Tail-anchored PEX26 targets peroxisomes via a PEX19-dependent and TRC40-independent class I pathway

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Tail-anchored (TA) proteins are anchored into cellular membranes by a single transmembrane domain (TMD) close to the C terminus and display their N-terminal domain to the cytosol (Kutay et al., 1993). These TA proteins are found in virtually all cellular membranes and play essential roles in various processes that range from protein translocation to vesicular trafficking, apoptosis, and many others. Therefore, their correct targeting and localization are of basic cellular importance across all eukaryotes (Borgese et al., 2007). Recent studies have increased our knowledge of the machineries and mechanisms by which TA proteins are targeted to and inserted into the ER membrane. Of several proposed pathways, the GET pathway that involves a cytosolic ATPase (mammalian TRC40 or yeast Get3) is now widely accepted as the dominant targeting pathway (Borgese and Fasana, 2011; Hegde and Keenan, 2011).

In contrast, the pathway and molecular mechanism for the delivery of TA proteins to peroxisomes remain elusive, mainly because two pathways are proposed for the import of peroxisomal membrane proteins (PMPs): a “direct import” pathway and an “ER to peroxisome trafficking” pathway, both of which are mediated by PEX3, PEX19, and in mammals, PEX16 (Fujiki et al., 2006; Ma et al., 2011; Nuttall et al., 2011; Rucktäschel et al., 2011). In the former pathway, PMPs are imported directly from the cytosol to peroxisomes. PEX19 functions as a chaperone and soluble receptor for PMPs (Jones et al., 2004; Matsuzono et al., 2006). PEX3 provides a docking site for PEX19, probably PMP-loaded PEX19, at the membrane (Fang et al., 2004). PEX16 acts as a membrane receptor for the soluble PEX3–PEX19 complex during PEX3 import (Matsuzaki and Fujiki, 2008). In contrast, in the latter pathway, PMPs are inserted into the ER and then sorted to peroxisomes. PEX3 and PEX19 mediate the sorting of PMPs from the ER to peroxisomes (Hoepfner et al., 2005; Lam et al., 2010; van der Zand et al., 2010). PEX16 was reported to recruit PEX3 to the ER (Kim et al., 2006).

Earlier studies on two peroxisomal TA proteins, yeast Pex15p and plant peroxisomal ascorbate peroxidase, suggested that they traffic through the ER en route to peroxisomes (Elgersma et al., 1997; Mullen et al., 1999; Schuldiner et al., 2008).

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Abbreviations used in this paper: AOx, acyl-CoA oxidase; Cyt b5, cytochrome b5; Fis1, fission1; MOM, mitochondrial outer membrane; mPTS, peroxisomal membrane-targeting signal; PMP, peroxisomal membrane protein; PTS1, peroxisomal targeting signal type 1; RRL, rabbit reticulocyte lysate; TA, tail anchored; TMD, transmembrane domain; VAMP2, vesicular-associated protein 2; WT, wild type.

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Recently, Get3 was shown to interact physically with Pex15p and, together with other components of the GET (guided entry of TA proteins) pathway, to mediate its insertion into the ER (Schuldiner et al., 2008; Jonikas et al., 2009; Costanzo et al., 2010). Moreover, the yeast Pex19p-dependent budding of Pex15p-containing vesicles from the ER was reconstituted in vitro (Lam et al., 2010). In contrast, studies using mammalian PEX26, a TA protein functionally homologous to Pex15p, showed that the import of PEX26 requires PEX19 (Halbach et al., 2006) and that cell-free synthesized PEX26 is transported to isolated peroxisomes in a PEX19-stimulated manner (Matsuzono and Fujiki, 2006), implying PEX19-dependent direct import. Indeed, two PEX19 binding sites, one overlapping with the TMD and the other in the hydrophilic luminal region (hereafter referred to as C segment), were identified in PEX26 as well as Pex15p (Halbach et al., 2006); however, the precise route and molecular mechanisms underlying the import of peroxisomal TA proteins in mammalian cells remain unclear, including the function of PEX19, the requirement of a membrane component, and the involvement of TRC40. Furthermore, the signal that directs TA proteins to mammalian peroxisomes remains to be characterized.

The present study analyzed the import of PEX26 using a semi-intact cell system and showed that PEX19 forms a complex with PEX26 in the cytosol and delivers it to peroxisomes in a manner dependent on the PEX3–PEX19 interaction. Neither the targeting nor the insertion of PEX26 requires ATP, indicating TRC40-independent import. Our results indicate that PEX26 follows the PEX19- and PEX3-mediated direct import pathway. Moreover, the data demonstrate that basic residues within the C segment of TA proteins are important but not sufficient for peroxisomal targeting. Peroxisomal TA proteins seem to require both a relatively hydrophilic TMD and a highly basic C segment to escape capture by TRC40 and ensure binding to PEX19. Based on these and earlier findings, a model for the selective targeting of TA proteins to the appropriate organelle membrane in mammalian cells is suggested.

**Results**

**PEX19 is required for peroxisomal targeting of PEX26**

PEX19, a predominantly cytosolic protein, was shown to be involved in the import of the mammalian peroxisomal TA protein PEX26 (Halbach et al., 2006). This result was confirmed by siRNA-mediated knockdown of PEX19. Transfection of PEX19-specific siRNAs into HeLa cells resulted in a prominent decrease in the level of PEX19, whereas the level of PEX14, a PMP, was not affected (Fig. S1 A). In control siRNA-treated cells, newly synthesized EGFP-fused PEX26 (EGFP-PEX26) was colocalized with proteins harboring peroxisomal targeting signal type 1 (PTS1), indicating the translocation of EGFP-PEX26 to peroxisomes (Fig. S1 B, a–c). In contrast, although the peroxisomal targeting of EGFP-PEX26 was still observed to some extent, EGFP-PEX26 was mistargeted to mitochondria in PEX19 siRNA-treated cells, as demonstrated by colocalization with cytochrome c, a mitochondrial marker protein (Fig. S1 B, d–e). The frequency of cells showing mislocalization of EGFP-PEX26 was >60% in PEX19 siRNA-treated cells, whereas that in control siRNA-treated cells was ~30% (Fig. S1 B, graph). Mislocalization observed in control siRNA-treated cells was probably caused by overexpression; overexpression of EGFP-PEX26 readily resulted in its targeting to mitochondria even in untreated cells (unpublished data). These observations are consistent with the previous study (Halbach et al., 2006) and demonstrate that PEX19 knockdown impairs the peroxisomal import of EGFP-PEX26.

