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PEX19 is a predominantly cytosolic chaperone and import receptor for class 1 peroxisomal membrane proteins

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Integral peroxisomal membrane proteins (PMPs) are synthesized in the cytoplasm and imported posttranslationally. Here, we demonstrate that PEX19 binds and stabilizes newly synthesized PMPs in the cytosol, binds to multiple PMP targeting signals (mPTSs), interacts with the hydrophobic domains of PMP targeting signals, and is essential for PMP targeting and import. These results show that PEX19 functions as both a chaperone and an import receptor for newly synthesized PMPs. We also demonstrate the existence of two PMP import mechanisms and two classes of mPTSs: class 1 mPTSs, which are bound by PEX19 and imported in a PEX19-dependent manner, and class 2 mPTSs, which are not bound by PEX19 and mediate protein import independently of PEX19.

Introduction

Peroxisomes are discrete, single membrane-bound organelles. All peroxisomal proteins are synthesized in the cytoplasm and imported posttranslationally, but the mechanisms used for importing peroxisomal membrane proteins (PMPs) are distinct from those that translocate soluble enzymes into the peroxisome lumen (Lazarow and Fujiki, 1985; Gould and Valle, 2000). This is reflected in the fact that most *pex* mutants are defective in peroxisomal matrix protein import but have no defect in PMP import (Chang et al., 1999; Hettema et al., 2000). Also, PMPs do not use either of the two peroxisomal targeting signals (PTSs) that direct proteins into the peroxisome matrix, the tripeptide PTS1, and the nonapeptide PTS2 (Gould and Valle, 2000). The functional properties of PMP targeting signals (mPTSs) and the mechanisms of PMP import remain obscure.

A common view of protein targeting signals is that they are independent elements that have little or no role in the protein's ultimate structure or function. It is difficult to reconcile this view with two well-established properties of targeting signals for integral PMPs. First, many polytopic PMPs contain multiple, nonoverlapping peroxisomal targeting signals, any one of which is sufficient to direct proteins into the peroxisome membrane (Biermanns and Gartner, 2001; Jones et al., 2001; Wang et al., 2001; Brosius et al., 2002). Second, PMP targeting signals are large, ~50–100 aa in length, and the aggregate size of the targeting signals in several PMPs comprises a significant fraction of the entire protein (Sacksteder et al., 2000; Biermanns and Gartner, 2001; Jones et al., 2001; Wang et al., 2001; Brosius et al., 2002).

We recently developed a hypothesis that can explain both the large size and functional redundancy of PMP targeting signals in the context of posttranslational PMP import (Jones et al., 2001). Specifically, we proposed the existence of a cytoplasmic PMP chaperone and import receptor that (a) binds to multiple sites along polytopic PMPs; (b) prevents PMP misfolding, aggregation, and destruction in the cytoplasm by masking their transmembrane domains; and (c) directs newly synthesized PMPs to the peroxisome membrane, and therefore plays a specific and essential role in PMP import. Although previous works have concluded that PEX19 cannot function as a PMP import receptor (Snyder et al., 2000; Fransen et al., 2001). We show here that PEX19 has all of the properties one would expect for a bifunctional PMP chaperone/import receptor. In addition, we establish the existence of two mechanistically distinct PMP import pathways: one that requires PEX19 and mediates the import of multiple PMPs; and one that is PEX19 independent and mediates the import of PEX3.

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Key words: peroxisome; protein import; posttranslational; Zellweger syndrome; PEX3

Abbreviations used in this paper: IPs, immunoprecipitates; mPTS, PMP targeting signal; PMP, peroxisomal membrane protein.

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Results

PEX19 has PMP chaperone activity

Molecular chaperones are proteins that mediate the correct assembly of other polypeptides but are not components of the functional assembled structures (Ellis and Hemmingsen, 1989). However, our hypothesis of PMP import requires a more restricted definition for the PMP chaperone. It demands that the PMP chaperone reside in the cytoplasm and both bind and stabilize newly synthesized PMPs before their insertion into the peroxisome membrane. PEX19 is a predominantly cytosolic, partly peroxisomal protein and is required for peroxisome biogenesis (Gotte et al., 1998; Matsuzono et al., 1999; Sacksteder et al., 2000). Therefore, we tested whether PEX19 binds and stabilizes PMPs in the cytoplasm. We examined the association of PEX19 and transiently expressed, epitope-tagged PMPs, including PMP34 (Wylin et al., 1998), PEX11β (Abe and Fujiki, 1998), and PMP24 (Reguenga et al., 1999), and two control proteins, the peroxisomal matrix protein PTE1 (Jones et al., 1999) and the viral envelope glycoprotein VSV-G (Adams and Rose, 1985). To simplify the analysis of PMP interactions in the cytoplasm, we performed our experiments in cells that lack peroxisomes and therefore cannot import PMPs: the PEX19-deficient human fibroblast line, PBD399-TI, and a PEX3-deficient human fibroblast line, PBD400-TI. Finally, we elevated PEX19 levels in the PBD400-TI cells by transient transfection to ensure that they were proportional to the levels of PMP expression in these experiments.

Immunoblot analysis revealed that integral PMPs were much more abundant in peroxisome-deficient cells that express PEX19 than in cells that do not express PEX19 (Fig. 1 A). These results reflect the status of soluble, cytoplasmic PMPs in detergent-free, membrane-free lysates. This was confirmed by immunofluorescence studies which revealed that all three PMPs shared the same cytoplasmic accumulation and nuclear exclusion as PEX19 (Fig. 1 B). In contrast, these PMPs could only rarely be detected in PEX19-deficient cells, and what little could be detected was mislocalized to the mitochondrion (unpublished data).

To determine whether the PEX19-mediated increase in PMP abundance reflected a change in PMP half-life, we expressed PMP34/13xmyc in the same two cell lines (+PEX19 or –PEX19), pulse labeled the cells with [^{35}S]methionine for 1 h, and chased with excess cold methionine for varying lengths of time. At each time point, a membrane-free cell lysate was subjected to immunoprecipitation with anti-myc antibodies. The levels of PMP34/13xmyc in each sample were determined by autoradiography and by immunoblot with anti-myc antibodies (Fig. 1, C and D). Densitometric analysis revealed that PEX19 increased the half-life of PMP34/13xmyc by 20-fold, an effect large enough to explain the PEX19-mediated increase in PMP abundance in these cells.

To determine whether PEX19 stabilized PMPs through physical interaction in the cytosol, we asked whether these cytoplasmic, stable PMPs could be recovered in PEX19 immunoprecipitates (IPs). We coexpressed each integral PMP with PEX19 and an epitope-tagged form of PEX19, 3xHA-PEX19. 1 d later, the cells were lysed and subjected to immunoprecipitation with anti-HA antibodies. The IPs were then separated by SDS-PAGE and immunoblotted with anti-myc antibodies. A higher level of each PMP was detected in the anti-HA IPs from cells expressing 3xHA-PEX19 (Fig. 1 E). Furthermore, the analysis of PMP levels in the samples before and after immunoprecipitation indicated that the majority of each PMP was associated with PEX19 (Fig. 1 F).

If PEX19 is a cytoplasmic PMP chaperone, it should also bind newly synthesized PMPs. To test this prediction we used PBD399 (*PEX19*-deficient) cells that stably express 3xHA-PEX19 at approximately the level of PEX19 in wild-type cells (these cells contain normal peroxisomes and import PMPs; unpublished data). These PBD399/3xHA-PEX19 cells were transiently transfected with pcDNA3-PMP34/13xmyc, pulse labeled with [^{35}S]methionine and chased with excess cold methionine for varying amounts of time. At each time point, half of the cells were lysed, membranes were discarded, and the samples were subjected to immunoprecipitation with an anti-HA mAb. The IPs were then solubilized with SDS, diluted, and subjected to a second immunoprecipitation with an anti-myc mAb. The other half of the cells were solubilized in 1% Triton X-100 and subjected to immunoprecipitation using an anti-myc mAb. After separation of the samples by SDS-PAGE, the labeled PMPs were detected by autoradiography (Fig. 1 G). Densitometric analysis of these films revealed that the half-time of association between cytosolic PMP34/13xmyc and PEX19 was ~15 min, whereas the half-life of total PMP34/13xmyc was ~300 min. Thus, PEX19 displays a kinetically restricted interaction with PMPs, preferentially binding newly synthesized PMPs in the cytosol.

PEX19 is an import receptor for multiple integral PMPs

Our hypothesis that PMP import requires a bifunctional PMP chaperone/import receptor led us to next ask whether PEX19 also functioned as a PMP import receptor. We first tested whether PEX19 binds to the mPTSs of multiple PMPs. We tested the c-terminal mPTS of PMP34 (Jones et al., 2001), an mPTS from PEX11β (Sacksteder et al., 2000), and both reported mPTSs of PMP22 (Brosius et al., 2002). We also tested two distinct mPTSs from PEX16, one reported previously (Fransen et al., 2001) and one (amino acids 221–336) identified in our laboratory (unpublished data). Finally, we examined three reported mPTSs of PMP70 (Sacksteder et al., 2000; Biermanns and Gartner, 2001). PEX3-deficient PBD400-TI cells, which lack peroxisomes, were cotransfected with plasmids designed to express the mPTS-containing proteins together with either PEX19 or 3xHA-PEX19. The next day the cells were lysed in hypotonic buffer and membranes were discarded after centrifugation. The resulting soluble protein lysates were subjected to immunoprecipitation with antibodies to the HA epitope tag, followed by immunoblot with antibodies specific for the mPTS-containing proteins. Each mPTS-containing protein was precipitated with 3xHA-PEX19, demonstrating the ability of PEX19 to bind these diverse mPTSs (Fig. 2 A).

