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Transmembrane Topology of the Peroxin, Pex2p, an Essential Component for the Peroxisome Assembly[1]

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The peroxisome biogenesis factor, peroxin Pex2p, is an integral membrane protein of peroxisomes [Tsukamoto, T., Miura, S., and Fujiki, Y. (1991) *Nature* 350, 77-81]. As a step toward elucidating the structure and biological function of Pex2p, we determined the transmembrane topology of Pex2p by expressing epitope-tagged rat Pex2p in COS-7 cells. Pex2p tagged with myc at the C-terminus was detected as a punctate staining pattern, when the cells were permeabilized with 50 μg/ml of digitonin, under which conditions intraperoxisomal proteins such as PTS1-proteins are inaccessible to exogenous antibodies. N-terminally flag-tagged Pex2p was likewise detected upon the same treatment. These results strongly suggest that both the N- and C-terminal parts of Pex2p are exposed to the cytosol. The transmembrane orientation of Pex2p was also assessed by using rat liver peroxisomes and Pex2p region-specific antibodies. The two types of antibodies used, raised to the N- (amino acid residues 1-131) and C-terminal part (residues 226 to the C-terminus), respectively, specifically recognized Pex2p and immunoprecipitated intact, whole peroxisomes. Pex2p was not recognized by the antibodies when the peroxisomes were treated with Proteinase K. Furthermore, *in situ* crosslinking studies involving bifunctional reagents revealed an apparently dimeric form of Pex2p. Therefore, Pex2p is anchored to the peroxisomal membrane by two membrane-spanning segments, with its N- and C-terminal regions exposed to the cytosol.

Key words: membrane topology, peroxin Pex2p, peroxisome assembly factor, peroxisome biogenesis.

Peroxisome deficiency in mammals comprises at least 16 complementation groups (CGs) (*1-4*), *i.e.* 12 CGs of human peroxisome-deficient disorders such as cerebrohepatorenal Zellweger syndrome (*1, 2, 5*), and 4 CGs, distinct from human CGs, of peroxisome biogenesis-defective Chinese hamster ovary (CHO) cell mutants (*1-4*). Expression of Pex2p (formerly peroxisome assembly factor-1, PAF-1), a 35-kDa peroxisomal integral membrane protein of 305 amino acids, restored the biogenesis of peroxisomes and complemented the peroxisomal function defect in a peroxisome-deficient Chinese hamster ovary (CHO) cell mutant, Z65 (*6, 7*). Pex2p cDNA, *PEX2*, also complemented peroxisome assembly in fibroblasts in CG-F (the same as CG-X in the USA and CG-5 in Europe) Zellweger syndrome (*8*). The cause of the syndrome in patients is a homozygous point mutation of a codon for ^{119}Arg that results in the premature termination of Pex2p (*8*). One homozygous patient inherited the mutation from the parents, each of whom was heterozygous for the mutation (*8*). The human PAF-1 gene has been assigned to chromosome 8q21.1 (*9*). Such findings allowed, for the first time, a prenatal diagnosis of Zellweger syndrome by means of DNA analysis (*10*).

Pex2p from three mammalian species, man (*8*), rat (*7*), and Chinese hamster (*11, 12*), show high sequence-homology, and contain two membrane-spanning segments (amino acid residues 138-140 to 159-160 and 195 to 213) and a conserved cysteine-rich sequence, C_3HC_4 RING finger (*13*), in the C-terminal region. Pex2p is encoded by free polysomal RNA (*11*), consistent with a general rule for the biogenesis of peroxisomal proteins including membrane polypeptides (*14*), implying posttranslational transport and integration of Pex2p into the peroxisomal membrane. Pex2p has also been identified in the yeasts, *Pichia pastoris* (*15*) and *Yarrowia lipolytica* (*16*), the fungus, *Posodena anserina* (*17*), and a parasite, *Leishmania* (*18*). However, no report has described the precise topology of Pex2p in peroxisomal membranes.

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Abbreviations: AOx, acyl-CoA oxidase; BS^3, bis[2-(succinimidyloxycarbonyloxyl)ethyl]sulfone; CETD, *N,N′*-bis(2-carboximidoethyl)-tartarimidedimethylester dihydrochloride; CG, complementation group; DMA, dimethyladipimidate dihydrochloride; DMS, dimethylsuberimidate dihydrochloride; GST, glutathione S-transferase; PBM, *N,N′*-(1,2-phenylene)bismaleimide; PBS, phosphate-buffered saline; Pex2pN and Pex2pC, N- and C-terminal parts of peroxin Pex2p; PTS1, peroxisome targeting signal type 1; TCA, trichloroacetic acid.

In this report, as a step toward elucidation of the relationship of the structure and biochemical function of Pex2p as well as interacting proteins, if any, we investigated the membrane topology of Pex2p, using epitope-tagged Pex2p, and antibodies to its N- and C-terminal regions. We evidently demonstrate that the N- and C-terminal regions of Pex2p are exposed to the cytosol.

