Domain Architecture and Activity of Human Pex19p, a Chaperone-like Protein for Intracellular Trafficking of Peroxisomal Membrane Proteins*

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Pex19p is a peroxin involved in peroxisomal membrane biogenesis and probably functions as a chaperone and/or soluble receptor specific for cargo peroxisomal membrane proteins (PMPs). To elucidate the functional constituents of Pex19p in terms of the protein structure, we investigated its domain architecture and binding affinity toward various PMPs and peroxins. The human Pex19p cDNA was overexpressed in Escherichia coli, and a highly purified sample of the Pex19p protein was prepared. When PMP22 was synthesized by cell-free translation in the presence of Pex19p, the PMP22 bound to Pex19p was soluble, whereas PMP22 alone was insoluble. This observation shows that Pex19p plays a role in capturing PMP and maintaining its solubility. In a similar manner, Pex19p was bound to PMP70 and Pex16p as well as the Pex3p soluble fragment. Limited proteolysis analyses revealed that Pex19p consists of the C-terminal core domain flanking the flexible N-terminal region. Separation of Pex19p into its N- and C-terminal halves abolished interactions with PMP22, PMP70, and Pex16p. In contrast, the flexible N-terminal half of Pex19p was bound to the Pex3p soluble fragment, suggesting that the binding mode of Pex3p toward Pex19p differs from that of other PMPs. This idea is supported by our detection of the Pex19p-Pex3p-PMP22 ternary complex.

Peroxisomes are organelles enclosed by a lipid bilayer membrane and are commonly found in a wide variety of eukaryotic cells. They serve as the compartment for lipid metabolism as well as the generation and degradation of peroxide (1). Peroxisomes are essential for human survival, and peroxisome biogenesis disorders cause severe metabolic diseases, such as Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease (2, 3). These are often attributed to the lack of peroxisomal proteins; therefore, these peroxins are thought to contribute to the assembly of the peroxisomal membranous structure and to be necessary for the correct translocation of peroxisomal membrane proteins (PMPs).

Pex19p is a pivotal molecule in the process of PMP translocation. Human Pex19p is a 33-kDa protein, which is farnesylated at the C-terminal CAAX-box (7, 8). Most of the Pex19p protein is cytosolic, with some on peroxisomal membranes (9–11). Several genetic engineering-based analyses have revealed that human Pex19p recognizes a variety of PMPs, such as PMP22, PMP70, adrenoleukodystrophy protein, and PMP34, as “cargo proteins” and transports them to the target peroxisomes (9, 12–15). The Pex19p-PMP complex presumably interacts with Pex3p on the peroxisomal membrane, and this PMP is integrated into the membrane by unknown mechanisms (16).

Pex19p would function as a cytosolic chaperone and/or a soluble receptor for PMPs (9). Similar post-translational translocations of membrane proteins have been observed in mitochondria, mediated by Hsp70 (70-kDa heat shock protein), and in Escherichia coli, mediated by SecB (17, 18). However, Hsp70 and SecB also interact with some soluble proteins, so Pex19p is unique with regard to its specificity for the cargo proteins. It is assumed that Pex19p specifically interacts with PMP via its internal basic amino acid clusters and the flanking transmembrane helix (12). This recognition is still ambiguous, because it is difficult to handle the insoluble PMPs experimentally.

One interesting question is how Pex19p recognizes not only a variety of cargo PMPs but also the other peroxins forming the translocational apparatus on the peroxisomal membrane (9, 19, 20). We expect that multiple binding regions for the respective peroxisomal proteins would exist within the Pex19p molecule. Previously, two-hybrid systems or pull-down assays have been used to investigate the functional and structural aspects of human Pex19p (20, 21). In addition, the domain structure of Pex19p was predicted by computational analyses (21). To discuss the functional regions of the Pex19p molecule in detail, however, experimental data are needed to understand the domain architecture of Pex19p on a structural basis and the structural components contributing to the recognition of PMPs and/or peroxins.

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¶The abbreviations used are: PMP, peroxisomal membrane protein; NTA, nitrilotriacetic acid; DTT, dithiothreitol; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; TPC, t-1-tosylamido-2-phenylethyl chloromethyl ketone.

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38486
In the present study, we investigated the domain structure of human Pex19p by limited proteolysis and the functions of each structural constituent toward either the cargo PMPs or the binding partner, Pex3p. In addition, we detected the Pex19p binding region for PMPs is separated from that for Pex3p.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic oligonucleotides were purchased from Prologi LLC (Boulder, CO). Restriction enzymes and DNA-modifying enzymes were obtained from Takara Bio, Inc. (Shiga, Japan), Toyobo Co., Ltd. (Osaka, Japan), and New England Biolabs (Beverly, MA). Plasmids pGEX-6P-1 and pET-11b, and the pT7Blue T-Vector were purchased from Novagen Inc. (Madison, WI). Plasmid pGEX-6P-1 was obtained from Amersham Biosciences. PROTEINS, a wheat germ cell-free protein synthesis core kit, including the plasmid pE3U3-NII (22, 23), was purchased from Invitrogen Co., Ltd. (Kyoito, Japan).