As an independent, in vivo test of PEX19-mPTS binding, we next asked whether PEX19 could control the distribution

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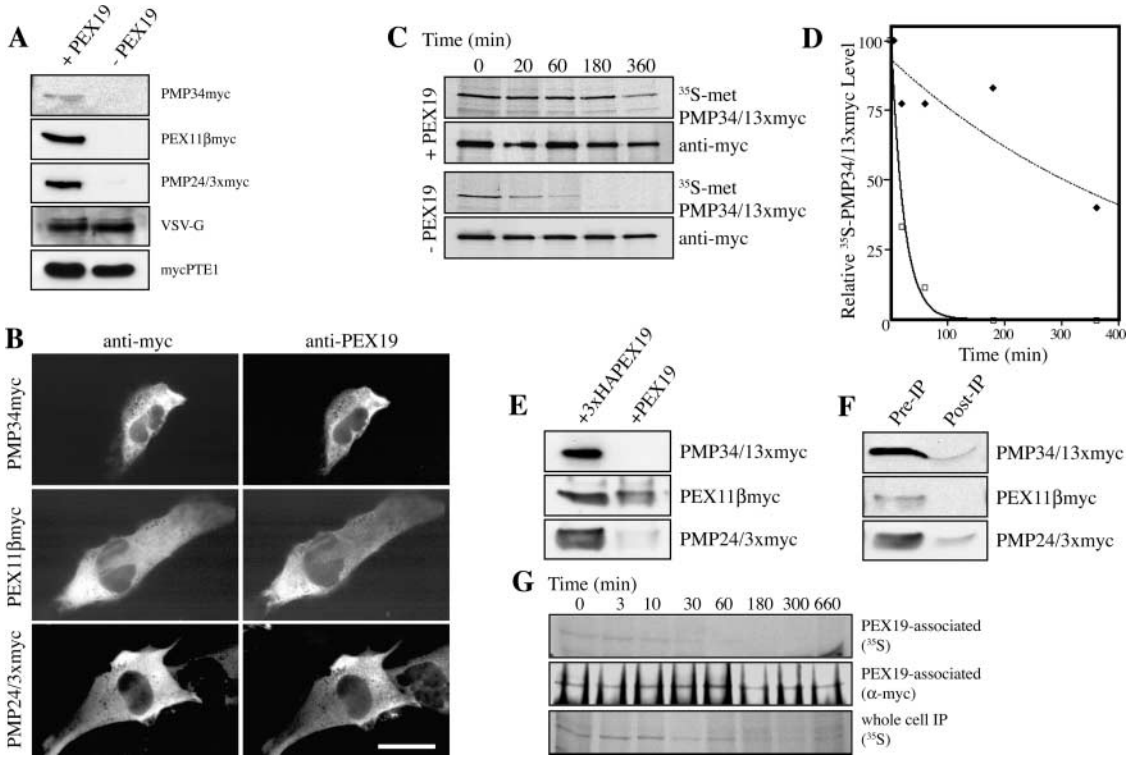

Figure 1. **Pex19 is a PMP chaperone.** (A) PEX19 expression results in increased abundance of PMPs in the cytosol. PEX3-deficient human fibroblasts were transfected with plasmids designed to express PMP34myc, PEX11βmyc, PMP24/3xmyc, VSV-G, mycPTE1, and PEX19 (+PEX19). PEX19-deficient human fibroblasts were transfected with plasmids designed to express the same test proteins and vector alone (−PEX19). Equal total protein from a membrane-free lysate of each cell sample was analyzed by immunoblot using antibodies to the c-myc epitope or to VSV-G as appropriate. (B) PEX3-deficient human fibroblasts transfected with plasmids designed to express PEX19 and either PMP34myc, PEX11βmyc, or PMP24/3xmyc were processed for indirect immunofluorescence using antibodies to the c-myc epitope (left) or PEX19 (right). Bar, 15 μM. (C) PEX19 stabilizes PMP34/13xmyc in the cytosol. PEX3-deficient human fibroblasts were transfected with plasmids designed to express PMP34/13xmyc and PEX19 (+PEX19); PEX19-deficient human fibroblasts were transfected with a plasmid designed to express PMP34/13xmyc and empty vector (−PEX19). Cells were pulsed for 15 min with [^{35}S]methionine and chased with excess cold methionine for the times indicated. A membrane-free cell lysate of each sample was subjected to immunoprecipitation with anti-myc antibodies. Equal fractions of each IP were analyzed by autoradiography (^{35}S) and immunoblot using anti-myc antibodies (anti-myc). (D) Relative ^{35}S-signal intensities for each time point in B versus time with best fit exponential curves. (□, solid line), +PEX19; (◆, dashed line), −PEX19; $t_{1/2\ (+PEX19)}$ = 300 min; $t_{1/2\ (-PEX19)}$ = 15 min. (E) PEX19 binds multiple PMPs in the cytosol. PEX3-deficient human fibroblasts transfected with plasmids designed to express PMP34/13xmyc, PEX11βmyc, PMP24/3xmyc, and either 3xHA-PEX19 (+3xHA-PEX19) or PEX19 (+PEX19). Equal total protein from a membrane-free lysate of each cell sample was subjected to immunoprecipitation with anti-HA antibodies. IPs were analyzed by immunoblot using anti-myc antibodies. (F) The majority of cytosolic PMP is bound to PEX19. Equal amounts of lysates of 3xHA-PEX19–expressing cells from E sampled before (Pre-IP) and after (Post-IP) immunoprecipitation were analyzed by immunoblot using anti-myc antibodies. (G) PEX19 interacts with newly synthesized PMPs. PEX19-deficient human fibroblasts stably expressing 3xHA-PEX19 were transfected with a plasmid designed to express PMP34/13xmyc. Cells were pulsed with [^{35}S]methionine for 10 min, chased with excess methionine for the indicated times, and split into two fractions. Membrane-free lysates of cells in the first fraction were subjected to immunoprecipitation with anti-HA antibodies. The samples were solubilized in SDS and subjected to a second immunoprecipitation with anti-myc antibodies. Immunoprecipitations were analyzed by autoradiography (PEX19-associated, ^{35}S) and immunoblot using anti-myc antibodies (PEX19-associated, anti-myc). Cells from the second fraction were solubilized in 1% Triton X-100 and subjected to immunoprecipitation using anti-myc antibodies; and PMP34/13xmyc was detected by autoradiography (whole-cell IP, ^{35}S). For PEX19-PMP34/13xmyc association, $t_{1/2}$ = 15 min. For the lifetime of PMP34/13xmyc in the whole cell, $t_{1/2}$ = 300 min.

of mPTS-containing proteins. We examined the mPTS-containing proteins in *PEX3*-deficient PBD400-TI cells that were also made to express high levels of either PEX19 or 3xNLS-PEX19, a form of PEX19 that is efficiently targeted to the nucleus (Jones et al., 2001). 1 d after transfection the cells were fixed and the mPTS-containing proteins were localized by immunofluorescence microscopy. PEX19 accumulates in the cytoplasm and is excluded from the nucleus, whereas 3xNLS-PEX19 is excluded from the cytoplasm but accumulates in the nucleus (Fig. 2 B). Proteins containing the mPTSs from PMP34, PEX11β, PEX16, PMP22, and PMP70 all accumulated in the cytoplasm when coexpressed with PEX19 but accumulated in the nucleus when coexpressed with 3xNLS-PEX19 (Fig. 2 C), demonstrating that PEX19-mPTS interactions can control the subcellular distribution of these mPTS-containing proteins in vivo.

PEX19 interacts with the transmembrane region of PMP targeting signals

The hypothesis that PMP import requires a bifunctional PMP chaperone/import receptor is rooted in the assumption that the hydrophobic transmembrane domains of PMPs must be masked as PMPs move through the cytoplasm to

