

Functional characterization of two missense mutations in Pex5p—C11S and N526K

Andreia F. Carvalho^{a,b}, Cláudia P. Grou^{a,b}, Manuel P. Pinto^{a,b}, Inês S. Alencastre^{a,b}, João Costa-Rodrigues^{a,b}, Marc Fransen^c, Clara Sá-Miranda^a, Jorge E. Azevedo^{a,b,*}

^a Instituto de Biologia Molecular e Celular (IBMC), Porto, Portugal

^b Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal

^c Katholieke Universiteit Leuven, Faculteit Geneeskunde, Departement Moleculaire Celbiologie, Leuven, Belgium

Received 16 March 2007; received in revised form 19 April 2007; accepted 19 April 2007

Available online 29 April 2007

Abstract

Most newly synthesized peroxisomal proteins are targeted to the organelle by Pex5p, the peroxisomal cycling receptor. Pex5p interacts with these proteins in the cytosol, transports them to the peroxisomal docking/translocation machinery and promotes their translocation across the organelle membrane. Finally, Pex5p is recycled back to the cytosol in order to catalyse additional rounds of transportation. Although several properties of this protein sorting pathway have been recently uncovered, we are still far from comprehending many of its basic principles. Here, we describe the mechanistic implications of two single-amino acid substitutions in Pex5p. The first mutation characterized, Cys11Ser, blocks the recycling of Pex5p back into the cytosol at the step in which stage 2 Pex5p is converted into stage 3 Pex5p. The mutation Asn526Lys, previously described in a child with neonatal adrenoleukodystrophy and shown to abolish the PTS1-binding capacity of Pex5p, results in a Pex5p protein exhibiting import capacity. Protease assays suggest that the Asn526Lys mutation causes conformational alterations at the N-terminal half of Pex5p mimicking the ones induced by binding of a PTS1-containing peptide to the normal peroxin. The implications of these findings on the mechanism of protein translocation across the peroxisomal membrane are discussed.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Pex5p; Peroxisomes; Protein translocation; Peroxisomal matrix proteins

1. Introduction

Peroxisomal matrix proteins are synthesized in cytosolic ribosomes and post-translationally targeted to the peroxisome. Specific targeting of these newly synthesized proteins to the organelle is warranted by the existence of peroxisomal targeting signals (PTSs) in their primary structure. There are two well-defined PTSs: the so-called PTS1 is a tripeptide with the sequence S–K–L (or similar) present at the extreme C-terminus of most matrix proteins [1,2]; the PTS2 is a degenerate

nonapeptide found at the N-terminus of a few matrix proteins [3–5]. In mammals, both classes of matrix proteins are targeted to the peroxisome by Pex5p [6,7]. PTS1-containing proteins are directly recognized (bound) by the C-terminal half of this peroxin, a region comprising several TPR domains [8,9]; PTS2-containing proteins are recognized indirectly by the large isoform of Pex5p, through the adaptor protein Pex7p [10–12].

After this recognition event, cargo-loaded Pex5p interacts with the so-called importomer, a large, but probably transient, protein complex present at the peroxisomal membrane [13–15]. This importomer comprises many different peroxins which can be divided into two functional groups: Pex2p, Pex10p, Pex12p, Pex13p, Pex14p and, in yeast, also Pex8p and Pex17p, are probably involved in the docking of the receptor–cargo protein complexes at the peroxisomal membrane and in the translocation of the cargo proteins across this membrane system; Pex1p, Pex6p and Pex26p (in mammals) or Pex15p (in yeast), are in

Abbreviations: PTS, peroxisomal targeting signal; PNS, postnuclear supernatant; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; ATP γ S, adenosine 5'-O-(thiotriphosphate); TPR, tetratricopeptide repeats

* Corresponding author. Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Rua do Campo Alegre, 823 4150-180 Porto, Portugal. Tel.: +351 226074900; fax: +351 226099157.

E-mail address: jazevedo@ibmc.up.pt (J.E. Azevedo).

charge of catalysing the recycling of peroxisomal Pex5p back into the cytosol, so that it can engage in additional rounds of protein transportation (reviewed in [16,17]).

Despite our knowledge on many of the properties of this protein sorting pathway, we are still far from understanding its basic principles. For instance, although the peroxisomal membrane lacks large pores and, accordingly, is impermeable to many small metabolites (reviewed in [18,19]), many peroxisomal proteins are translocated across the peroxisomal membrane in their folded, even oligomeric state [20–22]. The mechanism of this process remains completely unknown.

A classical approach that has been used for many years in the characterization of the mechanisms of protein translocation across biological membranes relies on the use of *in vitro* import systems. A few years ago we started to apply this strategy to study the Pex5p-mediated protein import process [23]. This *in vitro* system consists in incubating ³⁵S-labeled Pex5p with organelles from rat liver and it explores the fact that soluble (cytosolic) Pex5p is extremely sensitive to proteolysis due to the natively unfolded nature of a large portion of its polypeptide chain [24,25], a property that changes completely when Pex5p is inserted into the peroxisomal membrane. This strategy has been successfully applied and adapted to other organisms by other laboratories [26,27].

During recent years, the data obtained using this approach led to the conclusion that Pex5p is inserted into the peroxisomal membrane in a Pex13p-, Pex14p- and cargo-dependent process [23,28], an event that, unexpectedly, does not require ATP hydrolysis [26,27,29]. ATP is, in fact, needed in the Pex5p-mediated protein transport process but only during the export step [29], when the receptor is recycled back into the cytosol by the action of Pex1p and Pex6p [26,27]. It was also shown that the first 17 amino acid residues of Pex5p are absolutely required for this export step [25].

Interestingly, after insertion into the peroxisomal membrane of mammals two different membrane-bound populations of Pex5p can be detected [23,26]: the so-called stage 2 Pex5p exposes the majority of its mass into the matrix of the organelle and about 2 kDa of its N-terminus into the cytosol, as assessed by protease-protection experiments using proteinase K; stage 3 Pex5p is completely resistant to this protease and becomes a particularly prominent species under ATP-limiting conditions [23]. Furthermore, several independent observations established that stage 2 is the precursor of stage 3 ([23,28,29]; see also Discussion). These observations together with the finding that these two Pex5p populations are still associated with Pex14p [23], led us to propose that cargo proteins are translocated across the peroxisomal membrane by Pex5p itself in a process energetically driven by simple protein–protein interactions involving Pex5p on one side and the subunits of the importomer on the other [30,31]. This perspective is, thus, incompatible with the so-called extended version of the cycling receptor model, which predicts that cargo-loaded Pex5p is completely translocated across the peroxisomal membrane during the protein transport process (reviewed in [32]).

