

PEX19 Binds Multiple Peroxisomal Membrane Proteins, Is Predominantly Cytoplasmic, and Is Required for Peroxisome Membrane Synthesis

Katherine A. Sacksteder, Jacob M. Jones, Sarah T. South, Xiaoling Li, Yifei Liu, and Stephen J. Gould

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. Peroxisomes are components of virtually all eukaryotic cells. While much is known about peroxisomal matrix protein import, our understanding of how peroxisomal membrane proteins (PMPs) are targeted and inserted into the peroxisome membrane is extremely limited. Here, we show that PEX19 binds a broad spectrum of PMPs, displays saturable PMP binding, and interacts with regions of PMPs required for their targeting to peroxisomes. Furthermore, mislocalization of PEX19 to the nucleus leads to nuclear accumulation of newly synthesized PMPs. At steady state,

PEX19 is bimodally distributed between the cytoplasm and peroxisome, with most of the protein in the cytoplasm. We propose that PEX19 may bind newly synthesized PMPs and facilitate their insertion into the peroxisome membrane. This hypothesis is supported by the observation that the loss of PEX19 results in degradation of PMPs and/or mislocalization of PMPs to the mitochondrion.

Key words: Zellweger syndrome • organelle • peroxisome biogenesis receptor • protein import

Introduction

The elaborate architecture of eukaryotic cells allows for the concentration of integrated biochemical functions, the segregation of competing metabolic processes, and the creation of unique chemical environments. How the cell assembles and maintains this complex array of subcellular organelles is a central issue in cell biology. It is also a critical subject of evolutionary biology given that the genesis of each organelle represents a watershed event in the evolution of eukaryotic life. The peroxisome is a ubiquitous organelle of eukaryotic cells that participates in a wide variety of metabolic processes (Wanders and Tager, 1998). However, the mechanism of peroxisome biogenesis has yet to be elucidated, and there is no cogent model for the genesis of this organelle.

The formation of peroxisomes must involve the generation of the peroxisome membrane, the targeting and insertion of peroxisomal membrane proteins (PMPs)¹ into this membrane, and the transport of peroxisomal matrix proteins to and across this same bilayer. Of these processes, most is known about peroxisomal matrix protein import. Proteins destined for the peroxisome matrix are encoded by nuclear genes, synthesized in the cytoplasm (Lazarow

and Fujiki, 1985), and contain either of two peroxisomal targeting signals, PTS1 and PTS2 (Subramani, 1993). Import of these proteins is dependent upon PEX5 and PEX7, the receptors for the PTS1 and PTS2 (McCullum et al., 1993; Marzioch et al., 1994), and occurs in an ATP-dependent manner (Imanaka et al., 1987). The fact that the PTS receptors are predominantly cytoplasmic proteins (Marzioch et al., 1994; Dodt et al., 1995) has suggested models of import in which the receptors act as shuttles, delivering newly synthesized matrix proteins to the peroxisome (Braverman et al., 1995; Dodt and Gould, 1996; Hettema et al., 1999). These models also invoke a variety of other peroxins that catalyze the translocation of proteins across the peroxisome membrane and return the PTS receptors to the cytoplasm.

Although peroxisomes play critical roles in numerous metabolic pathways they are not essential for cell viability. Therefore, it is not surprising that defects in peroxisome biogenesis can cause human diseases by interfering with peroxisomal metabolic processes. Zellweger syndrome (ZS) and related diseases are characterized by defects in peroxisome biogenesis and the loss of multiple peroxisomal metabolic processes (Lazarow and Moser, 1995). Consistent with the complexity of organelle biogenesis, mutations in any of at least 12 different *PEX* genes can cause ZS. In most cases, loss of *PEX* gene activity disrupts peroxisomal matrix protein import, but has no effect on the synthesis of peroxisome membranes or import of peroxisomal membrane proteins (PMPs; Santos et al., 1988;

Address correspondence to Dr. Stephen J. Gould, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205. Tel.: (410) 955-3085. Fax: (410) 955-0215.

¹Abbreviations used in this paper: AD, activation domain; BD, binding domain; NLS, nuclear localization signal; ORF, open reading frame; PMP, peroxisomal membrane protein; ZS, Zellweger syndrome.

Chang et al., 1999b). However, there are a few rare ZS patients who lack detectable peroxisomal structures, indicating that the genes defective in these patients, *PEX3*, *PEX16*, and *PEX19*, are involved in synthesis of the peroxisome membrane or import of PMPs (Matsuzono et al., 1999; South and Gould, 1999; South, S.T., K.A. Sacksteder, S.J. Gould, manuscript in preparation). Studies in yeast have identified up to 20 *PEX* genes that are required for peroxisome biogenesis, and again, most mutants appear competent for PMP import (Hettema et al., 1999).

PMPs are synthesized on free polyribosomes and post-translationally inserted into the peroxisome membrane (Fujiki et al., 1984; Imanaka et al., 1996). However, the similarities between PMP import and peroxisomal matrix protein import appear to end there. PMPs lack recognizable PTS1- or PTS2-like features (Subramani, 1993), most *pex* mutants that disrupt matrix protein import have no effect on PMP import (Chang et al., 1999b), and PMP import appears to be ATP-independent (Diestelkötter and Just, 1993). To better understand the molecular mechanisms of PMP import and the synthesis of the peroxisome membrane, we examined the biochemical properties of *PEX19*, a protein that is required for the synthesis of peroxisome membranes in both human and yeast cells (Gotte et al., 1998; Matsuzono et al., 1999). We report here that human *PEX19* is a predominantly cytoplasmic PMP-binding protein, and we discuss the relevance of our findings for PMP import and peroxisome biogenesis.

Materials and Methods

Plasmids

The human *PEX19* open reading frame (ORF) was amplified from total human cDNA and inserted into pcDNA3 (Invitrogen Corp.) to create pcDNA3-*PEX19*. The plasmid pcDNA3-*PEX19*/C296A was created in the identical manner except that the 3' oligonucleotide encoded the C296A mutation. The WT *PEX19* ORF was also cloned into the following vectors: pPC86/LEU2, a dihybrid *GAL4* activation domain (AD) fusion vector, creating pPC86/LEU2-*PEX19*; pMBP, a modified form of the pMALc2 expression vector (New England Biolabs Inc.); and pT7-6xHis, a modified form of pET28A (Novagen, Inc.). pcDNA3-NLS/*PEX19* was constructed by appending a DNA fragment encoding the sequence MDKAEFLEAPKKRKRKVEDPRNSSVPGDPL upstream of the *PEX19* ORF in pcDNA3 (NLS underlined).

We have previously described the plasmids pcDNA3-*PEX10*, pcDNA3-*PEX10*myc (Warren et al., 1998), pcDNA3-*PEX11* β , pcDNA3-*PEX11* β myc (Schrader et al., 1998), pcDNA3-*PEX12*, pcDNA3-*PEX12*myc (Chang et al., 1997), pcDNA3-*PEX13* (Bjorkman et al., 1998), and pcDNA3-*PEX1* (Geisbrecht et al., 1999). The plasmids pcDNA3-*PEX3*, pcDNA3-*PEX14*, pcDNA3-*PMP34*, pcDNA1/Amp-*PMP70*, pcDNA3-*ALDP*, and pcDNA3-*ALDR* all contained the full-length ORF for the gene downstream of the cytomegalovirus promoter and T7 promoter in the appropriate vector. Myc tags were appended to these genes and to human *PMP22*, *PMP24*, *PEX11* α , and *PEX13* by amplification using primers designed to append Asp718 and BamHI sites at the 5' and 3' ends, respectively, and these fragments were cloned upstream of and in-frame with a 12-amino acid myc tag in the plasmid pcDNA3myc (Yahraus et al., 1996). The plasmids pJL59W-*PEX10*, pJL59W-*PEX11* β , pJL59W-*PEX12*, and pJL59W-*PEX13* contain the ORF of each gene in-frame with the *GAL4* DNA binding domain of pJL59W. Control plasmids used in the blot overlay experiments (SP6-*Tim23*, SP6-*Aac2*, SP6-*MIR1*, T7-*IBV-M*, and T7-*VSV-G*) were obtained from Drs. Robert Jensen and Carolyn Machamer (The Johns Hopkins University School of Medicine, Baltimore, MD). The plasmids pcDNA3-*PMP70*myc, pcDNA3- Δ C395*PMP70*myc, pcDNA3- Δ C476-*PMP70*myc, pcDNA3- Δ C535*PMP70*myc, pcDNA3- Δ C558*PMP70*myc, pcDNA3- Δ C578*PMP70*myc, and pcDNA3- Δ C598*PMP70*myc were created by amplifying the appropriate regions of the human *PMP70* cDNA

with oligonucleotides designed to append an Asp718 site immediately upstream of the start codon (GGTACCATG; start codon underlined) and a BamHI site after the last codon of the desired ORF. These fragments were cleaved with Asp718 and BamHI and inserted between the Asp718 and BamHI sites of pcDNA3myc. The plasmids pcDNA3- Δ N119-*PEX11* β myc, pcDNA3- Δ N180*PEX11* β myc, pcDNA3- Δ N210*PEX11* β myc, pcDNA3- Δ C231*PEX14*myc, and pcDNA3- Δ C270*PEX14*myc were all created in a similar fashion. Bacterial vectors designed to express 6xHis-*PEX14*, mutant forms of 6xHis-*PEX14*, 6xHis-PTE1, and 6xHis-XEC1 were created by transferring the coding regions of each clone into the bacterial expression vector, pT7-His (Geisbrecht et al., 1999). For all plasmids, any regions generated by PCR were sequenced to ensure the absence of any unintended mutations.

Dihybrid Analysis and Blot Overlay Assays

The yeast strain BY3168 (Vidal et al., 1996) was used for all dihybrid assays. Pairs of plasmids, one encoding the appropriate binding domain (BD) fusion, the other encoding the appropriate AD fusion, were used to transform the yeast to leucine and tryptophan prototrophy. After selection, the modified strains were incubated at 30°C for 2 d, transferred to BA-85 membranes (Schleicher and Schuell), grown for an additional 2 d, and assayed for β -galactosidase activity.

For blot overlay assays, all in vitro transcription/translation reactions were performed using TNT rabbit reticulocyte lysates (Promega). 5 μ g of 6xHis-*PEX19* and a nonspecific control protein (XEC1, a nuclear enoyl-CoA isomerase) were resolved by SDS-PAGE electrophoresis and transferred to PVDF membrane. The membrane was blocked for 2 h at room temperature in buffer A (500 mM Tris-HCl, pH 7.5, 50 mM NaCl, 100 mM sodium acetate, 100 mM KCl, 1 mM DTT, 5 mM MgCl₂, 1 mM EDTA, 0.3% Tween 20, 0.1 mM ZnCl₂, 5% milk, 0.1 M methionine). The membrane was incubated for 2 h at room temperature with 5 ml of buffer A containing ³⁵S-labeled PMPs that had been synthesized in vitro, washed, and exposed to film. For far Western analysis, membranes containing 5 μ g of each protein were blocked as before, incubated with 10 ml of buffer A containing 2 μ g of purified 6xHis-*PEX14*, washed, and probed with affinity-purified anti-*PEX14* antibodies.