**PEX19 forms soluble complexes with PEX26 in the cytosol and maintains it in an import-competent state**

As a step toward delineating the precise role of PEX19 in the biogenesis of PEX26, FLAG-tagged PEX26 (FLAG-PEX26) was expressed with or without HA-tagged PEX19 (HA-PEX19) in CHO-K1 cells, and its subcellular distribution was verified by cell fractionation. FLAG-PEX26 was detected exclusively in the organelle fraction when expressed alone, whereas it was found both in the cytosolic and in the organelle fractions when coexpressed with HA-PEX19 (Fig. 1 A, lanes 1–6). The steady-state level of FLAG-PEX26 was significantly higher in HA-PEX19–expressing cells than in mock-transfected cells, implying that FLAG-PEX26 was stabilized, most likely in the cytosol, by HA-PEX19. A further coimmunoprecipitation assay revealed that HA-PEX19 indeed formed a soluble complex with FLAG-PEX26 in the cytosol (Fig. 1 A, lanes 7–9). As suggested in a previous study (Halbach et al., 2006), the interaction of PEX19 with PEX26 is dependent on the peroxisomal membrane-targeting signal (mPTS); HA-PEX19 failed to interact with FLAG-PEX26ΔC, a variant lacking the TMD and the C segment (Fig. S2). These findings are consistent with previous studies suggesting that PEX19 functions as a chaperone and/or soluble receptor for nascent PMPs (Sacksteder et al., 2000; Jones et al., 2004; Rottensteiner et al., 2004; Shibata et al., 2004; Halbach et al., 2005; Matsuzono and Fujiki, 2006).

To examine whether the PEX19–PEX26 complex in the cytosol is an import-competent intermediate, import assays were performed in vitro using semi-intact cells. HeLa cells were treated with digitonin to selectively permeabilize the plasma membrane and subsequently incubated with the cytosolic fraction obtained from CHO-K1 cells coexpressing HA-PEX26 and FLAG-PEX19. Immunofluorescence microscopy analysis showed that HA-PEX26 colocalized with catalase, a peroxisomal matrix protein, indicating that HA-PEX26 was translocated to peroxisomes (Fig. 1 B, a and b). When semi-intact cells were incubated as a control with the cytosolic fraction of CHO-K1 cells expressing HA-PEX26 alone, peroxisomal targeting of HA-PEX26 was not observed (Fig. 1 B, c and d), which is consistent with the fact that PEX26 was recovered exclusively in the organelle fraction when expressed alone (Fig. 1 A). To confirm that PEX26 complexed with PEX19 is translocated to peroxisomes, in vitro import assays were performed using isolated PEX19–PEX26 complexes. FLAG-PEX19–HA-PEX26 complexes formed in the cytosol were immunopurified with the anti-FLAG antibody conjugated to agarose beads.
Given that the import of most mitochondrial outer membrane (MOM)–directed and several ER-directed TA proteins seems not to require any specific membrane component (Borgese and Fasana, 2011), it is of particular interest to investigate whether the PEX19-mediated import of PEX26 depends on a membrane component. Because PEX3 and PEX16 were proposed to function as a membrane receptor for PEX19–PMP complexes (Fang et al., 2004; Matsuzono and Fujiki, 2006; Matsuzaki and Fujiki, 2008), we focused on these two PMPs and verified the targeting of PEX26 in PEX3- or PEX16-overexpressing semi-intact cells. (anti-FLAG agarose beads; Fig. 1 C) and subjected to the import assay. HA-PEX26 was detected in peroxisomes, demonstrating that HA-PEX26 copurified with FLAG-PEX19 was specifically targeted to peroxisomes (Fig. 1 D). Importantly, HA-PEX26 targeted to peroxisomes was resistant to alkaline extraction, thereby suggesting that it was firmly anchored into the membrane (Fig. 1 E). These data clearly demonstrate that PEX26 in the cytosolic PEX19–PEX26 complexes is indeed transported to peroxisomes in semi-intact cells. Collectively, it is most likely that PEX19 forms a complex with PEX26 in the cytosol and maintains it in an import-competent state, thereby assisting its import into peroxisomes.
After the import reaction, the level of targeted HA-PEX26 was determined by immunoblotting. Results showed that overexpression of PEX3-EGFP, but not EGFP-PEX16, significantly increased the targeting of HA-PEX26 (Fig. 2 A). Conversely, the peroxisomal targeting of HA-PEX26 was markedly compromised in PEX3-depleted semi-intact cells in which the expression level of PEX3 was specifically reduced (Fig. 2, B–D). In line with these results, PEX3 knockdown gave rise to an increase in mislocalization of newly synthesized EGFP-PEX26 to mitochondria in vivo (Fig. 2 E). Together, these results strongly suggest that peroxisomal targeting of PEX26 depends on PEX3 and that PEX3 functions as a membrane receptor for cytosolic PEX19–PEX26 complexes.