**cDNAs**—The human Pex19p cDNA was generously provided by Dr. A. C. Muntau (Ludwig-Maximilians-University, Munich, Germany). The cDNAs encoding rat PMP22 and human PMP70 were a kind gift from Dr. T. Osumi (Himeji Institute of Technology, Hyogo, Japan). The human Pex3p and Pex16p cDNAs were isolated by PCR from a human kidney cDNA library (Invitrogen). Based on the published sequences for their cDNAs, oligonucleotides Pex3#Fw (5′-ATGCTGAGGTCTGTATG-3′), Pex3#Rev (5′-CTAGCCCCCAATCG-TAGAATA-3′) were used as primers for the cloning of Pex3p, and Pex16#Fw (5′-ATGGAGAAGCTGGCTTCTC-3′) and Pex16#Rv (5′-TCAGGCCCAATCG-TAGAATA-3′) were used as primers for the cloning of Pex16p.

**Plasmid Construction**—The plasmids constructed for the present study are listed in Table I. Their preparation was carried out by a series of DNA manipulations, as follows. To construct the His$_{16}$-Pex19p expression plasmid pET-16b/Pex19p, for instance, PCR was carried out with the forward primer Pex9#FNDel and the reverse primer Pex9#RBamHI (Tables I and II), to introduce an NdeI site at the N terminus and a BamHI site at the C terminus, respectively. The PCR product was ligated with the pETBlue T-Vector, and the DNA sequence of 1-m long dithiothreitol (DTT). The elution buffer was the starting one containing 10 mM glutathione.

**Pex19p variants, His$_{10}$-Pex19p, His$_{10}$-Pex19p(N-half), and His$_{10}$-Pex19p(C-half), were purified as follows. The bacterial pellets of BL21(DE3) were resuspended in 50 mM Tris-HCl, pH 7.5, 0.3 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT, and 0.1 mM PMSF. After 5 mM DTT was added to the protein solution, it was applied to a HiLoad 26/10 Sepharose 4 Fast Flow column equilibrated with the starting buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT, and 0.1 mM PMSF). The desired protein was eluted with a linear gradient of NaCl from 50 to 500 mM.

To purify the GST-fused Pex19p, the bacterial pellets of BL21(DE3) pGEX-6P-1/Pex19p from a 0.4-liter culture were resuspended in 10 ml of the starting buffer (50 mM Tris-HCl, pH 7.5, 0.3 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT, and 0.1 mM PMSF). The desired protein was eluted with a linear gradient of NaCl from 50 to 500 mM.
lyzed by SDS-PAGE. Detection of the cell-free synthesized PMP in the eluate readily reveals the interaction between Pex19p and PMP.

**Immunoprecipitation of PMP22**—Immunoprecipitation by anti-PMP22 antisemur was performed to reisolate the PMP22-unbound His<sub>10</sub>-Pex19 from the PMP22-His<sub>10</sub>-Pex19 complex in the cell-free translation products. The Ni<sup>2+</sup>-NTA eluate (1 ml) from 0.6 ml of the cell-free synthesized PMP22 in the presence of His<sub>10</sub>-Pex19p was mixed with the 4 µl of anti-PMP22 antisemur for 1 h at 4 °C. Next, the solution was added to the 80 µl of Protein A-agarose (50% slurry; Amersham Biosciences) and was further incubated for 1 h at 4 °C. Finally, the mixture was poured into an empty Pasteur pipette plugged with cotton to collect the supernatant fraction containing the PMP22-unbound His<sub>10</sub>-Pex19.

**Limited Proteolysis of Pex19p and Identification of the Digested Fragments**—His<sub>10</sub>-Pex19p (4 mg/ml) in buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, and 2 mM DTT) was treated with 1:1000 (w/w) TPCK-treated trypsin (Sigma) to start the proteolysis. After incubation at 37 °C for various periods, an aliquot of the mixture was withdrawn and mixed with SDS-PAGE sample buffer to stop the reaction. In the case of the V8 protease digestion, 4 mg/ml His<sub>10</sub>-Pex19p was treated with 1:200 (w/w) V8 protease (Pierce) in 0.1 M NH<sub>4</sub>HCO<sub>3</sub> and 2 mM DTT at 37 °C.

Each peptide fragment was identified by both mass spectrometry and N-terminal sequencing. To perform the mass spectrometry, the limited proteolysis products were desalted with ZipTip C18 (Millipore). The samples were applied to a Voyager-DE PRO spectrometer (Applied Biosystems), which is based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. At the same time, for the N-terminal sequence analysis, the peptide fragments were separated by SDS-PAGE and blotted onto an Immobilon-P transfer membrane. The fragments were stained with Ponceau S, excised from the membranes, and analyzed by a Procise 492HT protein sequencer (Applied Biosystems).