For quantitative analysis of *PEX19*-*PEX14* binding, ³⁵S-labeled 6xHis-*PEX14* was synthesized in vitro, mixed with bacterially expressed 6xHis-*PEX14*, and the resulting mixture was applied at various concentrations to membranes containing 100 ng of immobilized *PEX19*. After the binding reaction, the filters were washed briefly and the amount of *PEX14* bound to *PEX19* was determined using a PhosphorImager (MacBAS; Fuji). For competition assays, a small amount of labeled *PEX14* or *PEX3* (~1–10 nM) was mixed with the indicated concentrations of purified recombinant *PEX14*, PTE1, or mutant forms of *PEX14* and incubated with immobilized *PEX19* using the blot overlay technique. The labeled PMP bound was detected using a PhosphorImager. All 6xHis-tagged proteins were expressed and purified from bacteria as described (Geisbrecht et al., 1999).

Transfections, Immunofluorescence, and Antibody Production

Cell lines were cultured and transfected by electroporation and processed for indirect immunofluorescence 4 h after transfection as described (Chang et al., 1997, 1999b). Affinity-purified *PEX19* antibodies were used for all immunofluorescence experiments. Monoclonal mouse anti-myc antibodies were obtained from the tissue culture supernatant of the hybridoma 1-9E10 (Evan et al., 1985), guinea pig anti-*PMP70* antibodies were raised against the COOH-terminal 18 amino acids of human *PMP70*, and secondary antibodies were obtained from commercial sources. Antibodies directed against *PEX19* were generated by immunization of rabbits with an MBP-*PEX19* fusion protein and were affinity-purified against immobilized 6xHis-*PEX19*. The human skin fibroblast cell line GM5756-T was used for the expression of proteins in normal human fibroblasts. The cell line PBD399, which corresponds to the same Zellweger syndrome patient cell line examined by Matsuzono et al. (1999), was used for the functional complementation studies and for assessing PMP fates in the absence of *PEX19*. The functional complementation studies were performed by transfecting PBD399 cells by electroporation as described (Chang et al., 1997) with the plasmids pcDNA3, pcDNA3-*PEX19*, and pcDNA3-*PEX19*/C296A. 3 d after transfection, the cells were processed for indirect immunofluorescence using established protocols (Dodt and Gould, 1996) and antibodies specific for catalase, which is a peroxisomal matrix protein.

Relative rescue activity was calculated as described (Chang and Gould, 1998).

Rat Liver Fractionation, Differential Centrifugation, Differential Permeabilization, and Immunoblotting

Preparation of rat liver postnuclear supernatant, separation of the peripheral nervous system by Nycodenz centrifugation and assay of marker enzymes were performed as described previously using a 15–45% Nycodenz gradient (Mihalik, 1992). For differential centrifugation analysis, a rat liver was homogenized, a postnuclear supernatant was generated, and then spun at 25,000 *g* for 30 min. This supernatant was removed and subjected to further centrifugation at 200,000 *g* for 1 h. Equal proportions of the resulting homogenate, supernatants, and pellets were processed for immunoblot with anti-PEX19 antibodies. For the differential release assay, GM5756-T fibroblasts were harvested by trypsinization, washed, and divided into seven equal fractions. The cells were gently resuspended in 100 μ l of STE (10 mM Tris, 1 mM EDTA, 250 mM sucrose) that was adjusted to various concentrations of digitonin (0, 50, 100, 200, 300, and 400 μ g/ml) or 400 μ g/ml digitonin and 1% Triton X-100. After a 10-min incubation on ice, clarified supernatants were generated by centrifugation and analyzed by immunoblot. All immunoblots were performed using HRP-conjugated secondary antibodies and chemiluminescent detection (Amersham).

Results

PEX19 Binds Multiple Integral PMPs

One approach to understanding PEX19 function is to identify those proteins with which it interacts. We used the yeast two-hybrid assay as a crude screen to identify candidate PEX19-binding proteins. Full-length human PEX19 was expressed as a GAL4 AD fusion and screened against a panel of GAL4 DNA BD fusions to all 14 known human PEX proteins. Coexpression of AD-PEX19 and BD fusions with PEX10, PEX11 β , PEX12, or PEX13 led to activation of GAL4-regulated reporter genes, shown here by LacZ filter assays (Fig. 1 A). These results were particularly intriguing because PEX10, PEX11 β , PEX12, and PEX13 are all integral PMPs, but their roles in peroxisome biogenesis differ: PEX11 β participates in the regulation of peroxisome abundance (Schrader et al., 1998); PEX13 is thought to act in matrix protein import as part of the receptor docking apparatus (Hetteema et al., 1999); and PEX10 and PEX12 act in matrix protein import downstream of receptor docking (Chang et al., 1999a). Although we failed to detect an interaction signal in strains that express AD-PEX19 and BD fusions with five other peroxin PMPs (PEX2, PEX3, PEX11 α , PEX14, and PEX16), negative results in this assay can be caused by a variety of nonspecific problems, such as poor expression, improper folding, and failure to target to the nucleus, none of which were controlled for in this study. Furthermore, it should be noted that the yeast two-hybrid assay is generally not useful for assessing interactions with integral membrane proteins (Drees, 1999) such as the PMPs tested here.

The interaction of PEX19 with a diverse array of PMPs in the yeast two-hybrid assay raised the possibility that PEX19 might be a general PMP-binding protein, perhaps with a role in PMP biogenesis. However, an analysis of PEX19–PMP interactions in a biochemical assay system would be necessary to test this hypothesis. To this end, we expressed and purified recombinant PEX19 in bacteria and tested whether it could bind a variety of integral PMPs

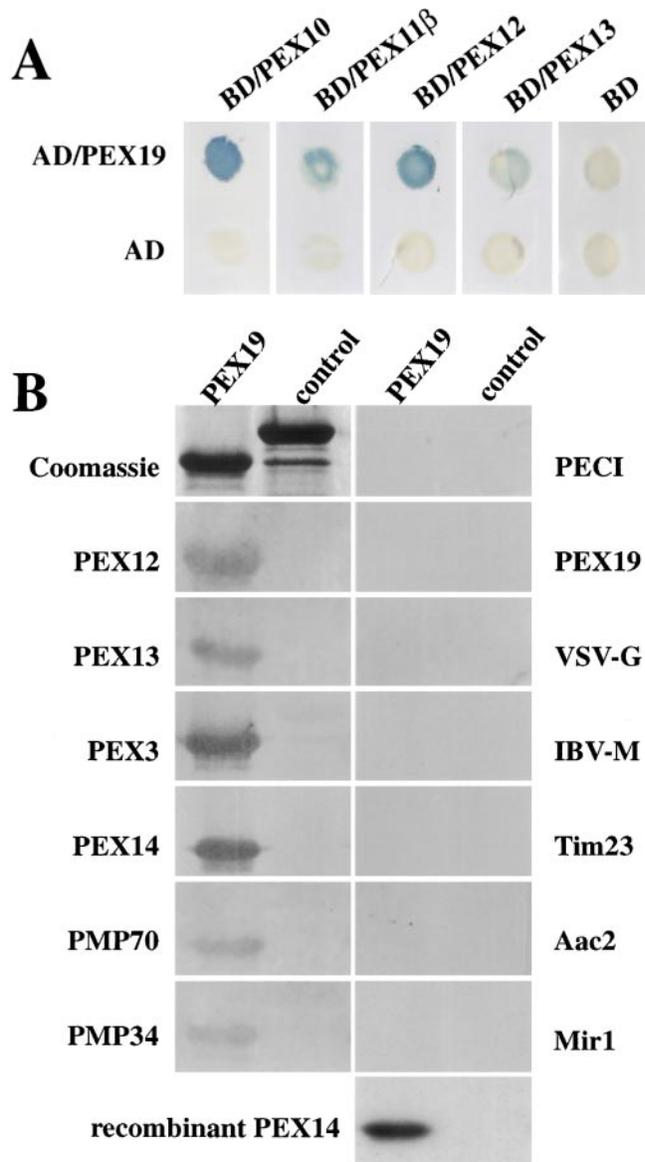


Figure 1. PEX19 displays specific PMP-binding activity. (A) Yeast strains expressing the GAL4 transcription activating domain (AD) or an AD-PEX19 fusion with the GAL4 DNA binding domain (BD) and BD-PEX10, BD-PEX11 β , BD-PEX12, and BD-PEX13 were grown on filters, lysed, and assayed for LacZ activity using the indicator substrate X-gal. (B) Purified recombinant 6xHis-PEX19 (left lanes) and 6xHis-XECI (right lanes) were resolved by SDS-PAGE and either stained with Coomassie blue (top left panel) or transferred to PVDF membranes and probed with 35 S-labeled, in vitro translated PEX12, PEX13, PEX3, PEX14, PMP70, PMP34, PEX1, PEX19, VSV-G, IBV-M, Tim23, Aac2, or Mir1. After washes, the filters were exposed to film. The bottom right panel shows a far Western blot in which a similar filter was incubated with purified recombinant PEX14, washed, probed with affinity-purified anti-PEX14 antibodies, and detected using HRP-conjugated secondary antibodies and chemiluminescent detection.

with diverse functions. Equal amounts of recombinant PEX19 and a nonspecific control protein (XECI; Geisbrecht, B.V., and S.J. Gould, unpublished observations) were separated by SDS-PAGE, transferred to membranes, and

renatured. Replicate filters were probed with seven different ^{35}S -labeled human integral PMPs at concentrations ranging from 1 to 10 nM. Recombinant PEX19 bound PEX12 and PEX13, two of the PMPs that interacted with PEX19 in the yeast two-hybrid assay, as well as to two other peroxin PMPs, PEX3 (Kammerer et al., 1998) and PEX14 (Fransen et al., 1998; Fig. 1 B). Importantly, PEX19 also bound three PMPs that do not appear to participate in peroxisome biogenesis: PMP70, a peroxisomal ATP-binding cassette transporter (Kamijo et al., 1990); PMP34, a peroxisomal solute carrier (Wylin et al., 1999; Fig. 1 B); and ALDR (data not shown), another ATP-binding cassette transporter of the peroxisome membrane (Lombard-Platet et al., 1996).

We also examined the specificity of PEX19 for integral PMPs (Fig. 1 B). We found that PEX19 does not bind peroxisomal matrix proteins, shown here by its inability to bind peroxisomal enoyl-CoA isomerase (PECI; Geisbrecht et al., 1999), nor does it bind to itself. PEX19 was also unable to bind to integral membrane proteins of other organelles, including an integral plasma membrane protein (VSV-G; Adams and Rose, 1985), an integral Golgi membrane protein (IBV-M; Bournsnel et al., 1984), and three integral mitochondrial membrane proteins (Tim23; Dekker et al., 1993), Aac2 (Lawson and Douglas, 1988), and Mir1 (Murakami et al., 1990). It should be noted that these control membrane proteins were added at approximately the same concentration as the PMPs that did bind to PEX19.

