Crystal structure of the conserved N-terminal domain of the peroxisomal matrix protein import receptor, Pex14p

Jian-Rong Su[a], Kazuki Takeda[a], Shigehiko Tamura[b], Yukio Fujiki[b,1], and Kunio Miki[a,c,1]

[a]Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan; [b]Department of Biology, Faculty of Sciences, Kyusyu University Graduate School, Higashi-ku, Fukuoka 812-8581, Japan; and [c]RIKEN SPring-8 Center at Harima Institute, Koto 1-1-1, Sayo, Hyogo 679-5148, Japan

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Pex14p is a central component of the peroxisomal protein import machinery, in which the conserved N-terminal domain mediates dynamic interactions with other peroxins including Pex5p, Pex13p, and Pex19p. Here, we report the crystal structure of the conserved N-terminal domain of Pex14p with a three-helix bundle. A hydrophobic surface is composed of the conserved residues, of which two phenylalanine residues (Phe-35 and Phe-52) protrude to the solvent. Consequently, two putative binding pockets suitable for recognizing the helical WXXXF/Y motif of Pex5p are formed on the surface by the two phenylalanine residues accompanying with positively charged residues. The structural feature agrees well with our earlier findings where F35A/L36A and F52A/L53A mutants were impaired in the interactions with other peroxins such as Pex5p and Pex13p. Pex14p variants each with Phe-to-Ala mutation at positions 35, 52, and 35/52, respectively, were defective in restoring the impaired peroxisomal protein import in *pex14* Chinese hamster ovary mutant ZP161 cells. Moreover, in GST pull-down assays His_6-Pex5pL bound only to GST-Pex14p(25–70), not to any of GST-Pex14p(25–70)F35A, GST-Pex14p(25–70)F52A, and GST-Pex14p(25–70)F35A/F52A. Endogenous Pex5p was recruited to FLAG-Pex14p on peroxisomes in vivo but barely to FLAG-Pex14pF35A, FLAG-Pex14pF52A, and FLAG-Pex14pF35A/F52A. Collectively, Phe-35 and Phe-52 are essential for the Pex14p functions, including the interaction between Pex14p and Pex5p.

peroxin | Pex5p | protein transport | WXXXF/Y motif

Peroxisome is an organelle in eukaryotic cells that functions in various metabolisms such as ß-oxidation of very long fatty acids and synthesis of plasmalogens (1). Peroxisomal matrix proteins synthesized in cytosol are imported into the peroxisome by a distinct dynamic system involving peroxins such as Pex5p, Pex7p, Pex13p, Pex14p, and Pex19p (2–5). The matrix proteins harbor the peroxisomal targeting signal-1 (PTS1) at the C terminus or cleavable presequence PTS2 at the N terminus. These topogenic signals are specifically recognized by the PTS1 receptor, Pex5p, and the PTS2 receptor, Pex7p (2–6). The soluble receptor–cargo protein complexes dock with Pex14p, the convergent membrane peroxin of peroxisomal matrix protein importomer (7–9).

Pex14p, a membrane-anchored peroxin with a molecular mass of 41 kDa, is a central component in the peroxisomal protein import system (7–14). A conserved domain of Pex14p comprising residues 21–70 interacts with Pex5p, Pex13p, and Pex19p (15, 16). Pex14p forms a homodimer by the coiled-coil domain or a larger oligomer by the GXXXG and AXXXA motifs in the transmembrane domain (15). Pex14p in different oligomeric states interacts with distinct partners.

The interaction between Pex14p and Pex5p is mediated via the WXXXF/Y motifs, seven in the mammalian longer form Pex5pL, located in the N-terminal half of Pex5p (9, 15, 17). The N-terminal half of Pex5p is natively unfolded (18), whereas the C-terminal half has a typical tetratricopeptide repeat (TPR) fold (19) involved in binding to the PTS1 motif sequence of peroxisomal matrix proteins.

Until now, no structural information on Pex14p has been elucidated. We report here the crystal structure of the N-terminal highly conserved domain of mammalian Pex14p at 1.8-Å resolution. Physiological importance of the "protruding" phenylalanine residues, Phe-35 and Phe-52, of Pex14p was verified by expressing Pex14p variants with Phe-to-Ala mutation at Phe-35, Phe-52, and Phe-35/Phe-52.

