Distinct Pores for Peroxisomal Import of PTS1 and PTS2 Proteins

Graphical Abstract

Highlights

- Import of folded proteins into peroxisomes requires distinct PTS-specific pores

- PTS2 pore is formed by the cytosolic co-receptor Pex18 and docking complex Pex14/Pex17

- The PTS2 receptor is not part of the unloaded pore

- Complex gating of the PTS2 channel is voltage and cargo dependent

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In Brief

Two peroxisomal targeting signals, PTS1 and PTS2, direct folded proteins to the peroxisomal matrix. Montilla-Martinez et al. (2015) identify a PTS2-specific pore, which contains the PTS2 co-receptor Pex18 and the Pex14/Pex17-docking complex as major constituents. The data demonstrate that import of PTS1 and PTS2 proteins is performed by distinct pores.

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Distinct Pores for Peroxisomal Import of PTS1 and PTS2 Proteins

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SUMMARY

Two peroxisomal targeting signals, PTS1 and PTS2, recognized by cytosolic receptors Pex5 and cooperating Pex7/Pex18, direct folded proteins to the peroxisomal matrix. A pore consisting of the PTS1 receptor Pex5 and the docking protein Pex14 imports PTS1 proteins. We identified a distinct PTS2-specific pore, which contains the PTS2 co-receptor Pex18 and the Pex14/Pex17-docking complex as major constituents. The estimated maximal pore size of ∼4.7 nm is large enough to allow import of folded PTS2 proteins. PTS2 cargo proteins modulate complex gating, open probability, and subconductance states of the pore. While the PTS1 channel is transiently activated by arriving receptor–cargo complexes, the reconstituted PTS2 channel is constitutively present in an open state. However, the cargo-loaded PTS2 channel is largely impermeable to solutes and ions. Our results demonstrate that import of PTS1 and PTS2 proteins does not converge at the peroxisomal membrane as previously anticipated but is performed by distinct pores.

INTRODUCTION

In contrast to other organellar protein import machineries, the peroxisomal protein translocons facilitate transport of folded and oligomeric proteins (Erdmann and Schliebs, 2005). Two peroxisomal targeting signals, designated PTS1 and PTS2, can direct newly synthesized peroxisomal proteins to peroxisomes and across the peroxisomal membrane. Central constituents of peroxisomal protein import are soluble import receptors, which cycle between the cytosol and peroxisomes. It has recently been demonstrated that the initially soluble import receptor of the PTS1 pathway, Pex5, becomes an integral constituent of a transient protein-conducting channel in the peroxisomal membrane (Meinecke et al., 2010). In particular, the PTS1 receptor recognizes and binds its folded cargo in the cytosol and then docks at the peroxisomal membrane via interaction with either Pex14 or Pex13 (Bottger et al., 2000). However, the detailed molecular mechanisms of pore formation and protein translocation remain elusive (Meinecke et al., 2010). The export-driven import model proposed that ATP- and ubiquitination-dependent release of the receptor from the peroxisomal membrane, performed by the AAA-ATPases Pex1 or Pex6 (Miyata and Fujiki, 2005; Platta et al., 2005), is coupled to translocation (Schliebs et al., 2010). The PTS1 signature is characterized by the carboxyterminal tripeptide sequence serine-lysine-leucine or a conserved variant thereof (Gould et al., 1989). PTS2 proteins carry a signal sequence defined by a nonapetide sequence RLx5HL located in close proximity to the amino terminus (Swinkels et al., 1991). The folded PTS2 proteins are recognized by the soluble receptor Pex7 (Glover et al., 1994), which cycles between peroxisomes and the cytosol, similar to the PTS1 receptor (Nair et al., 2004). However, unlike Pex5, the Pex7-mediated import pathway requires species-specific auxiliary proteins also known as co-receptors: Pex18 or Pex21 in Saccharomyces cerevisiae (Purdue et al., 1998). In human, an isofrom of Pex5 accomplishes this role (Braverman et al., 1998).