In order to gather more data on the mechanism of the Pex5p-mediated import pathway we have started to address the role of

specific domains/amino acid residues in the function of this peroxin. Here, we describe the functional implications of mutating a cysteine residue at position 11 of Pex5p. In addition, the unexpected properties of a Pex5p mutant version unable to bind PTS1-containing proteins are also presented.

2. Materials and methods

2.1. *In vitro* import reactions

Rat liver postnuclear supernatants (PNS) were prepared in SEM buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.4, 1 mM EDTA–NaOH, pH 7.4) supplemented with 2 μg/ml *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, as described before [23]. *In vitro* import reactions (100 μl) contained 300 μg of PNS protein and 0.3–1.0 μl of the relevant reticulocyte lysates in import buffer (0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH, pH 7.4, 3 mM MgCl₂, 0.2% (w/v) lipid-free bovine serum albumin, 20 μM methionine, and 2 μg/ml *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide). Unless otherwise specified, import reactions were incubated for 20 min at 37 °C. ATP and ATPγS were used at 10 mM final concentrations. In the experiment shown in Fig. 1B an ATP-regenerating system (10 mM creatine phosphate and creatine phosphokinase 5 units/ml, final concentrations) was also included. The cargo-dependence experiments were performed as described previously but using a His-tagged recombinant protein comprising amino acid residues 314–639 of Pex5p (TPRs-Pex5p; [24]) instead of the GST-fusion protein described before (see [33]). The concentration of TPRs-Pex5p in the import reactions was 0.17 μM. To reverse the inhibitory action of TPRs-Pex5p a PTS1-containing peptide (see below) was included in the import reactions at 8 μM final concentration. Proteinase K treatment of import reactions and processing of protein samples for SDS-PAGE and autoradiography was done exactly as described [23].

2.2. Construction of PEX5 cDNAs

A cDNA encoding the small isoform of Pex5p was obtained using a ligation-PCR strategy and pGEM4-Pex5 [23] as a template. The upstream and downstream flanking sequences of exon 8 of Pex5p were amplified with primers CCGGTCCGACATGGCAATGCGGGAGCTGGTGA (1F) and GGCATCTGATGTACCCTCAGAATTAGCCAATTTGGGGTCATCC (2R), and TTGGCTAATCTGAGGGTACATCAGATGCCTGGGTTGACCAGT (3F) and GCGGTCCGACCTGTACTGGGGCAGGCCAAACATAG (4R). The resulting PCR products were mixed and amplified with primers 1F and 4R and cloned into pGEM[®]-T Easy vector. The recombinant plasmid was digested with *Sal*I and the insert was cloned into the *Sal*I site of pGEM-4 (Promega), originating pGEM4-Pex5S.

Plasmids encoding Pex5L(C11S)p, Pex5L(N526K)p and Pex5S(N489K)p were obtained with the QuickChange[®] Site-Directed Mutagenesis Kit (Stratagene) using the primer pairs 5'-GGAGCTGGTGGAGGCCGAAAGCGG-GGGTGCCAACCCGC-3' and 5'-GCGGGTTGGCACCCCGCTTTCGGC-CTCCACCAGTCC-3' for the C11S mutation and 5'-GACTATTGCTGTG-GAAGAAGCTAGGCGCCACCCTGGC-3' and 5'-GCCAGGGTGGCGCC-TAGCTTCTCCACAGCAAATAGTC-3' for the N526/489K mutation. The templates used in the PCR step of the mutagenesis protocol were: pQE-Pex5, a plasmid encoding the large isoform of human Pex5p [25] to obtain pQE-Pex5L(C11S) and pQE-Pex5L(N526K); pQE-TPRs-Pex5 (a pQE-derivative encoding amino acid residues 314–639 of Pex5Lp [24]) to obtain pQE-TPRs-Pex5(N526K); and pGEM4-Pex5S to obtain pGEM4-Pex5S(N489K).

2.3. Synthesis of ³⁵S-labeled Pex5p proteins

cDNAs encoding Pex5L(C11S)p and Pex5L(N526K)p preceded by the T7 RNA polymerase promoter were produced by expression PCR [34] using as templates the plasmids described above. In the first PCR, the plasmids were amplified using the forward primer 5'-GGGAGAGCCACCATGG-CAATGCGGGAGCTG-3' and the reverse primer 5'-GCGGTCCGACT-CCTGGGGCAGGCCAAACAT-3'. In the second PCR, this reverse primer

