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Functional domain mapping of peroxin Pex19p: interaction with Pex3p is essential for function and translocation

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Summary

The peroxin Pex19p functions in peroxisomal membrane assembly. Here we mapped functional domains of human Pex19p comprising 299 amino acids. Pex19p mutants deleted in the C-terminal CAAx farnesylation motif, the C-terminal 38 amino acid residues and the N-terminal 11 residues, maintained peroxisome-restoring activity in *pex19* cells. The sequence 12-261 was essential for re-establishing peroxisome activity. Pex19p was partly localized to peroxisomes but mostly localized in the cytosol. Pex19p interacted with multiple membrane proteins, including the other two membrane biogenesis peroxins, Pex3p and Pex16p, those involved in matrix protein import such as Pex14p, Pex13p, Pex10p, and Pex26p, peroxisome morphogenesis factor Pex11pβ, and a PMP70 peroxisome-targeting signal region at residues 1-123. In yeast two-hybrid assays, Pex10p and Pex11pβ interacted only with full-length Pex19p. Of various truncated Pex19p variants active in translocating to peroxisomes, the mutants with the shortest sequence (residues 12-73 and 40-131) were localized to peroxisomes and competent in binding to Pex3p. Furthermore, membrane peroxins were initially discernible in a cytosolic staining pattern in *pex19* cells only when co-expressed with Pex19p and were then localized to peroxisomes in a temporally differentiated manner. Pex19p probably functions as a chaperone for membrane proteins and transports them to peroxisomes by anchoring to Pex3p using residues 12-73 and 40-131.

Supplementary material available online at
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Key words: Peroxisome biogenesis, Membrane assembly, Pex19p, Chaperone, Transporter

Introduction

Peroxisomal proteins are encoded by nuclear genes, translated on free polyribosomes in the cytosol, and imported to peroxisomes (Lazarow and Fujiki, 1985). The molecular mechanisms of peroxisomal import of matrix proteins are well understood, whereas those involving membrane protein transport and membrane vesicle assembly remain elusive (Fujiki, 2000; Lazarow, 2003; Sacksteder and Gould, 2000). In peroxisome-deficient mutant cell lines, including *pex3*, *pex16* and *pex19* Chinese hamster ovary (CHO) cell mutants and fibroblasts from patients with peroxisome biogenesis disorders (PBD) of complementation groups (CGs) 12, 9, and 14, respectively, peroxisome membrane assembly is severely impaired, hence membrane structures such as the so-called 'peroxisomal ghosts' or membrane remnants are morphologically and biochemically undetectable (Ghaedi et al., 2000b; Honsho et al., 2002; Honsho et al., 1998; Jones et al., 2001; Kinoshita et al., 1998; Matsuzono et al., 1999; Muntau et al., 2000; South and Gould, 1999). It is of interest to note that membrane particles are observed in *pex19* mutant cells of *Pichia pastoris* (Snyder et al., 1999), *Yarrowia lipolytica* (Lambkin and Rachubinski, 2001) and *Y. lipolytica pex16* cells (Eitzen et al., 1997). Peroxisome membrane assembly is initiated by at least three peroxins, Pex3p, Pex16p and Pex19p, followed by several distinct steps, including the import of membrane and matrix proteins as well as growth and division of peroxisomes. We earlier cloned human *PEX19* cDNA encoding the 299 amino acid, hydrophilic peroxin Pex19p with the farnesylation motif CAAx at the C-terminus, by functional complementation strategy using a mutant CHO cell line, ZP119, defective in the import of both matrix and membrane proteins (Kinoshita et al., 1998; Matsuzono et al., 1999). Pex19p is localized, mostly in the cytosol and partly associated with peroxisomes (Goette et al., 1998; James et al., 1994; Matsuzono et al., 1999; Snyder et al., 1999). Pex19p binds multiple peroxisomal integral membrane proteins (PMPs), including several peroxins such as Pex3p and Pex13p (Fransen et al., 2001; Ghaedi et al., 2000b; Snyder et al., 2000). Recent studies also reported that Pex19p specifically bound to peroxisome membrane targeting signal (mPTS) regions of multiple PMPs (Jones et al., 2001; Jones et al., 2004; Rottensteiner et al., 2004) or at regions distinct from the sorting sequences (Fransen et al., 2001). Furthermore, the farnesylation of Pex19p is an important determinant for the higher affinity of Pex19p to several peroxins such as Pex10p and Pex13p (Fransen et al., 2001). Thus, Pex19p has been proposed to function in recruiting newly synthesized PMPs from their site of synthesis on free polyribosomes to the peroxisomes as a soluble receptor and/or a chaperone in targeting of PMPs (Fransen et al., 2001; Jones et al., 2001; Matsuzono and Fujiki, 2006; Snyder et al., 2000).

As a further step toward understanding the molecular mechanisms of Pex19p function, in the present work we searched for the regions required for restoring peroxisome biogenesis, translocation to peroxisomes, and interaction with a wide-range of PMPs. We report that the sequence encompassing amino acid residues 12-261 is responsible for peroxisome-restoring activity in *pex19* cells. Pex19p variants comprising only amino-acid residues 40-131 and 12-73 were translocated to peroxisomes and were responsible for the interaction between Pex19p and Pex3p. Moreover, upon co-expression with Pex19p, integral membrane peroxins were initially maintained in the cytosol in *pex19* ZP119 cells and were then transported to newly formed peroxisomes. Taken together, Pex19p functions in the membrane-protein transport to peroxisomes, probably as a chaperone-like factor and a transporter.

Results

Functional domain mapping of Pex19p

As a step toward understanding the molecular mechanisms involved in peroxisome membrane biogenesis, we first searched for functional regions of Pex19p. We verified various truncated mutants of tandem double hemagglutinin A (HA)-tagged Pex19p, termed HA_2-Pex19p (Fig. 1), for the activity in restoring the impaired peroxisomal membrane biogenesis in CHO *pex19* ZP119 cells (Kinoshita et al., 1998; Matsuzono et al., 1999), deficient in endogenous Pex19p (supplementary material Fig. S1C, lane 1).

Deletions in the C-terminal region

Several mutants deleted in the C-terminal region of Pex19p (Fig. 1A) were separately expressed in ZP119 (Fig. 2A). Restoration of peroxisome biogenesis was assessed by immunofluorescent cell staining with antibodies recognizing matrix peroxisome-targeting signal type 1 (PTS1) proteins and the membrane peroxin Pex14p as markers for membrane proteins. A diffused PTS1 staining pattern was seen in mock-transfected ZP119 (data not shown), as reported (Matsuzono et al., 1999). In the cells expressing full-length Pex19p, PTS1- and Pex14p-positive particles were detectable in a superimposable manner (Fig. 2Aa,b), like those stained for other peroxisomal proteins such as thiolase, a PTS2 protein and PMP70 (data not shown), thereby indicating that peroxisomes were re-established (Matsuzono et al., 1999). Similarly, expression of residues 1-295 (the same as ΔC4) with deletion of the farnesylation motif CLIM from the C-terminus, showed peroxisomal restoration with slightly lower efficiency compared with the normal Pex19p (Fig. 2Ac,d). Expression of 1-261 (ΔC38) gave rise to fewer PTS1- and Pex14p-positive peroxisomes (Fig. 2Ae,f). However, 1-255 (ΔC44) and further truncated variants, 1-131 (ΔC168) and 1-73 (ΔC226), failed to restore peroxisome assembly (Fig. 2Ag,h; Table 1). Furthermore, internal deletion mutants, Δ256-260 and Δ256-295, lacking conserved residues conceivably involved in folding α-helix or loop structure (supplementary material Fig. S2), were significantly and completely eliminated in the peroxisome-restoring activity (Fig. 2Ai,j; Table 1).

The expression level of HA_2-Pex19p variants in ZP119 was comparable between the mutants and similar to the full-length Flag-Pex19p (supplementary material Fig. S1A, lanes 1-7).