In S. cerevisiae grown under peroxisome-proliferation-inducing conditions, the oleate-inducible 3-ketoacyl-CoA thiolase (Fox3) of the peroxisomal β-oxidation system is the major PTS2 cargo protein. In a first step of the topogenesis of Fox3, the cytosolic PTS2 receptor Pex7 recognizes the PTS2 of newly synthesized Fox3 in the cytosol. Next, Pex18 binds to the cargo-loaded receptor and thereby stabilizes the receptor-cargo complex and directs it to the peroxisomal membrane (Grunau et al., 2009; Pan et al., 2013). At the membrane, this ternary PTS2 pre-import complex associates with the docking complex Pex13/Pex14/Pex17. Pex14 has been described as the point of convergence for both Pex5- and Pex7-dependent import pathways (Albertini et al., 1997). However, it is a major question whether PTS1 and PTS2 proteins are imported via common or distinct import pores.

Here, we describe the identification and characterization of the peroxisomal translocation channel for PTS2 proteins, which is formed by the co-receptor Pex18 and the heterodimer Pex14/Pex17 and exhibits properties that would allow import of folded PTS2 proteins of various sizes.
RESULTS

The Pex18 Complex Constitutes a Large Water-Filled Pore

In order to identify the PTS2 import pore at the peroxisomal membrane, we isolated Digitonin-solubilized Pex18 complexes from total membrane preparations of oleate-induced Pex5-deficient yeast cells expressing Pex18-tobacco etch virus (TEV) cleavage site/Protein A (-TPA) fusions. Fluorescence microscopic analysis and protease protection assays revealed that these cells are functional for PTS2 import (Figures S1 A and S1B), supporting the conclusion that the PTS1 receptor is not required for maintenance of the PTS2 import machinery. After immunoglobulin G (IgG) affinity chromatography on IgG-Sepharose and eluted by TEV-protease cleavage, Proteins of the eluate were separated by SDS-PAGE followed by Coomassie staining (left lane) or immunoblotting using the indicated antibodies. Asterisk indicates TEV protease (A). The eluate of the Δpex5-Pex18-TPA strain was further fractionated by size-exclusion chromatography. Fractions were collected and subjected to immunoblot analysis with antibodies as indicated. Fractions containing the pore-forming 700-kDa complex (complex II) and the 150- to 250-kDa PTS2 preimport complex (complex I) were used for electrophysiological measurements (B). Alternatively, the eluate of the Δpex5-Pex18-TPA strain was immunoprecipitated using anti Pex14 antibodies. Precipitated proteins were separated by SDS-PAGE, stained with Coomassie, or analyzed by immunoblotting as indicated. Angle brackets label heavy and light IgG chains. Closed arrowheads point to positions of analyzed proteins (C).

The majority of membrane-bound Pex18 was found in a complex of a molecular size of 150–250 kDa, containing also Pex7 and the homodimeric cargo protein Fox3 and thus most likely representing the docking state of the PTS2 preimport complex (Figure 1B, complex I) (Grunau et al., 2009). When this low-molecular-weight complex (Figure 1B, complex I) was analyzed for single channel activity after reconstitution into liposomes and incorporation into planar bilayers, no channel activity at all was observed (Figure S2).

Of special interest was a complex with a molecular size of ~700 kDa, containing Pex18, Pex14, and Pex17, but lacking the cargo Fox3 and Pex7 (Figure 1B, complex II). Two broad Pex13 peaks were detected, which did not align with the 700-kDa complex. For a detailed analysis of the composition of this subcomplex, co-immunoprecipitation of Pex14 from the total Pex18 eluate was performed (Figure 1C). Major polypeptide bands could be identified by immunoblotting as Pex18, Pex14 and Pex17. The immunoblot analysis revealed that the precipitate also contained minor amounts of Pex7p and Pex13p. SDS-PAGE followed by Coomassie stain suggested a molar ratio of 1:1 for Pex14 and Pex18 (Figure 1C). Noteworthy, Fox3 and compounds of the receptor export machinery like E3 ubiquitin ligases Pex10 and Pex12 could not be detected by immunoblot analyses of the immunoprecipitated sample.