EXPERIMENTAL PROCEDURES

Materials—Expression vectors, pGEX4T-1 and pGEX-4T-3, and glutathione-Sepharose were purchased from Amersham Pharmacia Biotech. Affigel 10 and formalin-fixed *Staphylococcus aureus* cells (Pansorbin) were from Bio-Rad and Calbiochem, respectively. Bifunctional reagents, *N,N'*-(1,2-phenylene)bismaleimide (PBM), *N,N'*-bis(2-carboximidoethyl)tartarimidedimethylester dihydrochloride (CETD), dimethyladipimidate dihydrochloride (DMA), dimethylsuberimidate dihydrochloride (DMS), and bis[2-(succinimidyloxycarbonyoxyl)ethyl]sulfone (BS), were from Nacalai-Tesque (Tokyo).

Expression of Epitope-Tagged Pex2p—An epitope, myc, was used to tag the C-terminus of rat Pex2p by means of a PCR-based technique, using a forward primer: MT3C-S, 5′-CAGAAGCTTAAAGCCAAG-3′, a reverse one: Cend-Myc2, 5′-CCGCTCGAGTCACAAGTCTTCTTCAGAAAT-CAGCTTTTGTTCAAGAGCATTCACTTC-3′ (myc epitope and a stop codon, underlined), and rat PAF-1 cDNA clone T (*11*) as a DNA template. The *Hin*dIII-*Eco*RI fragment of the PCR product was replaced with the *Hin*dIII-*Eco*RI fragment of PAF-1 cDNA clone T with a codon, TTG, for ^{216}Leu that had been changed to CTT for Leu, hence creating a *Hin*dIII site, termed *myc*-Mutant 2. Plasmid pUcD2Hyg•*RnPEX2-myc* was constructed by inserting the *Bam*HI-*Eco*RI fragment of *myc*-Mutant 2 into the pUcD2SRαMCSHyg vector. *Flag*-tagged *RnPEX2, flag-RnPEX2*, was constructed in the pUcD2SRαMCSHyg vector by inserting a *Bam*HI-*Eco*RI fragment (blunt-ended) of PAF-1 cDNA clone T into a *Bam*HI-*Apa*I (blunted) vector fragment containing a flag-tag sequence, originating from pUcD2Hyg•*flag-HsPEX12* (*19*). Both plasmid constructs were assessed by nucleotide sequence analysis.

RnPEX2-myc and flag-RnPEX2 were separately transfected into COS-7 cells. Pex2p-myc and flag-Pex2p were detected using mouse monoclonal antibodies to human c-myc (9E10) (Santa Cruz Biotech) and flag (M2) (Scientific Imazing Systems), and Texas red-labeled sheep anti-mouse IgG second antibodies (Amersham Pharmacia Biotech), in cells that had been permeabilized with either 50 μg/ml of digitonin or 1% Triton X-100 (*19, 20*). Peroxisomes were stained with rabbit antibodies to the peroxisome targeting signal type 1 (PTS1) peptide (*21*).

Expression of a Glutathione S-Transferase Fusion Protein and Preparation of Antibodies—The N- and C-terminal regions of rat Pex2p, termed Pex2pN and Pex2pC, comprising amino acid residues 1-131 and 226-305, respectively, were fused to glutathione S-transferase (GST) at the DNA level in pGEX4T-1 and pGEX4T-3 (Amersham Pharmacia Biotech). GST-Pex2pN and GST-Pex2pC were expressed in *E. coli*, XL1 Blue (Stratagene). The sonicated *E. coli* lysate was centrifuged and the resulting supernatant was applied to a column of glutathione-Sepharose (Amersham Pharmacia Biotech), as described (*20*). After thorough washing, the GST-fusion protein was eluted with 10 mM glutathione and then further purified by conventional preparative SDS-PAGE. Antisera against Pex2pN and Pex2pC were raised in rabbits by subcutaneously injecting the purified GST-fusion proteins. Anti-Pex2pN and anti-Pex2pC antibodies were purified by applying the respective rabbit sera to Affigel 10 that had been covalently linked with GST-Pex2pN or GST-Pex2pC. Anti-GST antibodies were removed by passing the eluate through a GST-Affigel 10 column.

Isolation of Peroxisomes—Peroxisomes were isolated by isopycnic sucrose density-gradient centrifugation, from the livers of male Wistar rats that had been injected with WR-1339, as described (*22*). Peroxisomes were recovered by pelleting the highly purified peroxisomal fraction among sucrose-dense fractions that had been diluted 6 times with 0.25 M sucrose in 20 mM Hepes-KOH, pH 7.4.