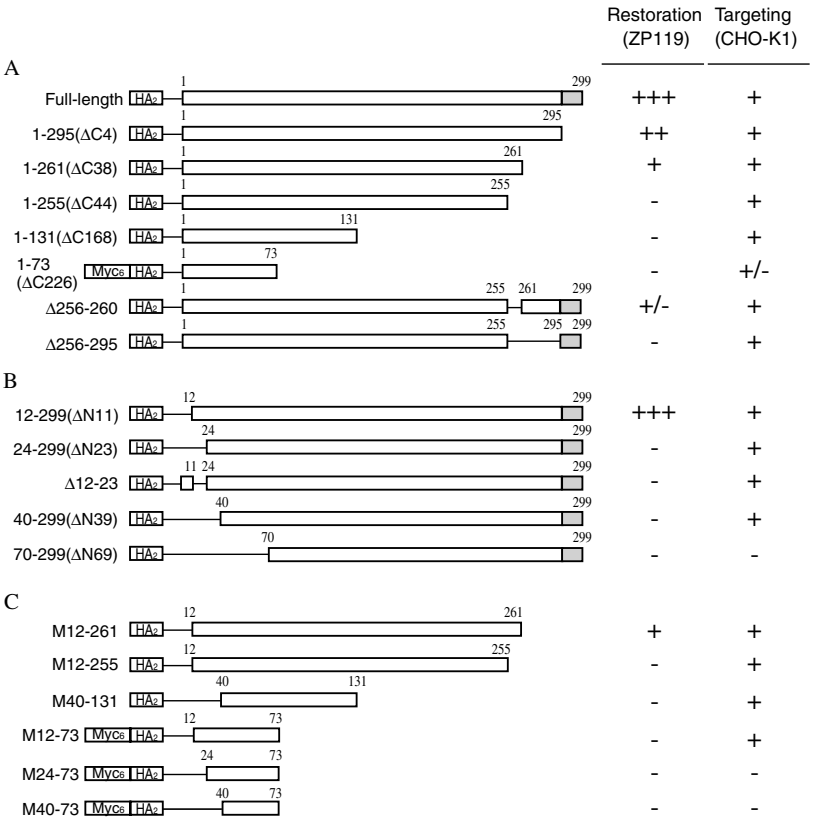

Fig. 1. Schematic representation of human Pex19p constructs. (A-C) Representative Pex19p constructs of deletions from C-terminus, N-terminus, and both terminal regions, respectively, used in this study. Numbers designate the positions in the amino acid sequence. Two tandem epitopes of hemagglutinin (HA_2) were tagged to the N-terminus of respective Pex19p variants. Several mutants with shorter sequences were fused to hexa-Myc tag, Myc_6, and HA_2. Farnesylation CAAx motif is shaded. The activities of the constructs in restoring peroxisomes in *pex19* ZP119 cells and translocating to peroxisomal membranes in CHO-K1 are summarized on the right: +++, strong; ++, medial; +, positive; +/–; weakly positive; –, negative.

Deletions in the N-terminal region

N-terminal truncation mutants of HA_2-Pex19p (Fig. 1B) were likewise verified in *pex19* cells as in Fig. 2A. In the cells expressing a Pex19p mutant with deletion of 11 amino acid residues from the N-terminus, termed 12-299 (the same as ΔN11), numerous PTS1- and Pex14p-positive punctate structures were observed (Fig. 2B,a,b), demonstrating that 12-299 was active in peroxisome restoration. By contrast, expression of 24-299 (ΔN23), 40-299 (ΔN39), and 70-299 (ΔN69) resulted in no punctate staining for PTS1 and Pex14p (Fig. 2Bc,d; Table 1), indicative of abrogation of peroxisome-restoring activity. Δ12-23 lacking the potential sequence involved in α-helix folding (supplementary material Fig. S2) also failed to re-establish peroxisome assembly (Fig. 2Be,f), suggesting the functional importance of this region. In ZP119 cells transfected with *M12-261* encoding the Pex19p mutant with deletion of amino-acid residues at 1-11 and 262-299 (Fig. 1C), punctate staining of both PTS1 and Pex14p was visible (Fig. 2Bg,h), whereas M12-255 and several shorter constructs such as M40-131 were inactive in complementing the ZP119 phenotype (Fig. 2Bi,j; Table 1). These constructs were expressed at levels similar to the C-terminal deletion mutants (supplementary material Fig. S1A, lanes 8-12). Collectively, these results strongly suggested that the minimal sequence required for restoring the peroxisome assembly resides in 12-261, including functionally important regions at 12-23 and 255-261. It is also noteworthy that Pex19p and its biologically active truncation mutants assessed above were partly localized to peroxisomes upon restoration of peroxisomes (data not shown).

Intracellular location of Pex19p

In CHO-K1 cells, expression of HA_2-Pex19p protein was discernible as a diffuse pattern in the cytosol in addition to numerous punctate structures (Fig. 3a). The Pex19p-positive particles were superimposable on those with Pex14p, hence indicating a peroxisomal localization (Fig. 3a,b). In immunoblots of CHO-K1-derived subcellular fractions with anti-Pex19p antibody (Matsuzono et al., 1999), less than 10% of endogenous Pex19p was detected in the organelle fraction and the majority was in the cytosol fraction (data not shown), consistent with the report describing the subcellular localization of Pex19p in rat liver (Sacksteder and Gould, 2000). Peroxisome-targeting activity of various HA_2- or Myc_6-HA_2-tagged Pex19p mutants was likewise assessed in CHO-K1 cells. Functionally active HA_2-1-295 (ΔC4), HA_2-1-261, HA_2-12-299 and HA_2-M12-261 were also detected in Pex14p-stained peroxisomes and in the cytosol, as the full-length Pex19p (Table 1). Pex19p variants defective in peroxisome-restoring activity, including 1-255, 24-299, 40-299, Δ256-260, Δ256-295, Δ12-23, and M12-255 (see Figs 1, 2; Table 1), and even shorter variants such as 1-131, 1-73, M40-131 and M12-73, were localized in the cytosol and to peroxisomes along with Pex14p (Fig. 3c-f,i-l; Table 1). Contrary to this, punctate HA staining was barely discernible in cells transfected with 70-299, M24-73 and M40-73 (Fig. 3g,h,m-p). The expressed

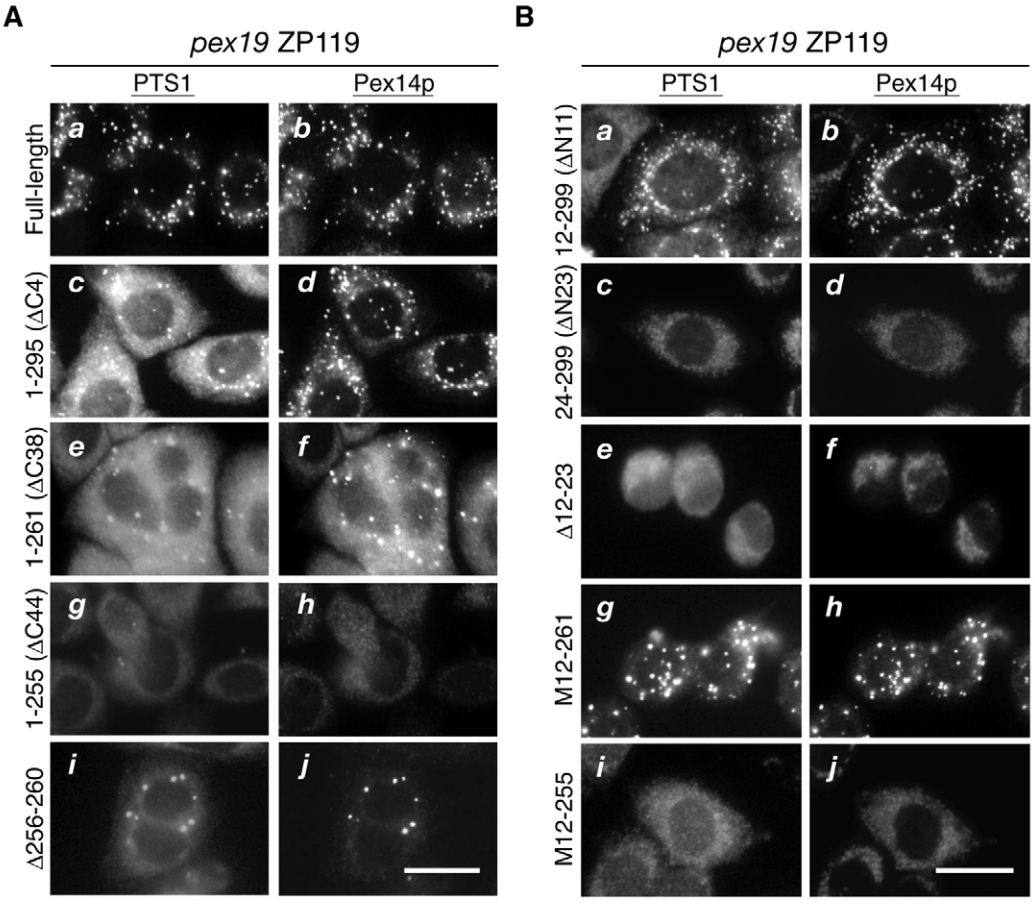

Fig. 2. Analysis of the region of Pex19p required for re-establishing peroxisome assembly. (A) HA_2-Pex19p variants shown in Fig. 1A were expressed in CHO *pex19* ZP119 cells: full-length Pex19p (a,b), 1-295 (the same as ΔC4) with deletion of four amino acid residues from the C-terminus (c,d), 1-261 (ΔC38) (e,f), 1-255 (ΔC44) (g,h), and Δ256-260 with internal deletion of amino-acid residues 256-260 (i,j). Cells were dual-stained with antisera against PTS1 and Pex14p for verifying peroxisome-restoring activity of Pex19p mutants. (B) HA_2-Pex19p deletion mutants in Fig. 1B,C were likewise expressed in ZP119 cells: 12-299 (ΔN11) lacking 11 amino-acid residues from the N-terminus (a,b), 24-299 (ΔN23) (c,d), Δ12-23 (e,f), M12-261 with deletion of 11 and 38 amino acid residues from the N- and C-termini (g,h) and M12-255 (i,j). Cells were stained as in A. Bars, 20 μm.

