Characterization of the 70-kDa Peroxisomal Membrane Protein, an ATP Binding Cassette Transporter*

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The 70-kDa peroxisomal membrane protein (PMP70) is one of the major components of rat liver peroxisomal membranes and belongs to a superfamily of proteins known as ATP binding cassette transporters. PMP70 is markedly induced by administration of hypolipidemic agents in parallel with peroxisome proliferation and induction of peroxisomal fatty acid β-oxidation enzymes. To characterize the role of PMP70 in biogenesis and function of peroxisomes, we transfected the cDNA of rat PMP70 into Chinese hamster ovary cells and established cell lines stably expressing PMP70. The content of PMP70 in the transfectants increased about 5-fold when compared with the control cells. A subcellular fractionation study showed that overexpressed PMP70 was enriched in peroxisomes. This peroxisomal localization was confirmed by immunofluorescence and immunoelectron microscopy. The number of immuno-gold particles corresponding to PMP70 on peroxisomes increased markedly in the transfectants, but the size and the number of peroxisomes were essentially the same in both the transfectants and the control cells. β-Oxidation of palmitic acid increased about 2-3-fold in the transfectants, whereas the oxidation of lignoceric acid decreased about 30–40%. When intact peroxisomes prepared from both the cell lines were incubated with palmitoyl-CoA, oxidation was stimulated with ATP, but the degree of the stimulation was higher in the transfectants than in the control cells. Furthermore, we established three Chinese hamster ovary cell lines stably expressing mutant PMP70. In these cells, β-oxidation of palmitic acid decreased markedly. These results suggest that PMP70 is involved in metabolic transport of long chain acyl-CoA across peroxisomal membranes and that increase of PMP70 is not associated with proliferation of peroxisomes.

Peroxisomes are organelles bounded by a single membrane that are present in almost all eukaryotic cells. The peroxisomes are involved in a variety of metabolic processes including per-oxide-based respiration, oxidative degradation of fatty acids and purine, and synthesis of plasmalogens and bile acids (1). Early studies on rat liver peroxisomes led to the concept that peroxisomes are freely permeable to compounds of low molecular weight (2, 3). However, Van Roermund et al. (4) suggested recently that some metabolites were unable to permeate the membrane of peroxisomes in Saccharomyces cerevisiae. The peroxisomal membrane in human fibroblasts has been shown not to be freely permeable to at least one of the substrates of acyl-CoA dehydrogenase phosphatase acyltransferase, unless ATP is present (5). Therefore, the exchange of metabolites between peroxisomes and cytosol seems to require specific transporters.

The 70-kDa peroxisomal membrane protein (PMP70) is a major component of peroxisomal membranes and markedly induced by the administration of hypolipidemic agents, and its induction parallels peroxisome proliferation and induction of peroxisomal β-oxidation enzymes (6, 7). We cloned and sequenced rat PMP70 and found that it was an ATP binding cassette (ABC) transporter (8). ABC transporters are a superfamily of membrane-bound proteins whose structure and function have been highly conserved from eubacteria to mammals and catalyze the ATP-dependent transmembrane translocation of a wide variety of substrates, including anti-tumor drugs and several kind of lipids (8, 10). They are composed of two homologous halves, each containing the following two parts: a domain containing six potential transmembrane segments and an ATP binding domain, which contains a particular type of nucleotide binding fold. PMP70 is half the size of an ABC transporter and seems to function as a dimer.

To date, four peroxisomal ABC transporters have been identified in mammalian peroxisomes: PMP70 (8, 11, 12), adrenoleukodystrophy protein (ALDP) (13), ALDP-related protein (ALDRP) (14, 15), and PMP70-related protein (P70R/PMP69) (16, 17). A defect in ALDP is known to be responsible for the X chromosome-linked neurodegeneration disorder adrenoleukodystrophy; this defect is an inborn error of peroxisome β-oxidation of very long chain fatty acids (18, 19). Mutations in the PMP70 gene have been identified in one complementation group (group 1) of Zellweger syndrome, an inborn error of peroxisome biogenesis, and it is suggested that PMP70 has an important role in peroxisome assembly (12). However, a recent
study of complementation group 1 probands by Southern blotting and single-strand conformation polymorphism (SSCP) of reverse transcription-PCR fragments failed to detect PMP70 mutations (20). Therefore, it is not clear whether PMP70 is associated with biogenesis of peroxisomes. On the other hand, the fact that inducibility of PMP70 parallels peroxisome proliferation and induction of peroxisomal \( \beta \)-oxidation enzymes suggests that PMP70 participates in proliferation of peroxisomal membranes and/or transport of substrates for peroxisomal \( \beta \)-oxidation enzymes.