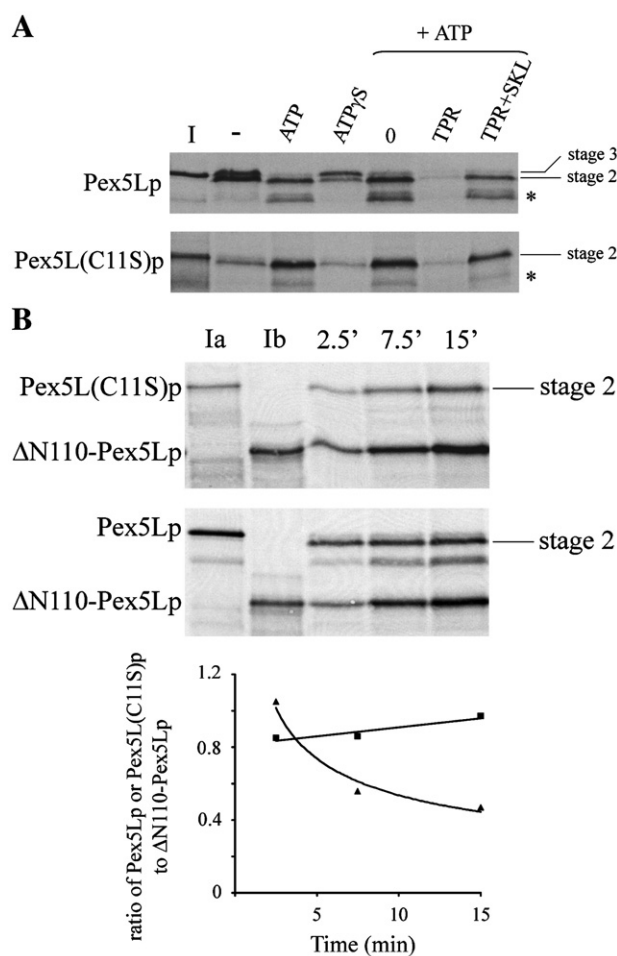


Fig. 1. The C11S mutation blocks export of Pex5p by blocking the stage 2 to stage 3 transition. (A) 35 S-labeled Pex5Lp and Pex5L(C11S)p were incubated with a PNS fraction from rat liver in the absence of exogenous nucleotides (–), in the presence of 10 mM ATP (ATP) or 10 mM ATP γ S (ATP γ S), as indicated. After 20 min at 37 °C, the import reactions were treated with proteinase K, the organelles isolated by centrifugation and analysed by SDS-PAGE/autoradiography. In order to verify that insertion of Pex5L(C11S)p into the peroxisomal membrane is cargo-dependent, three additional import reactions supplemented with 10 mM ATP were also performed. The first received 5 μ l of SEM buffer alone (lane “0”); the second was supplemented with 5 μ l of SEM containing recombinant TPRs-Pex5p (lane “TPR”; 0.17 μ M final concentration) to sequester PTS1-containing proteins present in the import reaction. The third (lane “TPRs+SKL”) contained in addition to this recombinant protein a PTS1-containing peptide (see Materials and methods) to reverse the inhibitory action of TPRs-Pex5p. I — 10% of the 35 S-labeled proteins used in each import reaction. The asterisk marks a truncated Pex5Lp protein occasionally present in the reticulocyte lysates used to synthesize Pex5p. This fragment most likely derives from translation initiation at an internal methionine because it becomes particularly enriched in import reactions performed in the presence of ATP (it lacks the domain necessary for the export step) and its insertion into the peroxisomal membrane is cargo-dependent (compare lanes “TPR” and “TPR+SKL”). (B) Import/Export kinetics of Pex5L(C11S)p. Mixtures of reticulocyte lysates containing Pex5L(C11S)p and Δ N110-Pex5Lp (upper panel) or Pex5Lp and Δ N110-Pex5Lp (lower panel) were subjected to import reactions in the presence of ATP for the indicated periods of time. After proteinase K treatment, the organelles were sedimented and analysed by SDS-PAGE/autoradiography. Ia and Ib, 10% of the 35 S-labeled proteins used in each import reaction. The graphic shows ratios of protease protected species normalized for the inputs as a function of time. Squares — ratio Pex5L(C11S)p/ Δ N110-Pex5Lp; triangles — ratio Pex5Lp/ Δ N110-Pex5Lp;

was used together with the forward primer 5'-GAATTCTAATACGACTCAC-TATAGGGAGAGCCACCATG-3'. 35 S-labeled proteins were synthesized using the TNT[®] T7 Quick Coupled Transcription/Translation kit (Promega) in the presence of Redivue[™] L- 35 S]methionine (specific activity > 1000 Ci/ mmol) following the manufacturer's instructions. 35 S-labeled Pex5Lp, Pex5Sp, Pex5S (N489K)p, Δ N110-Pex5Lp and Δ C1-Pex5Lp were synthesized using the strategies described before [28,33].

2.4. Protease assays

Recombinant proteins (5 μ g in 50 μ l of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT) were incubated at 37 °C for 10 min with 10 μ M of a PTS1-containing peptide (CRYHLKPLQSKL) or a control peptide (CRYHLKPLQLKS; see [33]), as indicated. The samples were placed on ice and treated for 15 min with proteinase K (1 μ g/ml) or trypsin (8 μ g/ml). After inactivation of the protease with phenylmethylsulfonyl fluoride (0.5 mg/ml), the proteins were precipitated with trichloroacetic acid (10% (w/v)), washed with acetone, and analysed by SDS-PAGE.

2.5. Miscellaneous

The purification of His-tagged Pex5p recombinant proteins was described before [24]. Densitometric analysis of X-ray films was performed using the UN-SCAN-IT automated digitizing system. Edman degradation of proteolytic fragments was performed by HHMI/Keck Biotechnology Resource Laboratory (New Haven, CT).

3. Results

3.1. Mutation of cysteine 11 in Pex5Lp leads to a block in its export pathway by blocking the stage 2 to stage 3 transition

In a previous work we have shown that the first 17 amino acid residues of Pex5p are essential for the ATP-dependent export step of peroxisomal Pex5p back into the cytosol [28]. This domain of Pex5p comprises a cysteine-containing motive that is conserved in all Pex5p sequences presently available and also in the functionally related peroxins, Pex18p, Pex20p and Pex21p from lower eukaryotes [35]. Given the ease with which (reduced) cysteine residues can be modified in vitro, we made a set of preliminary in vitro import experiments aiming at monitoring the export capacities of Pex5Lp and Δ C1-Pex5Lp (a truncated version of Pex5Lp containing only its first 324 amino acid residues; see [28,33]) pretreated with iodoacetamide. While the peroxisomal import rates of these two alkylated forms of Pex5Lp were normal (Δ C1Pex5Lp) or almost normal (Pex5Lp), the export step of both proteins was found to be severely affected (data not shown). Since Δ C1Pex5Lp contains only one cysteine residue, the one present in the above-mentioned motive, these observations prompted us to mutate Cys11 of Pex5Lp to the structurally related amino acid serine and to use this mutant protein [Pex5L(C11S)p] in in vitro import experiments. As shown in Fig. 1A, incubation of 35 S-labeled Pex5L(C11S)p with a PNS fraction in the presence of ATP results in a proteinase K-resistant species that is 2 kDa shorter than the complete protein. This species corresponds to stage 2 Pex5p (see Introduction). The same result was obtained with a C-terminally truncated version comprising the first 324 amino acid residues of Pex5L(C11S)p (data not shown). Insertion of Pex5L(C11S)p into the peroxisomal membrane is cargo protein-

dependent (compare lanes “TPR” and “TPR+SKL” in Fig. 1A) indicating that the C11S mutation does not interfere with the first steps of the Pex5p-mediated import pathway, as expected. However, and in agreement with the results of the iodoacetamide experiments, the fraction of the input protein that can be detected at stage 2 in the presence of ATP is higher for the Pex5L(C11S)p mutant protein than for the normal peroxin (23% versus 7%, respectively). As shown below, this phenomenon derives from the fact that the ATP-dependent export step of Pex5L(C11S)p back into the soluble fraction is blocked. Interestingly, in contrast to the results obtained with the normal Pex5Lp protein, no fully protease-protected species (corresponding to stage 3 Pex5p) can be observed with the Pex5L(C11S)p mutant protein, even when ATP γ S is used in these experiments. This ATP analogue blocks the export step leading to an accumulation of Pex5p at the peroxisomal membrane at stage 3 [23].