To test whether PEX19-PMP binding was direct, we expressed and purified a small amount of soluble PEX14 from bacteria (it was the only PMP that we were able to purify in soluble form). We performed the blot overlay assay using recombinant PEX14 rather than *in vitro* translated PEX14. The ability of PEX19 to bind recombinant PEX14 was determined by blotting with affinity-purified anti-PEX14 antibodies. This far Western assay revealed that recombinant PEX19 was indeed able to bind purified PEX14 in the absence of any other factors (Fig. 1 B).

The blot overlay assay was also used to examine the thermodynamic parameters of PEX19-PMP binding. Immobilized PEX19 was incubated with various concentrations of recombinant PEX14 spiked with ^{35}S -labeled PEX14, and the relative amounts bound were quantitated using a PhosphorImager (Fig. 2 A). Binding was saturated at concentrations of 3 μM and above in three independent experiments. The actual dissociation constants calculated in the three trials were 350, 800, and 500 nM, suggesting a PEX14-PEX19 dissociation constant of ~ 500 nM. To assess the specificity of PEX19 binding, we tested the ability of peroxisomal thioesterase (PTE1; Jones et al., 1999), a peroxisomal matrix protein, and PEX14 to compete ^{35}S -labeled PMPs from binding to PEX19 (Fig. 2 B). Excess unlabeled PEX14 effectively competed labeled PEX14 from PEX19, but excess PTE1 had no effect on PEX19-PEX14 binding. Specific competition was also observed when PTE1 and PEX14 were assayed for their ability to compete with labeled PEX3 for PEX19 binding.

Mislocalization of PEX19 Results in Mislocalization of Newly Synthesized PMPs

The specific binding of PEX19 to seven PMPs with diverse

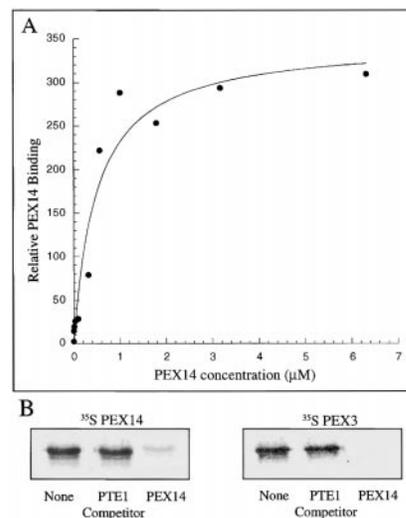


Figure 2. PEX19-PMP binding is saturable. (A) Purified 6xHis-PEX19 was separated by SDS-PAGE, transferred to PVDF membranes, and incubated with purified recombinant 6xHis-PEX14 spiked with ^{35}S -labeled 6xHis-PEX14 at various concentrations, washed, and analyzed using a PhosphorImager. The amount of PEX14 bound at each concentration was determined and plotted versus 6xHis-PEX14 concentration using Kaleida-Graph software. (B) Membranes containing 1 μg of immobilized 6xHis-PEX19 were incubated with ^{35}S -PEX14 (left) or ^{35}S -PEX3 (right) in buffer A (left lanes), buffer A containing 10 μM 6xHis-PTE1 (middle lanes), and buffer A containing 10 μM 6xHis-PEX14 (right lanes).

functions in metabolite transport (PMP70, PMP34, and ALDR), peroxisomal matrix protein import (PEX10, PEX12, and PEX14), and peroxisome membrane biogenesis (PEX3) strongly suggests that PEX19 is a broad specificity PMP-binding protein. We tested this hypothesis, as well as whether PEX19 could bind PMPs within human cells, using a completely independent assay. Specifically, we targeted PEX19 to the nucleus and asked whether this resulted in nuclear accumulation of newly synthesized PMPs. Wild-type PEX19 accumulates in the cytoplasm when overexpressed (Fig. 3 A), but a form of PEX19 containing a nuclear localization signal (NLS/PEX19) is instead targeted to the nucleus (Fig. 3, B and C). Normal human fibroblasts were cotransfected with plasmids designed to express a variety of epitope-tagged PMPs and either pcDNA3 or pcDNA3-NLS/PEX19. 4 h after transfection, the distribution of the newly synthesized PMPs was determined by immunofluorescence microscopy. As expected for a PMP, PEX14myc was imported into peroxisomes in cells cotransfected with pcDNA3. In contrast, PEX14myc was found in the nucleus of cells that were cotransfected with pcDNA3-NLS/PEX19 (Fig. 3, D-F). This PMP was never detected in the nucleus in the absence of NLS/PEX19 expression. NLS/PEX19 expression also resulted in the nuclear import of newly synthesized PMP34myc, ALDPmyc (a myc-tagged version of another nonperoxin PMP [Mosser et al., 1993, 1994]), PEX3myc, PEX11 β myc, and PEX12myc (Chang et al., 1997; Fig. 3, G-U). Similar results were observed for PMP70myc, ALDRmyc, PEX10myc (Warren et al., 1998), PEX11 α myc (Schrader

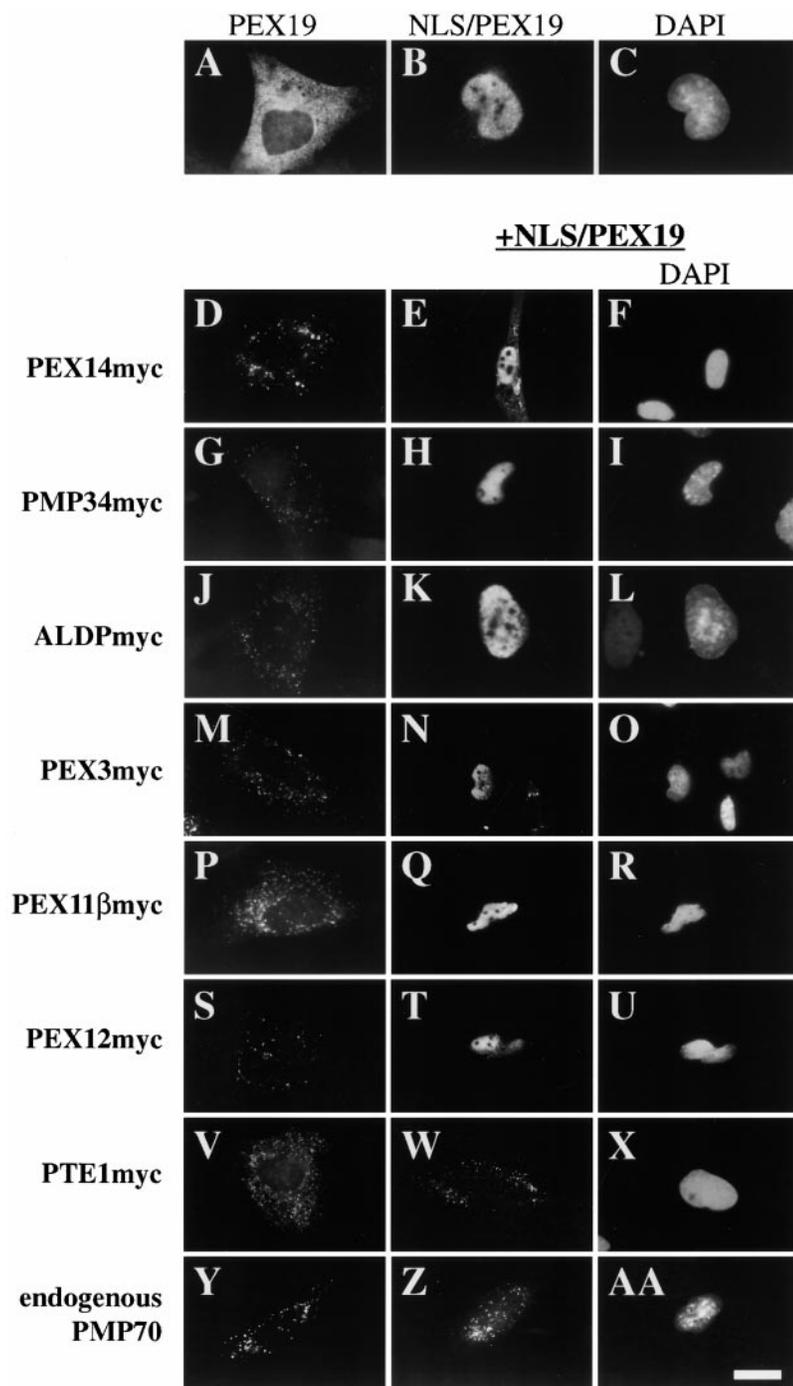


Figure 3. Targeting PEX19 to the nucleus leads to nuclear accumulation of PMPs. (A–C) Human fibroblasts expressing PEX19 (A) or NLS/PEX19 (B and C) were analyzed by indirect immunofluorescence using affinity-purified anti-PEX19 antibodies (A and B) and stained with DAPI (C). Human fibroblasts expressing the myc-tagged PMPs PEX14 (D–F), PMP34 (G–I), ALDP (J–L), PEX3 (M–O), PEX11 β (P–R), or PEX12 (S–U), or the peroxisomal matrix protein PTE1 (V–X) with pcDNA3 alone (left column) or with pcDNA3-NLS/PEX19 (middle and right columns) were analyzed by indirect immunofluorescence using monoclonal anti-myc antibodies with appropriate secondary antibodies (left and middle columns) and DAPI (right column). Fibroblasts transfected with pcDNA3 (Y) or pcDNA3-NLS/PEX19 (Z and AA) were analyzed by indirect immunofluorescence using anti-PMP70 antibodies (Y and Z) with appropriate secondary antibodies. Cells expressing NLS/PEX19 were also stained with DAPI (AA). Bar, 15 μ m.

et al., 1998), PEX13myc, PEX16myc (South and Gould, 1999), PMP24myc (Reguenga et al., 1999), and PMP22myc (Diestelkotter and Just, 1993; data not shown).

To control for the specificity of this nuclear mislocalization assay we also examined the effects of NLS/PEX19 on the distribution of PTE1, a peroxisomal thioesterase (Jones et al., 1999). PTE1 was imported into peroxisomes regardless of NLS/PEX19 expression (Fig. 3, V–X). Similar results were observed for PEI1, another peroxisomal matrix protein (data not shown). Furthermore, NLS/PEX19 did not affect the distribution of PMPs that al-

ready had been imported into peroxisomes, shown here by the presence of endogenously expressed PMP70 in vesicles with the typical morphology of peroxisomes in cells expressing NLS/PEX19 (Fig. 3, Y–AA). All together, the nuclear mislocalization assay provided evidence that PEX19 interacts with 14 PMPs of diverse functions in peroxisome biogenesis and metabolite transport, confirming all seven examples of PEX19-PMP binding obtained in the blot overlay experiments and all four examples of PEX19-PMP binding detected in the yeast two-hybrid assay.

