout all organisms studied. Alternatively, as a variation to the general import mechanism PEX5 might be completely released into the peroxisomal matrix or its export might be extremely slow under certain environmental conditions. In both cases PEX5 would be detected in the peroxisomal matrix of these organisms whereas in others this might be a short-lived transition state only.

Regarding all these results the dual location of PEX5 in the cytosol and at the peroxisomes resembles more a bimodal distribution. Therefore, import of PTS1-proteins into peroxisomes may function in a manner analogous to the post-translational import into the ER lumen with binding of the transport substrate to PEX5 in the cytosol and targeting of the complex to the translocation machinery. Upon release of the PTS1-protein at the organellar membrane with the help of other peroxin(s) the receptor recycles back to the cytosol for another round of interaction or becomes degraded by the proteasome. In this model it is conceivable, that PEX5 is able to associate with the peroxisomal membrane only if it is loaded with its PTS1-containing cargo as suggested [59].

4. Predicting a PTS1

Sequence-analytic tools were developed to screen for new potential peroxisomal matrix proteins based on the C-terminal consensus sequence. Efficiency and success essentially depend on both the data input and the prediction method. Is the PTS1 really a simple tripeptide or should more care be taken when predicting the location of PTS1-containing proteins? Some methods are only based on the analysis of the C-terminal tripeptide of proteins which may give rise to misleading results. PSORT applies an algorithm including the C-terminal sequences [A/C/S]–[H/K/R]–L with modest performance for peroxisomal proteins due to its restricted sequence selection, whereas PROSITE uses a pattern that is too promiscuous to restrict the results to peroxisomal proteins [A/C/G/N/S/T]–[H/K/R]–[A/F/I/L/M/V/Y]. Similarly, to detect peroxisomal matrix proteins *in silico*, a conceptually translated protein database of the entire yeast genome was analyzed searching for the peptides [S/A/C]–[K/H/R]–L, [S/A]–[Q/N]–L, and S–K–F at the C-terminus of proteins of 100 amino acids or longer [60]. Some of the novel proteins were tested for peroxisomal translocation via GFP-fusion at the N-terminus followed by the determination of the intracellular location of the fusion proteins, but not all of them were imported into the peroxisomal matrix. Clearly, fusion proteins may represent a particular problem in localization experiments, but in addition, the decision to limit the search to the tripeptide neglected some other features of the signal such as the physical properties of the amino acids involved or the signal accessibility. A more elaborate approach was performed by Emanuelsson et al. [61] feeding the C-terminal amino acid sequences of all truly peroxisomal proteins from databanks into a learning set in comparison with non-peroxisomal proteins harboring a PTS1-like C-terminus (relaxed consensus motif [A/C/H/K/N/P/S/T]–[H/K/N/Q/R/S]–[A/F/I/L/M/V]). The final PeroxiP prediction scheme excludes secreted and trans-membrane proteins prior to the motif identification and depending on the settings used is either restrictive missing known peroxisomal proteins or rather permissive with too many false positive results. A similar strategy i.e. searching whole genomes for the appearance of well-known PTS1s at the C-termini of *Arabidopsis thaliana* proteins was applied to create the subset of peroxisomal matrix proteins in the database AraPerox [62]. Kurochkin et al. [63] analyzed the whole rodent and primate GenBank of translated mRNA sequences for the occurrence of the C-terminal tripeptide [S/A]–K–L.

All these approaches are based on experimentally verified locations of peroxisomal proteins, which represent a limited set of proteins. Localization processes include not only the interaction with the corresponding receptor, which mainly depends on the signal and its accessibility, but also on the successful process of translocation itself. This in turn includes many other interactions and crossing barriers not directly associated with the function of the targeting signal. In fact, the potential of a given amino acid sequence to act as a targeting signal depends solely on its capability to initialize the translocation process through its interaction with the receptor. Therefore, approaches shielding the interaction between signal and receptor from all subsequent events should provide more reliable data on the putative targeting function of all possible C-terminal endings. Such a possibility is offered by the two-hybrid system employing PEX5 or its essential TPR domain as bait and a library expressing short peptides (16-mers) fused to the C-terminus of the GAL4 activation domain [36]. C-terminal amino acid sequences identified through this approach are able to interact with the TPR domain of the PEX5 protein fulfilling all requirements to act as PTS1. A comparison between peptides interacting with TPR domains from different organisms revealed species-specific recognition features as well as the influence of amino acid residues immediately upstream of the C-terminal peptide [37,49]. However, all peptides ending with the original consensus tripeptide are recognized by all PEX5 proteins tested, with the affinities varying according to the upstream regions.