When complex II was analyzed for single-channel activity by the planar lipid bilayer technique after reconstitution into liposomes,
pore-forming activity was observed (Harsman et al., 2011), whereas controls did not show any channel activity (Figure S2). After single fusion events of the complex II proteoliposomes with the bilayer and application of a voltage gate protocol (see Experimental Procedures for details), ion channel currents without pronounced gating activity were observed (Figure 2A). The current-voltage relation obtained from the application of a voltage ramp (Figure 2B) revealed a slight rectification with a ratio $r_{rec} = (i^+ / i^-) = 1.3$, meaning that the channel shows a slightly higher slope conductance at positive applied holding potentials ($G_V^+ = 1.61 \text{nS}$) than at negative holding potentials ($G_V^- = 1.23 \text{nS}$). When voltage-gate-induced currents were analyzed for their $I_{\text{max}}$ and $I_{\text{mean}}$, the resulting current-voltage relationship (Figure 2B) displayed nearly the same characteristics as the one

Figure 2. Analysis of Single-Channel Currents of Pex14/18/17 and Pex14/18/17 Cargo Complexes
Single-channel recordings in symmetrical buffer 250 mM KCl, 10 mM Mops-Tris (pH 7.0) (cis/trans) from bilayers containing the Pex18/14/17 complex (A), the Pex18/14/17 + Pex18/7/Fox3(Cargo1) complex (D), and the Pex18/14/17 + Pex18/7/Fox3-GFP(Cargo2) complex (G) in response to an applied voltage gate of 60 s duration with the indicated voltage amplitude. Corresponding current-voltage relations of the obtained maximal current amplitudes ($I_{\text{max}}$) and the mean currents ($I_{\text{mean}}$) as well as the corresponding current-voltage ramp are shown in (B), (E), and (H). The voltage-dependent open probabilities of the Pex18/14/17 channel, the Pex18/14/17-Cargo1 channel, and the Pex18/14/17-Cargo2 channel are shown in traces (C), (F), and (I). Data points in the ($I_{\text{max}}$)/($I_{\text{mean}}$) and the $P_{\text{open}}$ values are averages from three independent bilayers.

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obtained by the voltage ramp with slope conductance of $G_V = 1.64\text{ nS}$ and $G_V = 1.09\text{ nS}$, respectively (Figure 2B). Using the Ohmic model of Hille ($\text{Hille, 2001}$) and the approach of Smart et al. ($\text{Smart et al., 1997}$), a rough correlation between channel pore size and single-channel conductance may be deduced. The calculation leads to a maximal pore diameter of $d_{\text{pore}} \approx 4.7$ nm assuming a restriction zone of the pore with a length of 2 nm.

Channel fusion to the planar bilayer occurred mainly (>80%) in a single direction as displayed by the rectifying and therefore asymmetric, current-voltage relation of single-channel currents (Figure 2B). The voltage-dependent open probability ($P_{\text{open}}$) shows that the channel(s) closed symmetrically in a steep voltage-dependent manner at high membrane potentials with $P_{\text{open}}^\text{low} = \pm 120mV$ and a completely closed channel at $V_m = 140mV$ (Figure 2C).

Remarkably, at onset of the membrane potential, the channel opened completely in all cases to its maximal conductance state and closed at higher $V_m$ in multiple smaller and larger conductance steps with three to four frequent but hardly equidistant conductance steps (Table 1; Figures 2D and 4A–4D). These electrophysiological properties of the PTS2 channel and in particular the connectivity of the open channel states (Figures 2D, 3A–3C, and 4A–4F; see below) indicate that the pore can adopt different sizes up to a maximal diameter of $\sim 4.7$ nm, as deduced from the various conductance states (for more details, see below). The PTS2 channel was cation selective $P_{+}/P_{-} = 10 : 1$ and permeable to sodium, calcium, and tetrabutyl-ammonium (Table 1). This cation selectivity seems to be rather high for such a large pore. However, as shown for other large channels, this might mainly reflect the fact that within the channel, pore-preferential pathways exist where small cations can diffuse remarkably faster across the pore than the competing anions ($\text{Im and Roux, 2002}$; Phale et al., 2001). The significant selectivity also points to the anisotropic distribution of charged residues along the channel pore, which presumably is also important for the interaction of the inner channel surface with its substrates.