**Circular Dichroism Analysis**—Circular dichroism spectra were measured with a JASCO J-725 spectrometer (Jasco International Co., Ltd., Tokyo, Japan). The protein concentration was adjusted to 0.2 mg/ml in buffer (10 mM sodium phosphate, pH 7.5, and 1 mM DTT). A quartz cell with a 1-mm light path length was used.

**RESULTS**

**Overexpression and Purification of Pex19p**—To investigate the molecular properties of the human Pex19p protein, we prepared a highly purified sample. The Pex19p open reading frame encodes a C-terminal CAAX-box, which is modified by post-translational farnesylation (8). The three C-terminal residues of premature Pex19p are post-translationally processed during the farnesylation of Cys<sub>296</sub>. However, this farnesylation is not required for the binding of Pex19p to PMPs, Pex3p, or Pex16p (14, 20). To avoid the potential problem of incomplete farnesylation, we constructed expression plasmids for a Pex19p derivative, where Cys<sub>296</sub> was replaced with Ala and the three C-terminal residues were deleted. This modified Pex19p was overproduced in E. coli as an N-terminally histidine-tagged protein or a GST fusion protein. The purification of His<sub>10</sub>-Pex19p is summarized in Fig. 1.

**Cell-free Translation Analyses to Detect PMP22 Binding by Pex19p**—Since Pex19p is believed to function as a receptor and/or chaperone for PMPs, Pex19p should exhibit some features of the biological activity, such as binding to PMP synthesized on free ribosomes, keeping PMP soluble in the cytosol, and translocating PMP toward peroxisomes. In particular, the former two biological functions can be evaluated by a cell-free translation system, which can reveal the interaction between the purified Pex19p and the synthesized PMP.

We first examined whether a complex of His<sub>10</sub>-Pex19p and the cell-free synthesized PMP22 (22-kDa peroxisomal membrane protein) is formed and whether this complex is soluble. Using pEU-N1/PMP22 (Table I) as the template DNA, the PMP22 mRNA was synthesized by T7 RNA polymerase. Then, in the presence of this mRNA, PMP22 polypeptides were synthesized with the wheat germ extract cell-free translation system (Fig. 2A). In the presence of His<sub>10</sub>-Pex19p, a portion of the synthesized PMP22 was recovered in the supernatant. When this supernatant was applied to a Ni<sup>2+</sup>-NTA-agarose column, PMP22 co-eluted with His<sub>10</sub>-Pex19p. On the other hand, the translated PMP22 was completely insoluble in the absence of Pex19p, and no PMP22 was detected in the supernatant fractions. Therefore, the PMP22 synthesized in the cell-free system was bound to the purified His<sub>10</sub>-Pex19p and was solubilized by the complex formation. Additionally, we tested whether Pex19p recognizes the nascent polypeptide of PMP22 or the completely translated one as follows. PMP22, synthesized in the absence of Pex19p, was collected as a precipitate and was resuspended in the wheat germ extract system without the PMP22 mRNA. This solution was incubated with His<sub>10</sub>-Pex19p and applied to Ni<sup>2+</sup>-NTA-agarose to collect the imidazole eluate. The SDS-PAGE analysis revealed that no band corresponding to PMP22 was found in the imidazole eluate (lane −/+ in Fig. 2A). Therefore, Pex19p could not bind to the insoluble form of PMP22. We concluded that Pex19p interacts with a fraction of the nascent polypeptides of PMP22 and prevents their aggregation. This method is convenient for monitoring the Pex19p-mediated complex formation.

As shown in lane 6 in Fig. 2A, excess amounts of free His<sub>10</sub>-Pex19p were also present in the imidazole eluate from the Ni<sup>2+</sup>-NTA-agarose column. By removing the proteins that do not contribute to the complex formation, we further corroborated the formation of a specific complex between the synthesized PMP22 and Pex19p. First, GST-Pex19p was produced in E. coli and purified with glutathione-Sepharose. Second, His<sub>10</sub>-PMP22 was synthesized by the cell-free translation system in the presence of the purified GST-Pex19p. Finally, the complex was purified from the cell-free reaction mixture with Ni<sup>2+</sup>-NTA-agarose and glutathione-Sepharose. As shown in Fig. 2B, in the presence of GST-Pex19p and the His<sub>10</sub>-PMP22 mRNA, both Pex19p and PMP22 were detected by the SDS-PAGE analysis. In contrast, when either GST-Pex19p or the His<sub>10</sub>-PMP22 mRNA was absent, no bands were observed. Therefore, the cell-free synthesized PMP22 and Pex19p are both soluble and available for their specific complex formation.