Table 1. Summary of functional domain mapping of Pex19p

Pex19p variant	Restoration* (in *pex19* cells)	Translocation* (in CHO-K1)	Co-immunoprecipitation†			Yeast two-hybrid assay†					
			Pex3p	Pex14p	Pex16p	Pex3p	Pex26p	Pex10p	Pex11pb	Pex13p	PMP70/1-123
In vivo assay											
Full-length	+++	+	++	++	++	+++	++	++	+	+	+
1-295 (DC4)	++	+	++	++	++	+++	+	–	–	–	+/–
1-261 (DC38)	+	+	++	++	++	+++	+/–	–	–	–	–
1-255 (DC44)	–	+	++	++	+	+++	–	–	–	–	–
1-131 (DC168)	–	+	++	++	–	+++	–	–	–	–	–
1-73 (DC226)	–	+/–	+	+	–	–					–
D256-260	+/–	+	++	+	++	+++	+/–	–	–	–	+/–
D256-295	–	+	++	+	++	+++	–	–	–	–	+/–
12-299 (DN11)	+++	+	++	++	++	+++	+	–	–	+	+
24-299 (DN23)	–	+	++‡	++	++	–‡	+	+	–	–	–
D12-23	–	+	++‡	++	++	–‡	+	+	–	–	–
40-299 (DN39)	–	+	++‡	++	++	–‡	+				
70-299 (DN69)	–	–	–	–	–	–	–				
M12-261	+	+	++	++	+	+					–
M12-255	–	+	++	++	+	+					–
M40-131	–	+	+/–	++	–						
M12-73	–	+/–	+	+	–						
M24-73	–	–	–	+	–						
M40-73	–	–	–	+	–						
In vitro binding assay											
Full-length	+++	+	+	+	+						
1-131 (DC168)	–	+	+	+	–						
M12-131	–	+	+	+	–						
M24-131	–	+	–	+	–						
M40-131	–	+	–	+	–						
M12-73	–	+/–	+								
M24-73	–	–	–								
M40-73	–	–	–								

*Activities for restoring peroxisomes and translocating to peroxisomes were verified as in Figs 2 and 3.

†Interaction of Pex19p and its truncated mutants with integral membrane-type proteins were verified by (1) co-immunoprecipitation assays using the lysates of COS7 cells expressing Pex19p variants and respective proteins as in Fig. 5 and supplementary material Fig. S3, and (2) yeast two-hybrid assays as in Fig. 4, where the BD-fused peroxins without Pex19p and Pex19p-AD with mock vector showed no self-activation. In vitro binding assay was done by co-immunoprecipitation of cell-free synthesized [^{35}S]Pex19p variants with [^{35}S]Flag-Pex3p-EGFP, [^{35}S]Flag-Pex14p, and [^{35}S]Flag-Pex16p.

‡See the text for explanation.

levels of these Pex19p truncation mutants were at several to tenfold higher than the endogenous Pex19p in CHO-K1 cells (supplementary material Fig. S1B,C). Collectively, it is most likely that the sequences encompassing residues at positions 40-131 and 12-73 are sufficient for peroxisomal localization of Pex19p.

Domain mapping of Pex19p required for interaction with multiple PMPs

To determine whether Pex19p interacts with peroxisomal proteins, we performed the yeast two-hybrid assay. Expression of the *GAL4* DNA-binding domain (BD) fused to Pex3p and the activation domain (AD) fused to Pex19p gave rise to high β-galactosidase activity and colony growth in His^-/3-aminotriazole $(AT)^+$ medium, thereby indicating strong Pex19p-Pex3p interaction (Fig. 4A, Table 1), consistent with our earlier finding (Ghaedi et al., 2000b). Pex19p interaction was likewise observed with Pex10p, Pex11pβ, Pex13p and Pex26p, the recruiter of AAA ATPase Pex1p-Pex6p complexes (Matsumoto et al., 2003), but weaker than the binding to Pex3p, as verified by colony growth in His^- AT^+ medium (Fig. 4B, Table 1). Pex19p interacted with N-terminal sequence 1-123 of human PMP70, termed PMP70(1-123), sufficient for targeting to peroxisomes (Table 1), as reported (Biermanns and Gaertner, 2001; Gloeckner et al., 2000; Sacksteder et al., 2000; Shibata et al., 2004). By contrast, no interaction of Pex19p was detectable with other peroxisomal proteins including Pex1p, Pex6p, Pex11pα, Pex14p, Pex16p and PMP34 in this yeast two-hybrid system (data not shown).

Next, we determined the regions of Pex19p involved in the interaction with the peroxins. 1-295 and 1-261, which possess lower peroxisome-restoring activity in *pex19* cells, showed a high level of interaction with Pex3p in the yeast two-hybrid assays (Fig. 4A). 1-255 and 1-131, both defective in peroxisome-restoring activity, showed slightly lower and moderate binding to Pex3p, respectively (Fig. 4A). By contrast, 1-73 was inactive in this assay (Table 1). Together, the C-terminal region consisting of residues 132-299 of Pex19p was probably dispensable for the interaction with Pex3p. Furthermore, ΔC256-260 and Δ256-295 showed moderate binding to Pex3p. Moreover, 1-295 also interacted with Pex26p and PMP70(1-123), but less strongly than the full-length Pex19p (Fig. 4B, Table 1). However, further deletion from the C-terminus of Pex19p, such as 1-261, but not Δ256-260 and Δ256-295, eliminated the interaction with PMP70(1-123) (Table 1). 1-261 and Δ256-260 also weakly bound to Pex26p, whereas shorter Pex19p variants showed almost no detectable interaction with Pex26p (Fig. 4B). Thus, the Pex26p-binding activity of Pex19p C-terminal truncation mutants apparently correlated well with the re-establishment of peroxisome activity in *pex19* cells. Furthermore, other peroxins, including Pex10p, Pex11pβ, and Pex13p, did not

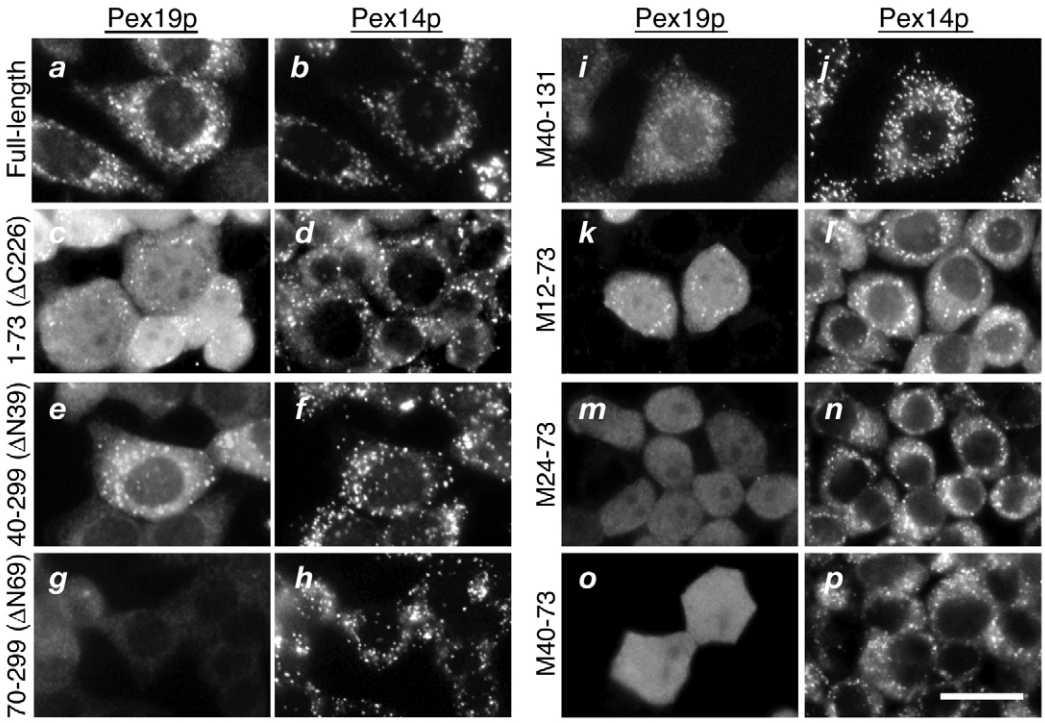

Fig. 3. Pex19p translocates to peroxisome membranes without N- and C-terminal parts. Full-length HA_2-Pex19p and its deletion mutants: 1-73 (ΔC226), 40-299 (ΔN39), 70-299 (ΔN69), M40-131, M12-73, M24-73, and M40-73 were expressed in wild-type CHO-K1 cells. Cells were double-stained with antisera to HA (left panels) and Pex14p (right panels). Bar, 20 μm.

interact with any of the C-terminal deletion mutants (Table 1). Conversely, Pex3p bound to all of these C-terminal truncated Pex19p variants except for 1-73, implying that Pex3p and other membrane peroxins bind mutually distinct sites of Pex19p.