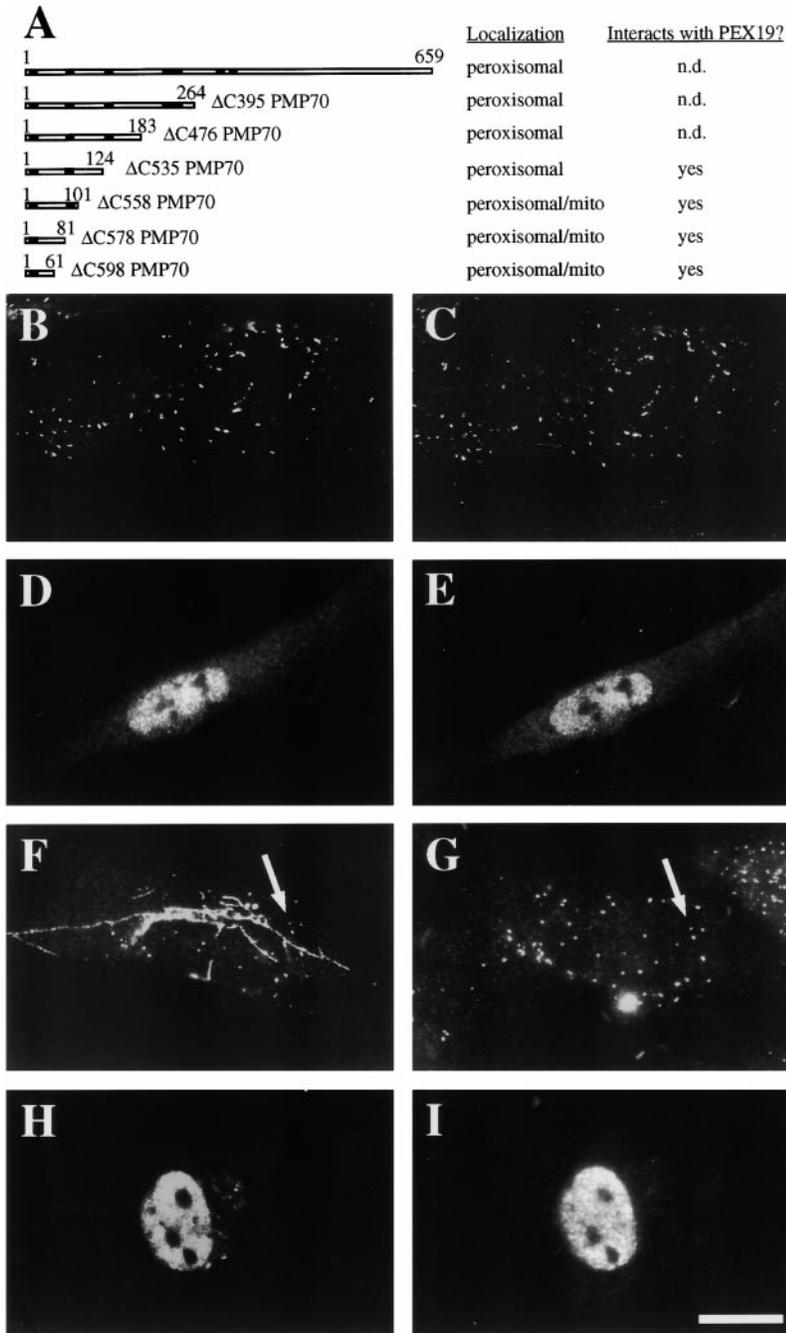


Figure 4. PEX19 binds the peroxisomal targeting element of PMP70. (A) Line diagram of PMP70 mutants. Full-length PMP70 is shown at the top, and predicted transmembrane domains are shown in black boxes. Subcellular distribution and interaction with PEX19 are indicated to the right. Normal human fibroblasts expressing the PMP70 mutant constructs Δ C535PMP70myc (B–E) or Δ C598PMP70myc (F–I) were cotransfected with either vector alone (B, C, F, and G) or pcDNA3-NLS/PEX19 (D, E, H, and I). The distribution of the PMP70 truncation mutants was determined by immunofluorescence using anti-myc antibodies (left). Cells cotransfected with vector alone were stained with antibodies to the COOH terminus of PMP70 (C and G), whereas cells cotransfected with pcDNA3-NLS/PEX19 were stained with DAPI (E and I). Bar, 15 μ M.

Targeting Elements of PMPs Retain Interaction with PEX19

The ability of PEX19 to bind PMPs of diverse function implicates PEX19 in the biogenesis of PMPs. One obvious possibility is that PEX19 may interact with regions of PMPs that are involved in targeting to peroxisomes. To test whether this might be true, we examined the regions of several human PMPs that are required for targeting to peroxisomes and tested whether they retained the ability to bind to PEX19. The integral PMPs, PMP70, PEX11 β , and PEX14, were chosen for these studies. COOH-terminal truncations of PMP70, each of which carried a COOH-terminal myc epitope tag, were expressed in human fibro-

blasts and their subcellular distributions were determined by immunofluorescence. The first 124 amino acids of PMP70 were sufficient to direct it into peroxisomes (Fig. 4, B and C). This same segment of PMP70 is efficiently misdirected to the nucleus in cells that overexpress NLS/PEX19 (Fig. 4, D and E). The first 101, 81, or 61 amino acids of PMP70 are also able to mediate peroxisomal targeting but only at reduced efficiency. These shorter pieces of PMP70 were localized to both peroxisomes and mitochondria, as shown here for Δ C598 PMP70, the smallest of the three proteins (Fig. 4, F and G). The positive identification of the tubulovesicular structures as mitochondria was confirmed by colocalization with a mitochondrial marker (Mi-

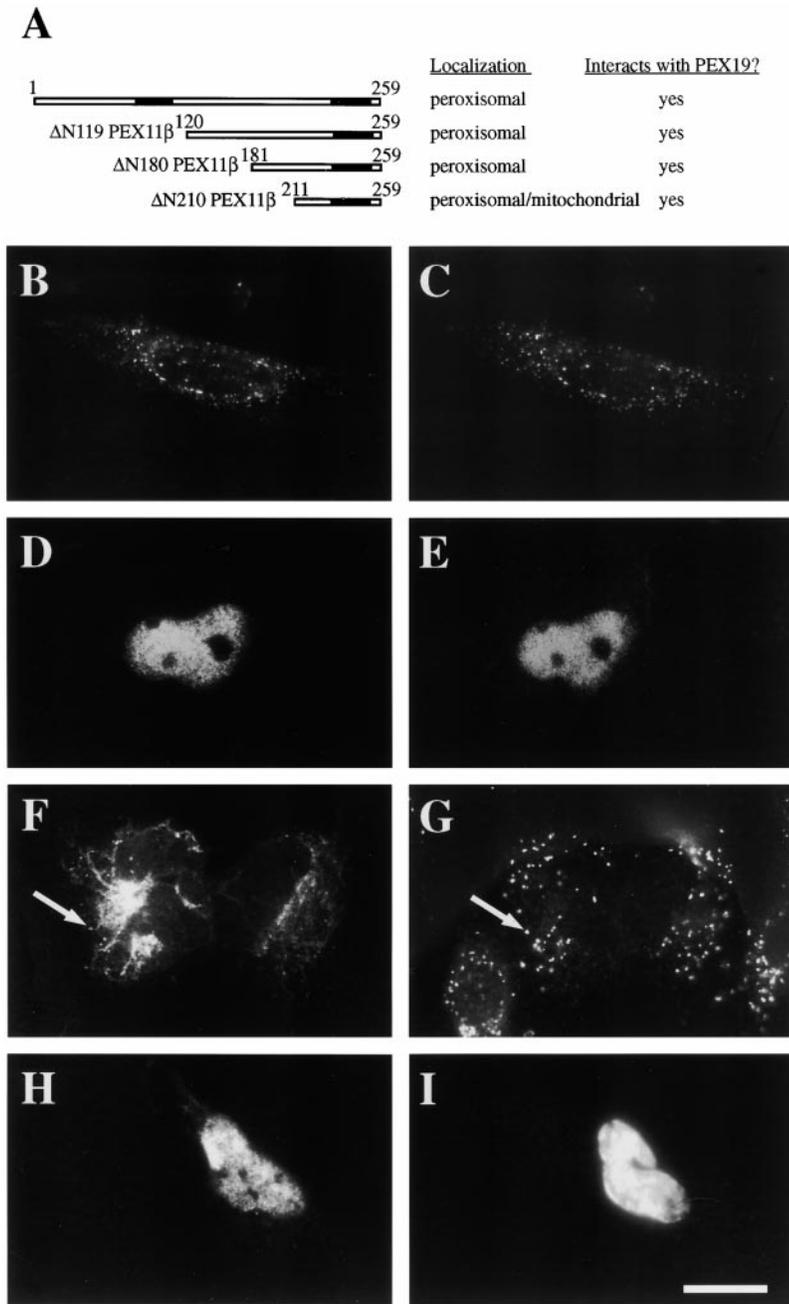


Figure 5. PEX19 binds the peroxisomal targeting element of PEX11β. (A) Line diagram of PEX11 (truncations, their subcellular distribution, and their ability to interact with PEX19 in the nuclear localization assay). Solid boxes show the position of the two transmembrane domains. (B–I) Subcellular distribution of two truncated forms of PEX11β in normal cells and in cells expressing NLS/PEX19. Normal human fibroblasts were transfected with pcDNA3-ΔN180/PEX11 (B–E) or pcDNA3-ΔN210/PEX11β (F–I) and cotransfected with a vector control (B, C, F, and G) or pcDNA3-NLS/PEX19 (D, E, H, and I). Cells were processed for indirect immunofluorescence using antibodies specific for the myc epitope tag (B, D, F, and H) and colabeled with antibodies specific for endogenously expressed PMP70 (C and G) or DAPI (E and I). Bar, 15 μm.

toTracker; data not shown). These three pieces of PMP70 retained their ability to interact with PEX19, as seen by the misdirection of ΔC598 PMP70 to the nucleus in cells overexpressing NLS-PEX19 (Fig. 4, H and I).

Similar results were observed for PEX11β. Targeting studies revealed that the COOH-terminal 78 amino acids of this 259-amino acid-long protein were sufficient for both peroxisomal localization and interaction with PEX19 (Fig. 5, A–E). The COOH-terminal 49 amino acids of PEX11β were also directed to the peroxisome, though at reduced efficiency, and also retained the ability to interact with PEX19 (Fig. 5, F–I).