**PEX3–PEX19 interaction is essential for peroxisomal targeting of PEX26**

PEX3 was shown to interact with PEX19 (Götte et al., 1998; Snyder et al., 1999; Soukupova et al., 1999; Ghaedi et al., 2000; Sacksteder et al., 2000), and the interaction has been proposed to play a prominent role in the biogenesis of peroxisomal membranes, probably by mediating the import of PMPs (Fang et al., 2004; Shibata et al., 2004; Matsuzono et al., 2006; Sato et al., 2008, 2010). Therefore, the role of the PEX3–PEX19 interaction in the peroxisomal targeting of PEX26 was investigated using the in vitro import assay system. A PEX3 mutant harboring a Trp104 to Ala substitution (PEX3-W104A), which is defective in binding to PEX19 (Sato et al., 2008), was first used. Results showed that unlike the wild-type (WT) PEX3, overexpression of PEX3-W104A-EGFP failed to stimulate the targeting of HA-PEX26 in semi-intact cells (Fig. 3 A). This result is interpreted to mean that the PEX3 mutant failed to recruit HA-PEX26. Next, chimeric constructs were generated in which the N-terminal mPTS of PEX3 was replaced by the first 69 amino acid residues of the MOM protein Tom20, which contain its MOM-targeting signal (Kanaji et al., 2000). The chimeric proteins, termed MitoPEX3-EGFP and Mito-PEX3-W104A-EGFP, were expressed in HeLa cells and subjected to the in vitro HA-PEX26 import assay. As expected, the chimeric proteins were localized to mitochondria (unpublished data). In semi-intact cells expressing MitoPEX3-EGFP, HA-PEX26 colocalized with MitoPEX3-EGFP in tubular and/or punctate structures as well as with catalase (Fig. 3 B, a–c), indicating that HA-PEX26 was...
recruited to mitochondria in addition to peroxisomes and supporting the idea that PEX3 functions as a receptor for cytosolic PEX19–PEX26 complexes. In sharp contrast, HA-PEX26 was translocated to peroxisomes in semi-intact cells expressing Mito-PEX3-W104A-EGFP, demonstrating that Mito-PEX3-W104A-EGFP failed to recruit HA-PEX26 to mitochondria (Fig. 3 B, d–f). In agreement with these findings, FLAG-PEX19ΔN23, which is a PEX19 variant lacking the N-terminal 23 amino acid residues and thereby defective in binding to PEX3 (Matsuzono et al., 2006), failed to deliver HA-PEX26 to peroxisomes (Fig. 3, C and D). Of note, PEX19ΔN23 formed soluble complexes with PEX26 as efficiently as WT (Fig. S3). Thus, these data strongly suggest that the import of PEX26 depends on the PEX3–PEX19 interaction and that PEX26 is transported directly from the cytosol to peroxisomes onto PEX3.

**ATP and TRC40 are dispensable for the import of PEX26**

We next asked whether ATP is required for the targeting and membrane integration of PEX26. EGFP-PEX26 was synthesized in a rabbit reticulocyte lysate (RRL) translation system supplemented with RRL-synthesized HA-PEX19 and then was incubated with semi-intact cells in the presence or absence of apyrase. It should be noted that the addition of RRL-synthesized PEX19 during, but not after, the synthesis of PEX26 resulted in efficient translocation of PEX26 to peroxisomes in semi-intact cells, whereas PEX26 synthesized in the absence of supplemental PEX19 was barely transported to peroxisomes (unpublished data; Pinto et al., 2006). To confirm that ATP is effectively depleted from the import reaction by treatment with apyrase, Myc-Sec61β, a well-defined substrate of TRC40, was synthesized together with EGFP-PEX26 and subjected to the import reaction, and its targeting to the ER was also assessed. As expected, without apyrase (i.e., in the presence of endogenous ATP), EGFP-PEX26 and Myc-Sec61β were transported to peroxisomes and ER-like structures, respectively (Fig. 4 A, top). In contrast, in the presence of apyrase, the import of Myc-Sec61β was almost completely inhibited, whereas peroxisomal targeting of EGFP-PEX26 was observed (Fig. 4 A, bottom). Furthermore, immunoblotting and alkaline extraction showed that the targeting to and integration into the membrane of EGFP-PEX26 were not affected at all by apyrase treatment (Fig. 4 B). These data clearly indicate that neither ATP nor TRC40 is required for the targeting and membrane insertion of PEX26.