N-terminally truncated 12-299, active in peroxisome restoration, interacted with Pex3p, Pex13p and PMP70(1-123), whereas 24-299 and Δ12-23, both devoid of peroxisome-restoring activity, did not bind to any of these three PMPs (Fig. 4A; Table 1), suggesting that the region at 12-23 plays an important role in the interaction of Pex19p with Pex3p, Pex13p and PMP70(1-123). By contrast, 12-299, 24-299, and Δ12-23 interacted with Pex26p (Fig. 4B). In binding to Pex3p, 40-299 and 70-299 were negative, whereas 40-299, but not 70-299, was positive in binding to Pex26p (Table 1). Collectively, the activities of Pex19p N-terminal deletion variants in binding to Pex3p, Pex13p and PMP70(1-123) agreed well with their peroxisome-restoring activity in *pex19* cells (Table 1). M12-261 competent in the peroxisome-restoring activity and incompetent M12-255 interacted with Pex3p, but not with PMP70(1-123) (Table 1). Therefore, considered together with the results of the C-terminal deletion mutants, it is more likely that the interaction of Pex19p with Pex3p is required for the peroxisome assembly.

Pex19p region involved in interaction with Pex3p

Pex3p interacted with N-terminal region of Pex19p in the yeast two-hybrid assay (Fig. 4A). To confirm these findings, we performed co-immunoprecipitation assays using cell lysates of COS7 cells expressing HA_2-Pex19p and its variants including Myc hexamer (Myc_6)-tagged shorter constructs together with Flag-Pex3p-fused enhanced green fluorescent protein (EGFP) (Flag-Pex3p-EGFP) (Fig. 5A). Flag-Pex3p-EGFP was co-immunoprecipitated with HA_2-Pex19p (Fig. 5A, lane 12). Flag-Pex3p-EGFP was likewise immunoprecipitated with Pex19p variants deleted from the C-terminus, 1-295, 1-261 and 1-255, and two middle-region deletions, Δ256-260 and Δ256-295 (Table 1). Flag-Pex3p-EGFP was also detected in the immunoprecipitates of Pex19p mutants, 1-131, 1-73, M12-261, M12-255 and M12-73, with less in M40-131, but not at all in those with M24-73 and M40-73 (Fig. 5A, lanes 13-20). Flag-Pex3p-EGFP was similarly immunoprecipitated with Pex19p N-terminal region truncation variants, 12-299, Δ12-23, 24-299 and 40-299, but not with 70-299 (Table 1), suggesting that N-terminal residues 1-39 are dispensable for binding to Pex3p. Of note, positive interaction of Δ12-23, 24-299 and 40-299 with Pex3p was inconsistent with the results of yeast two-hybrid assays, implying different types of their interactions in these two assay systems.

Next, to define a potential region of Pex19p involved in the direct interaction with Pex3p, cell-free synthesized HA_2- or Myc_6-HA_2-tagged [^{35}S]Pex19p variants were separately incubated with [^{35}S]Pex3p-EGFP. Upon immunoprecipitation of full-length [^{35}S]HA_2-Pex19p, [^{35}S]Pex3p-EGFP was specifically co-immunoprecipitated (Fig. 5B, lanes 11 and 12). [^{35}S]Pex3p-EGFP was likewise co-immunoprecipitated with 1-131, M12-131, 1-73 and M12-73, but not with M24-131, M40-131, M24-73 and M40-73 (Fig. 5B, lanes 13-20), suggesting that the N-terminal short peptide sequence at 12-23 is a direct Pex3p-binding site. The interaction of M40-131 with Pex3p detected by co-immunoprecipitation using cell lysates (Fig. 5A) is more likely to be mediated by other factor(s). Accordingly, M12-73 and M40-131 are the shortest sequences that are competent in translocation to the peroxisome (see Fig. 3) and binding to Pex3p. These results suggest that Pex19p interacts with Pex3p at its two regions and that Pex3p functions in recruiting Pex19p to peroxisomal membranes as a potential anchoring site in peroxisome

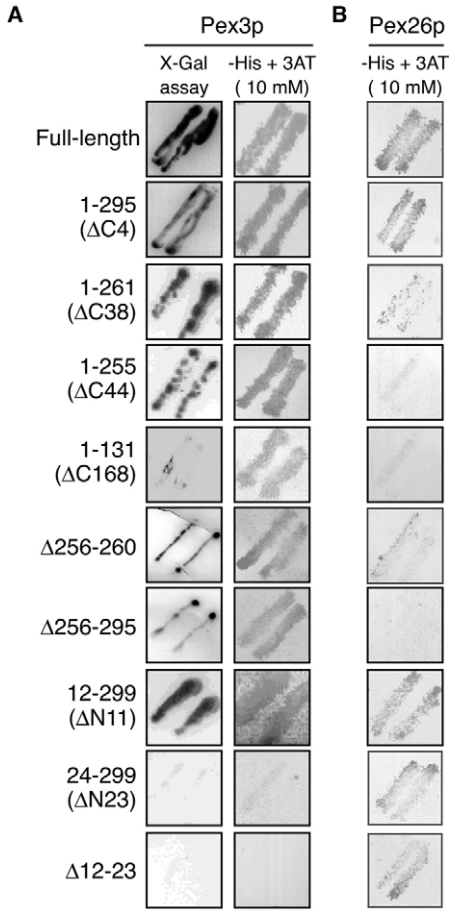

Fig. 4. Interacting region of Pex19p with Pex3p and Pex26p. Pex19p interaction was assessed by yeast two-hybrid assay. Host strain MaV203 was transformed with two plasmids, one encoding the DNA-binding domain (BD) of Gal4p fused to the full-length rat Pex3p (A) or human Pex26p (B) and the other encoding the Gal4p transactivation domain (AD) fused to Pex19p variants indicated on the left. Transformants were assayed for β-galactosidase expression (A, left panels) and growth on synthetic complete medium lacking His in the presence of 10 mM 3-aminotriazole (3AT) (A, right panels; B). BD-fused peroxins without Pex19p and Pex19p-AD with mock vector showed no self-activation.

membrane biogenesis. Such two-site interaction of Pex19p with Pex3p was recently suggested by a bacterial two-hybrid assay (Fransen et al., 2005).

The N-terminal region of Pex19p interacts with Pex14p and Pex16p

As model PMPs, we investigated Pex14p, the Pex5p-docking receptor (Albertini et al., 1997; Otera et al., 2000; Otera et al., 2002), and Pex16p, one of the membrane peroxins essential for peroxisomal membrane assembly (Honsho et al., 1998; South and Gould, 1999). We did not detect interaction of Pex19p with Pex14p and Pex16p in our yeast two-hybrid assays (data not shown), whereas such bindings were previously shown by mammalian and yeast two-hybrid assays (Fransen et al., 2001; Sacksteder et al., 2000). Therefore, we assessed any such interaction by co-immunoprecipitation assay from the lysates of COS7 cells expressing HA_2- or Myc_6-HA_2-Pex19p truncation mutants together with Flag-Pex14p or Flag-Pex16p (supplementary material Fig. S3; Table 1). The interactions of several Pex19p variants with Pex14p and Pex16p were also assessed by co-immunoprecipitation of cell-free synthesized [^{35}S]Flag-Pex14p and [^{35}S]Flag-Pex16p. [^{35}S]Flag-Pex16p was co-immunoprecipitated only with full-length [^{35}S]HA_2-Pex19p, not [^{35}S]HA_2-labeled 1-131, M12-131, M24-131 and M40-131, whereas [^{35}S]Flag-Pex14p was detected in the immunoprecipitates of all of these [^{35}S]HA_2-Pex19p variants (Table 1). Taken together, the minimum length sequences of Pex19p required for the interaction with Pex16p and Pex14p apparently reside at positions 40-255 and M40-73, respectively. With respect to Pex19p translocation to peroxisomes, Pex16p does not appear to be responsible, because several Pex19p truncation variants such as 1-131 and M12-131 are translocated to peroxisomes despite elimination of their activity in binding to Pex16p (Table 1). Pex14p is also less likely to be essential for peroxisomal localization of Pex19p, as noted that Pex19p is detectable in peroxisomal remnants in Pex14p-deficient CHO *pex14* mutant cells (see below, Fig. 6).