These results are consistent with the hypothesis that PEX19 may bind to PMP targeting elements. However, they are qualitative in nature since they rely on the nuclear

mislocalization assay for assessing PEX19 interaction. To apply a more quantitative PEX19 binding assay to this question, we performed a limited analysis of PEX14 targeting elements. Epitope-tagged forms of PEX14 were generated and their subcellular distributions were determined by immunofluorescence. Full-length PEX14 was efficiently imported into peroxisomes, but a fragment of PEX14, containing amino acids 1–108, did not associate with peroxisomes (Fig. 6, A–E). A segment of PEX14, containing amino acids 1–147, associated with peroxisomes but only poorly, with much of the protein being imported into mitochondria (Fig. 6, F and G). The affinity of PEX19 for these three forms of PEX14 was determined by expressing and purifying each protein from bacteria and assaying its ability to compete labeled PEX14 from

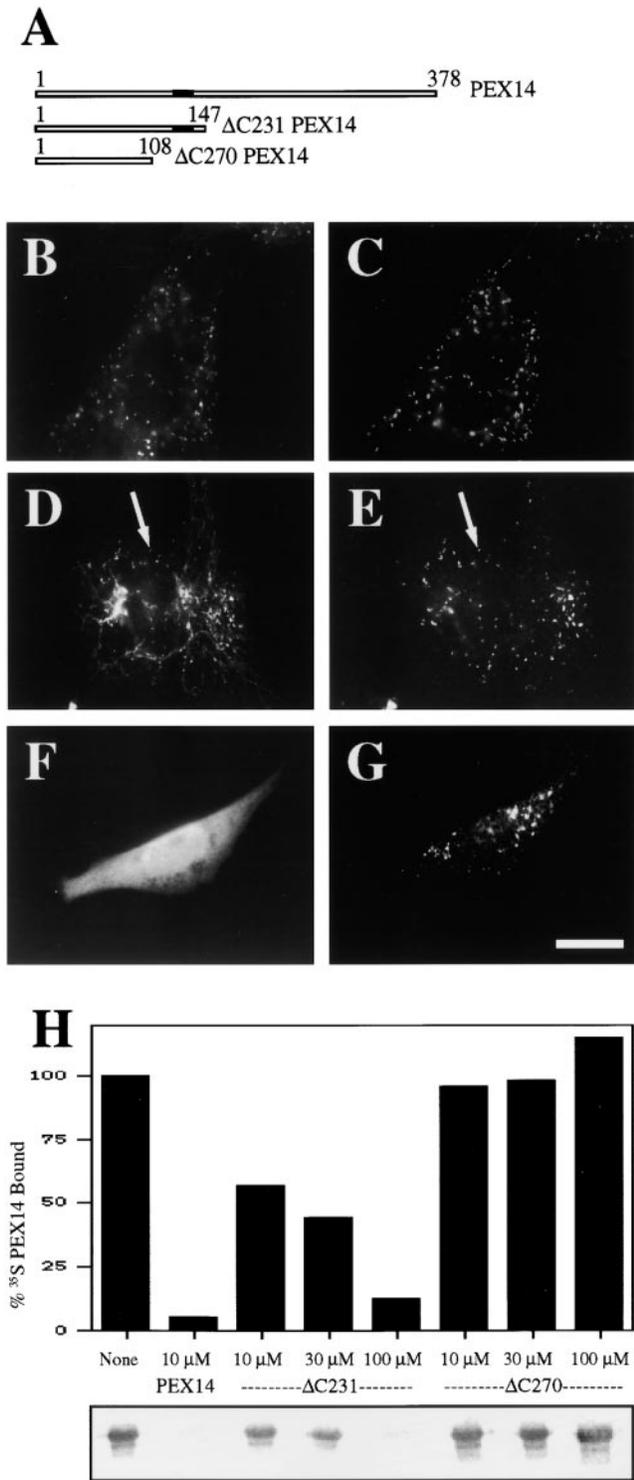


Figure 6. Analysis of PEX14 targeting and interaction with PEX19. (A) Line diagram of PEX14 truncation mutations. Solid boxes show the position of the transmembrane domain. (B–G) Normal human fibroblasts expressing PEX14myc (B and C), ΔC231PEX14myc (D and E), or ΔC270PEX14myc (F and G) were processed for indirect immunofluorescence using antibodies specific for the myc epitope tag (left) and PMP70 (right) using appropriate secondary antibodies. (H) Competition of PEX14–PEX19 binding by WT and mutant PEX14 proteins. Purified 6xHis-PEX19 was separated by SDS-PAGE, transferred to PVDF membranes, and probed with equal amounts of ³⁵S-labeled

PEX19. Unlabeled full-length PEX14 effectively displaced labeled PEX14 at a concentration of 10 μM, as expected (Fig. 6 H). The mutant containing amino acids 1–147 of PEX14, which was targeted to peroxisomes only poorly, also competed poorly: the 1–147-amino acid segment showed weak competition at 10 μM, competed 50% of the labeled PEX14 from PEX19 at a concentration of 30 μM, and reduced the amount of labeled PEX14 to 10% of the control at a concentration of 100 μM. The fragment of PEX14 that was not transported to peroxisomes, amino acids 1–108, failed to compete labeled PEX14 from PEX19 at all three concentrations. These results are consistent with the hypothesis that PMP targeting elements retain PEX19 binding. However, these results do not establish that PEX19 binds to PMP targeting signals, for we have yet to identify the targeting signals in these human PMPs.

PEX19 Is Bimodally Distributed between the Cytoplasm and Peroxisome

To better understand the biological basis of PEX19–PMP interactions we assessed the distribution of PEX19 within the cell. A rat liver postnuclear supernatant was separated by Nycodenz density gradient centrifugation, assayed for marker enzymes of peroxisomes, mitochondria, and cytosol, and blotted with affinity-purified anti-PEX19 antibodies. These experiments indicated that the majority of PEX19 was present in the cytoplasm, but that detectable levels were present in the peroxisomal fractions (Fig. 7 A). The presence of PEX19 in purified peroxisomes was also confirmed by blotting a sample of purified peroxisomes (Fig. 7 B). We also performed differential centrifugation experiments to determine whether any of the apparently cytosolic PEX19 can be pelleted at high speed. A small amount of PEX19 pellets at 25,000 *g*, as expected for a peroxisome-associated protein, and the PEX19 present in the 25,000-*g* supernatant cannot be pelleted even after centrifugation at 100,000 *g* (Fig. 7 C).

To control for the possibility that PEX19 might have been released from peroxisomes by homogenization, we also assessed the distribution of PEX19 using a differential permeabilization and release assay. Intact human fibroblasts were incubated with varying amounts of digitonin, which preferentially permeabilizes the plasma membrane, and the material released from the cells at each concentration was collected. These fractions were assayed for a cytosolic marker enzyme (LDH), PEX19, and a peroxisomal marker protein (catalase). Most PEX19 was released at concentrations that also released LDH from the cell, supporting the hypothesis that significant amounts of PEX19 reside in the cytoplasm (Fig. 7 C). It should be noted that 20% of LDH activity and 30–40% of PEX19 were released only after the addition of 1% Triton X-100.

PEX19 is a farnesylated protein and it is possible that PEX19 might use its farnesyl group to associate with per-

PEX14 in buffer A or buffer A containing various concentrations of 6xHis-PEX14, 6xHis-ΔC231PEX14, and 6xHis-ΔC270PEX14. Membranes were washed and the amount of ³⁵S-labeled PEX14 bound to PEX19 was determined using a PhosphorImager. These values were normalized to the control reaction and plotted on a bar graph. Bar, 15 μM.

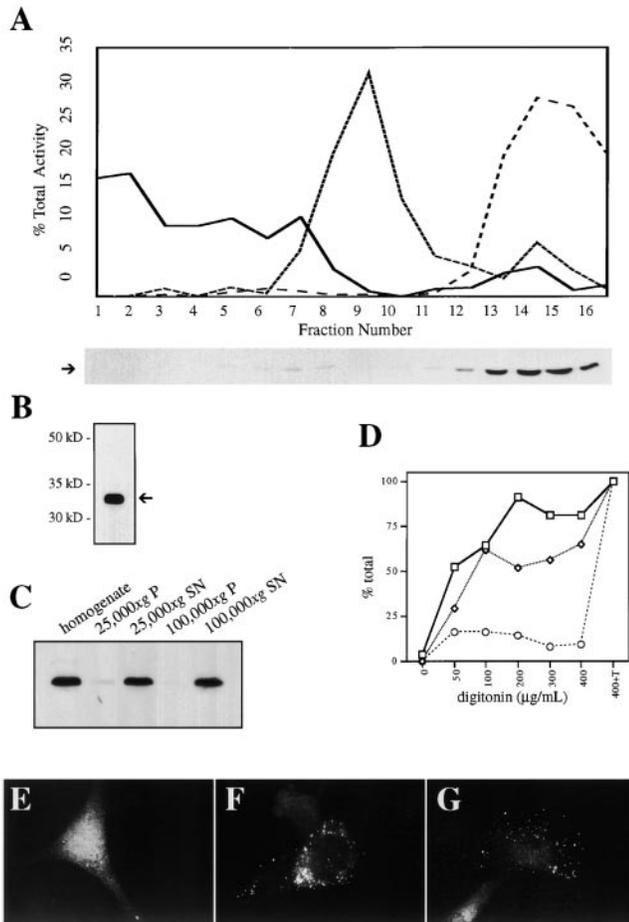


Figure 7. Subcellular distribution of mammalian PEX19. (A) A postnuclear supernatant of rat liver was fractionated by centrifugation on a 15–45% Nycodenz gradient and the position of the peroxisomal marker (catalase, solid line), the mitochondrial marker (succinate dehydrogenase, dotted line), and a cytoplasmic marker (lactate dehydrogenase, dashed line) are shown. The bottom of the gradient (most dense) is to the left, and the abundance of PEX19 in each fraction was determined by immunoblot (lower panel, arrow). (B) Highly purified rat liver peroxisomes were separated by SDS-PAGE and PEX19 was detected by immunoblot (arrow). (C) Rat liver was homogenized and subjected to differential centrifugation analysis. Equal proportions of homogenate, 25,000 *g* pellet, 25,000 *g* supernatant, 100,000 *g* pellet, and 100,000 *g* supernatant fractions were separated by SDS-PAGE and assayed by immunoblot for PEX19. (D) Human fibroblasts (GM5756-T) were harvested and incubated with varying amounts of digitonin, as well as digitonin and Triton X-100. Insoluble material was pelleted by centrifugation, and the supernatants were assayed for the release of cytoplasmic (LDH, square) and peroxisomal (catalase, circle) markers and for PEX19 by immunoblot (diamond). (E–G) Fibroblasts were transfected with pcDNA3 (E), pcDNA3-*PEX19* (F), or pcDNA3-*PEX19/C296A* (G) and processed by immunofluorescence using antibodies to catalase and the appropriate secondary antibodies.

oxisome membranes. If true, the homogenization and permeabilization experiments may have released PEX19 from peroxisomes without affecting the distribution of other peroxisomal proteins. Because it is virtually impossible to control directly for this possibility, we decided

instead to address this issue indirectly by determining whether farnesylation was critical for PEX19 function. PEX19 is farnesylated via the cysteine residue at position 296, and we generated a *PEX19* mutant in which this cysteine is replaced by an alanine (*PEX19/C296A*) and is, therefore, unable to accept the farnesyl moiety. We transfected a *PEX19*-deficient human fibroblast cell line with pcDNA3, pcDNA3-*PEX19*, and pcDNA3-*PEX19/C296A*, and assessed the ability of each plasmid to restore peroxisome biogenesis in these cells (Fig. 7, D–F). Transfection with the empty vector had no effect, expression of WT *PEX19* led to rescue of peroxisome biogenesis in ~50% of the cells, and expression of *PEX19/C296A* led to restoration of peroxisome biogenesis in a similarly high number of the cells. Quantitation of relative rescue activities revealed that the C296A mutation had only a mild effect on PEX19 activity, reducing it to ~80% the activity of wild-type PEX19. Therefore, it appears that farnesylation has an ancillary effect on PEX19 function and that PEX19 functions well in the absence of the farnesyl moiety.