Recently, yeast Get3 was shown to interact with the peroxisomal TA protein Pex15p, to mediate the delivery of Pex15p to the ER, and to be required for the peroxisomal localization of Pex15p as well as, to some extent, for the maintenance of functional peroxisomes (Schuldiner et al., 2008; van der Zand et al., 2010). To determine whether TRC40 is involved in the biogenesis of PEX26, the binding of TRC40 to PEX26 was examined. FLAG-tagged Sec61β and PEX26 were synthesized in RRL and subjected to immunoprecipitation under detergent-free conditions. Endogenous TRC40 in RRL was communoprecipitated with FLAG-Sec61β, but not with FLAG-PEX26, thereby indicating that TRC40 did not bind to PEX26 (Fig. 5 A). Moreover, FLAG-PEX26 coincided with cytochrome c upon expression in CHO pex19 ZP119 cells (Kinoshita et al., 1998; Matsuzono et al., 1999), whereas FLAG-Sec61β was colocalized with EGFP targeted to the ER (ER-EGFP; Fig. 5 B). This result suggests that PEX26 is targeted to mitochondria but not to the ER in PEX19-deficient mammalian cells where the GET pathway is functional. This is different from the finding that Pex15p is targeted
To assess the importance of basic amino acids in the C segment of PEX26, EGFP-PEX26 variants were generated in which one to four positively charged residues within the luminal PEX19 binding site were replaced by serine residues (termed K1S, RRK3S, and KRRK4S; Fig. 6 A). When WT EGFP-PEX26 was transiently expressed in HeLa cells, it was efficiently localized to peroxisomes, as demonstrated by colocalization with PTS1 proteins (Fig. 6 B, a and b). As noted in the previous section, however, in several cells, WT EGFP-PEX26 was partly localized to mitochondria probably because of overexpression (unpublished data). Upon reduction of the positive charges in the luminal PEX19 binding site, the efficiency of peroxisomal localization was decreased, and conversely, the extent of mitochondrial localization was increased (Fig. 6 B, c–h). These results suggest that positively charged residues in the C segment are essential for peroxisomal targeting of PEX26. Consistent with this finding and the previous study (Halbach et al., 2006), the variant lacking the C segment (ΔCS) was not localized to peroxisomes and was rather localized to mitochondria (Fig. 6 B, i and j). Interestingly, the variant in which only charged residues within the C segment were fused to the region downstream of the TMD (CHARGE) was localized to peroxisomes and partly to mitochondria (Fig. 6 B, k and l), implying that a C-terminal TMD and a following cluster of basic amino acids could function as an mPTS in mammalian cells.
Figure 6. **Basic amino acid residues in the C segment are essential for peroxisomal targeting of PEX26.** (A) Schematic representation of EGFP-PEX26 variants used. Amino acid sequences of the C segment are indicated by the single letter code. Basic and acidic amino acid residues are shown in red and blue letters, respectively, and mutated residues are underlined. (B) HeLa cells transiently expressing the respective EGFP-PEX26 proteins indicated on the left were fixed and then immunostained with the anti-PTS1 antibody. Bars, 10 µm. Intracellular localization of each EGFP-fused protein is shown on the right. Per, peroxisome; Mito, mitochondria; marks (= and >) represent equal frequency and higher frequency, respectively. For instance, the label “Mito & Per > Per” for RRK3S indicates that cells showing both mitochondrial and peroxisomal localization of the EGFP-fused protein are found more frequently than cells showing only peroxisomal localization. (C) FLAG-PEX26 variants indicated at the top were synthesized in RRL in the presence of RRL-synthesized HA-PEX19 and subjected to immunoprecipitation with anti-FLAG agarose beads. Immunoprecipitates (IP) and input (10%) were analyzed by immunoblotting (IB) using the indicated antibodies. Solid and open arrowheads indicate unmodified and farnesylated HA-PEX19, respectively. The asterisk indicates a cross-reactive, nonspecific band.
reduced peroxisomal targeting efficiency, such as RRK3S and KRRK4S, showed reduced binding affinity to PEX19 (Fig. 6 C), suggesting that the reduced peroxisomal targeting efficiency is accounted for by the lower binding affinity to PEX19 and that positively charged residues in the C segment of PEX26 are prerequisite to binding to PEX19.

**Basic amino acid residues in the C segment are important, but not sufficient, for peroxisomal targeting of TA proteins**

As described in the previous section, the TMD of PEX26 followed by a short cluster of basic amino acids serves as an mPTS (Fig. 6 B), thus raising the possibility that positive charges in the C segment determine the peroxisomal targeting of TA proteins. To assess this possibility, we selected a set of well-known TA proteins (Fig. 7 A; Borgese et al., 2003; Borgese and Fasana, 2011), including EGFP-fused constructs (WT) as well as their variants (R) in which the net positive charge in the C segment was increased to match that of PEX26 (Fig. 7 B, left), and determined their localization in HeLa cells. The WT form of a MOM-destined TA protein, OMP25, was localized to mitochondria, and those of ER-localized TA proteins, cytochrome b (Cyt b) and Sec61β, were localized to the reticular ER structures (Fig. 7 B, c, d, g, and h). The WT vesicular-associated protein 2 (VAMP2), which is transported from the ER to synaptic vesicles in neuronal cells (Kutay et al., 1995), was localized to the plasma membrane and intracellular membranes in HeLa cells (Fig. 7 B, m). Strikingly, the R variants, termed OMP25-R and Cyt b-R, were localized to peroxisomes (Fig. 7 B, c, d, g, and h). The aberrant peroxisome morphology observed in OMP25-R–expressing cells might be related to the function of OMP25 in the recruitment of synaptojanin 2A to mitochondria (Nemoto and De Camilli, 1999). In contrast, Sec61β-R and VAMP2-R were not localized to peroxisomes (Fig. 7 B, k, l, o, and p). These findings suggest that the basicity of the C segment is important, but not sufficient, for the peroxisomal localization of TA proteins. Notably, although EGFP–Sec61β-R seemed to be localized to ER-like structures, the pattern of EGFP fluorescence was not the same as that of the corresponding WT protein. For instance, EGFP–Sec61β–R and Sec61β, was localized to the nuclear envelope, but EGFP–Sec61β–R was not (Fig. 7 B, i and k). Thus, the basicity of the C segment may affect the postinsertional sorting of ER-localized TA proteins to a subdomain of the ER.

Next, we investigated whether the peroxisomal targeting of OMP25-R and Cyt b-R depends on PEX19. In *PEX19* siRNA-treated cells, EGFP–OMP25-R and EGFP–Cyt b-R were detected in both peroxisomes and mitochondria, as assessed by colocalization with PTS1 proteins and cytochrome c, respectively (Fig. 7 C and Fig. S4, A and B). These results suggest that *PEX19* knockdown impairs the targeting of OMP25-R and Cyt b-R to peroxisomes.

**TRC40 interferes with PEX19 in binding to Sec61β-R and VAMP2-R**

By increasing the basicity of the C segment, the C-terminal regions of OMP25 and Cyt b-, but not Sec61β and VAMP2, can be converted into targeting signals for peroxisomes (Fig. 7).