Pex3p deficiency enhances localization of Pex19p to the cytosol

Pex19p is partly localized to peroxisome membranes (see Fig. 3), although Pex19p contains neither a transmembrane domain nor known peroxisomal targeting signals (Matsuzono et al., 1999). To assess whether any peroxins play a role in membrane translocation of Pex19p, we expressed HA_2-Pex19p in wild-type CHO-K1 and peroxisome-deficient CHO *pex* mutants. In addition to a diffuse staining pattern, punctate staining with HA_2-Pex19p was observed, which was superimposable with PMP70, in CHO-K1 (Fig. 6Aa,b), *pex14* ZP161 (Shimizu et al., 1999) (Fig. 6Ac,d), and *pex26* ZP167 (Matsumoto et al., 2003) (Fig. 6Ae,f), indicating its localization to normal peroxisomes and peroxisomal ghosts. Thus, Pex14p is apparently not involved in translocation of Pex19p to peroxisome membranes, as addressed above in the results of Fig. S3 and Table 1. Furthermore, HA_2-Pex19p was likewise translocated to peroxisomal membrane remnants in CHO mutants defective in the matrix protein import (Fujiki, 2000), including *pex1* ZP107, *pex2* Z65, *pex5* ZP105, *pex6* ZP164, *pex7* ZPG207, *pex12* ZP109, *pex13* ZP128 and ZP114 (data not shown). By contrast, HA_2-Pex19p was detected only in a diffuse staining pattern in the cytosol in peroxisome-membrane-deficient *pex3* ZPG208 (Ghaedi et al., 2000b), where PMP70 was not discernible (Fig. 6A,g,h). Intracellular localization of HA_2-Pex19p was also determined in fibroblasts from a normal control and patients with PBD. In normal fibroblasts, HA_2-Pex19p was located in both the cytosol and peroxisomes as assessed by Pex14p-staining (Fig. 6Ba,b), as in CHO-K1 cells. HA_2-Pex19p was localized to Pex14p-stained peroxisome membrane remnants and the cytosol in fibroblasts from a *pex12* PBD patient of CG3 (Okumoto et al., 1998b) (Fig. 6Bc,d). On the other hand, HA_2-Pex19p was all diffused in the cytosol in *pex3* CG12 (Ghaedi et al., 2000a) and *pex16* CG9 (Honsho et al., 1998) fibroblasts absent from peroxisomal ghosts (Fig. 6Be-h), as in CHO *pex3* ZPG208. Accordingly, these results suggested that localizing Pex19p to peroxisomal membrane structures requires the presence of Pex3p and Pex16p.

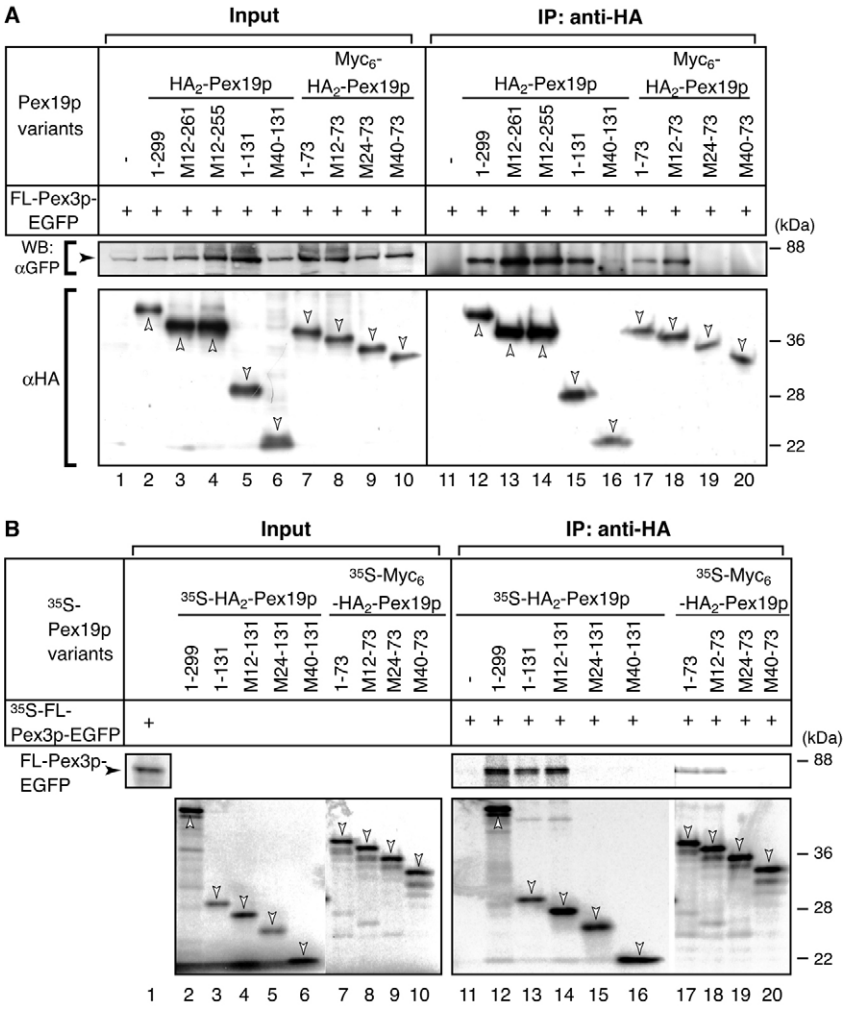

Fig. 5. A region of Pex19p encompassing amino acid residues 12-73 and 40-131 is sufficient for binding to Pex3p. (A) HA_2- or Myc_6-HA_2-tagged Pex19p truncation mutants were co-expressed with FL-Pex3p-EGFP in COS7 cells. HA_2-Pex19p and Myc_6-HA_2-Pex19p variants were immunoprecipitated using rabbit anti-HA antibody and analyzed by SDS-PAGE. HA_2-Pex19p and Myc_6-HA_2-Pex19p proteins were detected by immunoblot with mouse anti-HA antibody (lower panel). FL-Pex3p-EGFP co-immunoprecipitated with Pex19p was detected with anti-GFP antibody (upper panel). Lanes 1-10, 10% input used for immunoprecipitation; lanes 11-20, immunoprecipitates. Solid arrowhead indicates FL-Pex3p-EGFP and open arrowheads, HA_2- and Myc_6-HA_2-Pex19p variants. (B) Direct interaction of Pex19p N-terminal region with Pex3p. ^{35}S-labeled, HA_2- and Myc_6-HA_2-tagged Pex19p mutants and [^{35}S]FL-Pex3p-EGFP were separately synthesized in a cell-free system. [^{35}S]HA_2-Pex19p and [^{35}S]Myc_6-HA_2-Pex19p variants (2.5 μl each) were incubated with [^{35}S]FL-Pex3p-EGFP (2.5 μl) and immunoprecipitated with anti-HA antibody. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Lanes 1-10, 10% input used for immunoprecipitation; lanes 11-20, immunoprecipitates. Solid arrowhead indicates [^{35}S]FL-Pex3p-EGFP and open arrowheads indicate [^{35}S]HA_2-Pex19p and [^{35}S]Myc_6-HA_2-Pex19p mutants.

Pex19p functions as chaperone and transporter of PMPs in peroxisome biogenesis

In an attempt to address the Pex19p interaction with multiple PMPs at an early stage of peroxisome biogenesis, we separately expressed Flag-tagged PMPs, two transmembrane-type Pex14p (Shimizu et al., 1999) and Pex16p (Honsho et al., 1998), and C-terminal-tailed type-II Pex26p (Matsumoto et al., 2003), in *pex19* ZP119 cells and determined their intracellular localization at 12, 24 and 36 hours post-transfection (Ghaedi et al., 2000b; Matsuzono et al., 1999). These Flag-peroxins were mostly localized to mitochondria and/or some membrane-particle-like structures discernible in proximity to mitochondria at 12 hours post-transfection, as verified by staining with MitoTracker (Fig. 7A, left panels). By contrast, when these Flag-PMPs were co-expressed with HA_2-Pex19p, they were not localized to any intracellular membrane structures at 12 hours, apparently remaining in the cytosol as seen for HA_2-Pex19p (Fig. 7A, middle left panels). At 24 hours, Flag-Pex14p and Flag-Pex16p were partly visible in a punctate staining pattern, coincident with HA_2-Pex19p in punctate structures, thereby suggesting that peroxisome membranes were assembled, whereas Flag-Pex26p was mostly in the cytosol with HA_2-Pex19p (Fig. 7A, middle, right panels). At 36 hours, these PMPs became distinct in a manner superimposable on particle-bound HA_2-Pex19p, indicating that peroxisome membranes were assembled (Fig. 7A, right panels). Essentially the same results were obtained with Pex10p and Pex11pβ as for Pex14p and Pex16p (data not shown). Particles stained with Flag-PMP and HA_2-Pex19p were more readily discernible when the cytosol was washed out before cell fixation by digitonin treatment where only plasma membranes are permeabilized (Okumoto et al., 1998b) (data not shown). Flag-Pex26p- and HA_2-Pex19p-positive particles at 36 hours were confirmed as the re-established peroxisomes by dual staining of Pex14p and HA_2-Pex19p (Fig. 7As,t). HA_2-Pex19p and Pex14p were likewise discernible in a superimposable manner in ZP119 cells expressing the respective Flag-PMP peroxins (data not shown). Moreover, endogenous Pex14p in ZP119 showed the same profile as the ectopically expressed Flag-Pex14p upon transfection of HA_2-PEX19 (Fig. 7B). We interpreted these results to mean that Pex19p binds to newly synthesized PMPs in the cytosol, preventing them from mistargeting to other organelle membranes such as mitochondria, and then transports PMPs to peroxisome membranes, possibly in a temporally differentiated manner at least in the case of Pex26p, where peroxisomes are assembled. These findings are in good agreement with our most recent results (Matsuzono and Fujiki, 2006) reporting that