PMP Fates in the Absence of PEX19

It has been established previously that PMP70 is present at greatly reduced levels in *PEX19*-deficient mammalian cells, and that this is caused by an increased rate of PMP70 degradation (Kinoshita et al., 1998; Matsuzono et al., 1999). We examined the fates of additional PMPs in *PEX19*-deficient cells to assess the role of PEX19 in the biogenesis of other PMPs. Immunofluorescence studies showed that normal human fibroblasts contain hundreds of PMP70-containing peroxisomes but, as previously demonstrated, *PEX19*-deficient cells lack detectable PMP70-containing structures (Fig. 8, A and B). Similar results were observed when these cells were stained with antibodies specific for the integral PMPs PEX13 and PEX11 β (Fig. 8, C–F). However, not all PMPs were undetectable in *PEX19*-deficient cells. Immunoblot experiments revealed that the integral PMP PEX14 was present at similar levels in *PEX19*-deficient cells as in other *PEX* mutants (data not shown) and was easily detectable in these cells by immunofluorescence. However, the PEX14 in these cells was not present in peroxisome-like structures or other small vesicles, but was instead mislocalized to the mitochondria (Fig. 9, A–C). Similar mitochondrial mislocalization was observed for myc-tagged derivatives of several integral PMPs that were introduced by transfection. These included the integral PMPs PEX12 (Fig. 9, D–F) and ALDP (Fig. 9, G–I). Similar mitochondrial mislocalization was observed when PEX13 and PEX11 β were overexpressed in the *PEX19*-deficient cells (data not shown). Snyder et al. (1999) recently reported that minute, PEX3-containing minivesicles may exist in *PEX19*-deficient cells of the yeast *Pichia pastoris*. We could not directly test whether similar structures exist in human *PEX19*-deficient cells because we lack antibodies of sufficient titer to PEX3. However, myc-tagged PEX3, like the other PMPs we tested, was easily detected in peroxisomes of normal cells, but was mislocalized to mitochondria of *PEX19*-deficient cells (Fig. 9, J–L).

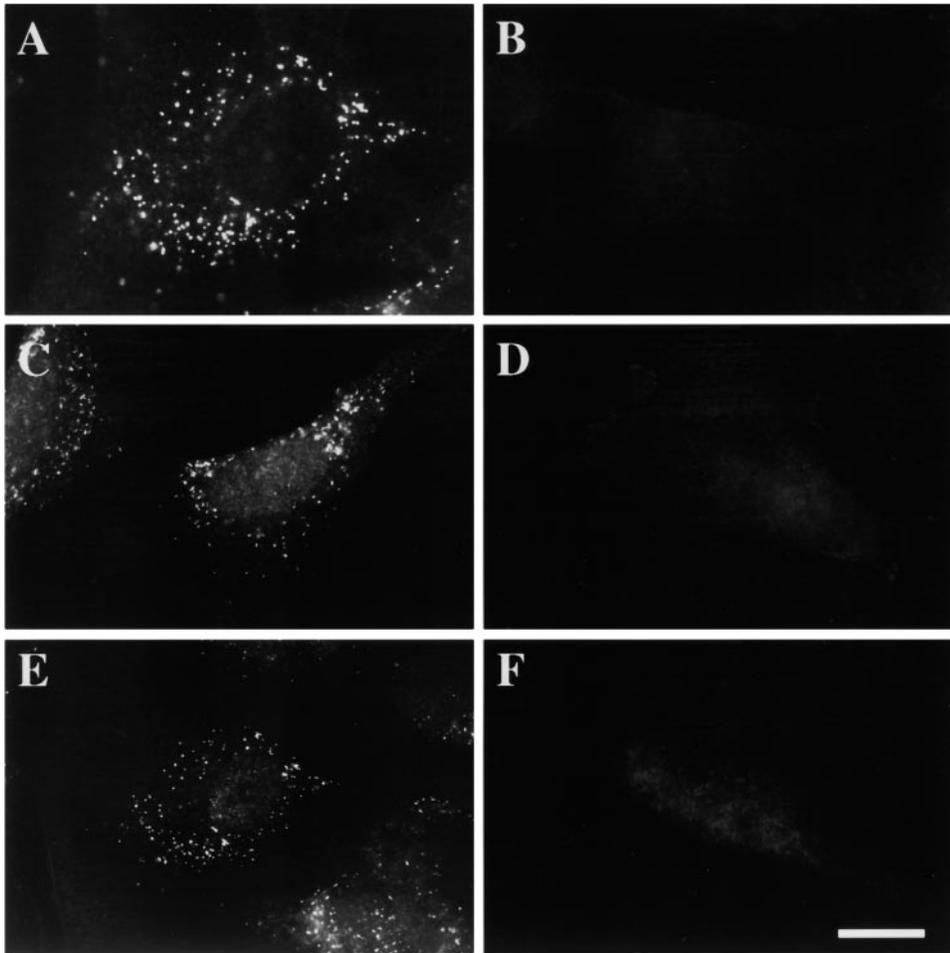


Figure 8. A subset of PMPs is not detectable in *PEX19*-deficient cells. Wild-type (left column) and PBD399 (right column) human fibroblasts were processed for indirect immunofluorescence using antibodies specific for PMP70 (A and B), PEX13 (C and D), and PEX11 β (E and F) with the appropriate secondary antibodies. Bar, 15 μ m.

Discussion

The import of nuclear-encoded peroxisomal proteins is a critical aspect of peroxisome biogenesis. Although much is known about peroxisomal matrix protein import, less is known about PMP import. PMPs, like peroxisomal matrix proteins, are synthesized on cytoplasmic ribosomes and imported posttranslationally, but it is unclear how the cell recognizes PMPs, directs them to the peroxisome, and inserts them into the peroxisome membrane bilayer. PMPs lack the PTS1 and PTS2 signals that direct proteins to the peroxisome matrix and do not require either the PTS1 or PTS2 receptors for import into peroxisome membranes (Chang et al., 1999b). Mutants that are defective in PMP import would be expected to lack detectable peroxisomal structures, and this phenotype has been reported for a Zellweger syndrome patient with mutations in *PEX19*, as well as a *Saccharomyces cerevisiae pex19* mutant (Gotte et al., 1998; Matsuzono et al., 1999). In this report, we observed that *PEX19* is a predominantly cytoplasmic PMP-binding protein that is required for the proper biogenesis of numerous PMPs.

PEX19 Is a Broad Specificity PMP-binding Protein

Using a blot overlay assay, we found that *PEX19* binds to a broad spectrum of PMPs with diverse functions in perox-

isome biogenesis (*PEX3*, *PEX12*, *PEX13*, and *PEX14*) and metabolite transport (*PMP70*, *PMP34*, and *ALDR*). The binding activity of *PEX19* appeared to be specific for PMPs as *PEX19* failed to bind peroxisomal matrix proteins or integral membrane proteins that reside in the plasma, Golgi, or mitochondrial membranes. The fact that *PEX19*-PMP binding could be assayed *in vitro* allowed us to further characterize the nature of *PEX19*-PMP binding. Using *PEX14* as a test PMP, we found that the *PEX19*-PMP interaction displayed a dissociation constant of ~ 500 nM, which is well within the range expected for a reversible protein-protein interaction. For comparison, it is interesting to note that the complex between PTS1-containing peroxisomal matrix proteins and *PEX5*, the PTS1 receptor, also has a dissociation constant of ~ 500 nM (Terlecky et al., 1995).

The interaction between *PEX19* and these same seven PMPs was also tested in an independent *PEX19*-binding assay in which a nuclear localization signal was appended to *PEX19* and the resulting NLS/*PEX19* protein was assayed for its ability to mislocalize newly synthesized PMPs to the nucleus. In this second assay, *PEX19* interacted with 14 human PMPs, including the same set of 7 PMPs that *PEX19* bound to in the blot overlay assay (*PEX3*, *PEX12*, *PEX13*, *PEX14*, *PMP70*, *PMP34*, and *ALDR*) as well as 7 more PMPs (*PEX10*, *PEX11 α* , *PEX11 β* , *PEX16*, *ALDP*,

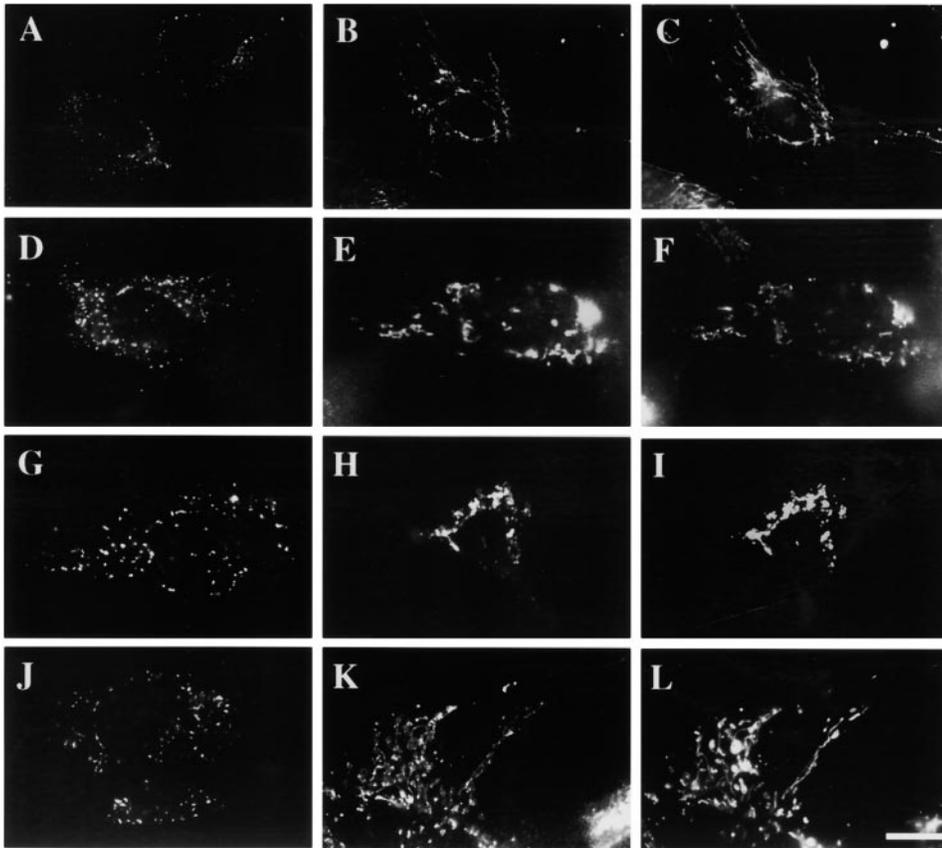


Figure 9. A subset of PMPs localizes to mitochondria in *PEX19*-deficient cells. The distribution of endogenous PEX14 was determined in wild-type (A) and PBD399 (B) fibroblasts using affinity-purified PEX-14 antibodies. The distribution of PEX12myc (D and E), ALDPmyc (G and H), and PEX3myc (J and K) was determined in wild-type (D, G, and J) and PBD399 (E, H, and K) fibroblasts using antibodies specific to the myc epitope. PBD399 fibroblasts were also stained with Mitotracker (C, F, I, and L). The peroxisomal nature of the punctate structures in wild-type fibroblasts was confirmed by double label with anti-PMP70 antibodies (data not shown). Bar, 15 μ m.