The affinity of the receptor PEX5 for PTS1 signals has been measured *in vitro* with bacterially expressed *Hs*PEX5L and a fluorophore-containing peptide lissamine-YQSKL. The dissociation constant was measured to be 35 nM as compared with an affinity 250 nM for *Pp*PEX5 with the same peptide [64]. This shows again that signal recognition appears to be species-specific and that different requirements might be set for PTS1 binding in different organisms. In this study PEX5 behaved as a

tetramer. Although there is no doubt that PEX5 binds to PTS1, *in vivo* one could expect the interaction to be facilitated by the presence of factors that alter the folding of the protein or enhance the presentation of its PTS1. A candidate for such action has been proposed to be Hsp70. However, Harper et al. (2003) demonstrated that the presence of Hsp70 had no effect on PEX5:PTS1 interaction. Similarly, the presence or absence of ATP did not affect interaction suggesting that if ATP is required in the import process this step happens downstream of the PEX5:PTS1 binding event [64].

In further studies PEX5 binding affinities were determined *in vitro* with peptides of different length and with some sequence variations [54,65,66]. Longer peptides bind to the TPR domain of PEX5 tighter than shorter ones (3–6 times), and amino acids upstream of the ultimate tripeptide influence the affinity between the C-terminal end and its receptor. These studies corroborate previous observations from 2-hybrid studies in yeast employing hexadecapeptide extension at the GAL4 activation domain that demonstrated that the amino acid context upstream of the C-terminal tripeptide does play a important role in the binding affinity to the receptor and some residues are favored such as leucine or valine at position −5 [37].

One should keep in mind that in these *in vitro*- or two-hybrid studies of interaction between PEX5 and peptides all potential interactions with PEX5 outside of the PTS1 binding pocket are abolished making the whole system artificially stable due to a lower structural demand. Considering the bipartite structure of the C-terminal part of PEX5 with two triplets of TPR domains building the contacts with the PTS1 signal [45], it is conceivable that other contacts exist with sequences upstream of the tripeptide that would account for the stability of the interaction and consequently for the efficiency of transport. For instance, the whole TPR domain could function as a trap for PTS1-containing proteins provided that it can close properly upon binding. Clearly, a tightly folded globular structure of a potential substrate protein would not accommodate the closure of the receptor. The extreme C-terminal end of the proteins carrying a PTS1 must be accessible and presented to the receptor. In the trapping scenario, the amino acid residues upstream of the C-terminal tripeptide would play an essential role in two ways: (1) structurally, because steric hindrance may be an important factor near the last three amino acid residues and (2) qualitatively, if other binding sites exist in PTS1-proteins that affect the affinity for PEX5. All studies on the PTS1 degeneracy and the extension of the original consensus suggest that the definition of the C-terminal targeting signal should be amended and residues upstream of the ultimate tripeptide should be included into the PTS1 [65,67].

C-terminal sequences identified solely by means of interaction with the TPR domain of PEX5 proteins yielded a list of peptides possibly recognized by the corresponding receptors, i.e. a list of potential signals regardless of their natural occurrence and unprejudiced by subsequent events. This set of C-terminal sequences together with the C-termini of experimentally verified peroxisomal matrix proteins represented a learning set to refine the PTS1-motif and to develop a novel and highly accurate predictor [68]. Based on the amino acid composition at the very end of peroxisomal matrix proteins compared to non-peroxisomal proteins and considering the structural constraints intrinsic to the contact with the TPR domain of PEX5, PTS1 was divided into three regions [68]. The most C-terminal tripeptide is responsible for interaction with the PEX5-cavity [45], a tetrapeptide immediately upstream is thought to interact with the surface of the PEX5 proteins, and a flexible hinge accounts for accessibility and flexibility, which corresponds altogether to a 12-amino acid sequence.