As an approach to determining the role of PMP70 in peroxisome assembly and function, we isolated CHO cells stably overexpressing PMP70 and its mutant forms. We found that PMP70 is involved in metabolic transport of long chain acyl-CoA across the peroxisomal membrane and is not required for proliferation of peroxisomes or peroxisome biogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—\( ^{125}\)I-Protein A (2.60–3.70 TBq/g), \( ^{1-14C}\)palmitic acid (1.85 GBq/mmol), \( ^{1-14C}\)lignoceric acid (1.92 GBq/mmol), and \( ^{1-14C}\)palmitoyl-CoA (1.46–2.22 GBq/mmol) were purchased from ICN Biomedicals Inc. (Irvine, CA). Moravek Biociences Inc. (Brea, CA), CEA (Gif-Sur-Yvette, France), and NEN Life Science Products, respectively. Palmitoyl-CoA, coenzyme A, NAD\(^+\), and dithiothreitol were obtained from Sigma. Preparation of antibodies against the C-terminal 15 amino acids of rat PMP70 was as described in (21). Anti-rat liver catalase antibodies were raised in guinea pigs (22), and anti-rat liver acyl-CoA oxidase antibodies were raised in rabbits (23). Other reagents were of analytical grade.

**Construction of PMP70 Expression Vector**—The plasmid pTZ18U/PMP70 in which the rat cDNA sequence encoding PMP70 was cloned into the EcoRI and HindIII sites of pTZ18U was described in (21). From this cDNA, a fragment containing the first 1971 bases of PMP70 was excised with EcoRI and recloned into pBluescript KS\(^+\) at the EcoRI site. This was cut with HpaI and PstI, and the linear plasmid containing the N-terminal 95% of PMP70 was purified by agarose gel electrophoresis. A 1.3-kilobase fragment containing the C-terminal 65% of PMP70 was then excised from pTZ18U/PMP70 with HpaI and EcoT221 and purified. The fragment was ligated into HpaI and PstI sites of the above plasmid, encoding the N-terminal 35% of PMP70 and the construct designated pBluescriptKS\(+\)/pMP70. The pBluescript KS\(+\)/pMP70 was cut with XhoI and PstI sites of the expression vector pME18S (24), which was then designated as pME18S/PMP70.

**Construction of Mutant cDNAs**—A mutant version of PMP70 containing mutations in EAA-like motif, designated as PMP70 (E277D/E278D), was constructed by asymmetric PCR using pME18S/PMP70 as the template. Two oligonucleotides (the site of substitution is underlined), 5\'-9\'-660AAGCCATTTTTAGACATAGTTTTGTA685-3\' and 5\'-9\'-1445GAAGAGGGAGCTGGCGCCACAGCCATTTGGACCACAAATTGGCTGAGT1745-3\', were used in the second step, and a 400-base pair fragment containing mutations in EAA-like motif, designated as PMP70 (E277D/E278D), was constructed by asymmetric PCR using pME18S/PMP70 as template. Two oligonucleotides (the site of substitution is underlined), 5\'-9\'-846-3\' and 5\'-9\'-1050-3\', were used in the first step, and a 220-base fragment containing the N-terminal 35% of PMP70 was purified and digested with HpaI and PstI. The fragment containing the mutations of E277D and E278D was ligated with XhoI and XbaI sites of expression vector pME18S (24), which was then designated as pME18S/PMP70.

**Expression Plasmids were Stably Transfected into CHO Cells**—CHO cells were cultured with Ham’s F-12 medium (100 units/ml penicillin and 100 \( \mu \)g/ml streptomycin) and transfected with 5.0 \( \mu \)g of pME18S/PMP70 plus 0.5 \( \mu \)g of pSVneo, which had been mixed with Transfectam\(^\text{TM}\) (Promega). The procedure was essentially the same as described (25). Surviving isolated colonies were picked up by cylinder technique and subjected to immunoblot analysis of PMP70. Cells overexpressing PMP70 were further purified by repeating the limited dilution twice. The same procedure was carried out to obtain cells overexpressing mutant PMP70.