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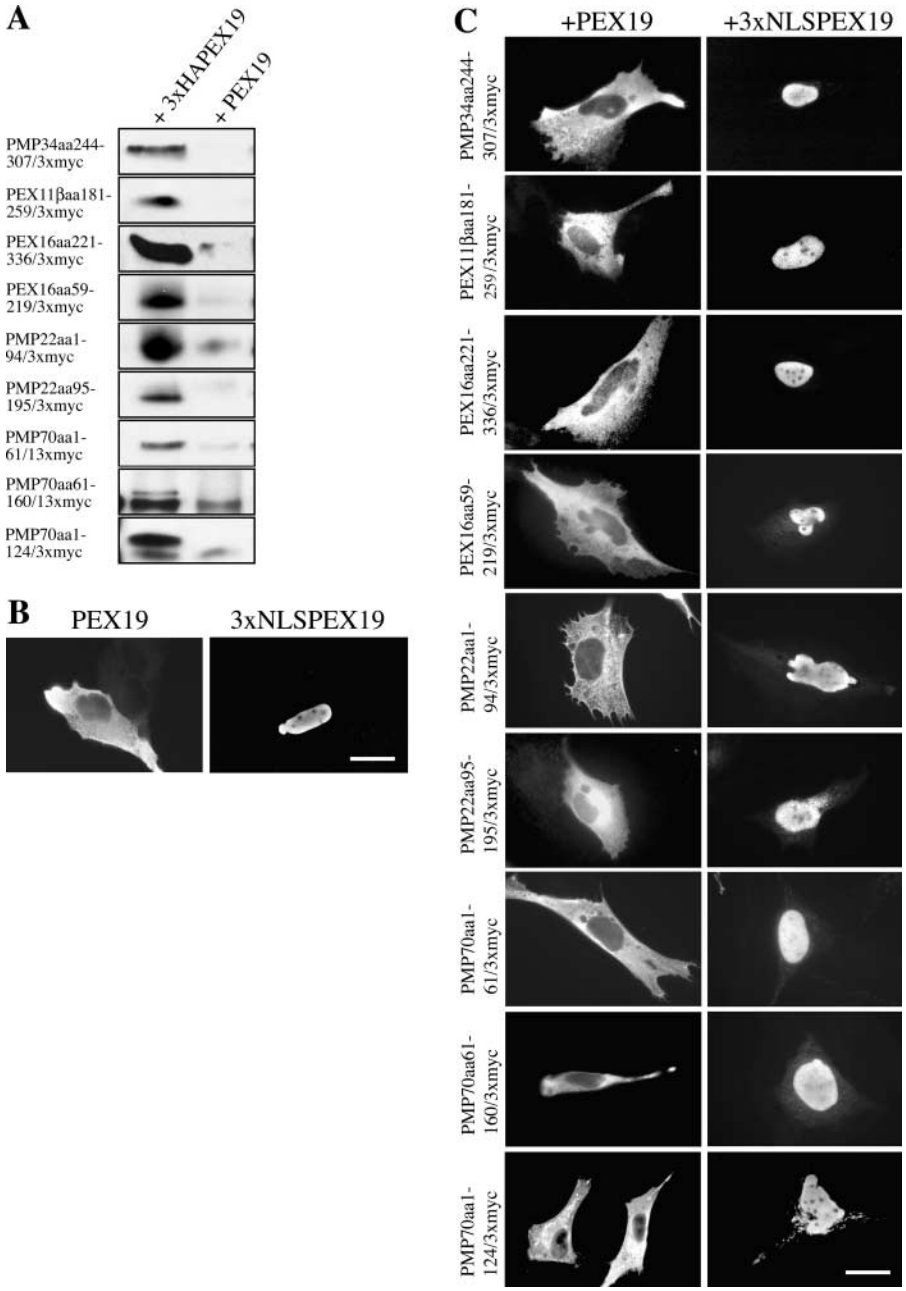

Figure 2. **PEX19 binds multiple PMP targeting signals.** (A) Multiple mPTSs coprecipitate with PEX19. PEX3-deficient human fibroblasts were transfected with plasmids designed to express PMP34aa244-307/3xmyc, PEX11βaa181-259/3xmyc, PEX16aa221-336/3xmyc, PEX16aa59-219/3xmyc, PMP22aa1-94/3xmyc, PMP22aa95-195/3xmyc, PMP70aa1-61/13xmyc, PMP70aa61-160/13xmyc, PMP70aa1-124/3xmyc, and either 3xHA-PEX19 (+3xHA-PEX19) or PEX19 (+PEX19). Equal total protein from a membrane-free lysate of each cell sample was subjected to immunoprecipitation with anti-HA antibodies. IPs were analyzed by immunoblot using anti-myc antibodies. (B) PEX3-deficient human fibroblasts transfected with plasmids designed to express PEX19 (left) or 3xNLS-PEX19 (right) were processed for indirect immunofluorescence using antibodies against PEX19. Bar, 15 μM. (C) PEX19 controls the subcellular distribution of multiple mPTSs. PEX3-deficient human fibroblasts transfected with plasmids designed to express the mPTSs from A and either PEX19 (left) or 3xNLS-PEX19 (right) were processed for indirect immunofluorescence using anti-myc antibodies. Bar, 15 μM.

the peroxisome membrane. If this is true, and if PEX19 is the PMP chaperone/import receptor of this hypothesis, then PEX19 should bind to subregions of mPTSs that contain transmembrane domains. We tested this prediction using the COOH-terminal mPTS of PMP34, which contains a single transmembrane domain. We generated a series of mutations in this mPTS and examined their effects on PEX19 interaction in vivo. Truncation mutants that retained the transmembrane domain retained their interaction with PEX19, whereas those that lacked part of the transmembrane domain were no longer efficiently bound by PEX19 (Fig. 3). The smallest fragment that retained full interaction with PEX19, PMP34aa270-307, contains a long hydrophobic stretch that is interrupted by a pair of charged residues (E289 and K290). Replacement of these charged residues with leucines inhibited the fragment's interaction with PEX19, as did replacement of three hydrophobic residues with hydrophilic amino acids (LMF283-285KKK). Replacing the flanking basic residues at the COOH-terminal side of the putative transmembrane domain with acidic residues (KR302-303EE) had no substantive effect on mPTS–PEX19 interaction. These results indicate that PEX19 interacts with the transmembrane domain of the PMP34 mPTS, and that the distribution of hydrophobic and charged residues within the transmembrane domain are important for mPTS–PEX19 interaction.

Inhibition of PEX19 activity results in a defect in PMP import

The data presented thus far support the hypothesis that PEX19 is a chaperone and import receptor for newly synthesized PMPs. However, this hypothesis demands that PEX19

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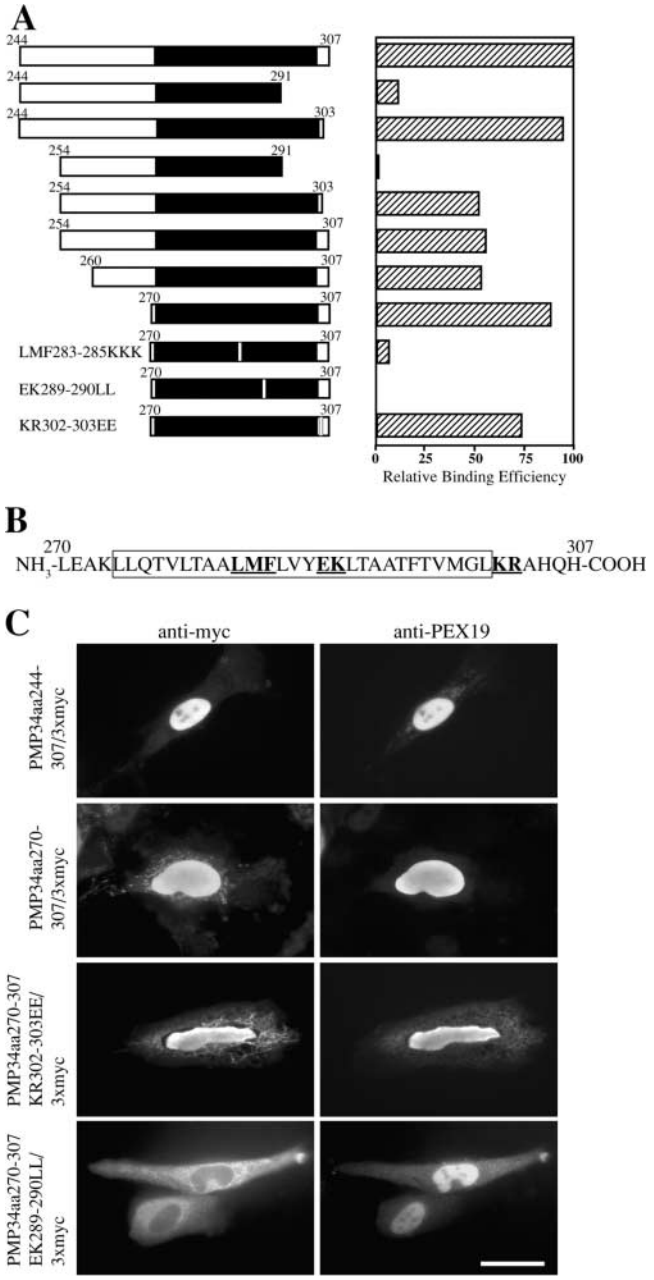

Figure 3. **PEX19 binds the transmembrane region of the PMP34 mPTS.** PEX3-deficient human fibroblasts expressing 3xNLS-PEX19 and myc-tagged mutant forms of the COOH-terminal PMP34 mPTS were processed for indirect immunofluorescence using antibodies to the myc epitope and PEX19. (A, left) Diagram showing amino acid range of each truncation mutant studied. Darkened regions indicated relative position of the putative transmembrane domain. Light vertical bars indicate relative positions of the engineered point mutations. All proteins fused to three tandem c-myc epitopes at the COOH terminus. (Right) Bar graph of relative binding efficiency of each mutant. Relative binding efficiency is the proportion of cells expressing both 3xNLS-PEX19 and PMP34 mPTS mutant in which the PMP34 mPTS mutant is seen in the nucleus. $n = 100$ cells for each sample. Values are normalized. (B) Amino acids 270-307 of PMP34. Putative transmembrane domain is boxed, sites of engineered point mutations are in bold and underlined. (C) Immunofluorescence images of cells expressing selected PMP34 mPTS mutants from the experiment in A. (Left) Anti-myc staining; (right) anti-PEX19 staining. Bar, 15 μM.