Results and Discussion

N-Terminal Conserved Domain of Mammalian Pex14p. Because Pex14p is a membrane protein with a multidomain structure (Fig. 1*A*), the full-length protein was less likely suitable for structure determination by X-ray crystallography (data not shown). However, it is known that amino acid sequences of a domain in the N terminus of Pex14p are highly conserved between among species (10, 15, 20) (Fig. 1*B*). Interactions with other peroxins such as Pex5p, Pex13p, and Pex19p are mediated by the domain (15, 16). We took advantage of this conserved domain as a target for crystallization experiments. The domain was suggested to form a helix-rich structure by secondary structure predictions (21, 22). In vitro protease degradation assays supported this prediction (data not shown). Therefore, we constructed three truncated variants containing the Pex14p conserved domain from *Rattus norvegicus* (rat), which were termed Pex14p(25–70), Pex14p(1–106), and His_6-Pex14p(1–106) (Fig. 1*A*). Pex14p(25–70) consists of only a helical region. The Pex14p(1–106) and His_6-tagged, His_6-Pex14p(1–106) variants contain multiple PXXP motifs near the helical region. Helix contents of the constructs were estimated to be 78% and 32% for Pex14p(25–70) and Pex14p(1–106), respectively, by means of circular dichroism (CD) spectroscopy (Fig. 1*C*). These results indicate that two peripheral regions (residues 1–24 and 71–106) at both ends of the middle region (residues 25–70) are disordered.

Structure Determination. The three constructs of the conserved N-terminal domain were supplied to crystallization experiments. Crystals of Pex14p(25–70) with approximate dimensions of 0.2 ×

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The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors for Pex14p(25–70) have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3FF5).

[1]To whom correspondence may be addressed. E-mail: yfujiscb@mbox.nc.kyushu-u.ac.jp or miki@kuchem.kyoto-u.ac.jp.

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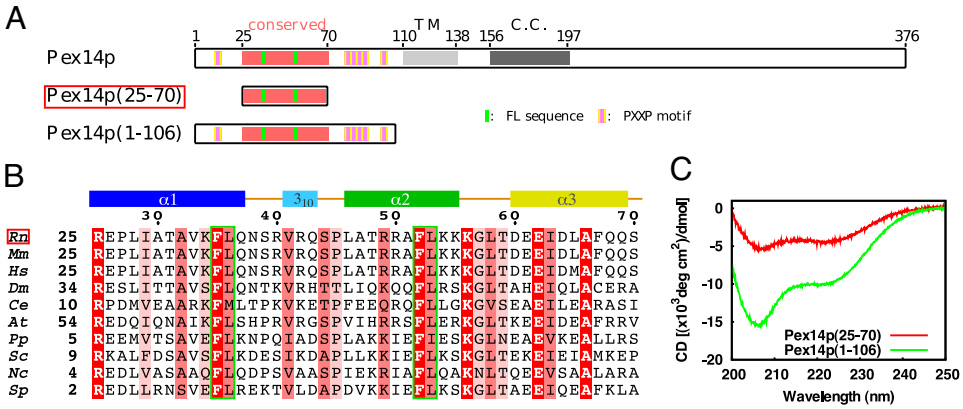

Fig. 1. Properties of Pex14p. (*A*) Domain architecture of mammalian Pex14p. TM, transmembrane; C.C., coiled-coil. The highly conserved region is indicated in red. (*B*) Sequence alignment of the functional domain of Pex14p. Fully, highly, and relatively conserved residues are shaded by red, light red, and pale red, respectively. The FL sequences are boxed in green. Rn, *Rattus norvegicus* (rat); Mm, *Mus musculus* (mouse); Hs, *Homo sapiens* (human); Dm, *Drosophila melanogaster* (fly); Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; Pp, *Pichia pastoris*; Sc, *Saccharomyces cerevisiae*; Nc, *Neurospora crassa*; Sp, *Schizosaccharomyces pombe*. Secondary structures were defined from the crystal structure with the DSSP program (30). The α1, α2, α3, and 3_{10} helices are schematically represented by blue, green, yellow, and cyan cylinders, respectively. (*C*) CD spectra for various constructs of the conserved domain. Pex14p(25–70) and Pex14p(1–106) are represented in red and green, respectively.