**Subcellular Fractionation of CHO Cells**—CHO cells were grown with Ham’s F-12 medium containing 10% fetal calf serum and cell homogenate and a postnuclear supernatant fraction were prepared with the same procedure described (21). Then a fraction combining both heavy and light mitochondrial fractions was obtained by centrifugation at 16,000 \( \times \) g for 20 min. This fraction (~10 mg of protein in 0.5 ml) was further subjected to equilibrium density centrifugation in a 10.6 ml linear sucrose gradient (1.10–1.20 g/ml) in a Hitachi RP55VF rotor. The gradient rested on 1.0 ml of 1.25 g/ml sucrose. All solutions contained 1 mM EDTA, 3 mM imidazole, and 0.1% (w/v) ethanol. Centrifugation was carried out at 50,000 rpm (193,000 \( \times \) g) for 90 min at 4 °C. Fractions of approximately 1.0 ml were collected in preweighed Eppendorf tubes, and the density of each fraction was determined by refractometry.

**Immunofluorescence**—CHO cells were seeded on LAb-TEK\(^\circ\) Chamber Slide\(^\text{TM}\) System that mounted 4 chambers on a glass slide (Nalge Nunc) in Ham’s F-12 medium with 10% fetal calf serum. After 18–24 h, the medium was replaced by a serum-free medium, and the culture was continued for an additional 15–18 h (22). Immunostaining microscopy was performed as described (26).

**Immunoelectron Microscopy**—CHO cells were fixed with 4% (w/v) paraformaldehyde, 0.2% (w/v) glutaraldehyde in 0.15 M cacodylate buffer, pH 7.4, for 1 h at room temperature. After washing with phosphate-buffered saline, the cells were dehydrated with graded dimethylformamide at ~20 °C and embedded in LR White. Polymerization of the resin was performed under UV light at ~20 °C for 24 h. Thin sections were cut with a diamond knife equipped with a Ultramicrotome (Amersham Pharmacia Biotech). Catalase was visualized by combination of guinea pig anti-catalase with a 15-nm protein A-gold probe applied to one side of the thin sections, and PMP70 was detected by rabbit anti-PMP70 antibody with a 3-nm protein A-gold probe applied to another side of the sections.

**β-Oxidation Assay in Cultured Cells**—β-Oxidation assay was done essentially as described (27). The cells cultured in 6-well flasks were preincubated for 1 h in Ham’s F-12 medium without fetal calf serum. Fatty acid oxidation reaction was initiated by adding 4 nmol of [1-14C]palmitic acid or 4 nmol of [1-14C]lignoceric acid to the freshly prepared Ham’s F-12 medium without fetal calf serum. Radioactive
palmitic and lignoceric acid dissolved in ethanol was evaporated under
a stream of nitrogen and dissolved in a solution of 0.1 M Tris-HCl, pH
8.0, containing 10 mM α-cyclodextrin. After incubation for 0–120 min,
the dishes were placed on ice, 0.15 ml of 10%(w/v) bovine serum albumin
and 0.2 ml of 3 M perchloric acid were added to the medium, and the
preparation was incubated for 30 min on ice. The medium was centri-
fuged, and the unreacted fatty acids in the supernatant were removed
by extraction 3 times with 3 ml of hexane. The acid-soluble radioactivity
such as in acetate and citrate in the medium was measured by a
scintillation counter.

β-Oxidation Assay Using Isolated Peroxisomes—Peroxisomal palmito-
oyl-CoA oxidation was measured with some modification of the meth-
ods in (28, 29). The oxidation was measured in a final volume of 100 µl
of 10 µM [1-14C]palmitoyl-CoA, 2 mM MgCl₂, 0.5 mM coenzyme A, 2 mM
NAD⁺, 2 mM KCN, 2 mM dithiothreitol, 0.1%(w/v) bovine serum albumin,
0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, with or without 2 mM ATP.
In some experiments, peroxisomes were disrupted with 0.1%(w/v) of
Triton X-100. After incubation for 10 min at 37 °C, reactions were
terminated by adding 700 µl of ice-cold H₂O, 0.15 ml of 10% (w/v) bovine
serum albumin, and 0.2 ml of 3 M perchloric acid to the reaction
mixture, and the mixture was incubated for 30 min on ice. The mixture
was centrifuged, and the supernatant was extracted 3 times with 3 ml
of hexane. The acid-soluble radioactivity in the medium was measured
by the scintillation counter.