To address whether this difference can be explained simply by binding to PEX19, the interaction between PEX19 and the EGFP-fused proteins used in Fig. 7 was examined. Digitonin lysates of HeLa cells overexpressing each of the EGFP-fused proteins together with Myc-PEX19 or FLAG-PEX19 were subjected to immunoprecipitation with anti-FLAG agarose beads followed by immunoblot analysis. All of the R variants were coimmunoprecipitated efficiently and specifically with FLAG-PEX19 (Fig. 8 A), indicating that PEX19 recognized and interacted with not only EGFP–OMP25-R and EGFP–Cyt b-R but also EGFP–Sec61β–R and EGFP–VAMP2-R. Furthermore, the amounts of R variants coimmunoprecipitated with FLAG-PEX19 were similar (Fig. S4 C). Therefore, the failure of Sec61β-R and VAMP2-R to localize to peroxisomes is unlikely to be caused by the defect in binding to PEX19. It should be noted that although the efficiency was quite low, a portion of WT EGFP–OMP25–R, Sec61β–R, and VAMP2 was also coimmunoprecipitated with FLAG-PEX19. The physiological significance of this interaction is unclear; however, this interaction may not occur under physiological conditions because none of the three proteins is targeted to peroxisomes (also see the following paragraph).

An intriguing question remains as to why Sec61β-R and VAMP2-R are destined for the ER but not for peroxisomes, despite the fact that they are recognized by PEX19. Importantly, OMP25 and Cyt b-R can spontaneously insert into the membrane, whereas both Sec61β and VAMP2 require TRC40 for insertion into the ER membrane (Fig. 7 A). In this context, association of TRC40 with its substrates was shown to depend primarily on TMD hydrophobicity (Mariappan et al., 2010), implying that TRC40 may bind to Sec61β-R and VAMP2-R. Moreover, cytosolic factors other than TRC40, including chaperones and quality control factors, may also interact with them (Abell et al., 2007; Leznicki et al., 2010; Hess et al., 2011). Therefore, we supposed that under physiological conditions and with endogenous levels of PEX19, PEX19 might have little chance to bind to Sec61β-R and VAMP2-R. To verify this issue, FLAG-tagged proteins were synthesized in RRL and then immunoprecipitated under detergent-free conditions. Endogenous TRC40 in RRL was coimmunoprecipitated with the WT as well as the R forms of Sec61β-R and VAMP2-R but not with PEX26 (Fig. 8 B), thereby demonstrating that TRC40 indeed recognizes and captures the R variants. Notably, the amounts of TRC40 coimmunoprecipitated with the R variants were comparable to those coimmunoprecipitated with the corresponding WT proteins.

A similar result was obtained when these variants were synthesized in the presence of RRL-synthesized HA-PEX19 (Fig. 8 C), implying that the addition of HA-PEX19 did not affect the binding of TRC40 to the R variants. Importantly, Sec61β-R and VAMP2-R did not appear to interact with HA-PEX19 under this experimental condition. First, because anti-FLAG agarose beads nonspecifically adsorbed HA-PEX19 under detergent-free conditions (not depicted), HA-PEX19 was found even in the immunoprecipitates of PEX26 lacking the PEX19-binding domains, though the amount of HA-PEX19 was lower than that in PEX26 immunoprecipitates (Fig. 8 C, lanes 1 and 2). Second, the amounts of HA-PEX19 found in immunoprecipitates of Sec61β-R and VAMP2-R variants were comparable to those
Figure 7. Basic residues in the C segment are important, but not sufficient, for peroxisomal targeting of TA proteins. (A) Amino acid sequences of the C-terminal part of several TA proteins and their insertion sites as well as their targeting pathways are shown. The TMDs are bolded, and basic and acidic amino acid residues are shown in red and blue letters, respectively. (B, left) Schematic representation of various EGFP-fused TA proteins [WT] and their variants [R] in which the net positive charge in the C segment was increased. Amino acid sequences of the respective C segments of the proteins are also indicated. (right) The WT and R forms of EGFP-OMP25 [a–d], EGFP–Cyt b3 [e–h], EGFP–Sec61β [i–l], and EGFP–VAMP2 [m–p] were transiently expressed in HeLa cells and assessed for intracellular localization. EGFP-fused proteins and peroxisomes were detected by EGFP fluorescence and immunostaining with the anti-PTS1 antibody, respectively. (C) HeLa cells were treated with PEX19 #1 siRNA for 44 h and then transfected with cDNA encoding EGFP–OMP25R [a–c] or EGFP–Cyt b3-R [e–f]. After a further 12-h incubation, cells were fixed and immunostained with antibodies to PTS1 and cytochrome c (Cyt c). Bars, 10 µm.
which is further supported by our earlier finding that cell-free synthesized PEX26 is imported into isolated peroxisomes in a PEX19-stimulated manner (Matsuzono and Fujiki, 2006).

The PEX19–PEX3-dependent direct import of PMPs, termed the class I pathway, was first proposed by Fang et al. (2004) and Jones et al. (2004). Most importantly, our results provide the first evidence that PEX3 indeed recruits PMP-loaded PEX19 and thereby mediates the direct import of PMPs. Whether the direct import requires any factors besides PEX3 and PEX19 remains to be defined. Remarkably, the PEX19–PEX26 complex immunoaffinity isolated from the cytosolic fraction of CHO-K1 cells contains the minimal factors required for PEX26 import (Fig. 1, C–E). The mechanistic basis of the membrane insertion also awaits future studies. Given the fact that TA proteins with moderately hydrophobic TMDs can insert spontaneously into protein-free liposomes (Brambillasca et al., 2005, 2006; Kemper et al., 2008), the membrane insertion of PEX26 could occur without assistance from any factors. Biochemical reconstitution studies might provide the answer to these questions.