### Table 1. Properties of the Pex18/14/17 Channel

<table>
<thead>
<tr>
<th></th>
<th>Pex18/14/17</th>
<th>Pex18/14/17 + Pex18/7/Fox3cyt</th>
<th>Pex18/14/17 + Pex18/7/Fox3-GFPcyt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main conductance</strong></td>
<td>pS</td>
<td>pS</td>
<td>pS</td>
</tr>
<tr>
<td>$G_{\text{main}}(+V_m)$</td>
<td>384 ± 59 (n ≥ 5)</td>
<td>558 ± 58 (n ≥ 4)</td>
<td>784 ± 76 (n ≥ 4)</td>
</tr>
<tr>
<td>$G_{\text{main}}(-V_m)$</td>
<td>364 ± 68 (n ≥ 5)</td>
<td>443 ± 63 (n ≥ 4)</td>
<td>763 ± 88 (n ≥ 4)</td>
</tr>
<tr>
<td><strong>Number of sublevels (resolved)</strong></td>
<td>15 (n ≥ 5)</td>
<td>9 (n ≥ 6)</td>
<td>9 (n ≥ 8)</td>
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### The PTS2 Import Channel Properties Are Modulated by PTS2 Preimport Complexes

It is known that the PTS1 pore is opened by the PTS1 preimport complex (Meinecke et al., 2010). To test for the influence of PTS2 preimport complexes on the properties of the PTS2 channel, we purified cytosolic Pex18-TPA complex from oleate-induced cells (Figures S3A). When Pex18/14/17-containing proteoliposomes, preincubated with Pex18/7/Fox3cyt complexes, were fused to the planar bilayer, the observed channel currents were significantly different with respect to gating frequency and $P_{\text{open}}$. In particular, higher gating frequencies were observed already at $V_m = -80$ mV, and the channel current dropped in roughly three main conductance steps to zero (Figure 2D; Table 1). Accordingly, $I_{\text{max}}$ and $I_{\text{mean}}$, as deduced from the current-voltage relationship and the voltage ramp, revealed decreased channel currents, particularly at negative $V_m$ (Figures 2D and 2E).

The observed slope conductances were $G_V = 1.55$ nS in the linear part of the $I_{\text{max}} / I_{\text{mean}}$ current-voltage relation and $G_V = 1.45$ nS for the voltage ramp, respectively (Figure 2E). Also, in addition to the frequent conductance transitions of $\Delta G_{\text{main}} = 500$ pS, smaller and less frequent larger conductance transitions than $\Delta G_{\text{main}}$ were observed (Figures 3A–3C and
The analysis of the voltage-dependent $P_{\text{open}}$ revealed channel closing mainly at negative voltages with $P_{\text{open}}^{\text{close}} = 0.98$ and $P_{\text{open}}^{\text{close}} = 0.04$ (Figure 2F). Such changes of the characteristic channel activity of the PTS2 pore could not be observed in the presence of proteins/peptides like CoxIV1–23 (Figure S2D) or BSA (Figure S2E) or PTS1-containing GST-SKL (Figure S2E). These data show that proteins other than PTS2 cargos do not modulate the PTS2 import pore.

**Gating and Channel States of the PTS2 Channel Are Affected by the Size of Cargo**

To analyze the dependence of the PTS2 channel on the size of the cargo, we expressed the fusion protein Fox3-GFP in yeast cells lacking endogenous Fox3, which is the major PTS2 protein under oleate-induced growth conditions. Growth assays and fluorescence microscopy studies revealed that the fusion tag affects neither the function of the enzyme/nos its import into peroxisomes (Figures S1A and S1C). The cytosolic PTS2 preimport complex containing Fox3-GFP (Pex18/7/Fox3-GFP) has an ~50 kDa larger size than the corresponding Fox3-containing complex (Pex18/7/Fox3-GFP) (Figure S3A).

The same experimental protocols as described above were applied to analyze potential ion channel activity of the PTS2 pore plus the larger Pex18/7/Fox3-GFP complex. The data are shown in Figures 2G–2I and Figures 4E and 4F. After single fusion events of the preincubated proteoliposomes, channel activity was again observed. However, the characteristics of this activity were strikingly different from the one observed for the PTS2 pore alone and the PTS2 pore plus Pex18/7/Fox3 complexes. Strong flickering of channel currents was observed even at membrane potentials of $V_m = \pm 20$ mV (Figures 4E and 4F). The $I_{\text{max}}$ and $I_{\text{mean}}$ analysis of voltage-gate-induced currents shows that both $I_{\text{max}}$ and $I_{\text{mean}}$ are close to zero at negative membrane potentials of $V_m > 20$ mV (Figure 2H).