Other Methods—Protein, catalase, N-β-D-glucosaminidase, cyto-
chrome c oxidase, and NADPH cytochrome c reductase were assayed as
described previously (21). Immunoblotting was done by the method of
Small et al. (30).

RESULTS

Overexpression of PMP70 in CHO Cells—The expression vec-
tor pME18/PMP70 was co-transfected with pSVneo into CHO
cells, and stable transformants were selected based on Geneti-
cin resistance. The clones were examined for the expression of
PMP70 by immunoblot analysis. Several cell lines transfected
with pME18S/PMP70 and pSVneo (#19 and #31) were analyzed
with anti-catalase (Cat) (A), anti-acyl-CoA oxidase (AOx; a and b mean
subunits a and b, respectively) (B), anti-hydratase-dehydrogenase (HD)
(C), and anti-thiolase (D).

Function of the 70-kDa Peroxisomal Membrane Protein

FIG. 2. Immunoblot analysis of several peroxisomal enzymes
in control CHO cells and CHO cells overexpressing PMP70. Cell
homogenates (150 µg of protein) prepared from wild type CHO cells
(Cont), the cells transfected with pSVneo (Neo), and the cells co-trans-
fected with pME18S/PMP70 and pSVneo (#19 and #31) were analyzed
with anti-catalase (Cat) (A), anti-acyl-CoA oxidase (AOx; a and b mean
subunits a and b, respectively) (B), anti-hydratase-dehydrogenase (HD)
(C), and anti-thiolase (D).

FIG. 3. Subcellular localization of
PMP70 in Neo and #19 cells. A, heavy
and light mitochondrial fractions from
Neo and #19 cells were fractionated by
equilibrium density centrifugation on su-
crose. The marker enzyme distributions
are plotted as described previously (21);
the recoveries varied between 70 and
120%. Catalase, cytochrome c oxidase
(Cyt Ox), NADPH cytochrome c reductase
(Cyt Red), and N-acetyl-β-D-glucosamini-
dase (NAGase) were measured as marker
enzymes of peroxisomes, mitochondria,
microsomes, and lysosomes, respectively.
Distribution of PMP70 was quantitated
by BAS 1500 imaging analyzer. B, immu-
noblot analysis of PMP70. An aliquot of
each fraction from the top (1) to the bot-
tom (12) of the sucrose gradient was ana-
lyzed by immunoblotting. One hundred µl
of each fraction was used in the case of
Neo cells and 25 µl was used in that of #19
cells.
PMP70 compared with Cont and Neo cells. To examine whether the overexpression of PMP70 affects the amount of other peroxisomal proteins in CHO cells, immunoblot analysis was carried out using #19 and #31 cells. As shown in Fig. 2, amounts of catalase, acyl-CoA oxidase, hydratase-dehydrogenase, and thiolase in these cells were essentially the same as those in Cont and Neo cells.

Subcellular Localization of PMP70—To investigate the roles of PMP70 in biogenesis and function of peroxisomes, we first examined whether PMP70 was enriched in peroxisomes in the cells overexpressing PMP70. When isolated subcellular fractions such as mitochondrial, light mitochondrial, microsomal, and cytosolic fractions were subjected to immunoblot analysis, a band corresponding to PMP70 was enriched in the light mitochondrial fraction in Cont, Neo, #19, and #31 cells (data not shown).

The subcellular distribution of PMP70 was further determined by equilibrium density centrifugation. In Neo cells, peroxisomes were well separated from mitochondria, endoplasmic reticulum, and lysosomes (Fig. 3A). The PMP70 was mainly recovered in fractions 10–11 on the sucrose gradient, which corresponded to the position of peroxisomes as shown by the catalase distribution (Fig. 3B). For #19 cells, peroxisomes were also separated from other organelles (Fig. 3A). The PMP70 was mainly recovered in fractions 9–10, and the distribution of PMP70 was similar to that of catalase (Fig. 3B). These results suggest that overexpressed PMP70 is enriched in peroxisomes of CHO cells. The distribution of PMP70 in #31 cells is essentially the same as that of #19 cells (data not shown).