Treatment with Proteinase K—Peroxisomes suspended at 1 mg/ml in 0.25 M sucrose/20 mM Hepes-KOH, pH 7.4, were treated with Proteinase K at 0°C for 30 min. After terminating the reaction at 0°C for 10 min by adding 2 mM phenylmethylsulfonyl fluoride, the incubation mixture was centrifuged. The resulting peroxisomal pellet was suspended in 0.1 M sodium carbonate on ice for 30 min (*23*), and then centrifuged at 70,000 × *g* for 40 min. The membrane fraction was solubilized in SDS-PAGE sample buffer containing 2% SDS; proteins in the soluble fraction were precipitated with 10% trichloroacetic acid (TCA), and then rinsed with ethyl ether. Both the membrane and soluble fractions were analyzed by SDS-PAGE and immunoblotting. Peroxisomes treated with Proteinase K in the presence of Triton X-100 were likewise analyzed after precipitation with TCA.

Immunoprecipitation of Peroxisomes with Domain-Specific Antibodies—Peroxisomes, 400 μg in 300 μl of 0.25 M sucrose/20 mM Hepes-KOH, pH 7.4 containing 3 mg/ml of BSA, were incubated with affinity-purified anti-Pex2p antibodies, at 0°C for 60 min. Pansorbin, 20 μl in the same buffer, was added to the reaction mixture, followed by incubation at 0°C for 40 min. The immunocomplexes were recovered by centrifugation at 1,200 × *g* for 1 min in a Hitachi microfuge Model CF 150 and then gently washed twice with 500 μl of 0.25 M sucrose/phosphate-buffered saline (PBS) containing 3 mg/ml BSA. The supernatant and the two wash fractions were pooled and termed Sup I. The peroxisome-containing pellet was solubilized with 1% Triton X-100 in PBS and then centrifuged at 15,000 rpm for 5 min, the resulting supernatant fraction being termed Sup II. Sup I and Sup II were assayed for catalase activity; a part of each of Sup I and Sup II was precipitated with acetone, and then analyzed by SDS-PAGE and immunoblotting using rabbit anti-rat acyl-CoA oxidase (AOx) antibodies (*6*).

Cross-Linking with Bifunctional Reagents—Peroxisomes were suspended at 1.2 mg/ml in PBS and then incubated at 0°C for 30 min with several bifunctional reagents, including 1 mM PBM, 1 mM DMA, 2 mM DMS, 2 mM CETD, and 2 mM BS. The reaction was terminated by incubation with 20 mM dithiothreitol or ammonium chloride at 0°C for 30 min. Peroxisomes were recovered by centrifugation as described above and the membrane fraction was prepared by the sodium carbonate method (*23*). Pex2p was detected by immunoblotting using anti-Pex2pC antibodies.

Other Methods—SDS-PAGE was performed on a 12% polyacrylamide gel, and proteins were transferred to a Biodyne nylon membrane (Bio-Rad) using a semi-dry electroblot system (Bio-Rad). Immunoblotting was performed with specific antibodies and an ECL Western blot detection system (Amersham Pharmacia Biotech) (*20*).

RESULTS

Pex2p Is Localized to Peroxisomes—The intracellular localization of Pex2p was determined by ectopic expression of epitope-tagged rat Pex2p and immunofluorescence microscopy. Expression of *RnPEX2-myc* fully restored peroxisome assembly in ZP65, as efficiently as that of *RnPEX2*, indicating that the C-terminal tagging did not interfere with the *PEX2* function (data not shown). In COS-7 cells transfected with *RnPEX2-myc*, Pex2p was detected as a punctate staining pattern, with anti-myc antibodies (Fig. 1a). The pattern was superimposable on that obtained with anti-PTS1 antibodies (Fig. 1b), thereby suggesting that Pex2p-myc was targeted to peroxisomes. Similar results were obtained when N-terminally flag-tagged rat Pex2p was expressed in COS-7 cells, and then assessed by staining using antibodies to flag and PTS1 (Fig. 1, c and d). Endogenous Pex2p was not detectable in CHO-K1 cells (data not shown), due to a lower level of Pex2p expression, as found on Northern analysis (*11*). Epitope-tagged Pex2p was barely detected when *flag-RnPEX2* and *RnPEX2-myc* were respectively transfected into Z65, suggesting a lower expression level of the exogenously introduced Pex2p in CHO cells (data not shown). We earlier demonstrated by means of a subcellular fractionation study involving rat liver that Pex2p is a peroxisomal integral membrane protein with two putative transmembrane segments (*7*), consistent with the findings in this study.