In our experimental conditions, not all of the synthesized PMP22 bound to His<sub>10</sub>-Pex19p even in the presence of the excess amount of His<sub>10</sub>-Pex19p (Fig. 2A). To exclude the possi-
TABLE I

| Plasmid               | Product                  | PCR primer pair (forward/reverse) |
|-----------------------|--------------------------|-----------------------------------|
| pET-16b/Pex19p        | His\textsubscript{10}-Pex19p | Pex19\#FNdeI/Pex19\#RBamHI         |
| pGEX-6P-1/Pex19p      | GST-Pex19p               | Pex19\#FBamHI/Pex19\#RSalI         |
| pET-11b/Pex19p        | Pex19p                   | Pex19\#FNdeI/Pex19\#RBamHI         |
| pET-16b/Pex19p(\text{N-half}) | His\textsubscript{10}-Pex19p-(1–156) | Pex19\#FNdeI/Pex19\#RBamHI         |
| pET-16b/Pex19p(\text{C-half}) | His\textsubscript{10}-Pex19p-(157–296) | Pex19\#FNdeI/Pex19\#RBamHI         |
| pGEX-6P-1/Pex3p-(34–373) | GST-Pex3p-(34–373)      | Pex19\#FNdeI/Pex19\#RBamHI         |
| pET-16b/PMP22         | His\textsubscript{10}-PMP22 | PMP22#FNdeI/PMP22#RBamHI           |
| pEU3-NII/PMP22        | PMP22                    | His\textsubscript{10}-PMP22         |
| pEU3-NII/His\textsubscript{10}-PMP22 | PMP22                  | His\textsubscript{10}-PMP22         |
| pEU3-NII/PMP70\textsuperscript{c} | PMP70                   | PMP70#FXhoI/pcDNA#Rv               |
| pEU3-NII/Pex16p       | Pex16p                   | Pex16#FSpeI/Pex16#RBglII           |
| pEU3-NII/Pex3p-(34–373) | Pex3p-(34–373)         | Pex3#FSpeI/Pex3#RBglII             |

\textsuperscript{a} PCR primers are listed in Table II.
\textsuperscript{b} Template for PCR was pET-16b/PMP22.
\textsuperscript{c} Template for PCR was pcDNA3.1(\text{+}) with the PMP70 cDNA inserted at the NotI sites.

**Fig. 2. Effects of Pex19p on the cell-free synthesis of PMP22.** Coomassie Brilliant Blue staining of a 12.5% polyacrylamide gel. A, in the presence (+) or absence (−) of the recombinant His\textsubscript{10}-Pex19p (0.5 mg/ml), the PMP22 mRNA was translated with the wheat germ extract. The product was centrifuged to collect the precipitates (ppt), and then the supernatant was applied to Ni\textsuperscript{2}\textsuperscript{+}-NTA-agarose to recover the pass-through fractions (sup\textsubscript{pass}) and the imidazole-eluate (sup\textsubscript{eluate}). The lane indicated as (−/+\textsubscript{+}) was the Ni\textsuperscript{2}\textsuperscript{+}-NTA-eluted sample prepared in the same way as the PMP22 precipitate, which was synthesized in the cell-free system without Pex19p, and then was incubated in the solution containing His\textsubscript{10}-Pex19p, as described under “Results.” The triangles and asterisks show the band of His\textsubscript{10}-Pex19p and the synthesized PMP22, respectively. B, in the presence of the recombinant GST-Pex19p (0.5 mg/ml), the His\textsubscript{10}-PMP22 mRNA was translated with the wheat germ extract. The product was successively applied onto Ni\textsuperscript{2}\textsuperscript{+}-NTA-agarose and glutathione-Sepharose, and the final eluate was analyzed by SDS-PAGE. The triangles and asterisks show the band of GST-Pex19p and the synthesized His\textsubscript{10}-PMP22, respectively. C, in the presence of the purified His\textsubscript{10}-Pex19p, PMP22 was synthesized by the cell-free translation system, and the free His\textsubscript{10}-Pex19p and the His\textsubscript{10}-Pex19p-PMP22 complex were collected with Ni\textsuperscript{2}\textsuperscript{+}-NTA-agarose (1st). The complex was then removed from the eluate by immunoprecipitation with anti-PMP22 antiserum. The given free His\textsubscript{10}-Pex19p was repurified with the Ni\textsuperscript{2}\textsuperscript{+}-NTA-agarose, added to the second cell-free synthesis of PMP22, and treated with Ni\textsuperscript{2}\textsuperscript{+}-NTA-agarose (2nd). The third experiment was further carried out in the same way (3rd). Each eluate was analyzed by Western blotting for the detection of PMP22 (upper row) and His\textsubscript{10}-Pex19p (lower row). PMP22 in the complex form (lane +) was compared with the control (lane −), where the PMP22 mRNA was eliminated.
The imidazole eluates were analyzed by SDS-PAGE. The triangles respectively. sized proteins were co-eluted with His10-Pex19p (Fig. 3). These eluates from the reaction mixtures showed that the synthesis of His10-Pex19p. SDS-PAGE analysis of the Ni2+ and then the translation was carried out in the presence of corresponding mRNA was prepared from the given plasmid, cDNA of each PMP was inserted into pEU3-NII (Table I). The proteins or peroxins, such as PMP70, Pex16p, and Pex3p. The binding affinity of Pex19p toward other peroxisomal membrane — Pex3p to Pex19p. aggregates could compete with the binding of PMP22 to same as that in the longer translation, such as a 16-h incubation of the precipitated PMP22 even in the early stage of the translation products and examined whether the isolated His10-Pex19p is able to bind to freshly synthesized PMP22. After the cell-free synthesis of PMP22 in the presence of His10-Pex19p, the His10-Pex19p-PMP22 complex was removed by immunoprecipitation with anti-PMP22 antisera. The given PMP22-unbound His10-Pex19p was once more applied to the second cycle of the cell-free PMP22 synthesis. As a result, the recycled His10-Pex19p also bound to and solubilized PMP22 at the same level as the fresh His10-Pex19p (Fig. 2C). Therefore, most of His10-Pex19p molecules in the present preparation seem to be active. The reason why parts of PMP22 were unbound to the excess amount of His10-Pex19p is ambiguous. However, a ratio of the precipitated PMP22 even in the early stage of the translation reaction, such as 2-h incubation, was essentially the same as that in the longer translation, such as a 16-h incubation (data not shown). Therefore, the binding of PMP22 to Pex19p could be not so tight, or the formation of the PMP22 aggregates could compete with the binding of PMP22 to Pex19p.