0.2×0.2 mm^3 were obtained by using a detergent, *n*-decylphosphocholine (DPC) [see *Materials and Methods* and supporting information (SI) Fig. S1*A*]. The crystals belong to the space group *I*23 with cell parameters of $a = (b = c =)$ 90.6 Å. The crystallographic data and statistics are summarized in Table S1. The structure was solved by the single-wavelength anomalous diffraction (SAD) method using an osmium derivative. The experimental electron density was of high enough quality for autotracing to construct an initial model (Fig. 2*A*). The structure was refined to 1.8-Å resolution with a good structural geometry (Table S1).

Overall Structure. The conserved N-terminal domain of Pex14p has three α helices (α1–α3) with a right-handed twist as shown in Fig. 2 *B*–*D*. In addition, a short 3_{10} helix is located between the α1 and α2 helices. The α1 and α2 helices lie in an antiparallel manner. The α3 helix links the two antiparallel helices. The helix content of this domain is calculated to be 72% from the determined crystal structure, which is consistent with the value (78%) determined by the CD spectra. Hydrophobic interactions between hydrophobic residues of different helices are observed by Leu-28, Ala-32, Phe-35, and Leu-36 in the α1 helix; Leu-46, Phe-52, and Leu-53 in the α2 helix; and Ile-63, Ala-66, and Phe-67 in the α3 helix (Fig. 3 *A* and *B*). Especially, it should be noted that Phe-67 of the α3 helix mediates remarkable interhelix interactions with both Leu-36 in the α1 helix and Leu-46 in the α2 helix. In addition, Val-41 of the 3_{10} helix interacts with Phe-35 in the α1 helix. Consequently, a rigid hydrophobic core is formed by these residues at the center of the domain. Therefore, it is expected that the arrangement of these three helices is mutually fixed in any actual states of Pex14p. Indeed, two molecules of the domain involved in the asymmetric unit have a vary small root mean square deviation of 0.3 Å for C_α atoms of 46 intrinsic residues (Fig. S1*B*). However, several side chains exposed on the molecular surface have different conformations in the two crystallographically independent polypeptide chains. Moreover, it is crystallographically determined that some amino acid residues such as Lys-34, Lys-56, and Gln-68 indicate double conformations. Therefore, side chains of the surface residues are probably able to adopt suitable conformations upon complex formation with other peroxins such as Pex5p. It is noteworthy that proteins possessing the DNA/RNA-binding three-helical bundle have significant structural similarities with Pex14p(25–70) as described in *SI Materials and Methods*.

FL Sequence. Two FL sequences (Phe-35–Leu-36 and Phe-52–Leu-53; Fig. 1 *A* and *B*) are highly conserved in Pex14p (Fig. 1*B*).

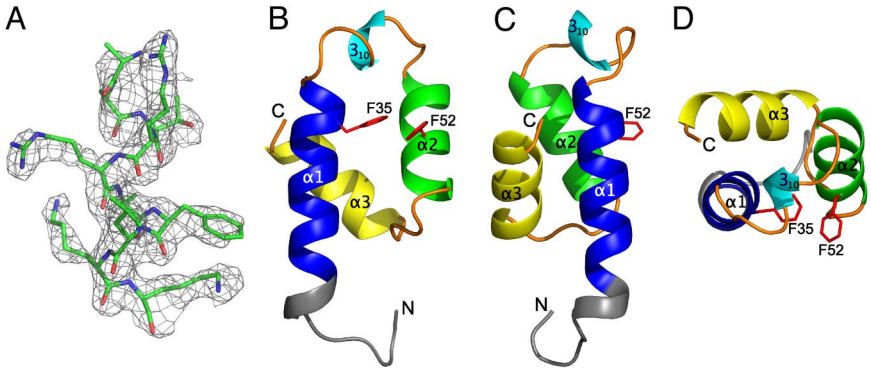

Fig. 2. Crystal structure of Pex14p(25–70). (*A*) The electron density map after density modification is represented as a gray mesh contoured at the 1σ level, and the model in the region of the α2 helix is shown as a stick model. (*B*) The Pex14p(25–70) molecule is shown as a ribbon model, in which the α1, α2, α3, and 3_{10} helices are shown in blue, green, yellow, and cyan, respectively. In addition, the vector-derived 8-amino acid residues at the N terminus are shown in gray. Side chains of Phe-35 and Phe-52 are represented as stick models in red. (*C*) Pex14p(25–70) in *B* is rotated by 90° around the vertical axis. (*D*) Pex14p(25–70) in *B* is rotated by 90° around the horizontal axis.