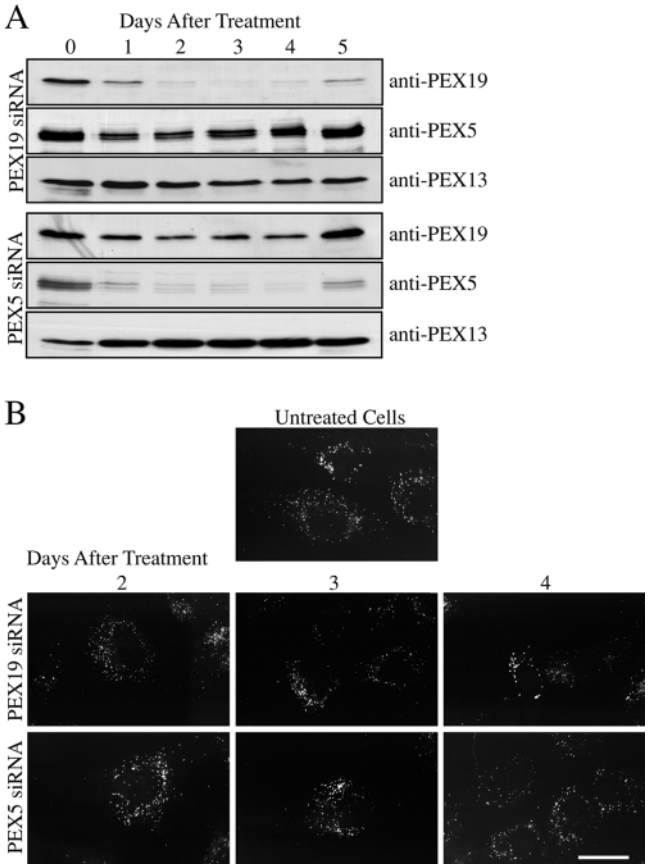

Figure 4. **Peroxisomes persist for multiple days after inhibition of PEX19.** (A) Treatment with PEX19 and PEX5 siRNA mediates specific reductions in PEX19 and PEX5 levels. Wild-type human fibroblasts were subjected to electroporation with PEX19 siRNA (top) or PEX5 siRNA (bottom). Equal amounts of protein from daily cell samples after siRNA treatment were separated by SDS-PAGE and processed for immunoblot using antibodies to PEX19, PEX5, and PEX13. (B) Peroxisomes persist for >3 d despite inhibition of PEX19. Daily cell samples after treatment with PEX19 siRNA (top) or PEX5 siRNA (bottom) were processed for indirect immunofluorescence using antibodies to the peroxisomal membrane marker PMP70. Bar, 15 μM.

play a direct role in PMP import. Although the absence of peroxisomes in PEX19-deficient cells is consistent with this hypothesis, this phenotype could also reflect a role for PEX19 in peroxisome division, lipid import, or peroxisome synthesis de novo. To determine whether PEX19 plays a direct role in PMP import, we used RNA interference (RNAi) to elicit a transient inhibition of PEX19 in normal human fibroblasts (Elbashir et al., 2001). A 21-bp siRNA duplex corresponding to a unique sequence of human PEX19 mRNA was transfected into 5756-TI cells and levels of PEX19 protein were followed over a span of 5 d. PEX19 protein levels were severely reduced by day two, remained low on days three and four, and rose slightly by day five (Fig. 4 A). In contrast, the levels of two other peroxins, the import receptor for peroxisomal matrix proteins, PEX5, and the integral PMP, PEX13, were relatively unaffected. Transfection with siRNA specific to PEX5 resulted in a reduction in PEX5 protein levels, with little or no change in PEX19 or

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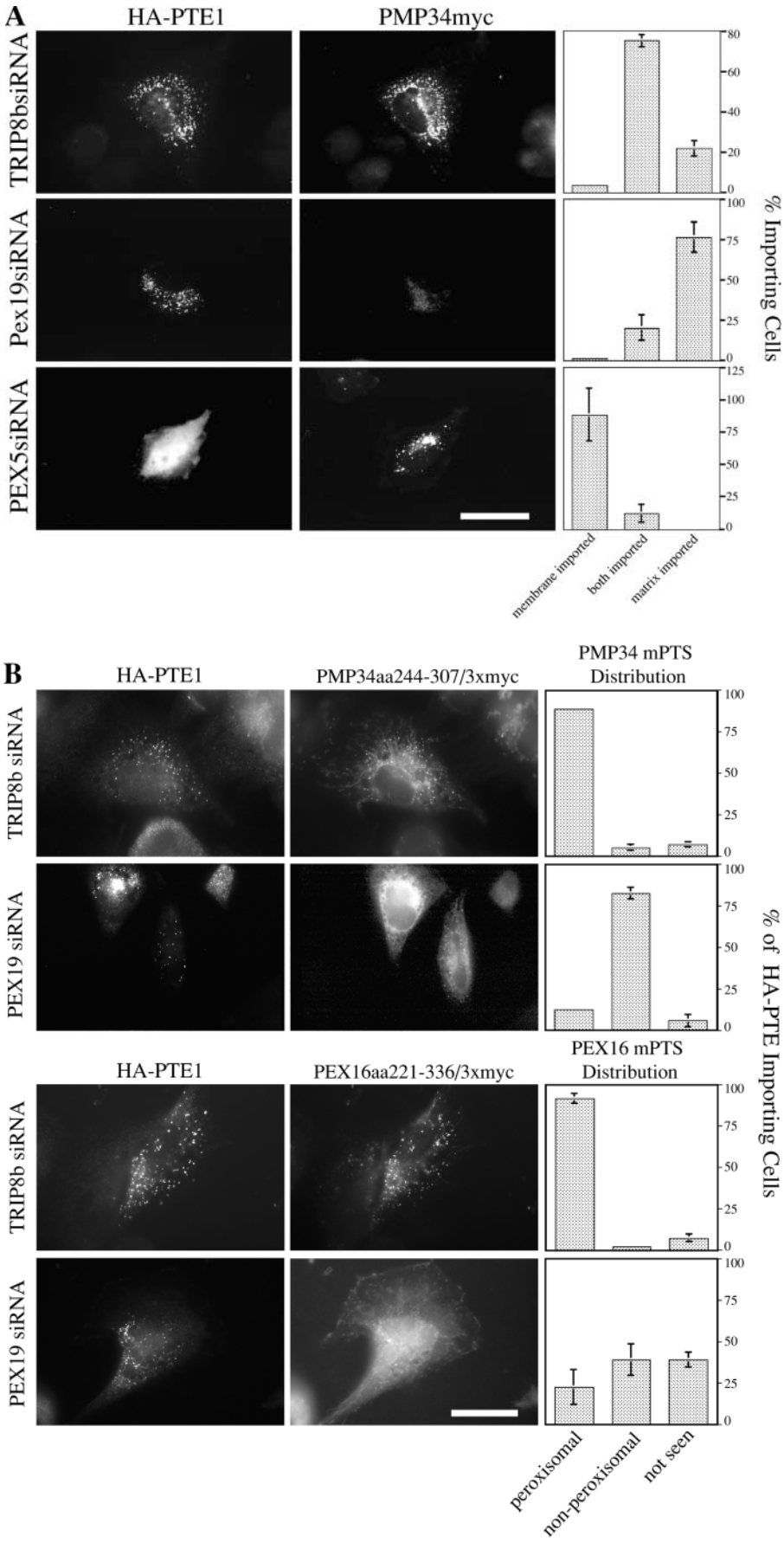

Figure 5. **Inhibition of PEX19 causes a specific PMP import defect.** (A) Inhibition of PEX19 disrupts import of PMP34 but not import of the peroxisomal matrix protein, PTE1. Wild-type human fibroblasts were subjected to electroporation with TRIP8b siRNA (top), PEX19 siRNA (middle), or PEX5 siRNA (bottom). Cells were transfected with plasmids designed to express HA-PTE1 and PMP34myc, and processed for indirect immunofluorescence with antibodies to the HA epitope (left) or the c-myc epitope (middle). Cells showing peroxisomal import were counted and scored for import of HA-PTE1 or PMP34myc (right). Means and SD of three independent trials are presented. TRIP8b siRNA $n = 314$; PEX19 siRNA $n = 303$; PEX5 siRNA $n = 67$. Bar, 15 μM. (B) Inhibition of PEX19 disrupts mPTS targeting. Wild-type human fibroblasts were subjected to electroporation with TRIP8b siRNA or PEX19 siRNA followed by transfection with plasmids designed to express HA-PTE1 and either PMP34aa244-307/3xmyc (top) or PEX16aa221-336/3xmyc (bottom). Cells were processed for immunofluorescence using anti-HA (left) or anti-myc (middle) antibodies. Cells that imported HA-PTE1 into peroxisomes were scored as to whether PMP34aa244-307/3xmyc or PEX16aa221-336/3xmyc was seen in peroxisomes, seen only in nonperoxisomal compartments, or not seen (right). Means and SD of three independent trials are presented. PMP34aa244-307/3xmyc: TRIP8b siRNA $n = 306$, PEX19 siRNA $n = 305$; PEX16aa221-336/3xmyc: TRIP8b siRNA $n = 301$, PEX19 siRNA $n = 306$. Bar, 15 μM.

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PEX13 abundance. Immunofluorescence studies of cells treated with PEX5 and PEX19 siRNAs showed numerous peroxisomes over the first 3 d (Fig. 4 B), demonstrating that PEX19 siRNA could be used to inhibit PEX19 in cells that still contained peroxisomes. Prolonged treatment with PEX19-specific siRNA caused a reduction in peroxisome number and the loss of peroxisomes from some cells (unpublished data). This finding is consistent with the known phenotype of PEX19-null cells, which lack peroxisomes.