Pex2p Is Exposed to the Cytosol—The membrane topology of Pex2p was determined by means of the differential permeabilization procedure, the detection of Pex2p being performed with antibodies to epitope-tags, myc and flag. When COS-7 cells expressing rat Pex2p-myc were permeabilized with 50 μg/ml of digitonin, under which conditions plasma membranes are selectively permeabilized but intra-peroxisomal proteins are inaccessible to exogenous antibodies (*19, 20*), Pex2p-myc was observed as a punctate staining pattern, whereas there was almost no staining of cells with anti-PTS1 antibodies (Fig. 1, e and f). Both Pex2p-myc and PTS1-proteins were detected upon treatment with Triton X-100, which solubilizes all cellular membranes (see above). Similar results were obtained for rat flag-Pex2p-expressing COS-7 cells, with a set of antibodies to flag and PTS1 (Fig. 1, c, d, g, and h). Taken together, these results strongly suggest that the both N- and C-terminal parts of Pex2p are exposed to the cytosol, being anchored by two membrane-spanning segments (*7*).

Domain-Specific Antibodies to Pex2p—Sera obtained from rabbits immunized with GST-Pex2pN (amino acid residues 1-131, Fig. 2A) or GST-Pex2pC (residues 226-305) were purified on an antigen-conjugated Affigel column. Anti-GST antibodies were then removed from the antibodies using GST-bound Affigel. The specificity of the affinity-purified antibodies was determined by immunoblotting. Anti-Pex2pN antibodies only reacted with a protein band corresponding to a molecular mass of ~35 kDa (*7*), presumably Pex2p, in rat liver peroxisomes (Fig. 2B, lane 1). The antibodies were also crossreactive with the purified GST-Pex2pN, but not with GST-Pex2pC or GST (Fig. 2B, lanes 2-4). Likewise, antibodies to GST-Pex2pC recognized authentic Pex2p and GST-Pex2pC, but neither GST-Pex2pN nor GST (Fig. 2B, lanes 5-8). Thus, antibodies raised against the N- and C-terminal parts of Pex2p are specific.

Protease Treatment of Peroxisomes—Freshly isolated rat liver peroxisomes were treated with various amounts of Proteinase K at 0°C for 30 min. Membrane and soluble fractions were separated by the sodium carbonate method (*23*). The integrity of Pex2p and AOx was monitored by

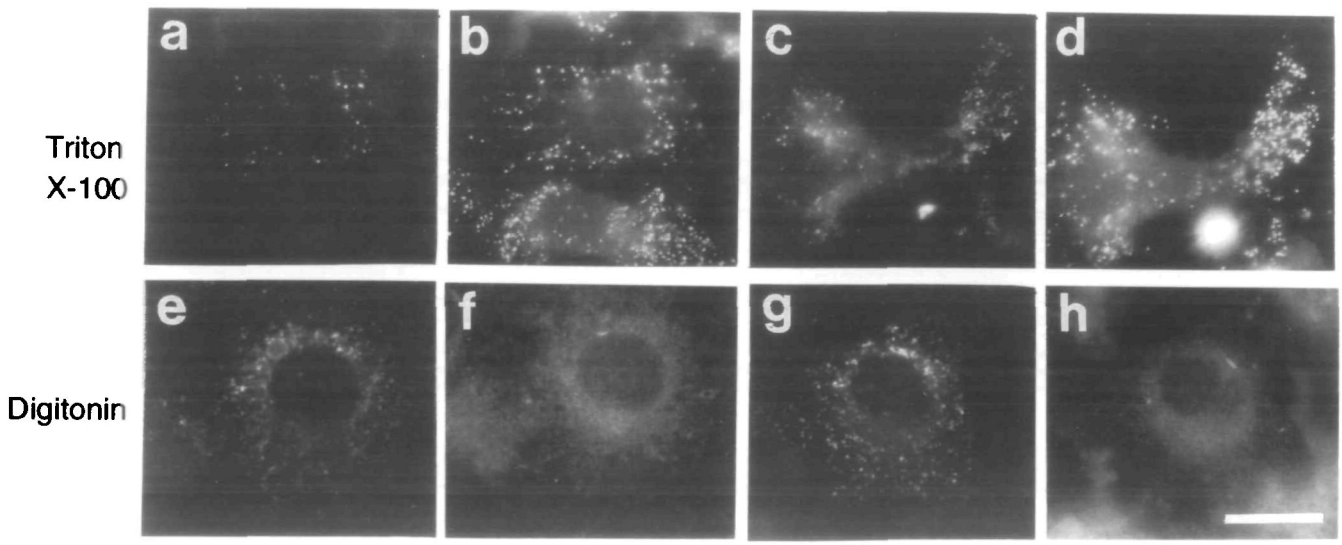

Fig. 1. **Intracellular localization and membrane topology of Pex2p.** Myc- or flag-tagged rat Pex2p was expressed in COS-7 cells. (a, b, e, and f) *PEX2-myc*-transfected COS-7 cells; (c, d, g, and h) *flag-PEX2*-transfected COS-7 cells. Cells were treated with 1% Triton X-100 (upper panels) or 50 μg/ml of digitonin (lower panels), and then stained with antibodies to myc (a and e), PTS1 (b, d, f, and h) and flag (c and g), respectively. Magnification, × 630; bar, 20 μm.