In order to quantify the degree of inhibition of the export step caused by the C11S mutation, we compared the kinetics of appearance of protease-protected Pex5L(C11S)p to the one obtained with Δ N110-Pex5Lp. These experiments were performed in the presence of ATP at 37 °C, conditions in which normal Pex5p continuously enters and exits the peroxisomal membrane displaying a half-life at the peroxisomal membrane of less than 2.5 min [28]. As shown in Fig. 1B, Pex5L(C11S)p displays the kinetic behaviour of Δ N110-Pex5Lp. This is particularly evident when the ratios of stage 2 Pex5L(C11S)p to protease protected Δ N110-Pex5Lp are plotted as a function of time: a straight line with a slope close to 0 is obtained, implying that the import kinetics of these two Pex5p versions are similar. In contrast the Pex5Lp/ Δ N110-Pex5Lp ratio decreases over time due to the fact that Pex5Lp (but not Δ N110-Pex5Lp) is efficiently exported from the peroxisomal membrane into the soluble (cytosolic) fraction, as described before [28]. Taken together these data indicate that the C11S mutation blocks the ATP-dependent export step of Pex5p from the peroxisomal membrane by inhibiting the stage 2 to stage 3 transition.

3.2. Pex5L(N526K)p, a mutant protein unable to interact with PTS1-containing proteins, is a substrate for the peroxisomal import and export machineries

Mutations in the gene encoding Pex5p define complementation group 2 of the peroxisome biogenesis disorders [8]. One of the patients belonging to this group was reported to produce normal amounts of a mutant Pex5p possessing a lysine at position 526 instead of an asparagine. Cells of this patient have an impaired PTS1-import pathway [8] and subsequent studies have shown that the N526K mutation blocks the binding of PTS1-containing cargo proteins to the C-terminal half of Pex5p [9]. We produced this mutant protein [Pex5L(N526K)p] and tested its functionality in our in vitro import system. Since in this system insertion of Pex5p into the peroxisomal membrane is a PTS1-dependent process no protease-protected species derived from Pex5L(N526K)p should be detected. Strikingly, the opposite result was obtained. As shown in Fig. 2A, import

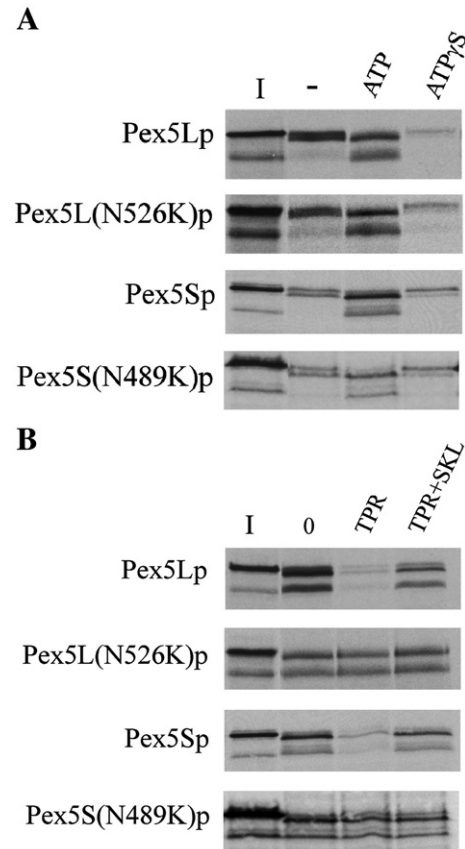


Fig. 2. Pex5L(N526K)p/Pex5S(N489K) are imported into peroxisomes in a cargo-independent process. (A) Standard import reactions in the absence of exogenous nucleotides (lanes “–”), in the presence of 10 mM ATP (lanes “ATP”) or 10 mM ATP γ S (lanes “ATP γ S”) were performed with 35 S-labeled Pex5Lp, Pex5L(N526K)p, Pex5Sp and Pex5S(N489K)p. (B) Insertion of Pex5L(N526K)p or Pex5S(N489K)p into the peroxisomal membrane is not inhibited when PTS1-containing proteins are sequestered from the import medium by addition of large amounts of TPRs-Pex5p. These experiments were performed as described in the legend of Fig. 1A.

experiments performed in the absence of exogenous nucleotides, in the presence of ATP or ATP γ S result in ratios of stage 2 to stage 3 Pex5L(N526K)p indistinguishable from the ones obtained for Pex5Lp indicating that once at the importomer, this mutant version of Pex5p displays the mechanistic properties of the normal Pex5p version. The only difference between the two proteins resides in the fraction of the input proteins entering this pathway. Indeed, 2–3 fold more Pex5L(N526K)p than Pex5Lp has to be added to these reactions so that similar amounts of protease protected species are obtained.

Considering that the 35 S-labeled proteins used in these experiments correspond to the large isoform of Pex5p, a protein that is also in charge of transporting PTS2-containing proteins into the peroxisome [10–12], one possibility to explain these results would be to assume that Pex5L(N526K)p is active in this assay because it interacts with Pex7p.PTS2-containing cargo protein complexes. Although unlikely (see [33]), we repeated these experiments using the small isoform of Pex5p (Pex5Sp). Due to alternative splicing of the PEX5 transcript, this version of Pex5p lacks the Pex7p-binding site and is unable to promote

import of PTS2-containing proteins [10–12]. As shown in Fig. 2A, Pex5S(N489K)p (residue 489 of Pex5Sp corresponds to residue 526 of Pex5Lp) is also a substrate for the peroxisomal importomer. This possibility excluded, we finally raised the hypothesis that Pex5L(N526K)p (or Pex5S(N489K)p) could still possess some residual PTS1-binding activity. If this were the case, then addition of a vast excess of the recombinant PTS1-binding domain of Pex5p to the import reactions to sequester PTS1-containing proteins should block the insertion of these proteins into the peroxisomal membrane. As shown in Fig. 2B, whereas the amounts of protease-protected Pex5Lp or Pex5Sp are highly decreased under these conditions, as described before [33], no inhibition can be observed for the two versions possessing the asparagine to lysine mutation. We

conclude that these mutant versions of Pex5p are inserted into the peroxisomal membrane in a cargo-independent process.