Wild-type cells were treated with siRNAs specific for *PEX19*, *PEX5*, or a control gene, *TRIP8b* (Chen et al., 2001). 60 h later, the cells were transfected with a pair of plasmids that express a PMP, PMP34myc, and a peroxisomal matrix protein, HA-PTE1. 100 min after transfection, the cells were fixed and processed for immunofluorescence to assess the import of these marker proteins. To ensure that intact, functioning peroxisomes were present, we examined only those cells that imported either PMP34myc or HA-PTE1 (Fig. 5 A). Under this scheme the majority (75%) of *TRIP8b*-treated cells showed import of both HA-PTE1 and PMP34myc. As expected from PEX5's well-established role in peroxisomal matrix protein import, inhibition of PEX5 induced a selective deficit in peroxisomal matrix protein import, with 88% of cells importing only PMP34myc and only 12% importing HA-PTE1. The fact that some cells imported HA-PTE1 is consistent with the fact that our electroporation protocol used for siRNA delivery is less than 100% efficient (Chang et al., 1997). Inhibition of PEX19 induced the converse phenotype, a selective defect in PMP import. In *PEX19* siRNA-treated cells, the majority of cells (76%) imported only HA-PTE1. Interestingly, rather than being distributed throughout the cytosol, like HA-PTE1 in *PEX5* siRNA-treated cells, PMP34myc was simply not seen in the majority of *PEX19* siRNA-treated cells. This was likely due to degradation of PMP34myc and is consistent with both the well-documented instability of PMPs in the absence of PEX19 (Hettema et al., 2000; Sacksteder et al., 2000) and the stabilizing effect of PEX19 on PMP34 observed in Fig. 1 G.

To quantify these effects we analyzed the phenotypes of hundreds of cells from each population and from a minimum of three trials. When no attempt is made to correct for the siRNA transfection efficiencies, the data show that inhibition of PEX19 caused a fourfold reduction in PMP import, whereas inhibition of PEX5 caused an eightfold reduction in matrix enzyme import. When one does correct for the apparent siRNA transfection efficiency (88%, the inverse of the percent of cells transfected with *PEX5* siRNA that still import peroxisomal matrix proteins), *PEX19* siRNA appears to cause a >10-fold reduction in PMP import.

Next, we tested whether the observed PMP import defect in cells treated with *PEX19* siRNA would hold true for mPTSs. We treated wild-type cells with *PEX19* and *TRIP8b* siRNAs, waited 60 h, and transfected the cells with plasmids that express HA-PTE1 and either PMP34aa244-307/3xmyc or PEX16aa221-336/3xmyc. Next, we assayed for import of the mPTS-containing proteins by identifying cells that had imported HA-PTE1 and scoring the distribution of the mPTS-containing protein in those cells as either peroxisomal (if any peroxisomes were identified), nonperoxisomal (if no peroxisomes were identified), or not seen (Fig. 5 B). Scoring those cells that effectively imported HA-PTE1 ensured that only those cells with peroxisomes able to import matrix proteins were counted in this analysis of PMP import. Both PMP34aa244-307/3xmyc and PEX16aa221-336/3xmyc were imported into peroxisomes in the majority of control cells (88% and 91%, respectively). In *PEX19* siRNA-treated cells, however, both mPTS-containing proteins were imported poorly. Specifically, only 12% of cells imported PMP34aa244-307/3xmyc and only 22% imported PEX16aa221-336/3xmyc. Of special interest here is that, rather than being degraded like full-length PMP34myc, PMP34aa224-337/3xmyc was mistargeted to other compartments of the cell in the majority of *PEX19* siRNA-treated cells. This result provides direct evidence that inhibition of PEX19 function disrupts PMP import not only by destabilizing PMPs but also by preventing appropriate PMP targeting.

A second PMP import pathway

The hypothesis that PEX19 is a PMP chaperone and import receptor contrasts with previous observations that PEX19 does not bind to the mPTS of PEX3, another integral PMP (Kammerer et al., 1998; Soukupova et al., 1999; Snyder et al., 2000; Fransen et al., 2001). Therefore, we tested whether PEX19 functioned as a chaperone and import receptor for PEX3 (Fig. 6). Using the same assays that showed strong interaction between PEX19 and other mPTSs, we found that the PEX3 mPTS (PEX3aa1-50/6xmet3xmyc) is neither bound by PEX19 (Fig. 6 B) nor is its subcellular distribution affected by PEX19 or 3xNLSPEX19 (Fig. 6 C). Rather, the PEX3 mPTS was targeted to mitochondria in cells that lack peroxisomes, as reported previously for PEX3 and the PEX3 mPTS (Soukupova et al., 1999; Sacksteder et al., 2000).

These results suggested that the PEX3 mPTS might represent a second, distinct class of mPTS. To test this hypothesis, we assayed the effect of PEX19 inhibition on the activity of the PEX3 mPTS. Normal human fibroblasts (5756-TI) were treated with either *PEX19* siRNA or *TRIP8b* siRNA, transfected with plasmids that express HA-PTE1 or the PEX3 mPTS, and processed for immunofluorescence. We identified those cells that had imported HA-PTE1 and scored whether or not they had imported the PEX3 mPTS. In contrast to the results with other mPTS-containing proteins, PEX19 inhibition had no effect on the targeting of the PEX3 mPTS (Fig. 6 D). These results suggest that there are at least two classes of functionally distinct mPTSs: class 1 mPTSs, which require PEX19 function, and class 2 mPTSs, which function independently of PEX19.

Discussion

Integral PMPs are synthesized on cytosolic ribosomes and imported into peroxisomes posttranslationally. We and others have shown that polytopic PMPs contain multiple targeting signals, each of which is sufficient for insertion into

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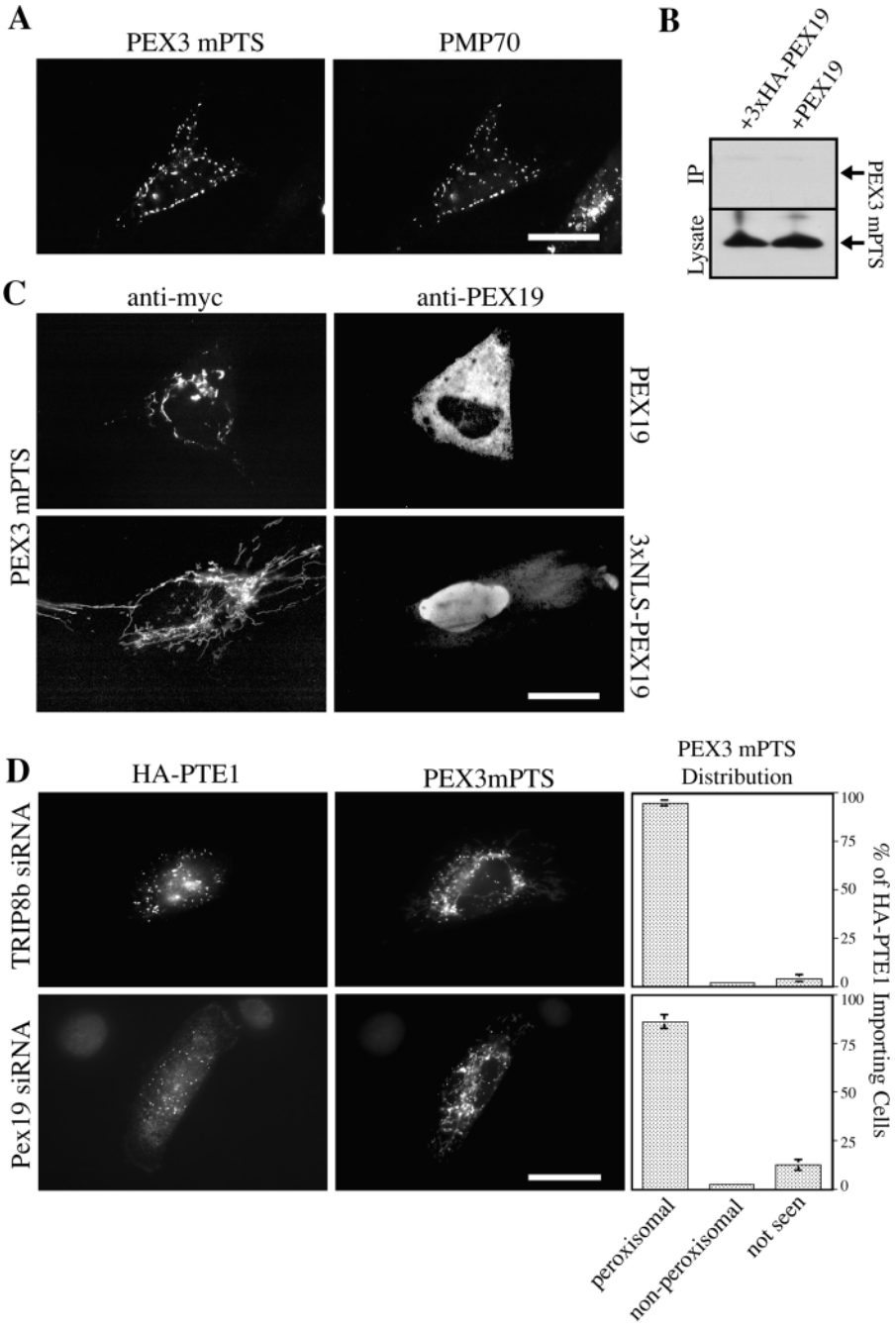