immunoblotting with specific antibodies to Pex2pN, Pex-2pC, and AOx, respectively (Fig. 3). Pex2p was detected by both types of anti-Pex2p antibodies in the membrane fraction after treatment of peroxisomes with 2 μg/ml of Proteinase K, at nearly the same level as in nontreated peroxisomes (Fig. 3, A and B, lanes 1 and 2). Upon digestion with 4 μg/ml of Proteinase K, a protein band corresponding to a molecular mass of ~27 kDa appeared with the concomitant disappearance of the authentic Pex2p, as assessed with anti-Pex2pN antibodies (Fig. 3A, lane 3). The 27-kDa polypeptide was not detected by antibodies to Pex2pC, suggesting that the C-terminal part of Pex2p was cleaved off (Fig. 3B, lane 3). Pex2p was barely detected with 6 μg/ml of Proteinase K and was not discernible at all with higher concentrations of the protease, as was the case for the digestion in the presence of Triton X-100 (Fig. 3, A and B, lanes 4 to 6). In contrast, AOx, a matrix enzyme, remained at nearly the same level as in intact peroxisomes on the treatment of peroxisomes with all concentrations examined, up to 8 μg/ml, of Proteinase K, while the addition of a detergent made AOx susceptible to the digestion (Fig. 3C). Taken together, the results suggest that Pex2p is located in the peroxisomal membrane, with its N- and C-terminal regions exposed to the cytosol, incompatible with the morphological findings described above.

Binding of Pex2p Antibodies to Intact Peroxisomes—Rat liver peroxisomes were incubated with anti-Pex2pN and -Pex2pC antibodies. The IgG fraction was recovered by adding formalin-fixed *Staphylococcus aureus* cells and examined as to whether or not peroxisomes were present in the complex, by determining catalase activity after solubilization with Triton X-100. Up to nearly 80% of the catalase activity was recovered in the immuno-complex, as anti-Pex2pC antibodies were increased to 15 μg (Fig. 4A). Conversely, catalase activity decreased in the supernatant as the antibodies increased, while about 80% of the activity remained in the supernatant with preimmune serum (data not shown). A very low level, ~5%, of catalase activity was detectable in the pellet, with preimmune serum (data not shown). Accordingly, the results strongly suggested that intact peroxisomes bound to *S. aureus* cells *via* anti-Pex-2pC IgG. This is consistent with the results of determination of the Pex2p topology by means of morphological analysis and protease treatment. Similar results were

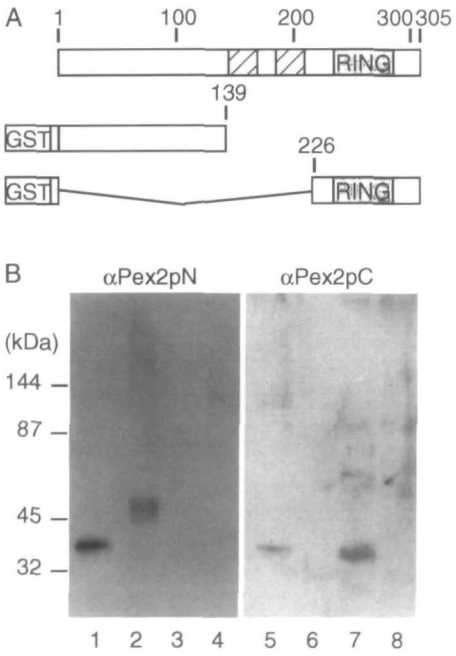

Fig. 2. **Specificity of Pex2p domain-specific antibodies.** A: diagrams of the rat Pex2p and GST-Pex2p fusion constructs, the N-terminal part, from the N-terminus to amino acid residue 139, and C-terminal residue 226 to the C-terminus of Pex2p, are separately fused to GST. B, immunoblots probed with affinity-purified rabbit antibodies against the N- and C-terminal regions of Pex2p, respectively. The antigen-antibody complexes were detected with horseradish peroxidase-conjugated goat anti rabbit IgG antibodies, using ECL Western blotting detection reagent. Lanes: 1 and 5, rat liver peroxisomes, 80 μg; 2 and 6, GST-Pex2pN fusion protein, 1 μg; 3 and 7, GST-Pex2pC, 0.1 μg; 4 and 8, GST, 0.1 μg.