3.3. The N526K mutation causes conformational alterations in regions preceding the C-terminal half of Pex5p that mimic the ones induced by binding of a PTS1-containing peptide to the normal peroxin

Previously, we proposed the existence of an auto-regulatory mechanism in Pex5p. According to this model, the peroxisomal targeting domain present at the N-terminal half of Pex5p is a target of negative regulation exerted by its C-terminal cargo-binding domain; binding of a PTS1-containing protein to Pex5p releases this inhibition [33]. Obviously, the observations described in the previous section challenge this idea. However, it is also possible that the effects of the asparagine to lysine mutation on the structure/function of Pex5p go beyond the simple disruption of its PTS1-binding capacity. For instance, this mutation could also interfere with the cross-talk between the N- and C-terminal halves of Pex5p resulting in a constitutively active peroxisomal targeting domain.

Presently the number of biochemical strategies that can be used to address the interdomain cross-talk of Pex5p is quite limited. In fact only two indirect methodologies have been used for this purpose. The first consists in detecting differences in the binding affinities of the N-terminal half of Pex5p for other peroxins in the presence or absence of cargo proteins. Although effects of this type have been shown for *Pichia pastoris* Pex5p and Pex14p [36], mammalian Pex5p binds Pex14p with apparently similar affinities both in the presence and absence of PTS1-containing peptide/proteins ([37], and unpublished

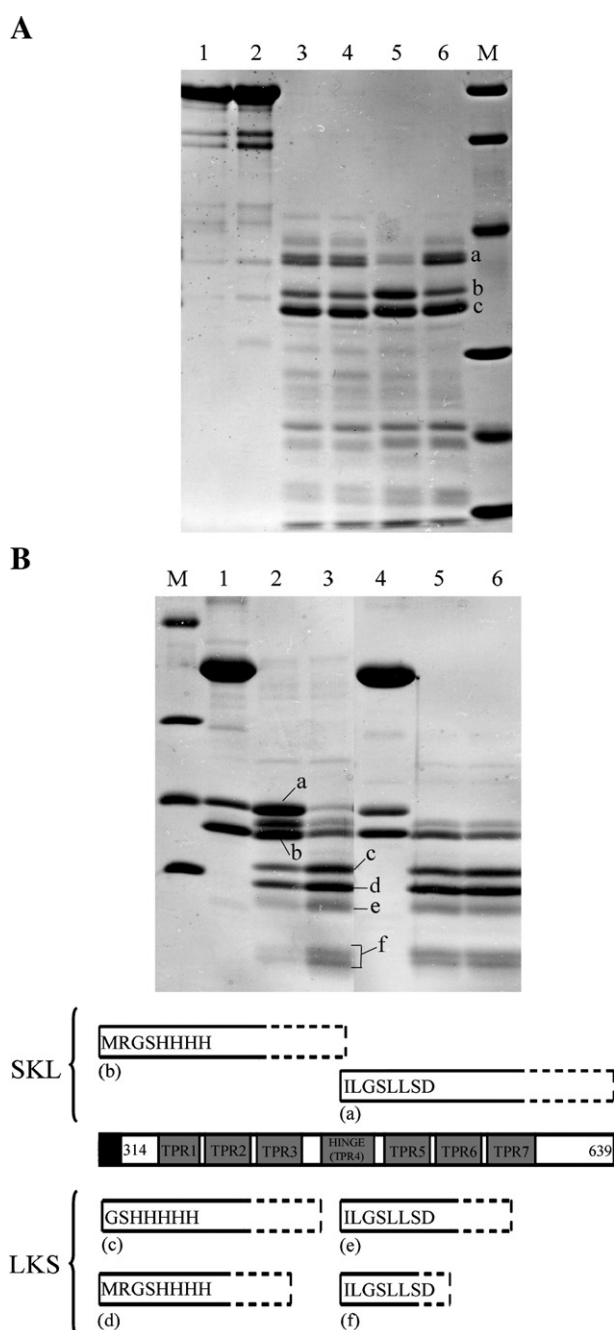


Fig. 3. The N526K mutation causes conformational alterations in regions preceding the C-terminal half of Pex5p that mimic the ones induced by binding of a PTS1-containing peptide to the normal peroxin. (A) Recombinant Pex5Lp (lanes 2, 5, 6) and Pex5L(N526K)p (lanes 1, 3, 4) were incubated with a PTS1-containing peptide (lanes 4 and 6) or a control peptide (lanes 3 and 5; see Materials and methods for details) and subjected to partial proteolysis using proteinase K (lanes 3–6). After inactivation of the protease, the samples were subjected to TCA precipitation and subjected to SDS-PAGE. The N-terminal sequences of polypeptides indicated by letters “a” and “b” were described before [25]. Band “c” represents a fragment starting at amino acid residue 308 of Pex5Lp. M, molecular mass standards (from top to bottom): 97, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa. (B) Recombinant proteins comprising amino acid residues 314–639 of the normal (lanes 1–3) or the mutant N526K (lanes 4–6) versions were incubated with the PTS1-containing peptide (lanes 2 and 5) or a control peptide (lanes 3 and 6; see Materials and methods) and subjected to partial proteolysis with trypsin (lanes 2, 3, 5, 6). A coomassie blue-stained gel is shown. Letters a–f indicate fragments that were subjected to Edman degradation. Note that a small amount of both recombinant proteins becomes cleaved, during the production and purification steps (protein fragments of 20 and 18 kDa). M, molecular mass markers (from top to bottom: 45, 31, 20 and 14.4 kDa). A schematic representation of this region of Pex5p as well as of the major tryptic fragments (fragments a–f) obtained in the presence of the PTS1-containing peptide (“SKL”) or a control peptide (“LKS”) is also shown. The N-terminal sequences of the fragments obtained with TPRs-Pex5p were determined by Edman degradation and are shown within the boxes representing the obtained fragments. The C-termini were estimated from the apparent sizes of the corresponding fragments on this gel. The seven TPR motives are indicated in grey. The black box represents the His-tag present in these proteins.

results). Positive results have been shown for the Pex5p/Pex13p protein pair in mammals. Pex13p binds stronger to free Pex5p than to the cargo-loaded form [37]. Unfortunately, we were unable to produce human Pex13p in a soluble state refraining us of using this strategy.