Figure 3. Analysis of Pex14/18/17 Channel Gating

(A) Mean variance plot of single-channel recording in symmetrical buffer 250 mM KCl, 10 mM Mops-Tris (pH 7.0) (cis/trans) from a bilayer after a single fusion event of Pex18/14/17 proteoliposomes incubated with Pex18/7/Fox3cyt (Pex18/14-Cargo1) in response to an applied voltage gate of 60 s duration of $V_m = -100$ mV. (B) Single-channel recording from a bilayer after a single fusion event of Pex18/7/14/17 proteoliposomes incubated with Pex18/7/Fox3-GFP$_{cyt}$ (Pex18/14/17-Cargo2) in response to an applied voltage gate of 60 s duration of $V_m = -120$ mV and the corresponding mean variance plot in (C). Mean variance and dwell time analysis of the Pex18/14/17 channel (D), the Pex18/14/17-Cargo1 channel (E), and the Pex18/14/17-Cargo2 channel (F).

The current-voltage relation between 20 and 100 mV, we obtained a conductance value of $G_{\text{slope}} = 1.01$ nS (Figure 2H), while the linear part of the voltage-ramp-induced current displayed a slope conductance of $G_{\text{slope}} = 1.09$ nS (Figure 2H). $P_{\text{open}}$ showed a symmetric course with a steep decrease at membrane potentials above $V_m = \pm 40$ mV ($P_{\text{open}}^{\text{close}} = 0.9$ and $P_{\text{open}}^{\text{close}} = 0.1$ (Figure 2I)). A fast gating frequency (flickering) was observed when the ionic strength during current recordings was increased to 1 M (Figures S4A–S4C). Increase in salt concentration is supposed to weaken protein-protein interactions and thus possibly release the cargo from the pore, which then reveals gating typically observed in the absence of cargo. (Figures 2A–2C). Accordingly, preincubation of the PTS2 pore proteoliposomes with receptor-cargo complexes under high ionic strength conditions also did not lead to channel flickering after fusion of the PTS2 proteoliposomes to the planar bilayer. This effect was reversible by perfusion to low-salt (250 mM KCl) conditions (Figures S4A–S4C). A possible explanation for the observed flickering might be that the bigger Fox3-GFP cargo is retained within the PTS2 pore. In agreement with this assumption, the amount of pore-associated protein complexes was measured by the size of proteoliposomes under high ionic strength conditions also did not lead to channel flickering after fusion of the PTS2 proteoliposomes to the planar bilayer. This effect was reversible by perfusion to low-salt (250 mM KCl) conditions (Figures S4A–S4C). A possible explanation for the observed flickering might be that the bigger Fox3-GFP cargo is retained within the PTS2 pore.
from the slope of the linear parts of the current-voltage relations from the $I_{\text{max}}$ analysis agreed well with the respective voltage ramp measurements at positive membrane potentials (Figures 2B, 2E, and 2H).

In order to characterize in more detail the channel features of the cargo-loaded pore complexes, we analyzed their voltage-dependent gating behavior in detail. One striking feature of the Pex18/17/Fox3cyt-induced channel complexes is the voltage-dependent closure in three frequent main conductance transitions of $\Delta G_{\text{main}} \equiv 500 \, \text{pS}$ with additional smaller and larger conductance transitions (Figures 2D, 3B, 4A, and 4F). Similar gating patterns were also observed for the PTS2 pore plus Pex18/17/Fox3-GFPcyt complex. However, in this case, additional fast gating of the channel, accompanied by very fast voltage-dependent closure of the channel in voltage gate experiments, led to a virtually decreased $G_{\text{max}}$ (see below for more details).