Meanwhile, the ER to peroxisome trafficking of TA proteins was suggested in yeast and plant cells (Elgersma et al., 1997; Mullen et al., 1999). More recent studies suggest that yeast Pex15p is inserted into the ER via the GET pathway and sorted to peroxisomes via a poorly characterized mechanism...
involving yeast Pex19p and ATP (Schuldiner et al., 2008; Jonikas et al., 2009; Costanzo et al., 2010; Lam et al., 2010; van der Zand et al., 2010). We demonstrate that ATP depletion abolishes the import of Sec61β but not that of PEX26 (Fig. 4) and that TRC40 fails to interact with PEX26 (Fig. 5 A). Moreover, the ER localization of PEX26 was not observed even in pex19 ZP119 cells (Fig. 5 B). These results argue against the involvement of the ER in PEX26 import. Thus, the targeting pathway of peroxisomal TA proteins may not be evolutionarily conserved. Nevertheless, the 1-h incubation used in our in vitro import assay might still leave room for an ER targeting before the peroxisomal targeting. Given that the direct import and ER to peroxisome trafficking pathways are not necessarily mutually exclusive, it is possible that these two pathways might operate simultaneously in one organism and that eukaryotic cells might use either one or both pathways depending on requirements.

In mammalian cells, several TA proteins, including fission1 (Fis1), are found in both mitochondria and peroxisomes (Koch et al., 2005; Kobayashi et al., 2007; Gandre-Babbe and van der Bliek, 2008; Dixit et al., 2010). A recent study suggested that peroxisomal targeting of Fis1 depends on PEX19 (Delille and Schrader, 2008). Hence, the PEX19–PEX3-dependent direct import pathway appears to deliver a small pool of nascent Fis1 to peroxisomes, although this remains to be demonstrated experimentally. The dual localization of Fis1 may be explained by its weak affinity for PEX19. Consistently, PEX26 variants with reduced affinity for PEX19 are indeed targeted to both peroxisomes and mitochondria (Fig. 6). Further studies are required to understand precisely how the dual targeting of Fis1 and the others is achieved and/or regulated.

**mPTS of peroxisomal TA proteins**

The targeting signal of PEX26 was also analyzed by focusing on the positively charged residues within the luminal PEX19 binding site (Halbach et al., 2006). The positive charges are indeed essential for peroxisomal targeting of PEX26 because a decrease in the positive charges lowers the efficiency of peroxisomal targeting (Fig. 6 B). The impaired peroxisomal targeting appears to mirror the decrease in binding to PEX19 (Fig. 6 C). The peroxisomal localization of PEX26-CHARGE, OMP25-R, and Cyt b5-R further supports the importance of the positive charges and suggests that a C-terminal TMD with a following short cluster of basic amino acids serves as an mPTS (Fig. 6 B and Fig. 7). Plant peroxisomal ascorbate peroxidase indeed harbors such a short, highly basic C segment, although such a C segment remains to be identified in mammals. Given that a short cluster of basic amino acids does not fit the predicted PEX19 binding motif (Rottensteiner et al., 2004; Halbach et al., 2005), the presence of a PEX19 binding site within the C segment may not be essential for peroxisomal targeting. In the case of PEX26, however, the luminal PEX19 binding site is likely to secure the efficient peroxisomal targeting (Fig. 6 B). The structural analysis of PEX19–PEX26 complexes would address the roles of the two PEX19 binding sites found in PEX26.

A highly basic C segment is important, but not sufficient, for the peroxisomal targeting of TA proteins, as noted for Sec61β-R and VAMP2-R, which are not localized to peroxisomes and rather are destined for the ER (Fig. 7). Surprisingly, PEX19 interacts with Sec61β-R and VAMP2-R under conditions of overexpression and detergent solubilization (Fig. 8 A). However, it is most likely that under physiological conditions, Sec61β-R and VAMP2-R are captured by TRC40, not PEX19 (Fig. 8, B and C), in accordance with the fact that the association of TRC40 with its substrate depends primarily on TMD hydrophobicity (Mariappan et al., 2010). Collectively, our results suggest that TA proteins that not only interact with PEX19 but also escape from capture by TRC40 are targeted to peroxisomes. Peroxisomal TA proteins would therefore require a less hydrophobic TMD to avoid capture by TRC40 and require a highly basic C segment to ensure binding to PEX19.

**Selective targeting of TA proteins to the correct membranes in mammalian cells**

Recent studies, including ours, have uncovered the targeting pathways of TA proteins in mammalian cells: the GET pathway for most ER-destined TA proteins (Stefanovic and Hegde, 2007), the PEX19–PEX3-dependent pathway for peroxisomal TA proteins (this study; Halbach et al., 2006), and the unassembled pathways for several ER-destined and most, if not all, MOM-targeted TA proteins (Brambillasca et al., 2005; Setoguchi et al., 2006; Kemper et al., 2008; Colombo et al., 2009). To achieve selective targeting, newly synthesized TA proteins must be sorted to the appropriate targeting pathway. Sec61β-R and VAMP2-R, showing significant affinity to PEX19, are captured by TRC40 and destined for the ER (Fig. 7 and Fig. 8), thereby suggesting that the GET pathway outcompetes the PEX19–PEX3-dependent pathway. Given that the GET pathway (a) appears to thoroughly hinder the potential targeting of ER-destined TA proteins to mitochondria (Schuldiner et al., 2008; Jonikas et al., 2009) and (b) succeeds in substrate loading onto TRC40 despite the presence of competing cytosolic factors (Mariappan et al., 2010), it is more likely that the GET pathway can uptake ER-destined TA proteins before other targeting pathways gain access to them (Fig. 9). Notably, the substrate recognition by the GET pathway seems to begin before the TMDs of TA proteins emerge from ribosomes (Mariappan et al., 2010).