The second approach to address this issue consists in probing PTS1-induced conformational alterations in Pex5p by partial proteolysis with proteinase K. Using this strategy, it was shown that the conformation/environment of a region of Pex5Lp that precedes its first TPR domain by 20–30 amino acid residues is altered when Pex5Lp is loaded with a PTS1-containing peptide/protein [25]. We tested recombinant Pex5L(N526K)p in this assay. As shown in Fig. 3A, while the proteolytic patterns obtained for Pex5Lp in the presence and in the absence of a PTS1-containing peptide are clearly different, treatment of Pex5L(N526K)p with the protease under both conditions results in a single proteolytic pattern. Remarkably, the pattern obtained for Pex5L(N526K)p corresponds to the one obtained with Pex5Lp in the presence of the PTS1-peptide. This observation suggests that the conformation/environment of the region that precedes the TPR domains of Pex5p is similar in both Pex5L(N526K)p and cargo-loaded Pex5Lp. Furthermore, since all the major Pex5p fragments detected in these experiments contain the C-terminal half of the peroxin (see [25] and legend to Fig. 3), these results also suggest that the conformation of the TPR domains of Pex5p is not dramatically changed by the N526K mutation. If this was the case then much smaller protein fragments would be expected as observed, for instance, when a Pex5Lp protein lacking the last 41 amino acid residues is used in this assay (data not shown).

One possibility to explain the properties of Pex5L(N526K)p would be to assume that this mutation causes conformational alterations in the C-terminal half of Pex5p mimicking the ones induced by the binding of a PTS1-containing cargo protein [38]. Again, we used a partial proteolysis approach to address this issue. Two recombinant proteins comprising amino acid residues 314–639 of the normal and mutated Pex5Lp versions (TPRs-Pex5p and TPRs-Pex5(N526K)p, respectively) were subjected to partial proteolysis using trypsin. As shown in Fig. 3B, this assay reveals the existence of PTS1-induced conformational alterations in the C-terminal half of Pex5p. Indeed, TPRs-Pex5p is more susceptible to the protease when the digestion is performed in the absence of the PTS1 peptide. This result is in agreement with the recently described structures of this Pex5p domain in its free and cargo-bound states, which reveal that the former is much more flexible and less compact than the latter [38]. Interestingly, the proteolytic pattern of TPRs-Pex5(N526K)p corresponds to the one obtained with TPRs-Pex5p in the absence of the PTS1 peptide. Apparently, the N526K mutation does not induce compactization of the C-terminal half of Pex5p.

4. Discussion

In this work we describe the mechanistic implications of two missense mutations in Pex5p. One of these mutations, the substitution of a cysteine by a serine at position 11 of Pex5Lp,

blocks the export step of peroxisomal Pex5Lp back into the cytosol. This finding is in complete agreement with our previous observations showing that a Pex5p version lacking the first 17 amino residues of this peroxin is not exported from the peroxisomal membrane. Those and other observations (see discussion in ref. [28]), together with the fact that cysteine 11 is one of the few residues conserved in this short region of Pex5p and Pex5p-like proteins [35,39] led us to propose that this domain of Pex5p comprises the binding site for the machinery catalysing the recycling of the PTS1-receptor back into the cytosol. Interestingly, recent steady-state data led to the discovery that the corresponding residue of *P. pastoris* Pex20p (a Pex5p-like peroxin) is also necessary for the export step [40].

Besides refining our previous observations, the properties of Pex5L(C11S)p should also clarify some doubts raised recently regarding the relationship between stage 2 and stage 3 Pex5p. Several findings from our laboratory suggest that stage 2 is the precursor of stage 3 Pex5p. These include: (1) the fact that stage 2 can be chased into stage 3 Pex5p in *in vitro* pulse-chase experiments performed in the presence of ATP- γ S; (2) the observation that the ATP-dependent export kinetics of stage 3 Pex5p is faster than the observed for stage 2 Pex5p; (3) the observation that only stage 2 Pex5p can be detected in an incomplete *in vitro* import system lacking an active export machinery and (4) that the transition of stage 2 into stage 3 Pex5p is blocked by low temperatures, conditions which also inhibit the export step of Pex5p [23,28,29]. However, it was recently argued that since stage 3 Pex5p is mainly detected under ATP-limiting conditions, which allow Pex5p import into peroxisomes but not its export, then it should precede stage 2 Pex5p [41]. Naturally, such interpretation would leave room for the extended receptor cycling model because the protein translocation step would be linked to a Pex5p population that is not accessible to exogenously added proteases, presumably the result of a peroxisomal matrix localization. The observation that no stage 3 Pex5p can be detected with Pex5L(C11S)p even when the import experiments are performed in the presence of ATP- γ S further invalidates this new interpretation. In fact, these results strongly suggest that peroxisomal Pex5p has to reach stage 3 in order to be exported from the peroxisomal membrane.

The properties displayed by Pex5L(C11S)p also raise new hypotheses regarding the mechanism of the export step. According to recent data obtained in yeast, export of Pex5p from the peroxisomal membrane may be linked to a ubiquitylation event [27,42,43] although ubiquitylation of Pex5p has also been interpreted by other authors as reflecting the existence of quality-control mechanisms at the peroxisomal importomer [44,45]. Despite several attempts we have been unable to detect a similar modification in our *in vitro* import experiments not even with the Pex5L(C11S)p mutant version (unpublished observations). Thus, if ubiquitylation is also linked to the export step of mammalian Pex5p back into the cytosol, then it may occur only after stage 2. Alternatively, cysteine 11 itself is ubiquitylated. These observations should encourage further experiments with yeasts (organisms in which Pex5p ubiquitylation is easily observed) aiming at clarifying this matter.

The second mutation characterized in this work disrupts the PTS1-binding activity of Pex5p [8,9]. One of the aims in producing this mutant version of Pex5p was in fact to obtain a protein that could be used as a control in our in vitro system. Strikingly, Pex5L(N526K)p proved to be quite efficient in these assays. This observation together with the results of the protease assays strongly suggests that the N526K mutation disrupts the interdomain cross-talk in Pex5p. This in turn raises the interesting possibility that the region of the C-terminal half of Pex5p that controls the peroxisomal targeting domain of Pex5p overlaps with the PTS1-binding site. We are convinced that Pex5L(N526K)p will be a helpful tool in any future attempt to clarify this issue.

Regardless of the mechanistic reasons behind the behaviour of Pex5L(N526K)p the results described here have two additional implications. First, they clearly show that the triggering mechanism to activate the peroxisomal docking/translocation machinery emerges basically from Pex5p and not from some interaction involving Pex5p-bound cargo proteins on one side and the membrane peroxins on the other. This provides one additional argument to support the notion that Pex5p itself is the protein translocase. Second, the properties of Pex5L(N526K)p also demonstrate that an hypothetical cargo-induced oligomerization of Pex5p at or in the peroxisomal membrane, the central concept of the so-called pre-implex model [46] and an idea that could be easily included also in the transient pore model [32], is not a sine qua non condition for insertion of Pex5p into the organelle membrane. This is a property that should be integrated in any future model aiming at describing the Pex5p-mediated protein import process.