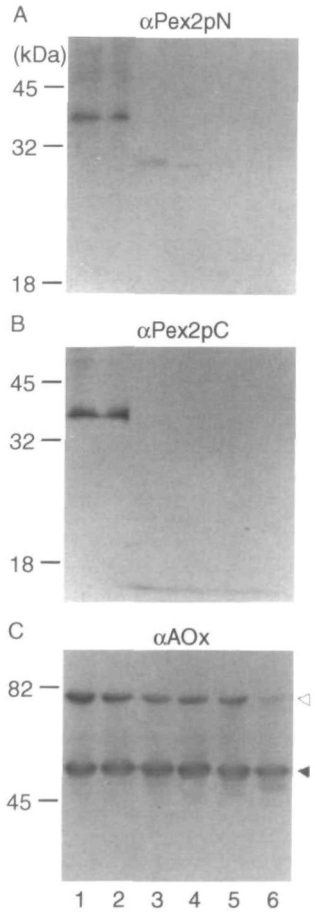

Fig. 3. **Protease treatment of peroxisomes.** Freshly isolated rat liver peroxisomes (400 μg) were treated with Proteinase K at 0°C for 30 min. After terminating the reaction with phenylmethylsulfonyl fluoride, the reaction mixture was treated with sodium carbonate as described under "EXPERIMENTAL PROCEDURES." The membrane pellet fraction (A and B, from 150 μg peroxisomes) recovered on centrifugation was solubilized in SDS-PAGE sample buffer. Soluble proteins in the supernatant were precipitated with TCA (C, from 150 μg peroxisomes). A-C, Western blots with antibodies to Pex2pN, Pex2pC, and AOx, respectively. Lanes: 1, mock-treated; 2-5, peroxisomes treated with 2, 4, 6 and 8 μg/ml of Proteinase K, respectively; 6, TCA-precipitated fraction from peroxisomes that had been treated with 8 μg/ml Proteinase K in the presence of 1% Triton X-100. The open and solid arrowheads indicate AOx-components A and B, respectively.

obtained with anti-Pex2pN antibodies, where less, 25%, catalase activity was found in the immunoprecipitate, 50% remaining in the supernatant (Fig. 4B). It is plausible that anti-Pex2pN antibodies are less potent in recognizing Pex2p or are not fully accessible to the antigen.

AOx in the immuno-pellets was examined by SDS-PAGE and immunoblotting. AOx was detected in the immunoprecipitate obtained with anti-Pex2pC antibodies (Fig. 4C, lanes 1 and 4–7), apparently in good accordance with the catalase activity recovered in an antibody dose-dependent manner. A faint AOx-B band was seen (lanes 2 and 3), presumably reflecting a small amount of peroxisomes recovered in the Sup II fraction or a nonspecific band. AOx was also recovered with anti-Pex2pN antibodies, but less efficiently than that with anti-Pex2pC antibodies (lanes 8–11).

The effect of protease-treatment on the immunoprecipitation of peroxisomes was investigated. Using anti-Pex2pC antibodies, with no addition of protease, almost full catalase activity was recovered in Sup II, i.e. little in Sup I (Fig. 5A). Nearly 80% of the catalase activity remained in Sup I, while there was only 7% in Sup II, in the mock experiment with preimmune serum. After treatment with 4 μg/ml of Proteinase K, 12% of the catalase activity was in Sup II and ~70% remained in Sup I. AOx in Sup II was determined by immunoblotting. AOx was quantitatively recovered in Sup II from undigested peroxisomes, as represented by the AOx-B component, while a small amount of AOx was found in Sup II obtained with preimmune serum, consistent with the finding shown in Fig. 4C (see lane 3) (Fig. 5B, lanes 1–3). The AOx band was not detectable when peroxisomes were treated with Proteinase K (lane 4).

Taken together, these results were interpreted to mean that of Pex2p the N- and C-terminal regions are exposed to the cytosol.