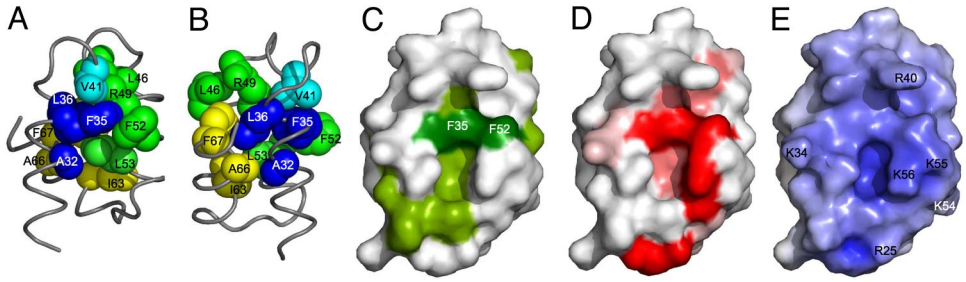

Fig. 3. Structural features of Pex14p(25–70). (*A*) Hydrophobic core of the domain. Residues involved in interhelix contacts are represented as CPK models. The view is the same as that in Fig. 2*B*. (*B*) Side view of *A*. (*C*) Hydrophobic property of the molecular surface. Aromatic residues (Phe, Trp, and Tyr) are shown in green, and other hydrophobic residues (Ala, Ile, Leu, Pro, and Val) in light green. (*D*) The molecular surface is colored by conservation of residues as the same manner as Fig. 1*B*. (*E*) The molecular surface is colored by electrostatic potential. Positive and negative values are represented in blue and red, respectively. Only the backside surface shows some red area.

As reported in our previous article (15), F35A/L36A and F52A/L53A mutants are impaired in the interaction with Pex5p and Pex13p. The two leucine residues participate in the hydrophobic core at the center of the domain, as shown above. The two phenylalanine residues contact with each other and protrude from the molecular surface (Fig. 2 *C* and *D*). These characteristic aromatic residues also exist on the surface of several similar proteins as discussed in *SI Materials and Methods* (Figs. S2 and S3*A*). Some of these mediate intra-protein interactions (Fig. S3).

Surface Properties. Many hydrophobic residues are exposed on one side of Pex14p(25–70) (Fig. 3*C*) in contrast to the opposite side (data not shown). In addition, this side consists of conserved residues (Fig. 3*D*) and has an electropositive potential because of the positively charged residues such as Arg-25, Lys-34, Arg-40, Lys-54, Lys-55, and Lys-56 (Fig. 3*E*). These features may imply the feasibility of functional interactions with other peroxins. The hydrophobic and conserved surface is divided into two pocket regions by protruding phenylalanine residues, Phe-35 and Phe-52 (Fig. 4*A* and *B*).

Implications for Pex5p Binding. The conserved domain of Pex14p interacts with other protein components of the peroxisomal protein import system such as Pex5p, Pex13p, and Pex19p (13, 15, 16). The PTS1 receptor, Pex5p, has the WXXXF/Y motif as its binding sites for Pex14p (9, 17). We therefore verified interactions between the crystallizable variant Pex14p(25–70) and synthetic WXXXF/Y peptides by Native PAGE. The result indicates that Pex14p(25–70) interacts with all peptides, especially with 5p-1, 5p-2, 5p-3, and 5p-5 (Fig. 4*D*). This is consistent with a result on human Pex14p(1–78) by fluorescence titration analysis (17).

The two pockets on the surface of Pex14p(25–70) can be estimated as binding sites for the WXXXF/Y motif of Pex5p. Both sites are surrounded by phenylalanine and positively charged residues (Phe-35 and Arg-40 for site 1; and Lys-34, Phe-52, Lys-55, and Lys-56 for site 2) (Fig. 4 *A* and *B*).