PMP22, and PMP24). Furthermore, we observed that PEX19 interacted with four human PMPs in the yeast two-hybrid assay: PEX10, PEX11 β , PEX12, and PEX13. All told, we observed interaction of PEX19 with nine human PMPs in at least two independent assays (PEX12 and PEX13 in all three assays; PEX3, PEX14, PMP70, PMP34, ALDR in the blot overlay and nuclear mislocalization assays; and PEX11 β and PEX10 in the two-hybrid and nuclear mislocalization assays) and 14 human PMPs altogether. It should be noted that ours are not the first evidence that PEX19 can bind PMPs, as previous reports have demonstrated that *S. cerevisiae* PEX19 binds PEX3 (Gotte et al., 1998) and that *P. pastoris* PEX19 binds PEX3 and PEX10 (Snyder et al., 1999). Although we did not detect interaction of PEX19 with several PMPs assayed in the yeast two-hybrid assay (PEX2, PEX3, PEX11 α , PEX14, and PEX16), these results are difficult to interpret in the absence of controls for the expression, folding, and nuclear targeting of the various GAL4BD-PMP fusion proteins.

PMP Targeting Elements Retain the Ability to Bind PEX19

The interaction of human PEX19 with a diverse array of human PMPs, the ability of NLS/PEX19 to draw newly synthesized, but not mature, PMPs into the nucleus, and the inability of *PEX19*-deficient cells to import PMPs all indicate that PEX19 plays an important role in PMP biogenesis, perhaps in the recognition of PMP targeting sig-

nals or as a chaperone for newly synthesized PMPs. If true, we would expect that regions of PMPs that were sufficient for targeting to peroxisomes would retain the ability to bind PEX19, and we tested this for three integral PMPs, PMP70, PEX11 β , and PEX14.

We found that the first 61 amino acids of PMP70 (<10% of the protein) are sufficient for peroxisomal localization and that this same segment of PMP70 retained the ability to interact with PEX19. The analysis of PEX11 β provided similar results, with the COOH-terminal 49 amino acids of PEX11 β being sufficient for both targeting and interaction with PEX19. We also used a more quantitative method for the analysis of PEX14-PEX19 binding. Targeting studies revealed that the first 147 amino acids of PEX14 targeted to peroxisomes, though weakly, and bound to PEX19 with a much lower affinity than full-length PEX14. We also found that the first 108 amino acids of PEX14 could not bind to PEX19, and that this fragment of PEX14 could not localize to peroxisomes. Taken together, these results are consistent with the hypothesis that targeting elements of PMPs retain the ability to bind PEX19.

It is important to note that the targeting elements described in this paper cannot be equated with the minimal targeting signals for these PMPs; thus, the question of whether PEX19 binds to PMP targeting signals remains to be addressed. However, our inability to identify the PMP targeting signals in PMP70 and PEX11 β was not due to a lack of effort. For both PMP70 and PEX11 β , we were unable to identify regions smaller than ~50–60 amino acids that could target to peroxisomes, and we have encoun-

tered similar problems during searches for targeting signals in the PMPs PMP34, PEX12, and PEX13 (data not shown). Such problems were not encountered during the search for targeting signals in peroxisomal matrix proteins (Subramani, 1993), and this difference is worth discussing. It is our opinion that the difficulty in identifying concise PMP targeting signals lies in the nature of protein targeting assays. Conventional targeting assays obviously measure the targeting abilities of the test proteins, but it is generally not appreciated that they also measure the retention of the protein in the organelle after the targeting event. In the case of peroxisomal matrix proteins, the retention is provided by the passive barrier of the peroxisome membrane and the assay, therefore, reflects just the targeting information of the protein. In contrast, it is difficult to envision how PMPs could be retained unless they were inserted into the peroxisome membrane, a process that is unlikely to be passive. Thus, biologically defined PMP targeting signals are likely to include signals for two potentially distinct processes: targeting, which may involve the recognition by a PMP receptor, and retention, which may require insertion of the protein into the peroxisome membrane. The targeting elements we identified in PMP70, PEX11 β , and PEX14 all contained a putative transmembrane domain, and it will be interesting to determine the nature of the PEX19-binding sites in these and other PMP targeting elements.

In addition to the test proteins we used for determining whether peroxisomal targeting elements retained the ability to bind to PEX19, it is necessary to discuss three proteins that we did not use in these particular experiments. The first is *Candida boidinii* PMP47. If we define a PMP targeting signal as the minimal regions that are sufficient for targeting to peroxisomes and for which the functional elements are known, the only PMP targeting signal in the literature that meets this criteria is the mPTS of *C. boidinii* PMP47 (Dyer et al., 1996). We considered testing whether PEX19 recognized this mPTS, but we found that this signal is not sufficient for peroxisomal membrane targeting in human cells (Gould, S.J., unpublished observations) and it was, therefore, unclear what benefit could come from such an analysis. The other two proteins that deserve mention are PEX3 and PEX16. We detected interactions between PEX19 and these two PMPs. Like any other integral PMPs we might have considered using PEX3 or PEX16 to test whether PEX19-binding sites reside within their PMP targeting elements. This is particularly true for PEX3 since previous studies have localized a low efficiency PMP targeting element to within the first 40 amino acids of this PMP (Kammerer et al., 1998; Soukupova et al., 1999). However, we consciously avoided using these PMPs for these particular experiments because they, like PEX19, are required for peroxisome membrane biogenesis along with PEX19 (Hettema et al., 1999; Matsuzono et al., 1999; South and Gould, 1999; South, S.T., K.A. Sacksteder, and S.J. Gould, unpublished observations). Thus, they may interact with PEX19 in multiple places and for multiple purposes. For example, it is possible that PEX19 could interact with targeting elements during the biogenesis of PEX3 and PEX16, but also bind to other regions of PEX3 and/or PEX16 as it mediates the biogenesis of other PMPs. This is not to say that an analysis of targeting signals and PEX19-

binding sites in PEX3 and PEX16 is unimportant. Rather, it reflects our view that such studies will be extremely important not only for what they will tell us about general PEX19-PMP interactions, but also for the information they will provide on the roles of these three proteins in the biogenesis of peroxisome membranes. In contrast, the analysis of targeting elements and PEX19-binding sites in PMPs that are not required for peroxisome membrane synthesis, such as PMP70 and PEX11 β , are likely to yield data that relates only to the general role of PEX19 binding in PMP biogenesis.

PEX19 Is Bimodally Distributed between Cytoplasm and Peroxisomes

To better understand how PEX19 facilitates peroxisome membrane biogenesis, we assessed its distribution within the cell. Subcellular fractionation, differential centrifugation, and differential permeabilization experiments all indicated that there is a large population of cytosolic, soluble PEX19, in addition to a peroxisomal pool of PEX19. Our results are consistent with previously published data that mammalian PEX19 is both cytoplasmic (James et al., 1994; Matsuzono et al., 1999) and peroxisomal (James et al., 1994; Kammerer et al., 1997; Matsuzono et al., 1999). A similar bimodal distribution has been observed for yeast forms of PEX19 (Gotte et al., 1998; Snyder et al., 1999). Quantitation of the PEX19 levels in cytosolic and peroxisomal fractions of our rat liver gradient fractions suggest that >95% of PEX19 may be cytoplasmic.

One potential caveat to our PEX19 distribution data is related to PEX19 farnesylation. It is formally possible that PEX19 utilizes its farnesyl moiety for association with peroxisomes and that our homogenization and permeabilization procedures released PEX19 from peroxisomes without disturbing peroxisome integrity in general. To assess the general relevance of farnesylation to PEX19 function we used site-directed mutagenesis to eliminate the farnesylation site. WT *PEX19* and the resulting *PEX19/C296A* mutant were assayed for their ability to restore peroxisome biogenesis in *PEX19*-deficient cells, and we found that the mutant was almost fully active (80% of WT activity). High activity also has been reported for an analogous CAAX box mutant of *P. pastoris PEX19* (Snyder et al., 1999). These studies strongly suggest that farnesylation has an ancillary rather than a central role in *PEX19* function, and makes it unlikely that PEX19 uses farnesylation as its primary means for associating with peroxisomes. Our results are based on the analysis of four independent, sequence-confirmed *PEX19/C296A* clones, and we have no explanation for the previous report that a C296S mutant of human *PEX19* had no activity (Matsuzono et al., 1999).

Implications for PMP Import and Peroxisome Biogenesis

In this report, we found that PEX19 binds to a diverse array of PMPs, including metabolite transporters and peroxins, and that regions of PMPs which are sufficient for targeting to peroxisomes retain the ability to interact with PEX19. We also observed that PEX19 is a predominantly cytoplasmic, partly peroxisomal protein, and that loss of PEX19 results in the absence of detectable peroxisomal

structures, the destabilization of many integral PMPs, and the mislocalization of other PMPs to the mitochondrion. One simple model that could explain these data is that PEX19 plays an important role in the biogenesis of PMPs. For instance, the bimodal distribution of PEX19 may represent a steady state view of a cycling PMP-binding protein that facilitates the early steps in PMP biogenesis, either as a chaperone or as a PMP receptor. Such a model provides many questions for future studies, such as whether PEX19 is indeed a PMP receptor, whether PEX19 binds PMPs in the cytoplasm before their import, whether PEX19 cycles between cytoplasm and peroxisome, and whether PEX19 has additional roles when present at the peroxisome membrane.

Existing data on peroxisome biogenesis in human cells are consistent with the ability of cells to generate peroxisomes in two ways, either by growth and division of preexisting peroxisomes or by synthesis from an as yet unidentified preperoxisomal vesicle. In this model (South and Gould, 1999), peroxisomes are defined as vesicles that contain a normal or nearly normal complement of PMPs (regardless of matrix protein content), whereas preperoxisomal vesicles are defined as vesicles that either lack PMPs altogether or have only one or a small number of PMPs. In our examination of *PEX19*-deficient cells, we did not see PMPs present in any vesicles reminiscent of peroxisomes or in any smaller vesicles. Instead, they were either completely absent or mislocalized to the mitochondrion. Although we did not find any evidence for the early remnants reported in *pex19* mutants of *P. pastoris* by Snyder et al. (1999), it is possible that we could have missed such structures with the techniques that we employed.