The Binding Affinity of Pex19p toward PMP70, Pex16p, and Pex3p—In the same manner as for PMP22, we examined the binding affinity of Pex19p toward other peroxisomal membrane proteins or peroxins, such as PMP70, Pex16p, and Pex3p. The cDNA of each PMP was inserted into pEU3-NII (Table I). The corresponding mRNA was prepared from the given plasmid, and then the translation was carried out in the presence of His10-Pex19p. SDS-PAGE analysis of the Ni2+-NTA-agarose eluates from the reaction mixtures showed that the synthesized proteins were co-eluted with His10-Pex19p (Fig. 3). These results indicate that the purified Pex19p was available for binding, to not only PMP22, but also PMP70, Pex16p, and Pex3p-(34–373). Thus, our preparation of Pex19p is believed to be biologically active.

In this experiment, we found that the full-length Pex3p synthesized with the cell-free system was mostly insoluble, even with His10-Pex19p, so that some of the His10-Pex19p proteins were involved in this pellet fraction (data not shown). This strong hydrophobicity of Pex3p would be caused by the N-terminal 33-residue segment, which presumably contains the transmembrane region. We expect that the removal of the hydrophobic segment from Pex3p would only slightly affect its structure and binding affinity to other proteins. Actually, Pex3p, lacking the N-terminal 33 residues, bound to His10-Pex19p (Fig. 3), so we used the Pex3p-(34–373) construct to estimate the binding of Pex19p toward Pex3p in the present study. This strategy is appropriate, because our results are consistent with those from previous studies on the in vivo effects of the N-terminal truncation of Pex3p (19, 20).

The Domain Structure of Pex19p Analyzed by Limited Proteolysis—To determine the functional regions in the Pex19p molecule, based on the protein structure, we carried out limited proteolysis experiments with trypsin and V8 protease. As shown in Fig. 4A, His10-Pex19p was readily digested by trypsin, which generated a fragment that migrated between the 14- and 20-kDa marker proteins on SDS-PAGE. The molecular mass of this fragment was measured as 15,766.5 by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Furthermore, the N-terminal amino acid sequence of this fragment was determined as Ala-Met-Glu-Gly-Leu. These data indicated that the accumulated fragment of Pex19p is the peptide from Ala156 to Ala296 at the C terminus, which has a calculated molecular weight of 15,765. These facts suggest that the C-terminal half of Pex19p is a relatively stable domain. On the other hand, the N-terminal half of Pex19p was susceptible to proteolytic degradation, which suggests that it has a flexible conformation. When a similar experiment was carried out with V8 protease, a comparable C-terminal fragment accumulated (Fig. 4B), which had Gly156 at its N terminus. Therefore, these results indicate that a domain boundary of Pex19p exists around Ala156.