The CD spectra of WXXXF/Y motif peptides indicate that the peptides do not fold into any helical structures in buffer solution (Fig. S4), whereas the motif is predicted to form helices (21, 22). However, the helix formation of the motif upon the binding to the SH3 domain of Pex13p was reported in *Saccharomyces cerevisiae* (23). Therefore, we speculate that the WXXXF/Y motif has a helical structure only in the case of binding with partners. Indeed, the helix content increases up to 60% in the presence of trifluoroethanol (TFE) (Fig. S4). The maximum value of the helix content is achieved in 50% (wt/vol) TFE (Fig. S4). It is considered that the solvent mimics the hydrophobic surface of the acceptor protein by its low dielectric constant of 27 (24). An amphiphilic α helix can be modeled from all of the synthetic WXXXF/Y-containing peptides of Pex5p. Two conserved aromatic residues, tryptophan and phenylalanine/tyrosine are located at the same side of the helix (Fig. 4*C*). Therefore, both aromatic residues can plug the two hydrophobic pockets of Pex14p, in which the π–π interactions (25) would be formed between Pex5p and Phe-35 and Phe-52 of Pex14p. The positively charged residues (Lys-34, Arg-40, Lys-55, and Lys-56) of Pex14p plausibly form cation–π interactions (26) with aromatic residues of the WXXXF/Y motif to enhance the binding affinity.

The calculated isoelectric point of the conserved domain of Pex14p is 10.4, whereas those of the WXXXF/Y motif peptides are in a range from 3.3 to 4.5. Therefore, it is more likely that opposite electrostatic properties also contribute to the interaction between Pex5p and Pex14p in the solution.

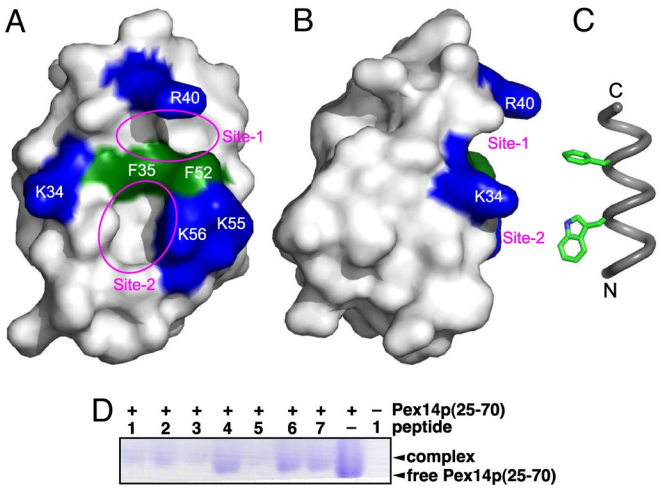

Fig. 4. Implications for Pex5p binding. (*A*) Putative binding sites for WXXXF/Y motifs of Pex5p are indicated by circles. Phe-35 and Phe-52 are colored in green, and Lys-34, Arg-40, Lys-55 and Lys-56 are in blue. (*B*) Side view of *A*. (*C*) Helix model of the 5p-1, a peptide of the WXXXF/Y motif. Side chains of aromatic residue in the motif are shown in green. (*D*) Native PAGE analysis of the complex formation between Pex14p(25–70) and the WXXXF/Y motif peptides of Pex5p.

Mutational Analysis on Phe-35 and Phe-52. To validate the functional role of Phe-35 and Phe-52 of Pex14p, we constructed and expressed in *pex14* Chinese hamster ovary (CHO) mutant ZP161 three FLAG-tagged Pex14p variants each with Ala mutation at F35A, F52A, and F35A/F52A. PTS1 proteins remained in the cytosol in ZP161 cells each transfected with *FLAG-PEX14F35A*, *FLAG-PEX14F52A*, and *FLAG-*