**The PTS2 Pore Display Complex Gating with Various Subconductance States**

Remarkably, in all experiments, single fusion events of the PTS2 pore and Pex18/17/Fox3cyt-containing proteoliposomes produced discrete channel-closing conductance transitions $(\Delta G_{\text{main}})$ after voltage-induced complete channel opening to $(G_{\text{max}})$ (see Figure 2D). Two fusion events produced a pattern with up to six main conductance transitions $2\Delta G_{\text{main}}$ and additional smaller $\Delta G_{\text{sub}}$ (see Figures 4A and 4B). From these results, the question arises whether the PTS2 translocon displays a channel unit with several individual pores or a single pore channel with three main gating states acting as an iris-diaphragm-like gating mechanism. To address this question, we performed mean variance analysis of single-channel current recordings (Patlak, 1993), which allows an unbiased evaluation of amplitudes, gating kinetics, and connectivity of channel states (Figures 3A–3C and 4). In addition, the relative occupation of channel states was determined (Figures 3D–3F) (Patlak, 1993). The mean variance analysis revealed statistically significant, multiple direct transitions between various combinations of $0.2 \times \Delta G_{\text{main}}$ to $2 \times \Delta G_{\text{main}}$ and voltage-dependent single-step complete channel closures. Moreover, the PTS2 pore is highly permeable for the large tetrabutyl-ammonium cation $(d \equiv 1.1 \, \text{nm})$ (Table 1). Together, these results clearly indicate that the basic channel unit of the PTS2 translocon is formed by a single large channel pore $(G_{\text{max}} \equiv 1.5 \, \text{nS})$ with various frequent main gating substrates with roughly equal conductance increments $(\Delta G_{\text{main}})$.

**DISCUSSION**

The PTS1- and PTS2-dependent peroxisomal protein import pathways have been described to converge at the peroxisomal membrane at the level of Pex14 (Albertini et al., 1997). Recently, the import pore for PTS1 proteins has been identified (Meinecke et al., 2010), and a major question was whether PTS1 and PTS2 proteins are imported via common or distinct import pores. In
support of distinct pores, previous data indicated that the PTS1 import is functional in absence of the PTS2 receptor and vice versa (Marzioch et al., 1994; Zhang and Lazarow, 1996). Moreover, the PTS2 co-receptors structurally and functionally resemble the N-terminal half of Pex5, which is supposed to contribute to pore formation in the PTS1-pathway (Hensel et al., 2011; Schäfer et al., 2004; Schliebs and Kunau, 2006). The identification of the PTS2 pore consisting of Pex18 and the Pex14/Pex17 heteromer demonstrates the existence of distinct pores for PTS1- and PTS2 proteins. Thus, the previously observed convergence of the two pathways at the level of Pex14 does not reflect a physical interaction within the same pore but is due to the fact that both pores contain Pex14 as constituent. Reports demonstrating that different E3 ligases are involved in ubiquitination of Pex5 and Pex18 and recycling of the receptor/co-receptor from the peroxisomal membrane support this notion (El Magraoui et al., 2013).

However, although the composition and oligomeric size resembles the protein import channel for PTS1 proteins, the basic channel properties of the newly identified PTS2 channel differ significantly. Whereas the PTS1 import pore was stably incorporated into the lipid bilayer in a nearly closed state and complete opening to up to 9 nm was induced by the cargo (Meinecke et al., 2010), the reconstituted PTS2 channel complex formed a constitutively open ~4.7-nm-wide pore. One possible explanation for this striking difference could be that the large PTS1-binding domain of Pex5 is involved in keeping the import gate in a closed state, whereas Pex7, the PTS2 binding partner of the receptor complex, is not associated with the open Pex18 channel. Rapid dissociation of Pex7 and Pex18 at the membrane has also been indicated in previous studies (Grunau et al., 2009; Hensel et al., 2011; Purdue et al., 1998). Recent structural observations suggest that release of Pex18 from the heterotrimeric Pex18/Pex7/cargo complex destabilizes the complex and results in dissociation of receptor/cargo complexes (Pan et al., 2013), which is a precondition for receptor recycling. However, it needs to be clarified whether dissociation of the complex takes place at the outside or inside of peroxisomes.

PT51 and PTS2 channels also differ remarkably in their gating properties. The PTS1 channel showed a multitude of conductance states with a rather broad distribution of individual open states (Meinecke et al., 2010). In contrast, the PTS2 channel without cargo resided in the fully open state without frequent gating, the cargo-loaded PTS2 channel showed an increase of gating, and the channel closed with three to four frequent current steps in a voltage- and cargo-dependent manner (Figures 2 and 3). This reflects the high dynamics of the pore and might be correlated with distinct steps of cargo translocation.