Figure 6. **The mPTS of PEX3 functions via a PEX19-independent pathway.** (A) The first 50 aa of PEX3 are sufficient for peroxisomal targeting. Wild-type human fibroblasts were transfected with a plasmid designed to express the PEX3 mPTS (PEX3aa1-50/6xmet3xmyc) and cells were processed for immunofluorescence using antibodies to the c-myc epitope (left) or PMP70 (right). Bar, 15 μM. (B) The mPTS of PEX3 does not coprecipitate with PEX19. PEX3-deficient human fibroblasts were transfected with plasmids designed to express the PEX3 mPTS and either 3xHA-PEX19 (+3xHA-PEX19) or PEX19 (+PEX19). Equal total protein from a membrane-free lysate of each cell sample was subjected to immunoprecipitation with antibodies to the HA epitope. IPs (top) and whole cell lysates (bottom) were processed for immunoblot using anti-myc antibodies. (C) The subcellular distribution of the PEX3 mPTS is not affected by PEX19. PEX3-deficient human fibroblasts were transfected with plasmids designed to express the PEX3 mPTS and either PEX19 (top) or 3xNLS-PEX19 (bottom). Cells were processed for indirect immunofluorescence using anti-myc (left) or anti-PEX19 (right) antibodies. Bar, 15 μM. (D) Inhibition of PEX19 does not affect peroxisomal targeting of the PEX3 mPTS. Wild-type human fibroblasts were subjected to electroporation with either TRIP8b siRNA (top) or PEX19 siRNA (bottom). Cells were transfected with plasmids designed to express HA-PTE1 and the PEX3 mPTS. Cells were processed for immunofluorescence using antibodies to the HA epitope (left) or the c-myc epitope (middle). Cells importing HA-PTE1 were scored as to whether the PEX3 mPTS was seen in peroxisomes, seen only in nonperoxisomal compartments, or not seen (right). Means and SD of three independent trials are presented. TRIP8b siRNA $n = 311$; PEX19 siRNA $n = 294$. Bar, 15 μM.

the peroxisomal membrane (Biermanns and Gartner, 2001; Jones et al., 2001; Wang et al., 2001; Brosius et al., 2002). To explain these features of PMP import, we proposed previously the existence of a cytosolic, PMP-specific chaperone and import receptor that (a) binds PMPs at multiple sites; (b) masks their hydrophobic transmembrane domains and prevents their misfolding, aggregation, and premature destruction; and (c) directs them to the peroxisome membrane (Jones et al., 2001).

PEX19 is a predominantly cytoplasmic, partly peroxisomal protein (Gotte et al., 1998; Matsuzono et al., 1999; Sacksteder et al., 2000); and we have shown here that PEX19 binds and stabilizes newly synthesized PMPs in the cytoplasm by interacting with their hydrophobic membrane-spanning domains. We have also shown that PEX19 binds the targeting signals of class 1 PMPs and is required for class 1 PMP import. These results demonstrate a direct and specific role for PEX19 in PMP import, that of a cytoplasmic PMP chaperone and a PMP import receptor.

This conclusion is supported by the prior observations that PEX19–PMP interactions can be detected with purified PMPs (Sacksteder et al., 2000) and in yeast two-hybrid systems (Gotte et al., 1998; Sacksteder et al., 2000; Snyder et al., 2000; Fransen et al., 2001), as well as the observation that PEX19 binds to all PMPs regardless of their function (Gotte et al., 1998; Sacksteder et al., 2000; Snyder et al., 2000; Fransen et al., 2001). Furthermore, PEX19 function appears to be specific to PMP import, as transient inhibition of PEX19 has no effect on peroxisomal matrix protein import and permanent loss of PEX19 in yeast and human cells

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generates peroxisome-specific cellular phenotypes (Gotte et al., 1998; Matsuzono et al., 1999; Sacksteder et al., 2000).

In this paper, we found that PEX19 does not bind the mPTS of PEX3 and that inhibition of PEX19 has no effect on its import into peroxisomes. These results suggest that PEX3 uses a distinct type of mPTS and a distinct import mechanism from the majority of PMPs. The fact that PEX3 contains a distinct mPTS may be related to its essential role in peroxisome membrane biogenesis. In both human cells and yeast, loss of PEX3 results in the absence of detectable peroxisomes and the rapid proteolysis of integral PMPs (Hettema et al., 2000; South et al., 2000). This and other observations have fueled speculation that PEX3 might mediate peroxisome formation from some other organelle, perhaps the ER (Baerends et al., 1996; Salomons et al., 1997; Kammerer et al., 1998; Kunau and Erdmann, 1998; Titorenko and Rachubinski, 1998), or might even catalyze de novo peroxisome synthesis (South et al., 2000, 2001). These models all imply that PEX3 occupies an early and central role in peroxisome membrane biogenesis, and our observation that PEX3 is imported by a mechanism that is distinct from that of several other PMPs is consistent with this possibility. Whether PEX3 is the sole class 2 PMP remains to be determined. PEX16 is the only other PMP known to be essential for peroxisome membrane biogenesis, but PEX19 does interact with both mPTSs from PEX16 and is required for the peroxisomal import of at least one of them.

The identification of PEX19 as a chaperone and import receptor for class-1 PMPs opens several areas for future investigation. For example, does PEX19 recognize newly synthesized PMPs cotranslationally at the ribosome or does a more general chaperone mediate the transfer of nascent PMPs from the protein synthesis machinery to PEX19? Also, what is the precise nature of PEX19–mPTS interactions? The extreme hydrophobicity of the known mPTSs has retarded our development of a quantitative in vitro PEX19–mPTS interaction assay, but such assays are clearly needed before we can truly understand the PEX19–mPTS interaction. The observation that a small fraction of PEX19 is found at peroxisome membranes (Sacksteder et al., 2000; Snyder et al., 2000) suggests that PEX19, together with other factors, might also participate in the insertion of PMPs into the peroxisome membrane. Of chief interest will be the improvement of our understanding of the relationship between PEX19 and PEX3.

It should be noted that some previous works have claimed that PEX19 cannot function as either a cytosolic chaperone or a PMP import receptor. Our results allow a fresh interpretation of many of the data used to support this opposing conclusion. Snyder et al. (2000) claimed that PEX19 cannot function as a cytosolic chaperone for newly synthesized PMPs. They based this conclusion on their inability to detect PEX19–PMP interactions in steady-state cross-linking experiments from cytosolic fractions. Aside from the general problems inherent in making conclusions solely from negative results, Snyder et al. (2000) failed to consider the differences in the steady-state abundance of PMPs in the cytoplasm as opposed to their steady-state abundance in the peroxisome membrane. This difference reflects the short half-time of import for PMPs (~3 min; Imanaka et al., 1996) relative to their half-life (~5 h; Fig. 1 G) and may be sufficient to explain the Snyder et al. (2000) result.

Reports have also claimed that PEX19 cannot function as an import receptor for newly synthesized PMPs (Snyder et al., 2000; Fransen et al., 2001). This claim is based largely on the observations that PEX19 does not bind all known mPTSs and that PEX19 has been observed to bind PMPs at locations distinct from known mPTSs. However, without additional information, this data can only be interpreted as evidence against a role for PEX19 as an import receptor if one assumes (a) that there is only one PMP import mechanism and therefore only one class of mPTS; and (b) that PMPs contain one and only one mPTS. Both of these assumptions are problematic. The first assumption flies in the face of many precedents in the field of cell biology, including the existence of two peroxisomal targeting signals and receptors for peroxisomal matrix proteins, and is refuted by the finding of two distinct classes of mPTSs in this paper. It is also now clear that many PMPs contain multiple mPTSs. Thus, the binding of PEX19 to a region of a PMP other than a known mPTS may simply suggest the presence of an additional, not yet identified, mPTS. As for the reported discordance between PEX19 binding sites and mPTSs in the yeast PMPs PEX10, PEX13, PEX22, and PEX17 (Snyder et al., 2000), and the human PMP PEX12 (Fransen et al., 2001), this discordance is based only on uncontrolled, negative results from two-hybrid assays using large protein fusions of unknown integrity. Thus, it is unclear whether these proteins lack a class 1 mPTS and are imported independently of PEX19 or whether more rigorous investigations would reveal them to also be class I PMPs. We are hopeful that the siRNA assays developed here for mammalian cells, or similar assays using temperature-sensitive mutants in yeast, will serve to distinguish between those PMPs whose import is PEX19 dependent and those whose import is PEX19 independent.

Another argument used to claim that PEX19 is not an import receptor for PMPs is that PEX19 sometimes binds to fragments of proteins or mutations thereof that do not function as mPTSs (Snyder et al., 2000; Fransen et al., 2001). However, this argument is also flawed, for it assumes (a) that the binding of a PMP to its import receptor is sufficient to mediate all facets of its localization, including both its targeting to the peroxisome surface and its insertion into the peroxisome membrane; and (b) that there is only one form of PEX19–PMP interaction. There is no evidence to support the first assumption, nor is there any a priori basis for this assumption. In fact, the mechanistic differences between import receptor-ligand binding and membrane protein insertion processes provide a compelling a priori basis for the opposite conclusion: correct PMP targeting likely requires mPTSs that possess multiple functional domains, only one of which mediates its binding to its cognate import receptor. For instance, other functional domains might mediate interaction with factors at the peroxisome surface or might be required for proper insertion in the peroxisome membrane. Disruption of these elements during a mutation analysis study would result in a failure to import, although receptor binding might be maintained. As for the second assumption, it seems likely that an import receptor, in addition to its interactions with its cargo, would also interact with factors at

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the peroxisome membrane required for its appropriate docking and the subsequent steps of membrane protein insertion. Therefore, the assumption that all of the interactions of a PMP import receptor must be with cargo molecules is unlikely to be true.

In summary, there is no credible evidence or argument that PEX19 does not or cannot function as a cytosolic chaperone for newly synthesized PMPs or as an import receptor for PMPs. Rather, the data presented in this paper offer significant mechanistic evidence that PEX19 does function as bifunctional chaperone/import receptor for class I PMPs. In addition, the data presented here also suggest the existence of a second, PEX19-independent PMP import pathway.