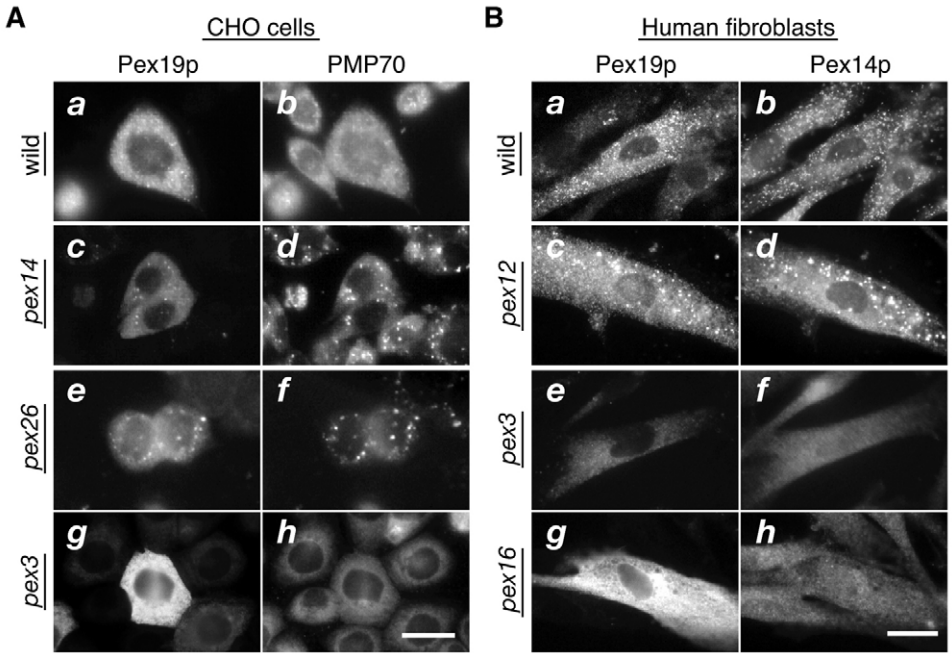

Fig. 6. Pex19p translocates to peroxisomes in a PMP-independent manner. (A) HA_2-Pex19p was expressed in CHO-K1 cells (a,b) and CHO mutants, pex14 ZP161 (c,d), pex26 ZP167 (e,f) and pex3 ZPG208 (g,h). Cells were stained with antisera to HA (left panels) and PMP70 (right panels). (B) Fibroblasts from a normal control (a,b) and PBD patients defective in PEX12 (CG3) (c,d), PEX3 (CG-G, CG12) (e,f), and PEX16 (CG-D, CG9) (g,h) were transfected with HA_2-PEX19. Cells were stained with antibodies against HA (left panels) and Pex14p (right panels). Bar, 20 μm.

Pex19p transports PMPs such as Pex16p and Pex26p to peroxisomes in vitro in an ATP-dependent manner. Furthermore, EGFP-fused Pex3p, a strong binding partner of Pex19p (Ghaedi et al., 2000b) (see Figs 4, 5; Table 1), was also mistargeted to mitochondria in the absence of Pex19p when expressed in ZP119 cells (Fig. 7C, left panels), consistent with the observation in human *pex19* fibroblasts (Sacksteder et al., 2000). Even upon co-expression with HA_2-Pex19p, Pex3p-EGFP remained mostly mislocalized to mitochondria together with Pex19p (Fig. 7C, right panels). These results implied that Pex19p was less likely to bind to newly synthesized Pex3p as a chaperone as seen in other PMPs at the early steps of peroxisome assembly (Fig. 7A). Strong interaction of Pex19p with Pex3p may occur at other distinct steps such as that involving Pex19p docking on peroxisome membranes.

Discussion

In several earlier studies (Goette et al., 1998; Matsuzono et al., 1999; Snyder et al., 1999), Pex19p is shown to play a central role in the early steps of peroxisomal membrane biogenesis. Defects of Pex19p function results in the impairment of peroxisome biogenesis in mammalian cells such as CHO cells and human fibroblasts, including the failure of peroxisomal membrane assembly occurring before the import of peroxisomal matrix enzymes (Kinoshita et al., 1998; Matsuzono et al., 1999; Sacksteder et al., 2000). Expression of Pex19p restores the impaired peroxisome biogenesis in CHO *pex19* mutant, ZP119 (Matsuzono et al., 1999) and CG-J (CG14) PBD patient-derived fibroblasts (Matsuzono et al., 1999; Sacksteder et al., 2000).

In the present work, we showed that the deletion of the CAAx box motif from the C-terminus of Pex19p slightly reduced the peroxisome-restoring activity, as noted in PTS1 proteins, which partly remained in the cytosol. Our findings in yeast two-hybrid assays indicated that the CAAx box was important for the interaction of Pex19p with several peroxins, such as Pex10p, Pex11pβ, and Pex13p, but not with Pex3p (Table 1), consistent with the previous observation (Fransen et al., 2001). Thus, the slight reduction in peroxisome-restoring activity of 1-295(ΔC4) of Pex19p apparently giving rise to fewer re-established peroxisomes is possibly due to the abrogation of its interaction with such peroxins. Several explanations can be considered for this observation. First, membrane targeting or assembly of these peroxins, including Pex10p, Pex11pβ and Pex13p, might not be a prerequisite for the early stages of peroxisomal membrane biogenesis. Pex19p may have an auxiliary role in targeting to peroxisome membranes or assembly of these peroxins during peroxisomal biogenesis. Second, Pex19p might not interact with these peroxins during the membrane biogenesis step but the interaction occurs at other biological steps, such as the maintenance of peroxisomes. Third, 1-295 might interact weakly with these peroxins, but such low activity is undetectable in the yeast two-hybrid system. It is also plausible that the impaired peroxisome membrane assembly in *pex19* ZP119 is restored simply by overexpression of 1-295. Fransen et al. (Fransen et al., 2002) reported that farnesylation is an important step for the affinity of Pex19p with Pex10p and Pex13p, and that the tetrapeptide CAAx is required for interaction with Pex11pβ. In the present work, Flag-Pex11pβ and Flag-Pex13p expressed in COS7 cells were indeed not co-immunoprecipitated with 1-295 (data not shown). However, both Δ256-260 and Δ256-295, lacking internal regions but containing the CAAx motif, lost the peroxisome-restoring activity and the interaction with Pex10p, Pex11pβ and Pex13p (Table 1). Therefore, the farnesylation may not play a major role in the peroxisome-restoring activity of Pex19p. Rather, it may promote a proper conformational change of the C-terminal domain of Pex19p by which the function of Pex19p is regulated, hence may not be involved in the direct interaction with PMPs and peroxisome-restoring activity of Pex19p. It is noteworthy that Pex19p mutants truncated in the C-terminal region and defective in the *pex19* cell-complementing activity can still interact with Pex3p (Figs 2, 4; Table 1). Together,