TA protein sorting in several targeting pathways is likely dictated by a combination of substrate properties and availability of binding partners. With respect to substrate features, TMD hydrophobicity is possibly a key determinant for the access to, or exclusion from, the GET pathway (Borgese et al., 2007; Rabu et al., 2008; Mariappan et al., 2010). TA proteins excluded from the GET pathway are more likely to be sorted based on C-terminal basicity (Fig. 9; this study; Isenmann et al., 1998; Kuroda et al., 1998; Borgese et al., 2001; Horie et al., 2002; Kaufmann et al., 2003); however, as there is some overlap in the hydrophobicity and basicity between TA proteins (Borgese et al., 2007), further biochemical and structural studies using a large number of bona fide and artificial sequences are required to envisage how each targeting pathway discriminates specific substrates from closely related TA substrates. Moreover, it should be also clarified whether and how the activities of the components of each targeting pathway are regulated. Addressing these issues should lead to a comprehensive understanding of the selective targeting of TA proteins.
Materials and methods

Antibodies

Rabbit antibodies to acetyl-CoA oxidase [AOx; raised against full-length rat AOX; Tsukamoto et al., 1990], catalase [raised against full-length human catalase; Shimozawa et al., 1992], PTS1 peptide [raised against aa 652–661 of rat AOX; Otera et al., 1998], PEX3 [raised against aa 356–373 of human PEX3; Ghaedi et al., 2000], PEX13 [raised against aa 256–403 of human PEX13; Mukai and Fujiki, 2006], PEX14 [raised against aa 358–376 of rat PEX14; Shimizu et al., 1999], HA [raised against HA epitope (YpyDVPDYY); Otera et al., 2000], and guinea pig anti-PEX14 antibody [raised against aa 241–376 of rat PEX14; Mukai et al., 2002] were described previously. Rabbit anti-TRC40 antibody [raised against full-length human TRC40; Shimozawa et al., 1999], HA [raised against HA epitope (aa 241–376 of rat PEX14; Shimizu et al., 1999), GFP (B-2; Santa Cruz Biotechnology, Inc.), cytochrome P450 reductase (F-10; Santa Cruz Biotechnology, Inc.), cytochrome b5 (H-20; Santa Cruz Biotechnology, Inc.), mossy fiber antigen (M-22; Santa Cruz Biotechnology, Inc.), and mouse monoclonal antibodies against FLAG (Sigma-Aldrich) and GFP (Medical Research Council Laboratory of Molecular Biology, Cambridge, England, UK). The following primary antibodies were purchased from the indicated vendors: rabbit antibodies against FLAG (Sigma-Aldrich) and GFP (Medical and Biological Laboratories); mouse monoclonal antibodies against FLAG (M2, Sigma-Aldrich), HA (1H12; Covance), c-Myc (PE10; Santa Cruz Biotechnology, Inc.), GFP (B2; Santa Cruz Biotechnology, Inc.), cytochrome P450 reductase (F10; Santa Cruz Biotechnology, Inc.), cytochrome c (BD), PEX19 (BD), and α-tubulin (Abcam); and goat anti-lactate dehydrogenase antibody (Rockland Immunochemicals). Secondary antibodies used for immunoblotting were HRP-linked sheep anti–mouse IgG, donkey anti–rabbit IgG (GE Healthcare), and rabbit anti–goat IgG (Invitrogen) antibodies. Secondary antibodies for immunostaining included Alexa Fluor 488–, 568–, or 647–conjugated goat anti–mouse IgG and anti–rabbit IgG antibodies and Alexa Fluor 647–conjugated goat anti–guinea pig IgG antibody (Invitrogen).

Cell culture, DNA transfection, and RNAi

CHO cells, including CHO-K1 and CHO pex19 ZP119 (Kinosita et al., 1998; Matsuzono et al., 1999), were maintained in Ham’s F-12 medium (Invitrogen) supplemented with 10% FBS, and HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS. All cell lines were cultured at 37°C under 5% CO2. DNA transfections were performed using Lipofectamine 2000 (Invitrogen) for CHO cells and Lipofectamine reagent (Invitrogen) for CHO cells according to the manufacturer’s instructions. siRNA-mediated knockdown of PEX3 and PEX19 in HeLa cells was performed using predesigned Stealth RNAi siRNAs (Invitrogen) with Stealth RNAi Negative Control Medium GC Duplex (Invitrogen) as a control. The sequences of siRNAs are listed in Table 1. HeLa cells were transfected twice at a 24-h interval with 33 nM siRNA duplexes using Lipofectamine 2000.

Plasmids

All plasmids used in this study (Table 2) were constructed by standard methods and verified by DNA sequencing. The cdNAs encoding human PEX26, human PEX19, rat PEX3, and EGFP were amplified by PCR from pCMVSPORT/PEX26 (Matsuzono et al., 2003), pL2D2Hyg/HA–PEX19 (Matsuzono et al., 2006), pcDNA2.1Neo/ RnpEX3 (Ghaedi et al., 2000), and EGFP vector (Takara Bio Inc.), respectively. The cdNAs encoding human Sec61β, human Hsp47, mouse OMP25, mouse Cyt b, mouse VAMP2, and rat Tom20 were obtained by RT-PCR using total RNA isolated from Hela cells, mouse brain tissue, and rat liver tissue, respectively.