Localization of PMP70 to peroxisomes in Neo, #19, and #31...
cells was also examined by immunofluorescence microscopy. As shown in Fig. 4, PMP70 exhibited a punctulate staining pattern in Neo cells. This pattern was completely superimposable on the distribution of catalase in the same cells (A and B). PMP70 also exhibited a punctulate structure with high intensity of fluorescence in #19 and #31 cells, and this pattern was also superimposable on the distribution of catalase on the same cells, although the intensity of cytosolic catalase increased (D and F). We observed cytosolic catalase activity increased slightly after digitonin permeabilization of these cells (data not shown). In immunoelectron microscopy (Fig. 5), gold particles corresponding to PMP70 were observed on peroxisomes, and their number increased in #19 and #31 cells (B and C) as compared with Neo cells (A). However the size of peroxisomes seems to be similar between Neo, #19, and #31 cells.

β-Oxidation of Fatty Acids—We have investigated the enzymatic β-oxidation activity of Cont, Neo, #19, and #31 cells using radiolabeled [1-14C]palmitic and [1-14C]lignoceric acids. 14C-labeled acid-soluble degradation products derived from both substrates increased linearly up to 2 h in all the cells. The rate of palmitic acid β-oxidation in #19 and #31 cells increased about 2–3-fold compared with control and Neo cells (Fig. 6A). On the other hand, the β-oxidation of lignoceric acid in these cells was reduced about 30–40% (Fig. 6B). However, when β-oxidation activities of palmitic acid and lignoceric acid were measured with 2 mM KCN in the lysates, prepared by freezing and thawing or treatment of 0.01% (v/v) Triton X-100, these activities were unchanged and indistinguishable among these cells, Cont, #19, and #31 cells, under those conditions where peroxisomes were disrupted (data not shown).

To evaluate peroxisomal β-oxidation, we measured β-oxidation of palmitic acid in cultured CHO cells in the presence of 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA), a potent inhibitor of mitochondrial carnitine palmitoyltransferase I (27, 31). However, POCA (50 μM) did not inhibit β-oxidation of palmitic acid in any of the cells, although under the same conditions palmitic acid oxidation was inhibited about 70 and 80% in rat hepatoma H4IIE cells and human fibroblasts. In addition, when a combined fraction of both heavy and light mitochondrial fractions prepared from CHO cells was separated on sucrose gradient centrifugation, about 80% of the activity was recovered in peroxisomal fraction, and it was not inhibited with 2 mM KCN (data not shown). We show, therefore, that peroxisomes contribute mainly to the formation of acid-soluble labeled products in CHO cells under our experimental conditions.

ATP Stimulates Palmitoyl-CoA Oxidation in Light Mitochondrial Fraction—As palmitic acid is activated to become palmitoyl-CoA outside of peroxisomes, we further examined whether overexpression of PMP70 on peroxisomal membranes stimulates β-oxidation of palmitoyl-CoA using light mitochondrial fractions in the presence of KCN. In some experiments, peroxisomes were disrupted with Triton X-100. As shown in Fig. 7, when β-oxidation was measured in the disrupted peroxisomes, the activity was essentially the same with or without ATP. On the other hand, if peroxisomes were intact, ATP stimulated β-oxidation of palmitoyl-CoA, and the degree of the stimulation in #19 cells was higher than that in Neo cells. Latency of catalase in both cells was essentially the same (about 90%) (data not shown). In addition, in our incubation, the rate of hydrolysis of palmitoyl-CoA to palmitic acid and CoA was about 10% and was not changed by ATP (data not shown). These results suggest that PMP70 is involved in transport of long chain acyl-CoA across peroxisomal membranes. It is unlikely that extensive hydrolysis of palmitoyl-CoA during incubation decreased its concentration and that the stimulative effect of ATP on the β-oxidation was because of reactivation of palmitoyl-

Mutation of Conserved Motifs in PMP70 Inhibited β-Oxidation of Palmitic Acid—ABC transporters possess highly conserved regions such as an EAA-like motif and Walker A and B motifs. The EAA-like motif seems to be important for ATP binding and hydrolysis (9, 34). As PMP70 is a half-sized ABC transporter and seems to function as a dimer, mutation of one of the conserved regions might cause loss of the PMP70 function. We chose to mutate the two glutamic acids (Glu291) in ALDP is known to be the site of mutation causing
adrenoleukodystrophy. Concerning the Walker A motif, we changed the corresponding lysine (Lys479) to alanine, because it was shown that mutation of the lysine abolishes or markedly reduces ATP hydrolysis and inhibits the protein function of several ATPases (35, 36). In addition, we constructed PMP70 (1–575) minus 84 C-terminal amino acids including the Walker B motif, because recently, Gärtnér et al. (12) suggested that the C-terminal sequence of PMP70 is important for the assembly of PMP70.