This refined PTS1 motif description served as a source to develop a prediction tool capable of detecting PTS1 C-termini in yet uncharacterized protein sequences. The score function S consists of two components, the S_{profile} evaluates the concordance with amino acid type preferences and S_{ppt} penalizes deviations from the physical property pattern derived from the learning set. Query sequences that generate scores larger or equal to 0 are considered “predicted”, sequences with scores below −10 are classified as “not predicted”, and sequences with scores between 0 and −10 are considered being situated in a “twilight zone” http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp).

The novel predictor was applied to protein sequences originally examined in mutational analyses to define the PTS1 and its variants. Although predictor performance exceeded by far the pattern-based methods of PSORT or PROSITE, it failed to recognize some unusual but already verified targeting signals on MDH3 terminating with E–K–L or S–Y–L [34]. An explanation could be that their targeting function is due to the presence of additional interactions which would improve the competence of this particular protein to be addressed to the peroxisomal matrix. Nonetheless, the addition of –SKL alone to the C-terminus of DHFR was correctly classified as “not-peroxisomal” since the score S_{ppt} was strongly negative. Accordingly, longer extensions at the C-terminus of DHFR consisting of amino acid sequences –KNIESKL or –GGKSKL resulted in positive prediction and experimentally in successful translocation. As an example we applied the predictor to a number of 2,4 dienoyl-CoA reductases identified through a BLAST search as homologues of *Sc*SPS19. In Table 1 the PTS1-predictor is compared with the established PSORTII [69].

When the predictor was applied to all SWISSPROT entries or to bacterial proteins it classified a number of proteins as targeted to peroxisomes that never encounter this organelle. In most cases the C-termini did not diverge from the derived consensus and could not be rationalized as false-positive predictions. Therefore, selected C-termini were tested for interaction with PEX5 in two-hybrid assays and *in vivo* the localization of the corresponding proteins fused to GFP at their N-termini was determined [20]. All tested C-termini interacted with the TPR domain of PEX5 proteins, and acted as functional PTS1 provided that the competing targeting signal was disguised or eliminated. The occurrence of a functional PTS1 signal does not convey evolutionary pressure since mislocalization is normally prevented by overriding the function of these C-terminal sequences either via exposure of N-terminal signals or through structural seclusion. Consequently, these signals remain silent under normal phy-

Table 1
2,4 dienoyl-CoA reductases and their intracellular location in various organisms