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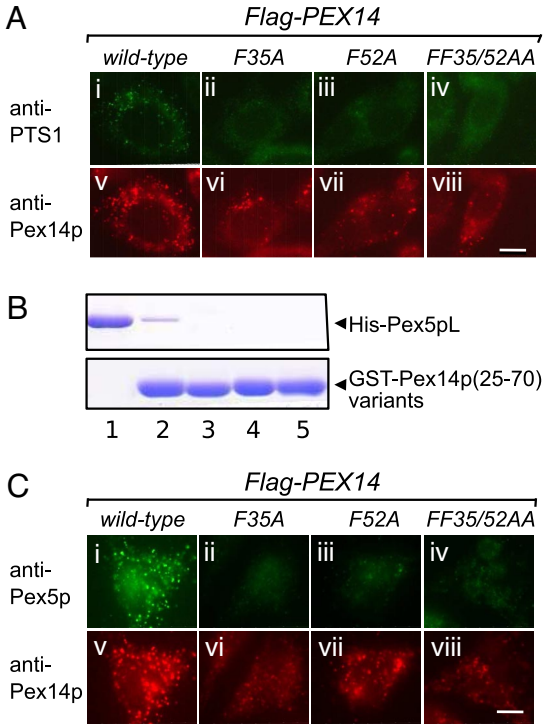

Fig. 5. Mutational analysis on Phe-35 and Phe-52. (*A*) Phe-35, Phe-52, and Phe-35/Phe-52 of rat FLAG-Pex14p were substituted with Ala, termed FLAG-Pex14pF35A, FLAG-Pex14pF52A, and FLAG-Pex14pF35A/F52A, respectively. Wild-type and FA mutants were expressed in *pex14* CHO mutant ZP161 cells and assessed for peroxisome-restoring activity. Cells were immunostained with antibodies to PTS1 (*i–iv*) and Pex14p (*v–viii*). (Scale bar: 30 μm.) Note that F35A, F52A, and F35A/F52A were inactive in complementing the impaired peroxisome biogenesis in ZP161. (*B*) Binding assays were performed by GST pull-down using GST-Pex14p(25–70) variants (0.5 μg each) and recombinant His_6-Pex5pL (0.4 μg) after incubation at 4 °C for 1 h. Proteins bound to the beads were analyzed by SDS/PAGE and staining with Coomassie brilliant blue. Lanes: 1, input, His_6-Pex5pL used for the binding assay; 2, GST-Pex14p(25–70); 3, GST-Pex14p(25–70)F35A; 4, GST-Pex14p(25–70)F52A; 5, GST-Pex14p(25–70)F35A/F52A. Note that only wild-type GST-Pex14p(25–70) bound to His_6-Pex5pL. (*C*) Wild-type and FA-mutants were expressed in normal CHO-K1 cells as in *A* and assessed for Pex5p-recruiting activity. Cells were immunostained with antibodies to Pex5p (*i–iv*) and Pex14p (*v–viii*). (Scale bar: 30 μm.) Note that Pex5p was discernible in a punctate staining pattern in CHO-K1 expressing the wild-type (*i*) but barely detectable in cells each expressing F35A, F52A, and F35A/F52A (*ii–iv*).

PEX14F35A/F52A, whereas PTS1 proteins were imported to peroxisomes by expression of normal FLAG-Pex14p (Fig. 5*A i–iv*). FLAG-Pex14p, FLAG-Pex14pF35A, FLAG-Pex14pF52A, and FLAG-Pex14pF35A/F52A were expressed nearly at the same level in respective transfectants (data not shown). Hence, all of the three mutants, FLAG-Pex14pF35A, FLAG-Pex14pF52A, and FLAG-Pex14pF35A/F52A were inactive in complementing the defective protein import in *pex14* ZP161 cells. With respect to subcellular localization of FLAG-Pex14p mutants, they were localized to peroxisomal membrane remnants (Fig. 5*A vi–viii*) as assessed by colocalization with a major membrane protein of peroxisomes, PMP70 (data not shown), hence indicating that the membrane targeting of FA mutants was normal.

In GST pull-down assays, His_6-Pex5pL was detected in the bound fraction only to GST-Pex14p(25–70), not any of GST-Pex14p(25–70)F35A, GST-Pex14p(25–70)F52A, and GST-Pex14p(25–70)F35A/F52A (Fig. 5*B*), strongly suggesting that the Phe-35 and Phe-52 played a pivotal role in the interaction between Pex14p and Pex5p. The result was in good agreement with in vivo assessment of the full-length Pex14p variants with respect to peroxisome-restoring activity described above (Fig. 5*A*).