The three mutant cDNAs were transfected into CHO cells, and stably expressing cells were selected. In the cells overexpressing PMP70 (E277D/E278D and K479A), the amount of PMP70 increased about 2–3-fold compared with that in Neo cells (Fig. 8, A and B). The activity in Neo cells and #19 cells was 2.07 nmol/h/mg of protein and 1.86 nmol/h/mg of protein, respectively.

When we investigated the β-oxidation of palmitic acid in the mutant cells, we found the rate of β-oxidation markedly decreased compared with that of Neo cells (Fig. 8D). In the case of E1 and E21 cells, β-oxidation of palmitic acid decreased almost 60–70%. These results support PMP70 involvement in β-oxidation of long chain fatty acids, and furthermore, EAA-like and Walker A motifs, as well as the C-terminal segment including the Walker B motif, are essential to the function of PMP70. Interestingly, the rate of lignoceric acid β-oxidation decreased slightly in all the mutant cells (Fig. 8E) and cells overexpressing wild type PMP70 (Fig. 6B). It might be that overexpression of mutant as well as wild type PMP70 inhibited the function of ALDP, which is thought to be involved in β-oxidation of very long chain fatty acids.

Overexpression of PMP70 Did Not Change the Number and Size of Peroxisomes in CHO Cells—Administration of hypolipidemic agents in rats induces peroxisome proliferation (6, 7). For example, in the above conditions where the number of peroxisomes was increased ~5-fold, the amount of PMP70 increased ~10-fold (37). This observation suggested that PMP70 induces peroxisomal membranes and increases the number of peroxisomes. To address this issue we compared the number and the size of peroxisomes between Neo, #19, and #31 cells by immunofluorescence and immunoelectron microscopy.

As shown in Fig. 9, a number of punctulate structures with high intensity of fluorescence representing acyl-CoA oxidase

![Fig. 6. β-Oxidation of [1-14C]palmitic acid and [1-14C]lignoceric acid in Neo, #19, and #31 cells. Cells in 3.5-cm dishes were incubated with [1-14C]palmitic acid and [1-14C]lignoceric acid for 30 min and 1 h, and 14C-labeled acid-soluble products were counted. The β-oxidation rates in Neo cells were taken as reference (100%). A, rate of formation of acid-soluble products from [1-14C]palmitic acid and β-oxidation in Neo cells was 1.90 pmol/h/100 μg of protein. B, rate of formation of acid-soluble products from [1-14C]lignoceric acid and β-oxidation in Neo cells was 4.80 pmol/h/100 μg of protein. Values are the mean ± S.D. of three experiments.](image)

![Fig. 7. Effect of ATP on β-oxidation of [1-14C]palmitoyl-CoA in light mitochondrial fraction prepared from Neo and #19 cells. Light mitochondrial fractions containing intact or disrupted peroxisomes were incubated with [1-14C]-palmitoyl-CoA in the absence or presence of ATP for 10 min at 37°C, and acid-soluble products were counted. Rates of [1-14C]palmitoyl-CoA β-oxidation are expressed as a percentage of the rates observed in disrupted peroxisomes in the presence of ATP. The activity in Neo cells and #19 cells was 2.07 nmol/h/mg of protein and 1.86 nmol/h/mg of protein, respectively.](image)
were detected in Neo, #19, and #31 cells. The number of fluorescent dots in #19 and #31 cells was similar or slightly decreased compared with that in Neo cells. In immunoelectron micrographs, the number of small gold particles corresponding to PMP70 located on peroxisomes in #19 and #31 cells markedly increased compared with that of Neo cells, but the size and the number of peroxisomes seemed to be similar between Neo cells and #19 and #31 cells. An increase in the size and number of peroxisomes as was observed in rat liver on the administration of hypolipidemic agents did not occur. Thus, it seems that overexpression of PMP70 is not associated with proliferation of peroxisomes.