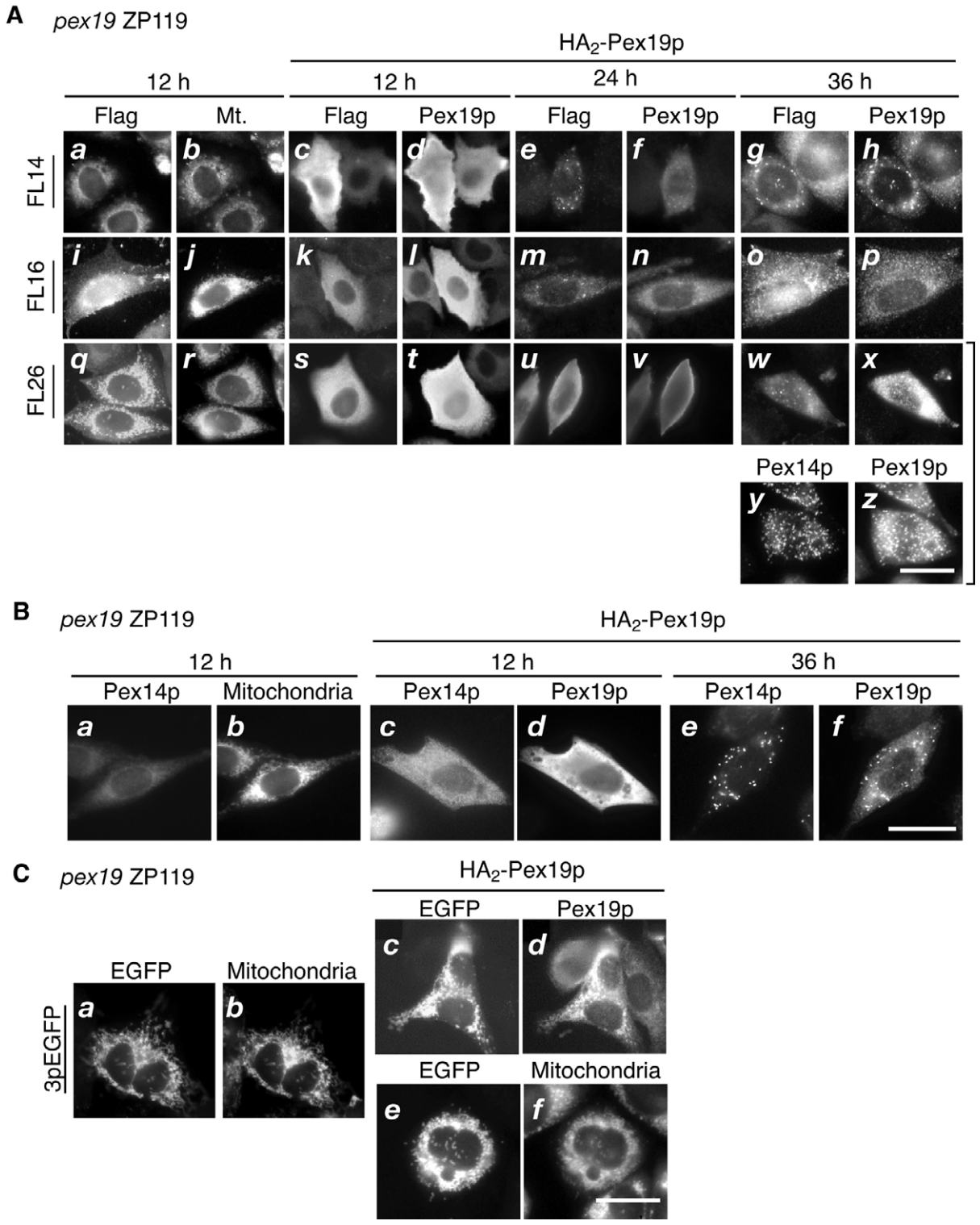

Fig. 7. Pex19p functions as a chaperone and transports PMPs in a temporally differentiated manner in peroxisome biogenesis. (A) Flag-tagged PMPs including Pex14p (FL14, a-h), Pex16p (FL16, i-p), and Pex26p (FL26, q-x) were expressed (two left panels each) or co-expressed with HA_2-Pex19p (middle and right panels) in pex19 ZP119 cells. Cells were stained with antibodies to Flag and HA and with MitoTracker, at the times indicated at the top after the transfection. ZP 119 cells co-transfected with Flag-PEX26 and HA_2-PEX19 were also stained with antibodies to Pex14p and HA at 36 hours post transfection (y,z). (B) ZP119 cells were likewise transfected with HA_2-PEX19 and were assessed for endogenous Pex14p by immunostaining with specific antibody, mitochondria by MitoTracker and Pex19p by HA staining. Note that Pex14p and HA_2-Pex19p were detectable in the cytosol at 12 hours post transfection. (C) ZP119 cells expressing Pex3p-EGFP alone (3EGFP, left panels) or with HA_2-Pex19p (right) were verified at 12 hours post transfection for Pex3p by EGFP, mitochondria and HA_2-Pex19p. Note that Pex3p-EGFP and HA_2-Pex19p were detectable in numerous particles, apparently mitochondria. Bar, 20 μm.

physiological consequences of the farnesylation of Pex19p may include: enhancement of the membrane targeting efficiency of Pex19p-PMP complexes; regulation of Pex19p in binding and release from cargo PMPs; control of the binding steps of Pex19p-PMP complexes to peroxisome membranes; and release of the PMP cargo-unloaded Pex19p from peroxisome membranes.

The Pex19p mutant 1-261 re-established peroxisomes with a reduced efficiency, whereas 1-255 was inactive. This is reminiscent of the finding that a homozygous inactivating, one-base insertion frameshift in a codon for Met255, inducing a 24-amino-acid sequence entirely distinct from normal Pex19p, is the genetic cause of Pex19p dysfunction in a patient with Zellweger syndrome of CG-J (CG14) (Matsuzono et al., 1999). Therefore, it is more likely that the region of highly conserved amino acid residues at 256-260, apparently responsible for folding the α-helix structure (Fig. S2) (Fransen et al., 2005), has an important role in peroxisome assembly. In the yeast two-hybrid assay, 1-261, but not 1-255, bound to Pex26p, the recruiter of Pex1p-Pex6p complexes (Matsumoto et al., 2003). This might also explain the difference in peroxisome-restoring activity between these two Pex19p variants. Moreover, the mutant 12-299, but not 24-299, re-established peroxisomes in *pex19* cells, indicating that the region of residues 12-23 involved in α-helix folding was essential for the complementing activity of Pex19p. The 12-299, not 24-299 and Δ12-23, was indeed as active as the full-length protein in binding to Pex3p in the yeast two-hybrid assays. Thus, the interaction of Pex19p with Pex3p is probably mediated by this α-helix region and is important for early stages of peroxisome membrane biogenesis. Taken together, we conclude that M12-261 comprises the minimal sequence for biologically active Pex19p. It is noteworthy that Δ12-23, 24-299 and 40-299, all defective in peroxisome-restoring activity, were co-immunoprecipitated with Pex3p. This may be explained by the findings that these three variants contain the sequence encompassing the residues at 40-131 apparently involved in the indirect interaction with Pex3p (see below).

Hydrophilic Pex19p is localized mostly in the cytosol and partly associated with peroxisomes (Goette et al., 1998; Jones et al., 2004; Matsuzono and Fujiki, 2006; Matsuzono et al., 1999; Sacksteder and Gould, 2000) (this study) and peroxisomal remnants in *pex* mutants defective in matrix protein import (Matsuzono and Fujiki, 2006) (this study). It is possible that Pex19p translocates to peroxisome membranes via association with other factors such as a Pex19p docking factor in peroxisome membranes. Such candidates could be Pex3p and Pex16p, the peroxins essential for membrane assembly, and Pex14p (Table 1). Pex3p is a strong interacting partner of Pex19p (Ghaedi et al., 2000b; Muntau et al., 2003; Snyder et al., 1999; Soukupova et al., 1999) (Fig, 4; Table 1). Pex19p also interacts with Pex16p (Fransen et al., 2001; Jones et al., 2004) (Table 1) and Pex14p (Fransen et al., 2002; Sacksteder et al., 2000) (Table 1). Two Pex19p variants, M12-73 and M40-131, are sufficient for translocation to peroxisomes in normal cells such as CHO-K1 (Fig. 3) and human fibroblasts (data not shown). M12-73 and M40-131 were co-immunoprecipitated with Pex3p and Pex14p, but not with Pex16p (Fig. 5 and supplementary material Fig. S3), suggesting that Pex3p and Pex14p are required for Pex19p docking to peroxisomes. Of note, direct binding of M12-73 to Pex3p is apparent, whereas M40-131 binding to Pex3p is probably indirect (Fig. 5). However, in a CHO *pex14* mutant, ZP110, which is deficient in Pex14p, Pex19p translocated to peroxisomal membrane remnants (see Fig. 6A), strongly suggesting that Pex14p is dispensable for Pex19p localization to peroxisomes and the peroxisomal ghost. The N-terminal residues 1-51 and 1-56 were shown to bind to Pex3p in the yeast (Fransen et al., 2001) and mammalian (Fang et al., 2004) two-hybrid assays, respectively. Pex3p is probably responsible for peroxisomal localization of Pex19p. This was supported by the findings using matrix-protein import-defective, CHO *pex* mutants and fibroblasts from patients with PBD, where Pex19p was localized to peroxisomal membrane remnants harboring PMPs such as Pex3p (Ghaedi et al., 2000b). On the other hand, Pex19p was exclusively localized in the cytosol in *pex3* CHO mutant cells and *PEX3*-deficient PBD patient-derived fibroblasts. Moreover, we found that co-expression with Pex19p of several membrane peroxins such as Pex14p, Pex16p, and Pex26p in CHO *pex19* cells prevented their mistargeting to mitochondria, rather maintaining them in the cytosol (Fig. 7A,B). Pex19p aided their translocation to peroxisome membranes, in a temporally differentiated manner, where peroxisomes were then assembled (Fig. 7A,B). PMPs such as Pex26p may be required at a later stage of biogenesis of functional peroxisomes. By contrast, co-expression of Pex3p with Pex19p in the *pex19* cells did not confer the cytosolic localization of Pex3p at the early step of restoration of peroxisome assembly. Pex3p remained mostly mistargeted to mitochondria, where Pex19p was colocalized (Fig. 7C), thereby implying a distinct mechanism of Pex3p transport.