Besides substrate-induced gating, voltage gating is also a common feature of large channels, including protein transport channels (Bainbridge et al., 1998; Harsman et al., 2011; Robertson and Tieleman, 2002). This also accounts for the PTS2 pore. However, for an interpretation of the physiological role of voltage gating for the PTS2, more knowledge on the membrane potential across the peroxisomal membrane is needed.

With respect to the size of dimeric Fox3 (Mathieu et al., 1997), and Pex7 (Pan et al., 2013), the water-filled cavity of the pore would be completely occupied during passage. This interpretation is corroborated by the fact that the channel remained even more in low conductance states when Fox3 was replaced with the larger PTS2 protein, Fox3-GFP (Figure 3F). The observed cargo-induced channel flickering (Figure 2G) can be caused by either partitioning of the cargo into the channel or binding of the cargo at the channel vestibule. In both cases, the flickering is due to an induced dynamic modulation of ionic currents through the channel pore. Although we cannot discriminate, whether the cargo was bound to the channel vestibule or within the channel, it is likely that the strongly bound bulky cargo penetrates at least partially into the pore thereby exerting strong steric and electrostatic impact on the permeating small ions.

Physiological regulation of both peroxisomal protein import translocans should avoid the passage of small solutes or ions. For the PTS1 pathway, the electrophysiological data up to now do not readily allow conclusions of how the physiological regulation of the pore permeability may be achieved. The electrophysiological data of the PTS2 complex show that the binding of the substrate combined with the gating of the PTS2 pore can keep the channel impermeable for solutes and ions (Figure S4F). Due to the broad range of different sizes of PTS1 proteins, it can be assumed that the PTS1 pore requires higher flexibility than the PTS2 import channel, which only accommodates Fox3 as a cargo protein under same growth conditions.

Although the PTS1 and PTS2 pores are distinct and independently working entities in yeast, they add up to a mechanistically unified concept of how completely folded proteins can cross the peroxisomal membrane. In the future, it will be challenging to find out whether other eukaryotic cells that use Pex5 for both PTS1 and PTS2 import use a single combined PTS1/PTS2 pore or two distinct Pex5-containing types of pores with different specificities.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**

Yeast strain UTL7A was used throughout this study for genetic manipulations (Züldörmann et al., 1989). The PEX5-deficient yeast strain pex5Δ and the double-deletion strain pex5Δfox3Δ were obtained by genomic replacement of these open reading frames (ORFs) by fox3Δ pamKΔ fox3Δ DNA cassettes (Güldener et al., 1996) using primers described previously (Girzalsky et al., 1999; Grunau et al., 2009).

Genomic integration of a TEV protease cleavage site/Protein A cassette behind the ORF of PEX5 as well as the procedure to delete the FOX3 gene has been described previously (Grunau et al., 2009). Fox3p-GFP was expressed from the C-terminal GFP fusion vector puG35 under control of the FOX3 promoter. For cloning, a genomic fragment consisting of the ORF of FOX3 and a 500-bp upstream region was amplified by primers RE3685 (5′-GATGAGCCGCTCGAGCGACCAGGCTGAACTCATAATCTAATGCTC3′) and RE3866 (5′-GATGAGCCGCTCGAGCGACCAGGCTGAACTCATAATGCTC3′) and subcloned into SacI/BamHI restricted puG35 plasmid.