DISCUSSION

In this study, we overexpressed PMP70 in CHO cells and examined roles of PMP70 in biogenesis and function of peroxisomes. First, subcellular localization of PMP70 overexpressed in CHO cells was investigated because information about peroxisomal localization is essential to the characterization of PMP70. The following findings suggest that the overexpressed PMP70 is enriched in peroxisomes. First, the distribution of overexpressed PMP70 in a sucrose gradient was similar to that of catalase (Fig. 3). Second, the numerous dots detected by immunofluorescent staining of PMP70 in #19 and #31 cells were superimposable on those stained with anti-catalase antibody (Fig. 4). Third, the enrichment of immunogold particles corresponding PMP70 on peroxisomes was shown in #19 and #31 cells by immunoelectron microscopy (Fig. 5). In light of these results, we further investigated roles of PMP70 in biogenesis and function of peroxisomes.

Function of PMP70—An early study demonstrated that ATP stimulated overall β-oxidation activity in isolated rat liver peroxisomes (29). In addition, it is well known that PMP70 is markedly induced by administration of hypolipidemic agents in parallel with the induction of peroxisomal fatty acid β-oxidation enzymes (6, 7, 37). These observations suggest the presence of an ATP-dependent acyl-CoA carrier in the peroxisomal membranes and PMP70 may be a carrier of fatty acids or their CoA ester. If PMP70 is a transporter of long chain fatty acids or their CoA ester, its overexpression might stimulate the β-oxidation in peroxisomes. On the other hand, as PMP70 is a half-sized ABC transporter, mutant PMP70 expressed in the cells might interact with wild type PMP70, resulting in the inhibition of peroxisomal β-oxidation. In fact, the rate of β-oxidation of palmitic acid in #19 and #31 cells increased 2–3-fold (Fig. 6). On the other hand, the rate of β-oxidation of palmitic acid was markedly decreased by overexpression of all the mutant PMP70 (Fig. 8). It seems unlikely that the increase of β-oxidation activity in #19 and #31 cells is because of the induction of peroxisomal β-oxidation enzymes in the cells overexpressing wild type and mutant PMP70 (Figs. 6 and 8), supporting the idea that increase in nonspecific permeability of fatty acids across peroxisomal membranes did not occur.

FIG. 8. Overexpression of mutant PMP70 in CHO cells and the β-oxidation of [1-14C]palmitic acid and [1-14C]lignoceric acid in the cells. A–C, immunoblot analysis of PMP70. A, missense mutation of EEA-like motif(E1-E22). Neo, CHO cells transfected with pSVneo. B, missense mutation of Walker A motif (W5-W19). C, deletion mutation of C-terminal sequence including the Walker B motif (S1-S15). Cell homogenates (150 μg of protein) were subjected to immunoblot analysis. In the case of C-terminal-truncated PMP70, antibody against purified PMP70 from rat liver peroxisomes (37) was used. The arrows indicate the position of PMP70 and C-terminal-truncated PMP70, respectively. D–E, the β-oxidation of palmitic acid (D) or lignoceric acid (E) in Neo and the mutant cells. Rates of fatty acid β-oxidation are expressed as a percentage of the rates observed in Neo cells. The rate of formation of acid-soluble products from [1-14C]palmitic acid and [1-14C]lignoceric acid in Neo cells was 2.80 pmol/h/100 μg of protein and 4.50 pmol/h/100 μg of protein, respectively. Values are the mean ± S.D. of three experiments.
Because the active site of long chain acyl-CoA synthetase is located outside of peroxisomes (38), the long chain fatty acids seem to be activated to change to acyl-CoA ester prior to β-oxidation in cells. To define whether PMP70 is involved in the transport of long chain acyl-CoA, we studied β-oxidation of palmitoyl-CoA using isolated peroxisomes prepared from Cont, #19, and #31 cells. Intact peroxisomes showed latency to palmitoyl-CoA oxidation. ATP stimulated the β-oxidation of palmitoyl-CoA when intact peroxisomes were used. The degree of stimulation was higher in the CHO cells overexpressing PMP70 than that in the Neo cells (Fig. 7). The effect of ATP was lost when the peroxisomal membranes were disrupted by the use of Triton X-100 (Fig. 7). Based on these results, we suggest that PMP70 functions as a transporter of long chain acyl-CoA across peroxisomal membranes to serve substrates for β-oxidation, and ATP facilitates the transport of the substrate by PMP70.