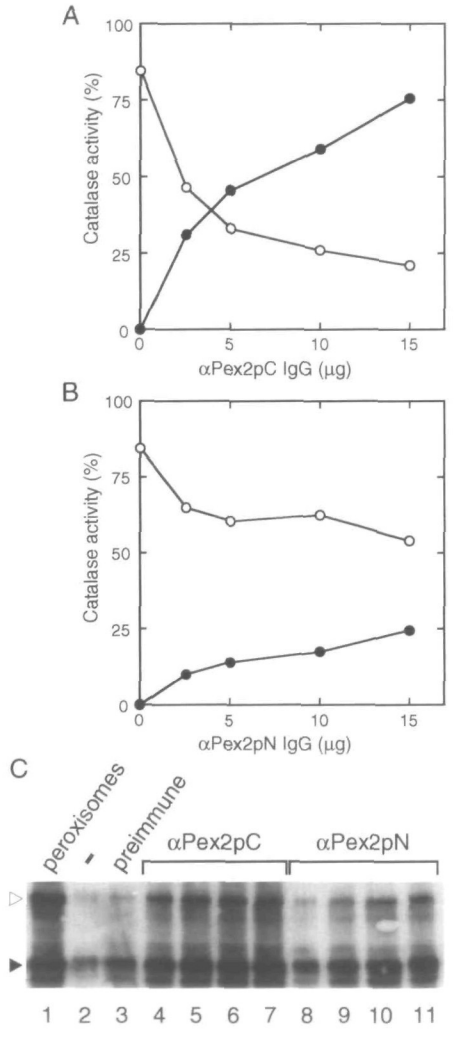

Fig. 4. **Immunoprecipitation of peroxisomes with antibodies specific for the N- and C-terminal parts of Pex2p.** Peroxisomes (200 μg) were incubated with 2.5, 5, 10, and 15 μg of anti-Pex2p antibodies at 0°C for 60 min, and then further for 40 min after the addition of Pansorbin. The "peroxisome-Pansorbin complex" was recovered by centrifugation at 1,200 × g for 1 min and then washed twice with isotonic PBS. The supernatant and two wash fraction were pooled, and termed Sup I. The peroxisome pellet was solubilized with 1% Triton X-100 and then centrifuged; the recovered supernatant was termed Sup II. Sup I and Sup II were analyzed for catalase activity (A, B) and AOx protein (C). A and B, immunoprecipitation was performed with antibodies to Pex2pC and Pex2pN, respectively; open and solid circles indicate Sup I and Sup II, respectively. Catalase activity is expressed as the average of duplicate assays, relative to the total input activity determined in the presence of 1% Triton X-100. The relative activities in Sup I and Sup II obtained with preimmune serum were 78 and 5%, respectively. C, immunoblotting with anti-AOx antibodies. Lanes: 1, total input peroxisomes, 1 μg; 2–11, Sup II (1/200 aliquot of the reaction mixture) recovered with no IgG added (lane 2), preimmune serum (1 μl, lane 3), and antibodies to Pex2pC (lanes 4–7) and Pex2pN (lanes 8–11). The amounts of antibodies used were 2.5 μg (lanes 4 and 8), 5 μg (lanes 5 and 9), 10 μg (lanes 6 and 10), and 15 μg (lanes 7 and 11). The open and solid arrowheads indicate AOx-components A and B.

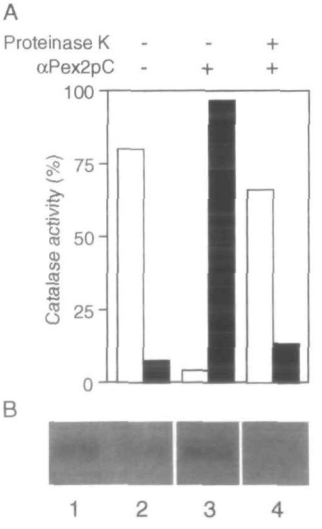

Fig. 5. **Protease-treatment of peroxisomes affects immunoprecipitation with anti-Pex2p antibodies.** The immunoprecipitation of peroxisomes (200 μg) was performed with 20 μg of anti-Pex2pC antibodies. Sup I and Sup II were prepared and analyzed as in Fig. 4. A, catalase activity in Sup I (open bars) and Sup II (solid bars); the experimental conditions are indicated at the top. Protease treatment was performed with 4 μg/ml of Proteinase K, as in Fig. 3. B, immunoblotting with anti-AOx antibodies. Only the most abundant B component of AOx is shown. Lanes: 1, total input peroxisomes, 4 μg; 2 and 3, Sup II (1/50 aliquot of the reaction mixture) from peroxisomes with preimmune serum (1 μl) and anti-Pex2pC antibodies, respectively; 4, the same as in lane 3 but from Proteinase K treated peroxisomes.

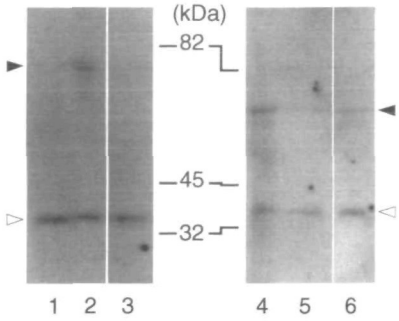

Fig. 6. **Cross-linking of Pex2p using bifunctional reagents.** Peroxisomes (120 μg) were incubated with various bifunctional cross-linkers, as described under "EXPERIMENTAL PROCEDURES." After terminating the reaction, peroxisomal membranes were prepared by the sodium carbonate procedure (*23*), and then analyzed by SDS-PAGE and Western blot with anti-Pex2pC antibodies. Lanes: 1, mock-treated; 2, peroxisomes treated with 1 mM PBM; 3, ones with 1 mM DMA; 4-6, ones with 2 mM DMS, 2 mM CETD, and 2 mM BS, respectively. The open and solid arrowheads indicate the positions of the Pex2p and 70-kDa protein bands (see text), respectively. The figure represents a composite of two separate experiments.