Acknowledgments

We thank Prof. Maria João Ramos (Faculdade de Ciências da Universidade do Porto) for a preliminary in silico analysis of the N526K mutation, and Chantal Brees (Katholieke Universiteit Leuven) for excellent technical assistance. The work done in Porto was supported by Fundação para a Ciência e Tecnologia (POCTI program) and FEDER funds, Portugal, and by European Union VI Framework program Grant LSHG-CT-2004-512018, Peroxisomes in Health and Disease. The work done in Leuven was supported through grants from the Research Foundation-Flanders (G.0237.4) and the Flemish government (GOA/2004/08).

References

- [1] S.J. Gould, G.A. Keller, N. Hosken, J. Wilkinson, S. Subramani, A conserved tripeptide sorts proteins to peroxisomes, *J. Cell Biol.* 108 (1989) 1657–1664.
- [2] C. Brocard, A. Hartig, Peroxisome targeting signal 1: is it really a simple tripeptide? *Biochim. Biophys. Acta* 1763 (2006) 1565–1573.
- [3] P.B. Lazarow, Chapter 3.1.7. The import receptor Pex7p and the PTS2 targeting sequence, *Biochim. Biophys. Acta* 1763 (2006) 1599–1604.
- [4] T. Osumi, T. Tsukamoto, S. Hata, S. Yokota, S. Miura, Y. Fujiki, M. Hijikata, S. Miyazawa, T. Hashimoto, Amino-terminal presequence of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisomal targeting, *Biochem. Biophys. Res. Commun.* 181 (1991) 947–954.
- [5] B.W. Swinkels, S.J. Gould, A.G. Bodnar, R.A. Rachubinski, S. Subramani, A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase, *EMBO J.* 10 (1991) 3255–3262.
- [6] N. Braverman, G. Dodt, S.J. Gould, D. Valle, An isoform of Pex5p, the human PTS1 receptor, is required for the import of PTS2 proteins into peroxisomes, *Hum. Mol. Genet.* 7 (1998) 1195–1205.
- [7] H. Otera, K. Okumoto, K. Tateishi, Y. Ikoma, E. Matsuda, M. Nishimura, T. Tsukamoto, T. Osumi, K. Ohashi, O. Higuchi, Y. Fujiki, Peroxisome targeting signal type 1 (PTS1) receptor is involved in import of both PTS1 and PTS2: studies with PEX5-defective CHO cell mutants, *Mol. Cell. Biol.* 18 (1998) 388–399.
- [8] G. Dodt, N. Braverman, C. Wong, A. Moser, H.W. Moser, P. Watkins, D. Valle, S.J. Gould, Mutations in the PTS1 receptor gene, PXR1, define complementation group 2 of the peroxisome biogenesis disorders, *Nat. Genet.* 9 (1995) 115–125.
- [9] G.J. Gatto Jr., B.V. Geisbrecht, S.J. Gould, J.M. Berg, Peroxisomal targeting signal-1 recognition by the TPR domains of human PEX5, *Nat. Struct. Biol.* 7 (2000) 1091–1095.
- [10] G. Dodt, D. Warren, E. Becker, P. Rehling, S.J. Gould, Domain mapping of human PEX5 reveals functional and structural similarities to *Saccharomyces cerevisiae* Pex18p and Pex21p, *J. Biol. Chem.* 276 (2001) 41769–41781.
- [11] H. Einwachter, S. Sowinski, W.H. Kunau, W. Schliebs, *Yarrowia lipolytica* Pex20p, *Saccharomyces cerevisiae* Pex18p/Pex21p and mammalian Pex5pL fulfil a common function in the early steps of the peroxisomal PTS2 import pathway, *EMBO Rep.* 2 (2001) 1035–1039.
- [12] H. Otera, T. Harano, M. Honsho, K. Ghaedi, S. Mukai, A. Tanaka, A. Kawai, N. Shimizu, Y. Fujiki, The mammalian peroxin Pex5pL, the longer isoform of the mobile peroxisome targeting signal (PTS) type 1 transporter, translocates the Pex7p.PTS2 protein complex into peroxisomes via its initial docking site, Pex14p, *J. Biol. Chem.* 275 (2000) 21703–21714.
- [13] 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.
- [14] C. Reguenga, M.E. Oliveira, A.M. Gouveia, C. Sa-Miranda, J.E. Azevedo, Characterization of the mammalian peroxisomal import machinery: Pex2p, Pex5p, Pex12p, and Pex14p are subunits of the same protein assembly, *J. Biol. Chem.* 276 (2001) 29935–29942.
- [15] K. Rosenkranz, I. Birschmann, S. Grunau, W. Girzalsky, W.H. Kunau, R. Erdmann, Functional association of the AAA complex and the peroxisomal importomer, *FEBS J.* 273 (2006) 3804–3815.
- [16] A. Baker, I.A. Sparkes, Peroxisome protein import: some answers, more questions, *Curr. Opin. Plant Biol.* 8 (2005) 640–647.
- [17] I. Heiland, R. Erdmann, Biogenesis of peroxisomes. Topogenesis of the peroxisomal membrane and matrix proteins, *FEBS J.* 272 (2005) 2362–2372.
- [18] V.D. Antonenkov, J.K. Hiltunen, Peroxisomal membrane permeability and solute transfer, *Biochim. Biophys. Acta* 1763 (2006) 1697–1706.
- [19] R.J. Wanders, H.R. Waterham, Biochemistry of mammalian peroxisomes revisited, *Annu. Rev. Biochem.* 75 (2006) 295–332.
- [20] J.R. Glover, D.W. Andrews, R.A. Rachubinski, *Saccharomyces cerevisiae* peroxisomal thiolase is imported as a dimer, *Proc. Natl. Acad. Sci. U. S. A.* 91 (1994) 10541–10545.
- [21] J.A. McNew, J.M. Goodman, An oligomeric protein is imported into peroxisomes in vivo, *J. Cell Biol.* 127 (1994) 1245–1257.
- [22] M.Q. Stewart, R.D. Esposito, J. Gowani, J.M. Goodman, Alcohol oxidase and dihydroxyacetone synthase, the abundant peroxisomal proteins of methylotrophic yeasts, assemble in different cellular compartments, *J. Cell Sci.* 114 (2001) 2863–2868.
- [23] A.M. Gouveia, C.P. Guimaraes, M.E. Oliveira, C. Reguenga, C. Sa-Miranda, J.E. Azevedo, Characterization of the peroxisomal cycling receptor, Pex5p, using a cell-free in vitro import system, *J. Biol. Chem.* 278 (2003) 226–232.
- [24] A.F. Carvalho, J. Costa-Rodrigues, I. Correia, J. Costa Pessoa, T.Q. Faria, C.L. Martins, M. Fransen, C. Sa-Miranda, J.E. Azevedo, The N-terminal half of the peroxisomal cycling receptor Pex5p is a natively unfolded domain, *J. Mol. Biol.* 356 (2006) 864–875.