Circular Dichroism Analysis of the Half-size Fragments of Pex19p—We analyzed the structural properties of the N- and C-terminal halves of Pex19p by circular dichroism. For the large scale preparation of the C-terminal half of Pex19p (Ala156–Ala296), His10-Pex19p (100 mg) was treated with trypsin. After the reaction was stopped with PMSF, the desired fragment was purified by HiLoad 26/10 Q-Sepharose HP anion exchange chromatography, as in the purification of the full-length protein. The final recovery of the purified fragment, Pex19p(C-half), was 16 mg. The N-terminal half of Pex19p (Met1–Ala156) was prepared by E. coli overproduction as an N-terminal His-tagged protein (Table II) and was purified in the same way as the full-length protein. Then circular dichroism spectra for Pex19p(C-half) and His10-Pex19p(N-half) as well as the full-length His10-Pex19p were measured (Fig. 5). In comparison with the full-length His10-Pex19p, Pex19p(C-half) exhibited a larger amount of secondary structure. The α-helix contents calculated by the mean residual ellipticity at 222 nm were evaluated as 22 and 55% for the full-length and C-half, respectively. On the other hand, His10-Pex19p(N-half) displayed little secondary structure; its α-helix content was estimated as 6%. These results are consistent with the consequences of the limited proteolysis. The Pex19p(C-half) protein has an abundance of secondary structure, which conferred its resistance to proteases. In contrast, Pex19p(N-half) has much less secondary structure, and thus the flexible structure was susceptible to the proteolytic attacks. Moreover, the additive spectrum of His10-Pex19p(N-half) plus Pex19p(C-half) almost coincided with that of the full-length His10-Pex19p (data not shown). Therefore, we conclude that both halves of Pex19p maintain their respective three-dimensional structures as in
The Binding Affinity of the Pex19p-Pex3p Complex toward PMP22—Since the recognition of Pex3p by Pex19p differed from those of the other PMPs, we expected that a Pex19p-Pex3p-PMP22 ternary complex could be formed. To test this idea, we tried to synthesize PMP22 in the presence of the Pex19p-Pex3p-PMP22 complex. GST-Pex3p-(34–373) and tagless Pex19p were overexpressed independently, and then both cell harvests were mixed and disrupted together to form the GST-Pex3p-(34–373)-Pex19p complex. The complex was purified on a glutathione-Sepharose affinity column (Fig. 7A). In the presence of the Pex19p-GST-Pex3p-(34–373) complex, the His$_{16}$-PMP22 mRNA was translated in vitro by the wheat germ system, and the reaction mixture was chromatographed on Ni$_{2+}$-NTA-agarose and subsequently on glutathione-Sepharose to purify the complex containing His$_{16}$-PMP22 and GST-Pex3p-(34–373). As shown in Fig. 7B, the final eluate included the tagless Pex19p as well as His$_{16}$-PMP22 and GST-Pex3p-(34–373), indicating that a Pex19p-GST-Pex3p-(34–373)-His$_{16}$-PMP22 ternary complex was formed. On the other hand, when GST-Pex3p-(34–373) without Pex19p was included in the cell-free synthesis of His$_{16}$-PMP22, only a slight amount of GST-Pex3p-(34–373) was detected, whereas very little His$_{16}$-PMP22 was observed. These results suggest that Pex3p cannot form a stable complex with PMP22. In addition, when the GST-Pex3p-(34–373)-Pex19p complex was incubated without the synthesis of His$_{16}$-PMP22, the tagless Pex19p was not found in the final affinity-purified eluates. In the latter two cases, a band corresponding to GST-Pex3p-(34–373) was faintly visible. This is probably GST-Pex3p-(34–373) that nonspecifically bound to the columns, due to its propensity to form aggregates. In essence, the data indicate that Pex3p and PMP22 make an indirect interaction only when Pex19p is present. In other words, the recognition site on Pex19p for Pex3p is independent of that for PMP22.

**DISCUSSION**

Pex19p is a cytosolic protein that specifically interacts with PMPs during their intracellular translocation. The present biochemical studies revealed that Pex19p consists of a rigid C-terminal domain and a flexible N-terminal region. When the purified Pex19p was gently digested with trypsin, a stable 16-kDa fragment accumulated, which was identified as the C-terminal half of the protein (Ala$_{156}$-Ala$_{206}$). On the other hand, the N-terminal half of Pex19p was readily digested into smaller fragments with trypsin. In the case of the limited proteolysis with V8 protease, a similar stable fragment also accumulated, which was the C-terminal peptide starting from Gly$_{159}$. These results highlight the different structural properties between the N-terminal and C-terminal halves of Pex19p. Circular dichroism analyses showed that the majority of the corresponding regions of the full-length His$_{16}$-Pex19p.

**The Binding Affinity of Pex19p Domains toward PMPs**—To examine the binding activity of the two halves of Pex19p toward PMPs, we performed cell-free translation assays for each half as well as the full-length protein. The sample of the N-terminal half of Pex19p was prepared as His$_{16}$-Pex19p(N-half), which was the form used in the circular dichroism spectra measurement. The sample of the C-terminal domain of Pex19p was prepared as His$_{16}$-Pex19p(C-half), which was produced by the expression vector pET-16b/Pex19p(C-half) and then was purified in the same way as the full-length His$_{16}$-Pex19p. Each half of Pex19p was mixed with the mRNA encoding PMP22, PMP70, or Pex16p, and then the translation was performed with the wheat germ system. When the reaction was finished, the supernatant from the mixture was applied to the Ni$_{2+}$-NTA-agarose, and the imidazole eluate was analyzed by SDS-PAGE (Fig. 6). Pex19p(C-half) showed no binding to any PMP examined in the present study. On the other hand, Pex19p(N-half) bound to Pex3p-(34–373), but not to the other PMPs. These results demonstrate that only the half-size fragment is unable to bind to PMP22, PMP70, and Pex16p. In addition, even if both Pex19p fragments were mixed together with the solution for the cell-free synthesis of these PMPs, no translational products were observed in the imidazole eluates from the Ni$_{2+}$-NTA column. This result indicates that binding of Pex19p to PMP22, PMP70, and Pex16p requires more than the C-terminal half-sized domain of Pex19p. On the other hand, the N-terminal region (Met$^1$-Ala$_{156}$) of Pex19p is sufficient to interact with Pex3p. Thus, the binding mode of Pex3p toward Pex19p seems to be distinct from those of PMP70, PMP22, and Pex16p.