Recent studies from yeast have suggested that the ER may contribute to peroxisome synthesis (for reviews see Kunau and Erdmann, 1998; Titorenko and Rachubinski, 1998), and the ER could, in fact, represent preperoxisomal vesicles of our model. However, the hypothesis that the ER plays a direct and essential role in peroxisome biogenesis still lacks evidence from multiple experimental systems that would be necessary for its general acceptance. The results of this study do not directly address this question, but it is worth noting that our results offer no clear support for an ER role in peroxisome membrane synthesis. For instance, we failed to observe accumulation of any PMPs in the ER in the absence of PEX19, a result that would be predicted for PMPs that transit through the ER before their import into peroxisomes, as has been suggested for PEX3 and PEX16 (for review see Kunau and Erdmann, 1998; Titorenko and Rachubinski, 1998). Also, the fact that PEX19 is a predominantly cytoplasmic PMP-binding protein with an important role in PMP biogenesis does lend some support to the model that PMPs, or at least most PMPs, are synthesized on free polyribosomes and imported directly from the cytoplasm into peroxisomes (Lazarow and Fujiki, 1985).

We would like to thank Jim Morrell for excellent technical assistance, Gail Stetten for aid with fluorescence microscopy, Tony Guerrero (all from The Johns Hopkins University School of Medicine, Baltimore, MD) for analysis of binding data, and Skaidrite Krisans (San Diego State University, San Diego, CA) for the gift of purified rat liver peroxisomes.

This work was supported by grants from the National Institutes of Health

and the March of Dimes to S.J. Gould (DK45787, HD10981, and MOD FY93/94-0514).

Submitted: 27 May 1999

Revised: 5 January 2000

Accepted: 21 January 2000

References

- 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.
- Bjorkman, J., G. Stetten, C.S. Moore, S.J. Gould, and D.I. Crane. 1998. Genomic structure of PEX13, a candidate peroxisome biogenesis disorder gene. *Genomics* 54:521-528.
- Bournsnel, M.E., T.D. Brown, and M.M. Binns. 1984. Sequence of the membrane protein gene from avian coronavirus IBV. *Virus Res.* 1:303-313.
- Braverman, N., G. Dodt, S.J. Gould, and D. Valle. 1995. Disorders of peroxisome biogenesis. *Hum. Mol. Genet.* 4:1791-1798.
- Chang, C.-C., and S.J. Gould. 1998. Phenotype-genotype relationships in complementation group 3 of the peroxisome-biogenesis disorders. *Am. J. Hum. Genet.* 63:1294-1306.
- 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., D.S. Warren, K.A. Sacksteder, and S.J. Gould. 1999a. PEX12 binds PEX5 and PEX10 and acts downstream of receptor docking in peroxisomal matrix protein import. *J. Cell Biol.* 147:761-764.
- Chang, C.-C., S. South, D. Warren, J. Jones, A.B. Moser, H.W. Moser, and S.J. Gould. 1999b. Metabolic control of peroxisome abundance. *J. Cell Sci.* 112: 1579-1590.
- Dekker, P.J., P. Keil, J. Rassow, A.C. Maarse, N. Pfanner, and M. Meijer. 1993. Identification of MIM23, a putative component of the protein import machinery of the mitochondrial inner membrane. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 330:66-70.
- Diestelkotter, P., and W.W. Just. 1993. In vitro insertion of the 22 kD peroxisomal membrane protein into isolated rat liver peroxisomes. *J. Cell Biol.* 123: 1717-1725.
- Dodt, G., and S.J. Gould. 1996. Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. *J. Cell Biol.* 135: 1763-1774.
- 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.
- Drees, B.L. 1999. Progress and variations in two-hybrid and three-hybrid technologies. *Curr. Opin. Chem. Biol.* 3:64-70.
- Dyer, J.M., J.A. McNew, and J.M. Goodman. 1996. The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop. *J. Cell Biol.* 133:269-280.
- Evan, G.E., G.K. Lewis, G. Ramsay, and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5:3610-3616.
- Fransen, M., S.R. Terlecky, and S. Subramani. 1998. Identification of a human PTS1 receptor docking protein directly required for peroxisomal protein import. *Proc. Natl. Acad. Sci. USA.* 95:8087-8092.
- Fujiki, Y., R.A. Rachubinski, and P.B. Lazarow. 1984. Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes. *Proc. Natl. Acad. Sci. USA.* 81:7127-7131.
- Geisbrecht, B.V., D. Zhang, H. Schulz, and S.J. Gould. 1999. Characterization of PECL1, a novel monofunctional delta3,delta2-enoyl-CoA isomerase of mammalian peroxisomes. *J. Biol. Chem.* 274:21797-21803.
- 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.
- Hettema, E.H., B. Distel, and H.F. Tabak. 1999. Import of proteins into peroxisomes. *Biochim. Biophys. Acta.* 1451:17-34.
- Imanaka, T., G.M. Small, and P.B. Lazarow. 1987. Translocation of acyl-coA oxidase into peroxisomes requires ATP hydrolysis but not a membrane potential. *J. Cell Biol.* 105:2915-2922.
- 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.
- James, G.L., J.L. Goldstein, R.K. Pathak, R.G.W. Anderson, and M.S. Brown. 1994. PxF, a prenylated protein of peroxisomes. *J. Biol. Chem.* 269:14182-14190.
- 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.
- Kamijo, K., S. Taketani, S. Yokata, T. Osumi, and T. Hashimoto. 1990. The 70 kDa peroxisomal membrane protein is a member of the Mdr (P-glycoprotein)-related ATP-binding protein superfamily. *J. Biol. Chem.* 265:4534-

- Kammerer, S., N. Arnold, W. Gutensohn, H.W. Mewes, W.H. Kunau, G. Hofler, A.A. Roscher, and A. Braun. 1997. Genomic organization and molecular characterization of a gene encoding HsPXF, a human peroxisomal farnesylated protein. *Genomics* 45:200–210.
- 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 (Fed. Eur. Biochem. Soc.) Lett.* 429:53–60.
- Kinoshita, N., K. Ghaedi, N. Shimozawa, R.J.A. Wanders, Y. Matsuzono, T. Imanaka, K. Okumoto, Y. Suzuki, N. Kondo, and Y. Fujiki. 1998. Newly identified Chinese hamster ovary cell mutants are defective in biogenesis of peroxisomal membrane vesicles (peroxisomal ghosts), representing a novel complementation group in mammals. *J. Biol. Chem.* 273:24122–24130.
- Kunau, W.H., and R. Erdmann. 1998. Peroxisome biogenesis: back to the endoplasmic reticulum? *Curr. Biol.* 8:R299–R302.
- Lawson, J.E., and M.G. Douglas. 1988. Separate genes encode functionally equivalent ADP/ATP carrier proteins in *Saccharomyces cerevisiae*. Isolation and analysis of AAC2. *J. Biol. Chem.* 265:14812–14818.
- Lazarow, P.B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489–530.
- Lazarow, P.B., and H.W. Moser. 1995. Disorders of peroxisome biogenesis. In *The Metabolic and Molecular Bases of Inherited Disease*. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill, New York. 2287–2324.
- Lombard-Platet, G., S. Savary, C.O. Sarde, J.L. Mandel, and G. Chimini. 1996. A close relative of the adrenoleukodystrophy (ALD) gene codes for a peroxisomal protein with a specific expression pattern. *Proc. Natl. Acad. Sci. USA* 93:1265–1269.
- Marzoch, M., R. Erdmann, M. Veenhuis, and W.-H. Kunau. 1994. PAS7 encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4908–4918.
- 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.
- McCullum, D., E. Monosov, and S. Subramani. 1993. The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells. The PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal and is a member of the TPR protein family. *J. Cell Biol.* 121:761–774.
- Mihalik, S.J. 1992. Involvement of both peroxisomes and mitochondria in the alpha-oxidation of phytanic acid. *Prog. Clin. Biol. Res.* 357:239–244.
- Mosser, J., A.M. Douar, C.O. Sarde, P. Kioschis, R. Feil, H. Moser, A.M. Poustka, J.L. Mandel, and P. Aubourg. 1993. Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature* 361:726–730.
- Mosser, J., Y. Lutz, M.E. Stoeckel, C.O. Sarde, C. Kretz, A.M. Douar, J. Lopez, P. Aubourg, and J.L. Mandel. 1994. The gene responsible for adrenoleukodystrophy encodes a peroxisomal membrane protein. *Hum. Mol. Genet.* 3:265–271.
- Murakami, H., G. Blobel, and D. Pain. 1990. Isolation and characterization of the gene for a yeast mitochondrial import receptor. *Nature* 347:488–491.
- 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.
- Santos, M., T. Imanaka, H. Shio, G.M. Small, and P.B. Lazarow. 1988. Peroxisome membrane ghosts in Zellweger syndrome—aberrant organelle assembly. *Science* 239:1536–1538.
- Schrader, M., B.E. Reuber, J.C. Morrell, G. Jimenez-Sanchez, C. Obie, T. Stroth, 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., K.N. Faber, T.J. Wenzel, A. Koller, G.H. Luers, L. Rangell, G.A. Keller, and S. Subramani. 1999. PEX19 interacts with Pex3p and Pex10p and is essential for peroxisome biogenesis in *Pichia pastoris*. *Mol. Biol. Cell* 10:1745–1761.
- 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., and S.J. Gould. 1999. Peroxisome synthesis in the absence of preexisting peroxisomes. *J. Cell Biol.* 144:255–266.
- Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol.* 9:445–478.
- Terlecky, S.R., W.M. Nuttley, D. McCollum, E. Sock, and S. Subramani. 1995. The *Pichia pastoris* peroxisomal protein Pas8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:3627–3634.
- Titorenko, V.I., and R.A. Rachubinski. 1998. The endoplasmic reticulum plays an essential role in peroxisome biogenesis. *Trends Biochem. Sci.* 23:231–233.
- Vidal, M., R.K. Brachman, A. Fattaey, E. Harlow, and J.D. Boeke. 1996. Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. *Proc. Natl. Acad. Sci. USA* 93:10315–10320.
- Wanders, R.J., and J.M. Tager. 1998. Lipid metabolism in peroxisomes in relation to human disease. *Mol. Aspects Med.* 19:69–154.
- Warren, D.S., J.C. Morrell, H.W. Moser, D. Valle, and S.J. Gould. 1998. Identification of PEX10, the gene defective in complementation group 7 of the peroxisome-biogenesis disorders. *Am. J. Hum. Genet.* 63:347–359.
- Wyllin, T., M. Baes, C. Brees, G.P. Mannaerts, M. Fransen, and P.P. Van Veldhoven. 1999. 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.
- Yahraus, T., N. Braverman, G. Dodt, J.E. Kalish, J.C. Morrell, H.W. Moser, D. Valle, and S.J. Gould. 1996. The peroxisome biogenesis disorder group 4 gene, *PXAAA1*, encodes a cytoplasmic ATPase required for stability of the PTS1 receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2914–2923.