With regard to its physiological role, Pex19p is thought to function as a PMP chaperone (Hettema et al., 2000; Shibata et al., 2004) and an import receptor that mediates the transport of PMPs to peroxisomes (Fransen et al., 2004; Snyder et al., 2000) by interacting with their PTS (Jones et al., 2004). However, Fang et al. (Fang et al., 2004) recently reported that Pex3p was required for recruiting and docking of Pex19p-PMP complexes to peroxisomes. Our findings in the present work favor such models. Furthermore, in our recently established in vitro Pex19p-dependent PMP transport system (Matsuzono and Fujiki, 2006), we also showed the release of Pex19p to the cytosolic fraction from peroxisomes, suggesting that Pex19p was a shuttling PMP receptor (Matsuzono and Fujiki, 2006).

Materials and Methods

Construction of *PEX19* variants

HA_2-*PEX19* and its truncated mutants were amplified by PCR using pUcD2Hyg•*PEX19* as a template and respective set of primers (supplementary material Table S1), in which *Sal*I and *Xho*I sites were introduced at the 5′ and 3′ ends, respectively. Amplified products were cloned in pGEM-T Easy vector (Promega, Madison, WI). *Sal*I-*Xho*I fragments were inserted into the *Sal*I-*Apa*I site of pUcD2Hyg•HA_2-*PEX10* (Honsho et al., 1998; Okumoto et al., 1998a). Primers used were sense primer, N2F/SalI, and antisense, R1/XhoI-NotI, for the full-length *PEX19*. To construct *PEX19* variants encoding C-terminally truncated Pex19p mutants, ΔC4 (the same as 1-295), ΔC38 (1-261), ΔC44 (1-255), ΔC168 (1-131) and ΔC226 (1-73), PCR was done with each set of primers: a forward primer N2F/SalI and respective reverse primer C299R/XhoI-NotI, C261R/XhoI-NotI, C255R/XhoI-NotI, C133R/XhoI-NotI and C73R/XhoI-NotI. To construct *PEX19* mutants encoding N-terminally truncated mutants, including ΔN11 (the same as 12-299), ΔN23 (24-299), ΔN39 (40-299) and ΔN69 (70-299), PCR was done using respective forward primers, N12F/SalI, N24F/SalI, N40F/SalI and N70F/SalI, and the reverse primer C299R/XhoI-NotI. For construction of *PEX19* variants encoding Pex19p with deletion of amino acid residues 1-11 and 262-299 (named M12-261), M12-255, M40-131 and M40-73, PCR was done using N12F/SalI or N40F/SalI and C261R/XhoI-NotI, C255R/XhoI-NotI, C131R/XhoI-NotI and C73R/XhoI-NotI,

respectively. Δ256-295 was likewise constructed with N2F/SalI and C255-CLIMR/XhoI-NotI. Primers used were N2F/SalI, C255R, N262F and C299R/XhoI-NotI for construction of Δ256-261; N2F/SalI, C11R, N23F and C299R/XhoI-NotI were used for Δ12-23.

HA_2-*PEX19* variants encoding HA_2-tagged full-length Pex19p, 1-131 (ΔC168), M12-131, M24-131, and M40-131 were cloned into pcDNA3.1 vector for an in vitro transcription and translation (see below). For M12-131, M24-131 and M40-131, PCR was done using forward N12F/SalI, N24F/SalI, and N40F/SalI, and a reverse C131R/XhoI-NotI. HA_2-*PEX19* variants were also cloned into pcDNA3.1•Myc_6 vector. Myc_6-HA_2-*PEX19* was constructed by PCR using HA_2-*PEX19* as template and primers, HAF/EcoRI and C299R/XhoI-NotI. Myc_6-HA_2-*PEX19* variants encoding 1-131, 1-73 (ΔC226), M12-73, M24-73 and M40-73, was constructed by PCR using Myc_6-HA_2-*PEX19* as template and a set of primers, N2F/SalI, N12F/SalI, N24F/SalI and N40F/SalI, with C131R/XhoI-NotI and C73R/XhoI-NotI.

Cell culture, plasmids transfection, and morphological analysis

CHO cells, including CHO-K1 and peroxisome-deficient CHO mutants, *pex3* ZPG208 (Ghaedi et al., 2000b), *pex14* ZP161 (Shimizu et al., 1999), *pex26* ZP167 (Matsumoto et al., 2003) and *pex19* ZP119 (Kinoshita et al., 1998; Matsuzono et al., 1999), were cultured as described (Tsukamoto et al., 1990). COS7 cells (Okumoto et al., 1998b) and human fibroblasts from a normal control and PBD patients each deficient in *PEX3* (Ghaedi et al., 2000a), *PEX12* (Okumoto et al., 1998b) and *PEX16* (Honsho et al., 1998) were also used. DNA transfection to CHO cells and fibroblasts was done by lipofection and electroporation, respectively (Matsuzono et al., 1999). At 3 days post transfection, peroxisomes in cells were detected by indirect immunofluorescence light microscopy (Tamura et al., 1998), with rabbit antibodies to PTS1 (Otera et al., 1998), HA (Otera et al., 2000) and PMP70 (see below), guinea pig anti-Pex14p antibody (Mukai et al., 2002), or mouse monoclonal anti-Flag antibody (Sigma, St Louis, MO). Antigen-antibody complexes were detected using Fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (IgG) (MP Biomedicals-Cappel, Irvine, CA) and goat anti-guinea pig IgG (Molecular Probes, Eugene, OR) antibodies and with Texas-Red-labeled goat anti-rabbit IgG antibody and anti-guinea pig IgG antibody (Leinco Technologies, St Louis, MO). Mitochondria were visualized with MitoTracker Red CMXRos (Molecular Probes).

Yeast two-hybrid assay

Maintenance and transformation of the yeast *Saccharomyces cerevisiae* cells were done using a Proquest two-hybrid system (Life Technologies, Gaithersburg, MD), according to the manufacturer's protocol. The full-length *PEX19* and its truncated mutants were excised from pGEM/T-easy vector with *Sal*I and *Not*I. The fragments were separately inserted into the *Sal*I-*Not*I sites downstream of the *GAL4-AD* in the pPC86 plasmid. For the constructs encoding fusion proteins with rat *PEX3* (Ghaedi et al., 2000b), human *PEX10* (Okumoto et al., 1998a), human *PEX11β* (Abe and Fujiki, 1998), human *PEX13* (Toyama et al., 1999) and human *PEX26* (Matsumoto et al., 2003), PCR amplification was done using each plasmid as a template and resulting products were inserted downstream of the *GAL4-BD* in pDBLeu. Expression of Pex19p-fused AD with empty pDBLeu vector or respective peroxins each fused to the DNA-binding domain (DB) with empty pPC86 vector did not show any self-activation activity. Co-transformation of two-hybrid vectors into *S. cerevisiae* MaV203, screening of transformants and β-galactosidase activity of the *lacZ* marker gene were done as described (Okumoto et al., 2000).

Co-immunoprecipitation assay

PEX3 in an EGFP expression vector pEGFP-C1 (Clontech, Tokyo, Japan) was cloned into pcDNA3.1 and expressed with full-length or various deleted mutants of HA_2-Pex19p in COS7 cells. After 2 days, cells were lysed in 300 μl of ice-cold binding assay buffer (Otera et al., 2002), incubated for 60 minutes on ice, and centrifuged to remove the cell debris. Protein-A-Sepharose and rabbit anti-HA antibody were added to the supernatant and the mixture was further rotated for 2 hours at 4°C. Protein-A-Sepharose beads were collected by centrifugation and washed three times with the binding assay buffer. Bound fractions were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting as described (Matsuzono and Fujiki, 2006), with mouse monoclonal antibodies to GFP (Molecular Probes) and HA (16B12: Covance, Berkeley, CA), and a second antibody, sheep anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham Biosciences, Tokyo, Japan).

[^{35}S]HA_2-Pex19p variants and [^{35}S]Pex3p-EGFP were separately synthesized in vitro using respective cDNA in pcDNA3.1 (Matsuzono and Fujiki, 2006; Miyata and Fujiki, 2005). [^{35}S]HA_2-Pex19p variants (2.5 μl each) were incubated with [^{35}S]Pex3p-EGFP (2.5 μl) in 300 μl of binding buffer at 4°C for 2 hours and [^{35}S]HA_2-Pex19p and Myc_6-HA_2-Pex19p variants were immunoprecipitated with rabbit anti-HA antibody. Immunoprecipitates were analyzed by SDS-PAGE and ^{35}S-labeled proteins were detected as described (Matsuzono and Fujiki, 2006).

Anti-PMP70 antibody

Rabbit anti-PMP70 antibody was raised by subcutaneous injection of human C-terminal 18-amino-acid peptide, as described (Ghaedi et al., 2000b).

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