**Oligonucleotides used for construction of plasmids**

| Primer | Sequence (from 5’ to 3’) |
|--------|--------------------------|
| Pex19#FNdeI | CATATGCCTCCCGCTAGGG |
| Pex19#RBamHI | GATCTTCACGCTCTGCAACCTGCCAG |
| Pex19#5156#RBamHI | GATCTTCACGCTCTGCAACCTGCCAG |
| Pex19#157#FNdeI | CATATGCCTCCCGCTAGGG |
| Pex19#FBamHI | CGCGGACCTTGGCCCGCTAGGG |
| Pex19#FRsalI | ACGCGTCTACATAGCTTCCACCTGCGCCAG |
| Pex3344#FSpeII | AGATCTTCACGCTCTGCAACCTGCCAG |
| Xpc#RSalI | GATCTTCACGCTCTGCAACCTGCCAG |
| PMP22#FNdeI | CATATGCCTCCCGCTAGGG |
| PMP22#RBamHI | ACGCGTCTACATAGCTTCCACCTGCGCCAG |
| PMP22#FSpeII | ACGCGTCTACATAGCTTCCACCTGCGCCAG |
| Hisuus#FSpeII | ACGCGTCTACATAGCTTCCACCTGCGCCAG |
| PMP70#FXhoI | TTTAAATCGACCGAGCTGCGGGCCCT |
| PcdNA#Rv | ATGGTGGCAAATGAGAGCG |
| Pex16#FSpeII | ACGCGTCTACATAGCTTCCACCTGCGCCAG |
| Pex16#RBgII | AGATCTTCACGCTCTGCAACCTGCCAG |
| Pex3344#FSpeII | ACGCGTCTACATAGCTTCCACCTGCGCCAG |
| Pex3#RBgII | AGATCTTCACGCTCTGCAACCTGCCAG |

*Fig. 4. Time course of limited proteolysis of His$_{16}$-Pex19p. Coomassie Brilliant Blue staining of a 10–20% gradient polyacrylamide gel is shown. A, the purified His$_{16}$-Pex19p (4 mg/ml) was digested with 4 μg/ml TPCK-treated trypsin at 37 °C. B, the purified His$_{16}$-Pex19p (4 mg/ml) was digested with 20 μg/ml V8 protease at 37 °C.*
secondary structure resided in the C-terminal fragment, as compared with that of the N-terminal fragment. Therefore, we propose that Pex19p consists of the compact C-terminal half, which acts as a scaffold, and the N-terminal half, with a disordered conformation. These are the first biochemical data about the structural aspects of Pex19p. Our model of the Pex19p molecular architecture is supported by the following experimental results. Analytical ultracentrifugation indicated that Pex19p disperses as a monomer in solution, although a gel filtration analysis revealed that His$_{10}$-Pex19p (35 kDa) elutes at a retention time corresponding to a molecular weight of 111 kDa. These facts suggest that Pex19p is not a simple globular protein but rather one with a certain distorted shape, caused by the disordered N-terminal region.

We found that the division at Ala$^{156}$ of Pex19p into two half-sized fragments destroyed its ability to bind to the cargo PMPs, such as PMP22 and PMP70. The present binding assay, based on the cell-free translation system, demonstrated that the recombinant full-length His$_{10}$-Pex19p interacts with PMP22 and PMP70. On the other hand, neither His$_{10}$-Pex19p(N-half) nor His$_{10}$-Pex19p(C-half) forms a complex with PMP22 and PMP70. In addition, even in the presence of both Pex19p fragments, no binding was observed between Pex19p and the cargo PMPs. Therefore, the N-terminal flexible region of Pex19p must be covalently attached to the subsequent C-terminal core domain. The N-terminal region of Pex19p, based on the C-terminal core scaffold, could accommodate a variety of PMPs, and each complex could have a fixed conformation. Considering the previous report by Mayerhofer and colleagues (21), where a Pex19p splice variant lacking the N-terminal 90 amino acids binds to PMP70, the region between Met$^{91}$ and Lys$^{155}$ in the Pex19p N-terminal half may play a role in adapting to PMPs.

On the other hand, the present experiments suggest that Pex3p, which is anchored on the peroxisomal membrane in the cell, is recognized by Pex19p in a distinct manner from the cargo PMPs, such as PMP22 and PMP70. We found that the ability of His$_{10}$-Pex19p(N-half) to bind to the soluble Pex3p-(34–373) is similar to that of the full-length His$_{10}$-Pex19p. Actually, the complex of GST-Pex3p-(34–373) and Pex19p(N-half) could be purified by glutathione-Sepharose affinity resin (data not shown) in a similar way as the complex of Pex3p-(34–373) and the full-length Pex19p (Fig. 7A). Therefore, this flexible N-terminal region of Pex19p, Met$^{1}$–Ala$^{156}$, is sufficient for the interaction with Pex3p. In addition, it is noteworthy that a truncated form of Pex3p, lacking the N-terminal hydrophobic region, interacted with Pex19p. This hydrophobic region contains the peroxisomal targeting signal of Pex3p (19). Therefore, Pex19p would not recognize the targeting signal of Pex3p. We suggest that Pex19p recognizes Pex3p not as one of its cargo proteins but as a docking protein on the peroxisomal membranes. Our viewpoint on the significance of this Pex19p-Pex3p interaction is consistent with that described in a recent report (28), which proposed that there are two types of targeting signals for peroxisomal membrane proteins; i.e. the class 1 proteins translocated by Pex19p, and the class 2 proteins, like Pex3p, which are independent of Pex19p during the translocation.