**Purification of the Pore-Forming Complex and Reconstitution into Liposomes**

Soluble and membrane-bound Pex18-TEV (tobacco etch virus protease cleavage site)/Proteina complexes were purified from oleate-induced yeast cells as described earlier (Agne et al., 2003; Grunau et al., 2009). Pex18-associated protein subcomplexes were fractionated on a Superose 6 10/300 size-exclusion chromatography column (GE Healthcare) equilibrated with lysis buffer with or without 0.1% (w/v) Digitonin (Merck, Darmstadt) at 4°C using the ÄKTA-purifier system (GE Healthcare). 500 μl Pex18 TEV-protease eluate...
fractions (total 640 µl) were loaded, and 500 µl fractions were collected. The high-molecular-weight-low-molecular-weight-gel filtration calibration kit (GE Healthcare) contained thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), RNase A (13.7 kDa), and aprotinin (6.5 kDa). Pex18 complexes were reconstituted into liposomes by detergent depletion. For this purpose, synthetic phospholipids (70% 1,2-dioleoyl-sn-glycero-3-phosphocholine, 30% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; Avanti Polar Lipids) were dried under an argon stream and hydrated with designated fractions of size-exclusion chromatography by gentle shaking. Digidition (0.1% [w/v]) was removed from the buffer (20 mM HEPES, 100 mM KAc, and 5 mM MgAc [pH 7.5]) by batch hydrophobic adsorption with Calbiosorb resin (Calbiochem). After formation of proteoliposomes, samples were passed through a 200 nm polycarbonate membrane in a Mini-Extruder (Avanti Polar Lipids) prior to use.

Preincubation of the Pex18/14/17-proteoliposomes with soluble cargo protein complexes Pex18/7/Fox3C and Pex18/7/Fox3-Grp-U was performed by mixing the Pex18/14/17-proteoliposomes and the soluble cargo protein complexes 1:1 (v/v) for 30 min on ice. After preincubation, the mixture was directly used for bilayer experiments.

**Electrophysiological Measurements**

Electrophysiological characterization of the peroxisomal importor was carried out using the planar lipid bilayer technique (Harsman et al., 2011), described in detail elsewhere (Krüger et al., 2012). A simultaneous characterization of multiple copies of active PTS2 channels revealed that the PTS2 pore can be considered as fully open only up to Vm = ±30 mV (Figure 4D). The voltage-dependent closings of individual channels at higher Vm impedes a detailed analysis of PTS2 channel properties in these measurements. In our study, the analysis was therefore more easily achieved from single-active-channel current recordings (Figure 4E). Purified reconstituted protein complexes were applied directly below the bilayer in the cis compartment. Buffer conditions for osmotically driven fusion were asymmetrical with 250 mM KCl, 10 mM MOPS-Tris (pH 7.0) in the cis compartment and 20 mM KCl, 10 mM MOPS-Tris (pH 7.0) in the trans compartment. Two Ag/AgCl electrodes enclosed by 2 M KCl-agar bridges were inserted into each chamber with the trans chamber electrode being the reference for exported membrane potentials as it was connected to the headstage (Cv-S-1GUL) of a Geneclamp 500 current amplifier (Axon Instruments). Current recordings were carried out using a Digidata 1200 A/D converter. Data analysis was performed by a self-written program ("Ion channel Master" [ICM]) based on the mean variance analysis of single-channel current recordings (Patlak, 1993) in combination with Origin 7.0 (Microcal Software). Current recordings were performed at a sampling interval of 50 µs, filtered with a low-pass filter at 5 kHz.

**Analysis of Channel Gating**

Representative current recordings in response to a voltage gate of 60 s duration from the Pex18/14/17 complex and the two different Pex18/14/17 Cargo1, Cargo2 complexes as shown in Figures 2A, 2D, and 2G and mean variance plots (Figures 3A and 3C) were analyzed according to Patlak (1993). The connectivity of gating was analyzed comparatively for the three different complexes at Vm = ±120 mV and the relative time of occupation of a particular open state was determined by statistical analysis (Patlak, 1993; Figures 3D–3F). The connectivity of gated channel currents in the first possible case of three different independent channel pores would produce gating transitions across two open channel states at the assigned instrumental time resolution of rsa = 50 µs (20 kHz) with a probability of W = 50·10^-4 ·50·10^-4 = 2·10^-7/s = 250·10^-7/s; thus, it would take 12.7 years to observe such a simultaneous conductance transition across two independent channel pores. However, as shown in different figures (Figures 3A and 3C), we observed frequent transitions with larger conductances according to ∆m = 2 ∆m_max and ∆m = 3 ∆m_max. Moreover, the gating frequency of full single-step channel closures in the presence of cargo increased with increasing membrane potential.

**SUPPLEMENTAL INFORMATION**

Supplemental information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.016.