The cdNAs encoding full-length PEX26, PEX26ΔC (aa 2–246), and PEX26ΔNCS (aa 2–270) were PCR amplified and cloned into a modified pcDNA3.1/Zeo(+) vector (Invitrogen) encoding an N-terminal FLAG tag (pcDNA3/FLAG) or EGFP (pcDNA3/EGFP) via the BamHI–NotI sites. The cdDNA coding for full-length PEX26 was also cloned into a modified pcDNA3.1/Zeo(+) vector encoding an N-terminal 2×HA tag. The cdDNA encoding PEX26ΔK1S, PEX26ΔK2S, and PEX26ΔKKS were generated by overlap extension PCR (Ho et al., 1989) and ligated into the BamHI–NotI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNAs encoding PEX26ΔK1S, PEX26ΔK2S, and PEX26ΔKKS were generated by overlap extension PCR (Ho et al., 1989) and ligated into the BamHI–NotI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding Sec61β was also cloned into a modified pcDNA3.1/Zeo(+) vector encoding an N-terminal 6×Myc tag.

The cdDNAs encoding PEX19 and PEX19ΔN3 (aa 24–299) were cloned into the BamHI–Xhol sites of pcDNA3/Flag and a modified pcDNA3.1/Zeo(+) vector encoding a N-terminal Myc tag. The cdDNA encoding full-length rat PEX3 was cloned into the BamHI–XhoI sites of pcDNA3/Flag and a modified pcDNA3.1/Zeo(+) vector encoding a N-terminal Myc tag. The cdDNA for full-length rat PEX3 was cloned into the BamHI–XhoI sites of pcDNA3/Flag and a modified pcDNA3.1/Zeo(+) vector encoding a C-terminal EGFP tag. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP. The cdDNA encoding a fusion of aa 1–69 of rat Tom20 and aa 40–573 of rat PEX3 was generated by overlap extension PCR (Horton et al., 1989) and ligated into the BamHI–XhoI sites of pcDNA3/Flag and pcDNA3/EGFP.
mutation was introduced by overlap extension PCR. To generate pcDNA3.1-Flag/ER-EGFP, the cDNA encoding aa 1–414 of human Hsp47 was ligated into the NheI–BamH I sites of the pcDNA3.1-Zeo(+) vector in which a BamH I–SpeI fragment encoding EGFP followed by a KDEL retention signal had been inserted into the BamH I–XbaI sites. pcDNA3.1-Zeo/HA-PEX19 (Matsuzono et al., 2006) and pEGFP-C1/EGFP-PEX16 (Matsuzaki and Fujiki, 2008) were as described previously.

**Immunoblotting**

Immunoblotting was performed as described previously (Otera et al., 2000). In brief, protein samples were separated by SDS-PAGE and electro-transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories). After blocking in PBS containing 5% nonfat dry milk and 0.1% Tween 20, blots were probed with appropriate primary and HRP-conjugated secondary antibodies, developed with ECL Western blotting detection reagents (GE Healthcare), and exposed to an x-ray film (Hyperfilm ECL; GE Healthcare).

For densitometric analysis, immunoblot films were scanned in translucent mode with a scanner (GT-X900; Epson), and the intensity of protein bands was analyzed using the gel analysis tool in ImageJ software (National Institutes of Health). The relative amounts of proteins were determined as described in the figure legends.

**Immunofluorescence microscopy**

Cells on glass coverslips were fixed with 4% PFA in PBS for 15 min at RT, permeabilized with 1% Triton X-100 in PBS for 5 min at RT, and blocked with PBS-BSA (PBS containing 1% BSA) for 30 min at RT. Subsequently, cells were incubated for 60–90 min at RT with primary antibodies diluted in PBS-BSA, washed extensively with PBS, and incubated for 45 min at RT with appropriate Alexa Fluor 488–, 568–, or 647–conjugated secondary antibodies diluted in PBS-BSA. After extensive washing with PBS, coverslips were rinsed with ultrapure water and mounted on slides with aqueous mounting medium (PermaFluor; Thermo Fisher Scientific). Images were acquired using a fluorescence microscope.

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Table 2. **Plasmids used in this study**

<table>
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<tr>
<th>Name</th>
<th>Expressed protein</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>pcDNA3.1-Zeo/HA-PEX26</td>
<td>Full-length human PEX26 with an N-terminal 2xHA tag</td>
<td>This study</td>
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<td>Full-length human PEX26 with an N-terminal FLAG tag</td>
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<td>pcDNA3.1-Zeo/FLAG-VAMP2-R</td>
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<td>Matsuzono et al., 2006</td>
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<td>This study</td>
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<td>pcDNA3.1-Zeo/PEX3-W104A-EGFP</td>
<td>PEX3 carrying the W104A mutation with a C-terminal EGFP</td>
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<td>pcDNA3.1-Zeo/Mito-PEX3-EGFP</td>
<td>A chimeric protein, termed Mito-PEX3-EGFP, comprising aa 1–69 of rat Tom20, ao 40–373 of rat PEX3, and EGFP</td>
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<tr>
<td>pcDNA3.1-Zeo/Mito-PEX3-W104A-EGFP</td>
<td>Identical to Mito-PEX3-EGFP except that PEX3 in this construct harbors the W104A mutation</td>
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<tr>
<td>pcDNA3.1-Zeo/ER-EGFP</td>
<td>A fusion protein comprising aa 1–414 of human Hsp47, EGFP, and a KDEL retention signal</td>
<td>This study</td>
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</table>

pEGFP-C1/EGFP-PEX16 is based on pEGFP-C1 (Takara Bio Inc.), whereas other plasmids are based on pcDNA3.1-Zeo(+) (Invitrogen).
Online supplemental material

Fig. S1 demonstrates that PEX19 knockdown impairs peroxisomal targeting of newly synthesized PEX26. Fig. S2 shows that the C-terminal region of PEX26 is required for binding to PEX19. Fig. S3 shows the interaction between PEX19ΔN23 and PEX26 in the cytosol. Fig. S4 shows that peroxisomal targeting of EGF–OMP25-R and EGFP–Cyt b2-R depends on PEX19 and also shows the interaction of PEX19 with EGFP-fused OMP25-R, Cyt b2-R, Sec61α-R, and VAMP2-R. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201211077/DC1.

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