Species	GI	Dodecapeptide	PTS1-predictor (Score)		PSORT-II	Reference
Yarrowia lipolytica	CAG81681	–MLIAQGNDTPKL	Twilight	(−5.180)	70% Cyto	
	CAG79530	–TVIVQGNKPPKL	Targeted	(2.226)	48% Cyto	
	CAG80482	–MLKHMGEDKSKI	Twilight	(−0.701)	57% Cyto	
Candida albicans	XP_712761	–VIHQNEDPQGKL	Twilight	(−7.433)	61% Cyto	
	XP_713778	–GTEGMSRLITFK	Not targeted	(−49.126)	61% Cyto	
	XP_713744	–LNAINAPKGGKL	Twilight	(−9.456)	52% Cyto	
Candida glabrata	XP_447149	–PDLLFKDPKAKL	Targeted	(5.439)	33% Px	
Saccharomyces cerevisiae	NP_014197	–PEALIKSMTSKL	Targeted	(2.239)	78% Px	[72] Px
Aspergillus nidulans	XP_681039	–FQNVKGKKKAKL	Targeted	(10.023)	44% Cyto	
Neurospora crassa	XP_328664	–KNLKDGRQKSKL	Targeted	(13.266)	67% Px	
Kluyveromyces lactis	XP_453899	–PKELNYAFDSKL	Twilight	(−7.045)	78% Px	
Caenorhabditis elegans	CAA90268	–NLIRGRTGKEKA	Not targeted	(−33.897)	44% Cyto	[73] Predicted Mito
	CAA91310	–NVIRGRTGKEKP	Not targeted	(−32.941)	35% Cyto 35% Mito	
	CAB02118	–RGRTGKEKKSKL	Targeted	(8.457)	67% Px	[73] Predicted Px
Mus musculus	NP_036063	–QLLEFESFSAKL	Targeted	(4.290)	44% Cyto	[74] Px
	NP_076012	–RIKESFKKKAKL	Targeted	(6.083)	39% Cyto	[75] Px
	NP_080448	–IIEGLIRKTKGS	Not targeted	(−83.937)	44% Nu	[76] Mito
Rattus norvegicus	NP_741993	–RLLEFESSSAKL	Twilight	(−0.324)	48% Cyto	[77] Px
	NP_579833	–KVKESLKKQARL	Targeted	(−5.84)	48% Nu	
	NP_476545	–VIEGLIRKTKGS	Not targeted	(−83.334)	44% Mito	[78] Mito
Homo sapiens	NP_001350	–TLEELIRKTKGS	Not targeted	(−81.551)	65% Mito	[79] Mito
	NP_065715	–GLPDFASFSAKL	Targeted	(−6.964)	39% Cyto	[80] Px
	NP_060911	–KMKETFKEKAKL	Targeted	(−4.383)	44% ER	[81] Px
Arabidopsis thaliana	BAD94126	–RAKPVGLPTSKL	Targeted	(−16.448)	78% Px	

The locations indicated in the columns are: Cytosolic (Cyto), Endoplasmic reticulum (ER), Mitochondrial (Mito), Nuclear (Nu) or Peroxisomal (Px).

siological conditions but preserve the potential to become important in future evolutionary scenarios or in pathological situations. In line with these observations, it seems that cells only exploit a fraction of the potential molecular capacities of their proteins.

5. Concluding remarks

In summary, PTS1s are defined as C-terminal dodecamers able to interact with the TPR domain of PEX5 proteins. These sequences comprise the potential to initialize a peroxisomal translocation event via their interaction with PEX5 proteins. This interaction alone is certainly not sufficient to bring the entire translocation process to an end. Accessibility, lack of competition with other hierarchically more important signals, or simply the presence of target organelles are just as important, but without the potential to interact with PEX5 the translocation process of PTS1-containing proteins to peroxisomes would not even resume.

A sequential model for the initial steps of import includes a first contact between the extreme C-terminus, possibly just a tripeptide, of a potential cargo with some residues of the TPR domain of a PEX5 protein. This primary interaction would lead to the closure of the clamp formed by the TPR domain, the state observed in the crystal structures. Further contacts between the 12-mer and the TPR domain are consistent with the final affinity describing the ability of a particular sequence to act as a PTS1. Partial or complete inaccessibility or residues detrimental to the interaction would preclude the closure of the TPR clamp and thus inhibit the flow of the import process. Once the cargo is bound to the TPR domain the translocation starts, but finally the cargo has to be released and the interaction of PTS1 with the TPR domain needs to be reversed. In the future, crystal structures of full-length PEX5 proteins in its loaded and unloaded state will certainly help to elucidate the releasing step.

Understanding sub-cellular location and transport of protein has greatly increased the knowledge of genetically inherited diseases. In fact, mutated signal peptides can target proteins to the incorrect place in the cell leading to pernicious clinical symptoms. For instance cystic fibrosis is a lethal illness caused by defective channel protein transport to the plasma membrane [70]. Mistargeting of peroxisomal proteins can also have dramatic effects on health as exemplified by the mislocalization of alanine:glyoxylate aminotransferase which has been reported to be the cause of the hereditary disease primary hyperoxaluria responsible for the formation of kidney stones in youth [71].

In conclusion, the exact knowledge of proteins with their signals and their mode of transport in the cell is a prerequisite for the comprehension of innumerable illnesses and the subsequent discovery of new treatments. Indeed, studies around protein targeting have direct practical applications in our society such as the production of curative proteins, or the development of efficient and affordable diagnosis along with the advancement of therapeutic strategies.

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