Another important point addressed by the present study is the functional domain of PMP70. All ABC transporters characterized so far contain the consensus sequence for an ATP binding site (Walker motifs A and B (34, 35)). Walker A is characterized by the simple motif GXXGXGKT/S, and the lysine as a source of the positive charge is invariant. In the case of PMP70, mutation of this lysine to alanine resulted in marked inhibition of the palmitic acid β-oxidation (Fig. 8D). The fact that the lysine is critical for the PMP70 function suggests that PMP70 possesses ATP binding and hydrolytic activity, which couples to the transport of palmitoyl-CoA. EAA-like motif is a conserved sequence located in the region between the fourth and fifth putative membrane-spanning domains of ABC transporters. Studies of prokaryotic ABC transporters suggest missense mutations, which alter conserved residues, cause loss of transporter function (32–34). Our mutant demonstrated that this motif is also critical for the PMP70 function.

Recently, two ABC transporters called Pat1p (Pxa2p) and Pat2p (Pxa1p) were identified on peroxisomal membranes of S. cerevisiae (39–43). Disruption of PAT1 and PAT2 resulted in impaired growth on oleic acid and reduced ability to oxidize long chain fatty acids such as oleic acid. Furthermore, a possibility that pat2p is involved in the transport of long chain acyl-CoA was suggested using digitonin-permeabilized yeast cells (44). Therefore, PMP70 seems to be a counterpart of yeast peroxisomal ABC transporter of Pat1p or Pat2p. However, the functional unit of PMP70 and yeast peroxisomal ABC transporters Pat1p and Pat2p seems to be different. Disruption of PAT1 or PAT2 in yeast cells resulted in impaired growth in oleic acid medium, suggesting that Pat1p and Pat2p function as heterodimers (43–46). On the other hand, overexpression of PMP70 alone in CHO cells induced β-oxidation of palmitic acid, suggesting that PMP70 can function as homodimer.

Unexpectedly, overexpression of wild type as well as mutant PMP70 suppressed the β-oxidation of lignoceric acid. We don’t know the reason for this at present, but one possibility is that because of the overexpression of PMP70, a part of PMP70 may associate with ALDP, although the interaction might be weak and such a heterodimer might reduce the transport activity of lignoceric acids. This possibility is now under investigation.

**Effect of Overexpression of PMP70 on Structure of Peroxisomes**—Proliferation of peroxisomes parallels induction of PMP70 (6, 7, 37). A subset of patients with Zellweger syndrome, a peroxisome biogenesis disorder (complementation group 1), has defects at the locus encoding PMP70 (12). This observation led us to examine whether PMP70 is involved in proliferation and biogenesis of peroxisomes. To answer this, we analyzed the number and the morphology of peroxisomes by immunofluorescence and immunoelectron microscopy using CHO cells overexpressing PMP70. The following results suggest that PMP70 is not involved in such a process. First, the number of punctulate structures corresponding to peroxisomes did not increase on overexpression of PMP70 (Fig. 9). Second, the number of gold particles against PMP70 located on peroxisomes was markedly increased by overexpression of PMP70, but the size and the number of peroxisomes did not increase (Fig. 5). Furthermore, CHO cells expressing C-terminal truncated PMP70 as well as the cells possessing missense mutations of EAA-like and Walker A motifs had morphologically normal peroxisomes (data not shown). Therefore, PMP70 seems not to be necessary for the biogenesis and proliferation of peroxisomes but to be required for the normal function of peroxisomes. Recently, it was revealed that peroxisomes in mammalian cells preferentially oxidized several kinds of lipids, prostanooids such as prostaglandin E2, F2α, and leukotriens (45, 46) and dicarboxylic acids (47), trihydroxycholestanic acid (48), and phytic acid (49, 50). These substances must be transported into peroxisomes to be oxidized. It would be interesting to know whether PMP70 is involved in the transport of these...
substances.

In this study we found that PMP70 is involved in metabolic transport of long chain fatty acyl-CoA across the peroxisomal membrane and is not required for proliferation of peroxisomes or peroxisome biogenesis using CHO cells stably overexpressing wild and mutant type PMP70. The mechanism by which PMP70 transports long chain acyl-CoA will be the subject of further research.

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