Cross-Linking of Pex2p by Bifunctional Reagents—To determine if Pex2p interacts in a homomeric and/or heteromeric manner with other proteins, peroxisomes were incubated at pH 7.4 in the presence of bifunctional reagents and then Pex2p in membranes was analyzed by immunoblotting using anti-Pex2pC antibodies. A protein band corresponding to an apparent molecular mass of ~70 kDa appeared upon the reaction with an SH-reactive crosslinker, PBM (Fig. 6, lane 2). A faint protein band with a mobility similar to that of the 70-kDa Pex2p conjugate was noted for the mock-treated peroxisomes (lane 1), plausibly representing a Pex2p-bound protein, including a dimeric form of Pex2p, that presumably resulted from the oxidation of cysteine residues but not dissociation by reduction. The 70-kDa conjugate band decreased in amount, with a concomitant increase in aggregates with higher masses, as the concentration of PBM was increased (data not shown). Similarly, the 70-kDa polypeptide band was detectable for peroxisomes that had been incubated with other bifunctional NH_2-reactive agents, such as DMS and BS (Fig. 6, lanes 4 and 6). Incubation with NH_2-linkers, DMA and CETD, did not result in the 70-kDa band (lanes 3 and 5). The 70-kDa protein conjugate formed in these assays was not crossreactive with antibodies to 40-kDa peroxin, Pex12p (*19*), with a similar mass to Pex2p (data not shown). Collectively, it is possible that Pex2p forms a homodimer or is localized in proximity.

DISCUSSION

In the present work, we revealed the peroxisomal localization of Pex2p by means of morphological determination, which was in good agreement with our earlier findings on subcellular fractionation of rat liver (*7*). We also demonstrated the transmembrane topology of Pex2p by means of several different, but mutually complementary, approaches: a morphological one involving the use of epitope-tagged Pex2p, trimming of isolated peroxisomes with externally added protease, and immunoadsorption of peroxisomes with Pex2p region-specific antibodies. The findings were consistent between the methods used, thereby leading us to conclude that both the N- and C-terminal parts of Pex2p are exposed to the cytosol. The orientation of the C-terminal part, including the RING domain, of Pex2p toward the cytosolic face, observed in this study, agrees with our earlier finding that the C-terminal part of Pex2p of isolated rat liver peroxisomes is highly sensitive to exogenously added protease (*7*). However, a mechanistic insight into the Pex2p function has not been obtained, while Pex2p was shown to be required for the biogenesis of peroxisomes, including the import of the PTS1 and PTS2 proteins, from yeast to humans (*7, 8, 11, 12, 15-17*), and the assembly of glycosomes (*18*), a family of peroxisomes. Moreover, both the N- and C-terminal regions are conserved in all species, implying the importance of these parts for the biological function of Pex2p. With the deletion of 19 amino acids from the N-terminus of Pex2p the peroxisome assembly-restoring activity was maintained when it was overexpressed in both Z65 and fibroblasts from a CG-F Zellweger patient, whereas the deletion of 27 residues eliminated the activity (*11*). Thus, the cytosolic topology of the N-terminal part and residues 19-27 of Pex2p are likely to be essential for the Pex2p function. One point missense mutation in codon 246 for Tyr was found in a CHO cell mutant of the same CG as Z65 (*12*), suggesting the functional significance of the C-terminal region of Pex2p. It is noteworthy, however, that Pex2p truncated at residues 214-305 complemented peroxisome assembly in Z65 in an overexpressing system (*11*). It is plausible that elimination of this part may be compensated for by overexpression of the truncated form.

We found that 35-kDa Pex2p apparently formed a 70-kDa conjugate *via* crosslinkers, possibly a homodimer of Pex2p, or a heteromeric oligomer with other components. The C-terminal Cys-rich region is possibly located in proximity between Pex2p, where SH-reactive crosslinkers are accessible. The C-terminal domain, including the Cys-rich RING finger, may function in the interaction between Pex2p molecules, as suggested (*13*). Mutations in this region may hinder dimerization of Pex2p, thereby resulting in a dysfunction of Pex2p. It is equally possible that a functionally important region in Pex2p may reside in the N-terminal part, which is conserved in mammals (*7, 8, 11*), a parasite (*18*), a fungus (*17*), and yeast (*15, 16*). Pex2p may function in the peroxisome assembly process as a component of the matrix protein-import machinery comprising several peroxins, including Pex14p (*20, 24*). Pex2p appears not to be involved in the import of membrane proteins, as peroxisomal remnants called ghosts are detectable in CG-F mutants including human patient fibroblasts. It would be of interest to determine whether or not Pex2p interacts with other peroxisome biogenesis factors such as the RING peroxins, Pex10p (*25, 26*) and Pex12p (*19, 27, 28*).

We also developed in the present work an efficient immunochemical method for determination of the transmembrane topology of an integral peroxisomal membrane protein: antibodies to a cytosolic part of the integral membrane protein of interest can precipitate intact, whole peroxisomes into which the membrane protein is integrated. A similar method was developed to determine the localization of NADPH-cytochrome P-450 reductase in rat liver Golgi membranes (*29*), where an antibody-coated polyacrylamide gel adsorbed organelle membranes bearing

the antigen. The method we developed in the present study will also be useful for examining the membrane topology of endomembrane proteins.

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