Furthermore, we were able to reproduce complex formation between Pex3p and Pex19p harboring PMP22, as a step in intracellular peroxisomal translocation. When His$_{10}$-PMP22 was synthesized with the cell-free translation system in the presence of the GST-Pex3p-(34–373)-Pex19p complex, their ternary complex was detected. This complex formation was mediated by Pex19p, because in the absence of Pex19p, little interaction occurred between Pex3p-(34–373) and PMP22. These results indicate that Pex19p simultaneously serves the respective binding sites to Pex3p and the cargo PMPs. If the binding of PMPs requires Met$^{91}$–Ala$^{296}$ of Pex19p, as mentioned above, then the binding site for Pex3p could be included in the N-terminal 90-residue region of Pex19p. At the end of the translocation of PMPs mediated by Pex19p, this N-terminal portion of Pex19p seems to be captured by the membranous Pex3p. Recently, Muntau et al. (16) detected the interaction between Pex19p and Pex3p on the peroxisomal membrane by a fluorescence resonance energy transfer analysis. We believe that this intracellular event corresponds to our in vitro observation of the interaction between Pex3p and Pex19p harboring PMP22.

The present studies propose that Pex19p would function as a specific chaperone to prevent the precipitation of PMPs during intracellular translocation. During the cell-free translation, the synthesized PMP22 was solubilized by the complex formation with Pex19p, whereas the synthesized PMP22 alone was an insoluble membrane protein. In fact, the purified Pex19p exhibited quite high solubility, with concentrations of more than 200 mg/ml remaining in solution. This property of Pex19p...
would allow the quite hydrophobic PMP to remain in a soluble form. In addition, considering that the precipitated PMP22 was unable to bind to Pex19p, Pex19p is available for interactions with nascent polypeptides on ribosomes and probably stabilizes the bound PMP during intracellular translocation. Our results are consistent with the earlier observations as follows. PMP22 forms two types of complexes in the postribosomal supernatant \((29)\). One of them includes TCP1 ring complex, which is the eukaryotic GroEL equivalent, and the other includes a 40-kDa protein, which is expected to be Pex19p. Recently, Jones et al. \((28)\) also indicated that the cytosolic Pex19p interacts with PMP34, another type of PMP, so that PMP34 is stabilized during translocation.

Consequently, we suggest a model for the molecular architecture and the function of human Pex19p. Pex19p consists of the core domain, composed of the C-terminal half, and the flexible region of the rest of N-terminal half, with the domain boundary located around Ala\(^{156}\). The C-terminal core and the domain boundary side of the N-terminal region in concert bind to PMP synthesized on free ribosomes. This complex migrates to the target peroxisomes, and the head side of the N-terminal region interacts with the Pex3p, anchored in the membrane to form a Pex19p-cargo PMP-Pex3p ternary complex. This model demonstrates that the Pex19p-dependent translocation of PMPs is a unique system, as compared with other post-translational translocation systems observed in mitochondria from eukaryotic cells or in bacterial cells. To transport membrane proteins toward mitochondria, Hsp70 (70-kDa heat shock protein) chiefly functions as a cytosolic receptor/chaperone \((17)\). In bacterial cells, in contrast, SecB plays similar roles in post-translational membrane protein trafficking \((18)\). However, Hsp70 and SecB are available for binding to an extended
polypeptide (30, 31). This hypothesis suggests that Hsp70 and Sec61 recognize cargo membrane proteins in denatured (trans-
location-competent) states. On the other hand, Pex19p would be a PMP-specific cytosolic receptor/chaperone, which recog-
nizes not only a particular internal sequence, which could be a basic residue cluster, but also at least one transmembrane helix (12). Therefore, Pex19p seems to distinguish some char-
acteristic secondary structures of PMPs, rather than the ex-
tended form of a polypeptide. We propose that Pex19p adapts its N-terminal flexible region to such secondary structures of PMP, so that the overall architecture of Pex19p could be fixed. Such lenient recognition may allow Pex19p to interact with a variety of PMPs, which have dissimilar amino acid sequences. These properties of Pex19p are quite different from those of Pex5p and Pex7p, which are assumed to strictly recognize the short targeting signals of peroxisomal matrix proteins.

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Domain Architecture and Activity of Human Pex19p, a Chaperone-like Protein for Intracellular Trafficking of Peroxisomal Membrane Proteins

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