Materials and methods

Plasmids

The plasmids pcDNA3-PMP34aa244-307/3xmyc, pcDNA3-PMP34aa254-307/3xmyc, pcDNA3-PMP34aa260-307/3xmyc, pcDNA3-PMP34aa270-307/3xmyc, pcDNA3-PMP34aa244-303/3xmyc, pcDNA3-PMP34aa244-291/3xmyc, pcDNA3-PMP34aa254-291/3xmyc, pcDNA3-PMP34aa254-303/3xmyc, p3xNLS-PEX19 (Jones et al., 2001), pcDNA3-ΔN180-PEX11βmyc, pcDNA3-ΔC535PMP70myc, pcDNA3-ΔC598PMP70myc, pcDNA3-PMP34myc, pcDNA3-PEX19 (Sacksteder et al., 2000), pNmyc-PTE1 (Jones et al., 1999), and pcDNA3-PEX11βmyc (Schrader et al., 1998) have been described. New plasmids were generated by PCR amplification of the appropriate ORF region with primers that append a 5′ Asp718 site, a 5′ start codon where necessary, and either a 3′ BamHI or 3′ BglII site. PCR products were then inserted into the corresponding sites in the base vectors pcDNA3/3xmyc (Geisbrecht et al., 1998), pcDNA3/13xmyc (Jones et al., 2001), or pcDNA3/6xmet3xmyc. The plasmid pcDNA3/6xmet3xmyc is a modified form of pCDNA3/3xmyc designed to express proteins upstream of and in fusion with the sequence NH_3-GSMMGMMKMMRS-COOH followed by three consecutive c-*myc* epitopes. For mutagenesis in the plasmids pcDNA3-PMP34aa244-307EK289-290LL/3xmyc and pcDNA3-PMP34aa244-307KR300-301EE/3xmyc, the 3′ primers 5′-CCCGGATCC-GTGTTGGTGTGCACGCTTAGCCCCATAACTGTGAAGGTGGCAGCTGTC-AGT**AA**C**AG**ATAAACAAGGAAC-3′ and 5′-CCCGGATCCGTGTTGGTGT-GC**TT**C**C**TCCAGCCCCATAACTGTG-3′, respectively, were used (BamHI sites underlined, altered bases in bold). The plasmid pcDNA3-PMP34aa244-307LMF283-285KKK/3xmyc was made using the 5′ primer 5′-CTGCT-GCAGACAGTCCTCACTGCTGCT**AAG**AA**G**A**AG**CTTGTTTATGAG-3′ (PstI site underlined, altered bases in bold) and a 3′ primer with a BamHI site and insertion between the PstI and BamHI sites of pcDNA3-PMP34aa224-307/3xmyc. Inserts for the plasmids pcDNA3-PMP34aa270-307KR300-301EE/3xmyc, pcDNA3-PMP34aa270-307LMF283-285KKK/3xmyc, and pcDNA3-PMP34aa270-307EK289-290LL/3xmyc were created by amplification from the above palsmids. All constructs were sequenced to ensure that no unintended mutations were introduced.

Cell lines, transfections, and immunofluorescence studies

The cell lines used in this paper were wild-type human fibroblasts (GM 5756-TI), the PEX3-deficient fibroblast line PBD400-TI, and the PEX19-deficient fibroblast line PBD399-TI. These are immortalized derivatives of 5756-T, PBD400-T (South et al., 2000), and PBD399-T (Sacksteder et al., 2000) cells, and were generated by stable transfection with a vector that constitutively expresses human telomerase (pBabePuro/hTERT; Hahn et al., 1999). Cells were cultured, transfected, and processed for immunofluorescence as described (Chang et al., 1997). We used the following antibodies: an anti–c-*myc* mAb from the mouse hybridoma line 1-9E10 (Roche), sheep anti-PMP70 antibodies (South et al., 2000), rabbit anti-PEX19 antibodies (Sacksteder et al., 2000), anti-catalase antibodies (The Binding Site), and polyclonal anti-HA antibodies (Santa Cruz Biotechnology, Inc.). Fluorescent secondary antibodies used either FITC or Texas red fluorochromes and were from Jackson ImmunoResearch Laboratories.

Immunofluorescence images were obtained on an BH2-RFCA microscope (Olympus) with an Olympus SplanApo 60x 0.40 oil objective and a Sensicam QE (Cooke) digital camera using IPLab 3.6.3 software (Scanalytics, Inc.) at room temperature. Images were imported into Photoshop 7.0 software (Adobe Systems, Inc.) for figure use, and contrast was adjusted to approximate the original IPLab image.

Immunoblots, cell lysates, immunoprecipitations, pulse-chase studies

We used the Bradford protein assay (Bio-Rad Laboratories) for protein quantification and Complete™ protease inhibitor cocktail (Roche Diagnostics) for protease inhibition. For immunoprecipitation experiments, tubes were pretreated with 10 mg/ml BSA and preclear was with 15 μl of protein G agarose for 30 min at 4°C. Immunoblots were performed using standard protocols and the following antibodies: rabbit anti-PEX19 antibodies (Sacksteder et al., 2000), rabbit anti-PEX5 antibodies (Dodt et al., 1995), rabbit anti-c-*myc* antibodies (Santa Cruz Biotechnology, Inc.), and mouse monoclonal anti–VSV-G antibodies obtained from C. Machamer (The Johns Hopkins University School of Medicine). Rabbit anti-PEX13 antibodies were generated against the COOH-terminal 13 aa of human PEX13.

For comparison of cytosolic PMP levels, PEX3-deficient PBD400-TI cells were transfected with the appropriate PMP expression plasmid and pcDNA3-PEX19. PEX19-deficient PBD399-TI cells were transfected with the appropriate PMP expression plasmid and vector alone. 1 d later, cells were washed in PBS, lysed by harsh resuspension in 400 μl hypotonic lysis buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA, protease inhibitors), and centrifuged at 200,000 *g* for 30 min. Equal amounts of total protein from supernatant were analyzed by immunoblot.

For immunoprecipitations, PBD400-TI cells were transfected with the appropriate PMP or mPTS expression plasmid and either pN3xHA-PEX19 or pcDNA3-PEX19. Cells were washed, lysed, and centrifuged as in the previous paragraph. Equal total protein from each supernatant was brought to a final volume of 1 ml with PBS. Lysates were precleared and samples were incubated for 3 h at 4°C with 15 μl of anti-HA mAb agarose beads (Santa Cruz Biotechnology, Inc.). Beads were washed three times in PBS and PMPs were detected by immunoblot.

For the determination of PEX19's affect on the stability of PMP34/13xmyc, *PEX3*-deficient PBD400-TI cells were transfected with pcDNA3-PMP34/13xmyc and pcDNA3-PEX19, whereas *PEX19*-deficient PBD399-TI cells were transfected with pcDNA3-PMP34/13xmyc and vector alone. Cells were divided into five equal fractions, incubated overnight in complete medium, incubated for 1 h in methionine-free medium, pulsed for 15 min with 2 ml of methionine-free medium containing 0.5 mCi ^{35}S-labeled methionine per fraction, and chased with excess cold methionine. Cells were washed in cold PBS, lysed, and centrifuged as above. The entire supernatant from each sample was brought to 1 ml with PBS plus 0.5% Triton X-100. Lysates were precleared then incubated for 3 h at 4°C with 15 μl of anti-myc antibody agarose beads (Santa Cruz Biotechnology, Inc.). Beads were washed three times in PBS plus 0.5% Triton X-100 and analyzed by autoradiography and immunoblot.

For the detection of PEX19–PMP interaction during PMP biogenesis, *PEX19*-deficient PBD399-TI cells were made to stably express 3xHA-PEX19 by transfection with pN3xHA-PEX19 and selection with 400 μg/ml G418. Cells were transfected with pcDNA3-PMP34/13xmyc, separated into eight equal fractions, incubated overnight in complete medium, incubated in methionine-free medium for 1 h, pulsed for 10 min with 0.66 mCi per sample, and chased with excess methionine. Cells were washed twice in ice-cold PBS and resuspended in cold STE (250 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA, protease inhibitors). Cells were lysed using a ball bearing homogenizer and centrifuged at 3,000 *g* for 5 min. Supernatants were centrifuged at 25,000 *g* for 30 min and the resulting supernatants were diluted 1:1 in PBS and centrifuged at 200,000 *g* for 30 min. The resulting supernatants were precleared and the samples were incubated with 15 μl of anti-HA antibody beads for 16 h at 4°C. Beads were pelleted, washed in PBS, resuspended in 100 μl of release buffer (1% SDS, 50 mM Tris, pH 7.5, and 1 mM EDTA), and heated in boiling water for 10 min. The beads were pelleted and the supernatant was brought to 1 ml in 2% BSA, 50 mM Tris, pH 7.5, 1 mM EDTA. Samples were incubated with 15 μl of anti-myc antibody beads for 3 h at 4°C. The beads were pelleted, washed once in 0.1% SDS, 2% BSA, 50 mM Tris, pH 7.5, 1 mM EDTA, twice in PBS plus 1% Triton X-100, and analyzed by autoradiography and immunoblot.

Studies using siRNA-mediated inhibition

The following RNA oligonucleotide pairs (Dharmacon Research, Inc.) were used in the creation of siRNA duplexes according to the manufacturer's instructions: *PEX19*, 5′-GAGAUCGCCAGGAGACACUdTdT-3′, 5′-AGUGUCUCCU-GGCGAUCUCdTdT-3′; *PEX5*, 5′-AGAAGCUAC-UCCCAAAGGCdTdT-3′, 5′-GCC-UUUGGGAGUAGCUUCUdTdT-3′; *TRIP8b*, 5′-GCAGGGAAAAGGCUCUAG-GdTdT-3′, 5′-CCUAGAGC-CUTTTCCCUGCdTdT-3′. 50 μl of a 20 μM RNA duplex stock in annealing buffer (100 mM KAc, 30 mM Hepes-KOH, pH7.4, and 2 mM MgAc) was mixed with normal human fibroblasts (GM 5756-TI) from a confluent 75 cm^2 flask in 500 ml Hepes buffered saline (21 mM Hepes, pH 7.15, 137

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mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM dextrose). Cells were electroporated (model BTX ECM600; Genetronics) at 220 V, 1,500 μF, 129 Ω. For characterization of siRNA effect on cell phenotype, cells were grown for 5 d after siRNA treatment. Every 24 h, samples of cells were either fixed in 3% formaldehyde or stored at –20°C for study by immunofluorescence or immunoblot.