Interactions with Pex5p of FLAG-Pex14p, FLAG-Pex14pF35A, FLAG-Pex14pF52A, and FLAG-Pex14pF35A/F52A were also verified in vivo. Wild-type and FA mutants were expressed in normal CHO-K1 cells and assessed for Pex5p-recruiting activity (Fig. 5*C*). Endogenous Pex5p was discernible in a punctate staining pattern, in a manner superimposable on Pex14p localized on peroxisomes in CHO-K1 cells expressing the wide-type apparently at a higher level (Fig. 5*C i* and *v*), but barely detectable in cells each expressing F35A, F52A, and F35A/F52A (*ii–iv* and *vi–viii*), thereby indicating that the translocation of Pex5p only to FLAG-Pex14p. Similar recruitment of Pex5p was observed on peroxisome remnants (data not shown) in *pex1* CHO mutant ZP107 cells (27). These results confirmed the finding in the in vitro pull-down assay (Fig. 5*B*) supported the complementing activity as assessed with *pex14* ZP161 cells (Fig. 5*A*).

Materials and Methods

Preparation of Proteins and Peptides. Truncation variants of rat Pex14p were expressed in *Escherichia coli* and purified. The WXXXF/Y motif peptides of the Pex5p were synthesized by the Fmoc solid-phase method. The detailed descriptions about preparation of proteins and peptides are in *SI Materials and Methods*.

CD Spectroscopy. A quarts cuvette with a pass length of 1 mm was filled with a solution containing 0.1 mg/mL Pex14p in 10 mM Tris·HCl buffer (pH 7.5). CD spectra were measured by using a J-805 CD spectropolarimeter (JASCO) in a range from 200 to 250 nm at 20 °C. Scan speed and scan step were set to be 10 nm/min and 0.1 nm, respectively. Five scans were averaged. The secondary structure content was analyzed with the program JWSSE-408 (JASCO) by using a reference dataset (28). CD spectra of WXXXF/Y motif peptides were likewise measured and analyzed in the same manner.

Crystallographic Study of Pex14p. Crystals of Pex14p(25–70) were obtained at 4 °C in the solution containing 1.2 M sodium citrate, 100 mM Hepes (pH 7.5), 10%(vol/vol) glycerol, and 0.2% (wt/vol) DPC. The structure was solved by the SAD method using an osmium derivative and refined to 1.8-Å resolution (R_{free} = 21.1% and R_{work} = 17.9%). Further details are given in Table S1 and *SI Materials and Methods*.

Native PAGE for in Vitro Binding Assay. Pex14p(25–70) (0.3 μM) was incubated in the presence or absence of 1.5 μM WXXXF/Y peptides for 30 min at 25 °C. Native PAGE was carried out by using a 15% gel and a running buffer containing 30 mM histidine and 30 mM Mes (pH 6.1) at 4 °C. Each lane was loaded with 5 μL of sample solution. The gel was stained with Coomassie brilliant blue.

Morphological Analysis. Tagging of FLAG epitope to the N terminus of Pex14p was done as described in *SI Materials and Methods*. Peroxisome-deficient *pex14* CHO mutant ZP161 and wild-type CHO-K1 cells were transfected with cDNAs each encoding FLAG-Pex14p and its FA mutants and cultured for 2 days at 37 °C as described (10, 29). Peroxisomes in cells were visualized by indirect immunofluorescence light microscopy with mono-specific rabbit antibodies and fluorescein isothiocyanate- or Texas red-labeled goat anti-guinea pig IgG antibody (Cappel) (9).

Assay for Binding of Pex14p(25–70) and Its FA Mutants to Pex5pL. Binding assays (100 μL each) of GST-Pex14p(25–70), GST-Pex14p(25–70)F35A, GST-Pex14p(25–70)F52A, and GST-Pex14p(25–70)F35A/F52A (2 μg each) to recombinant His_6-Pex5pL (1.5 μg) were performed by GST pull-down using glutathione-linked Sepharose 6B beads (GE Healthcare), essentially as described in ref. 9. Proteins bound to the beads were analyzed by SDS/PAGE and staining with Coomassie brilliant blue.

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