- [25] J. Costa-Rodrigues, A.F. Carvalho, M. Fransen, E. Hambruch, W. Schliebs, C. Sa-Miranda, J.E. Azevedo, Pex5p, the peroxisomal cycling receptor, is a monomeric non-globular protein, *J. Biol. Chem.* 280 (2005) 24404–24411.
- [26] N. Miyata, Y. Fujiki, Shuttling mechanism of peroxisome targeting signal type 1 receptor Pex5: ATP-independent import and ATP-dependent export, *Mol. Cell. Biol.* 25 (2005) 10822–10832.
- [27] H.W. Platta, S. Grunau, K. Rosenkranz, W. Girzalsky, R. Erdmann, Functional role of the AAA peroxins in dislocation of the cycling PTS1 receptor back to the cytosol, *Nat. Cell Biol.* 7 (2005) 817–822.
- [28] J. Costa-Rodrigues, A.F. Carvalho, A.M. Gouveia, M. Fransen, C. Sa-Miranda, J.E. Azevedo, The N terminus of the peroxisomal cycling receptor, Pex5p, is required for redirecting the peroxisome-associated peroxin back to the cytosol, *J. Biol. Chem.* 279 (2004) 46573–46579.
- [29] M.E. Oliveira, A.M. Gouveia, R.A. Pinto, C. Sa-Miranda, J.E. Azevedo, The energetics of Pex5p-mediated peroxisomal protein import, *J. Biol. Chem.* 278 (2003) 39483–39488.
- [30] J.E. Azevedo, J. Costa-Rodrigues, C.P. Guimaraes, M.E. Oliveira, C. Sa-Miranda, Protein translocation across the peroxisomal membrane, *Cell Biochem. Biophys.* 41 (2004) 451–468.
- [31] J.E. Azevedo, W. Schliebs, Pex14p, more than just a docking protein, *Biochim. Biophys. Acta* 1763 (2006) 1574–1584.
- [32] R. Erdmann, W. Schliebs, Peroxisomal matrix protein import: the transient pore model, *Nat. Rev. Mol. Cell Biol.* 6 (2005) 738–742.
- [33] A.M. Gouveia, C.P. Guimaraes, M.E. Oliveira, C. Sa-Miranda, J.E. Azevedo, Insertion of Pex5p into the peroxisomal membrane is cargo protein-dependent, *J. Biol. Chem.* 278 (2003) 4389–4392.
- [34] K.C. Kain, P.A. Orlandi, D.E. Lanar, Universal promoter for gene expression without cloning: expression-PCR, *BioTechniques* 10 (1991) 366–374.
- [35] W. Schliebs, W.H. Kunau, PTS2 Co-receptors: diverse proteins with common features, *Biochim. Biophys. Acta* 1763 (2006) 1605–1612.
- [36] A.J. Urquhart, D. Kennedy, S.J. Gould, D.I. Crane, Interaction of Pex5p, the type 1 peroxisome targeting signal receptor, with the peroxisomal membrane proteins Pex14p and Pex13p, *J. Biol. Chem.* 275 (2000) 4127–4136.
- [37] H. Otera, K. Setoguchi, M. Hamasaki, T. Kumashiro, N. Shimizu, Y. Fujiki, Peroxisomal targeting signal receptor Pex5p interacts with cargoes and import machinery components in a spatiotemporally differentiated manner: conserved Pex5p WXXXF/Y motifs are critical for matrix protein import, *Mol. Cell. Biol.* 22 (2002) 1639–1655.
- [38] W.A. Stanley, F.V. Filipp, P. Kursula, N. Schuller, R. Erdmann, W. Schliebs, M. Sattler, M. Wilmanns, Recognition of a functional peroxisome type 1 target by the dynamic import receptor Pex5p, *Mol. Cell* 24 (2006) 653–663.
- [39] J.J. Smith, R.A. Rachubinski, A role for the peroxin Pex8p in Pex20p-dependent thiolase import into peroxisomes of the yeast *Yarrowia lipolytica*, *J. Biol. Chem.* 276 (2001) 1618–1625.
- [40] S. Leon, S. Subramani, A conserved cysteine residue of *Pichia pastoris* Pex20p is essential for its recycling from the peroxisome to the cytosol, *J. Biol. Chem.* 282 (2007) 7424–7430.
- [41] S. Leon, J.M. Goodman, S. Subramani, Uniqueness of the mechanism of protein import into the peroxisome matrix: transport of folded, co-factor-bound and oligomeric proteins by shuttling receptors, *Biochim. Biophys. Acta* 1763 (2006) 1552–1564.
- [42] A. Kragt, T. Voorn-Brouwer, M. van den Berg, B. Distel, The *Saccharomyces cerevisiae* peroxisomal import receptor Pex5p is monoubiquitinated in wild type cells, *J. Biol. Chem.* 280 (2005) 7867–7874.
- [43] H.W. Platta, W. Girzalsky, R. Erdmann, Ubiquitination of the peroxisomal import receptor Pex5p, *Biochem. J.* 384 (2004) 37–45.
- [44] J.A. Kiel, K. Emmrich, H.E. Meyer, W.H. Kunau, Ubiquitination of the peroxisomal targeting signal type 1 receptor, Pex5p, suggests the presence of a quality control mechanism during peroxisomal matrix protein import, *J. Biol. Chem.* 280 (2005) 1921–1930.
- [45] J.A. Kiel, M. Otzen, M. Veenhuis, I.J. van der Klei, Obstruction of polyubiquitination affects PTS1 peroxisomal matrix protein import, *Biochim. Biophys. Acta* 1745 (2005) 176–186.
- [46] S.J. Gould, C.S. Collins, Opinion: peroxisomal-protein import: is it really that complex? *Nat. Rev. Mol. Cell Biol.* 3 (2002) 382–389.