For assay of peroxisomal protein import in siRNA-treated cells, cells were treated a second time with siRNA at 16 h after initial treatment to optimize treatment efficiency. At 60 h after initial siRNA treatment, cells were transfected with pNHA-PTE1 and either pcDNA3-PMP34myc, pcDNA3-PMP34aa244-307/3xmyc, pcDNA3-PEX16aa221-336/3xmyc, or pcDNA3-PEX3aa1-50/6xmet3xmyc. 100 min after this transfection cells were processed for immunofluorescence. For comparison of PEX19 and PEX5 siRNA effects on import, all cells showing import of either marker were scored as to whether they imported HA-PTE1, PMP34myc, or both. For the comparison of PEX19 siRNA effects on mPTS targeting, cells importing HA-PTE1 were scored as to whether the mPTS-containing protein was imported into peroxisomes, was only seen in nonperoxisomal compartments, or was not seen. This methodology ensured that only those cells with intact, import-competent peroxisomes were scored.

This work was supported by grant DK59479 from the National Institutes of Health to S.J. Gould.

Submitted: 25 April 2003
Accepted: 15 October 2003

References

Abe, I., and Y. Fujiki. 1998. cDNA cloning and characterization of a constitutively expressed isoform of the human peroxin Pex11p. *Biochem. Biophys. Res. Commun.* 252:529–533.

Adams, G.A., and J.K. Rose. 1985. Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. *Cell.* 41:1007–1015.

Baerends, R.J.S., S.W. Rasmussen, R.E. Hilbrands, M. van der Heide, K.N. Faber, P.T.W. Reuvekamp, J.A.K.W. Kiel, J.M. Cregg, I.J. van der Klei, and M. Veenhuis. 1996. The Hansenula polymorpha PER9 gene encodes a peroxisomal membrane protein essential for peroxisome assembly and integrity. *J. Biol. Chem.* 271:8887–8894.

Biermanns, M., and J. Gartner. 2001. Targeting elements in the amino-terminal part direct the human 70-kDa peroxisomal integral membrane protein (PMP70) to peroxisomes. *Biochem. Biophys. Res. Commun.* 285:649–655.

Brosius, U., T. Dehmel, and J. Gartner. 2002. Two different targeting signals direct human peroxisomal membrane protein 22 to peroxisomes. *J. Biol. Chem.* 277:774–784.

Chang, C.C., W.H. Lee, H.W. Moser, D. Valle, and S.J. Gould. 1997. Isolation of the human PEX12 gene, mutated in group 3 of the peroxisome biogenesis disorders. *Nat. Genet.* 15:385–388.

Chang, C.C., S. South, D. Warren, J. Jones, A.B. Moser, H.W. Moser, and S.J. Gould. 1999. Metabolic control of peroxisome abundance. *J. Cell Sci.* 112: 1579–1590.

Chen, S., M.C. Liang, J.N. Chia, J.K. Ngsee, and A.E. Ting. 2001. Rab8b and its interacting partner TRIP8b are involved in regulated secretion in AtT20 cells. *J. Biol. Chem.* 276:13209–13216.

Dodt, G., N. Braverman, C. Wong, A. Moser, H.W. Moser, P. Watkins, D. Valle, and S.J. Gould. 1995. Mutations in the PTS1 receptor gene, PXR1, define complementation group 2 of the peroxisome biogenesis disorders. *Nat. Genet.* 9:115–124.

Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 411:494–498.

Ellis, R.J., and S.M. Hemmingsen. 1989. Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem. Sci.* 14:339–342.

Fransen, M., T. Wylin, C. Brees, G.P. Mannaerts, and P.P. Van Veldhoven. 2001. Human pex19p binds peroxisomal integral membrane proteins at regions distinct from their sorting sequences. *Mol. Cell. Biol.* 21:4413–4424.

Geisbrecht, B.V., C.S. Collins, B.E. Reuber, and S.J. Gould. 1998. Disruption of a PEX1-PEX6 interaction is the most common cause of the neurologic disorders Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. *Proc. Natl. Acad. Sci. USA.* 95:8630–8635.

Gotte, K., W. Girzalsky, M. Linkert, E. Baumgart, S. Kammerer, W.-H. Kunau, and R. Erdmann. 1998. Pex19p, a farnesylated protein essential for peroxisome biogenesis. *Mol. Cell. Biol.* 18:616–628.

Gould, S.J., and D. Valle. 2000. The genetics and cell biology of the peroxisome biogenesis disorders. *Trends Genet.* 16:340–344.

Hahn, W.C., C.M. Counter, A.S. Lundberg, R.L. Beijersbergen, M.W. Brooks, and R.A. Weinberg. 1999. Creation of human tumour cells with defined genetic elements. *Nature.* 400:464–468.

Hettema, E.H., W. Girzalsky, M. van Den Berg, R. Erdmann, and B. Distel. 2000. *Saccharomyces cerevisiae* pex3p and pex19p are required for proper localization and stability of peroxisomal membrane proteins. *EMBO J.* 19:223–233.

Imanaka, T., Y. Shiina, T. Hashimoto, and T. Osumi. 1996. Insertion of the 70-kDa peroxisomal membrane protein into peroxisomal membranes in vivo and in vitro. *J. Biol. Chem.* 271:3706–3713.

Jones, J.M., K. Nau, M.T. Geraghty, R. Erdmann, and S.J. Gould. 1999. Identification of peroxisomal acyl-CoA thioesterases in yeast and humans. *J. Biol. Chem.* 274:9216–9223.

Jones, J.M., J.C. Morrell, and S.J. Gould. 2001. Multiple distinct targeting signals in integral peroxisomal membrane proteins. *J. Cell Biol.* 153:1141–1150.

Kammerer, S., A. Holzinger, U. Welsh, and A.A. Roscher. 1998. Cloning and characterization of the gene encoding the human peroxisomal assembly protein Pex3p. *FEBS Lett.* 429:53–60.

Kunau, W.H., and R. Erdmann. 1998. Peroxisome biogenesis: back to the endoplasmic reticulum? *Curr. Biol.* 8:R299–R302.

Lazarow, P.B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489–530.

Matsuzono, Y., N. Kinoshita, S. Tamura, N. Shimozawa, M. Hamasaki, K. Ghaedi, R.J. Wanders, Y. Suzuki, N. Kondo, and Y. Fujiki. 1999. Human PEX19: cDNA cloning by functional complementation, mutation analysis in a patient with Zellweger syndrome, and potential role in peroxisomal membrane assembly. *Proc. Natl. Acad. Sci. USA.* 96:2116–2121.

Reguenga, C., M.E. Oliveira, A.M. Gouveia, C. Eckerskorn, C. Sa-Miranda, and J.E. Azevedo. 1999. Identification of a 24 kDa intrinsic membrane proteins from mammalian peroxisomes. *Biochim. Biophys. Acta.* 1445:337–341.

Sacksteder, K.A., J.M. Jones, S.T. South, X. Li, Y. Liu, and S.J. Gould. 2000. PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. *J. Cell Biol.* 148:931–944.

Salomons, F., I.J. van der Klei, A. Kram, W. Harder, and M. Veenhuis. 1997. Brefeldin A interferes with peroxisomal protein sorting in the yeast Hansenula polymorpha. *FEBS Lett.* 411:133–139.

Schrader, M., B.E. Reuber, J.C. Morrell, G. Jimenez-Sanchez, C. Obie, T. Stroh, D. Valle, T.A. Schroer, and S.J. Gould. 1998. Expression of PEX11β mediates peroxisome proliferation in the absence of extracellular stimuli. *J. Biol. Chem.* 273:29607–29614.

Snyder, W.B., A. Koller, A.J. Choy, and S. Subramani. 2000. The peroxin Pex19p interacts with multiple, integral membrane proteins at the peroxisomal membrane. *J. Cell Biol.* 149:1–7.

Soukupova, M., C. Sprenger, K. Gorgas, W.H. Kunau, and G. Dodt. 1999. Identification and characterization of the human peroxin PEX3. *Eur. J. Cell Biol.* 78:357–374.

South, S.T., K.A. Sacksteder, X. Li, Y. Liu, and S.J. Gould. 2000. Inhibitors of COPI and COPII do not block PEX3-mediated peroxisome synthesis. *J. Cell Biol.* 149:1345–1360.

South, S.T., E. Baumgart, and S.J. Gould. 2001. Inactivation of the endoplasmic reticulum protein translocation factor, Sec61p, or its homolog, Ssh1p, does not affect peroxisome biogenesis. *Proc. Natl. Acad. Sci. USA.* 98:12027–12031.

Titorenko, V.I., and R.A. Rachubinski. 1998. The endoplasmic reticulum plays an essential role in peroxisome biogenesis. *Trends Biochem. Sci.* 23:231–233.

Wang, X., M.J. Unruh, and J.M. Goodman. 2001. Discrete targeting signals direct Pmp47 to oleate-induced peroxisomes in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 276:10897–10905.

Wylin, T., M. Baes, C. Brees, G.P. Mannaerts, M. Fransen, and P.P. Van Veldhoven. 1998. Identification and characterization of human PMP34, a protein closely related to the peroxisomal integral membrane protein PMP47 of *Candida boidinii*. *Eur. J. Biochem.* 258:332–338.

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