Biogenesis of Peroxisomes: Immunocytochemical Investigation of Peroxisomal Membrane Proteins in Proliferating Rat Liver Peroxisomes and in Catalase-Negative Membrane Loops

Eveline Baumgart,* Alfred Völkl,* Takashi Hashimoto,† and H. Dariush Fahimi*

*Department of Anatomy and Cell Biology, University of Heidelberg, 6900 Heidelberg, Federal Republic of Germany; and †Department of Biochemistry, Shinshu University, Asahi, Matsumoto, Nagano 390, Japan

Abstract. Treatment of rats with a new hypocholesterolemic drug BM 15766 induces proliferation of peroxisomes in pericentral regions of the liver lobule with distinct alterations of the peroxisomal membrane (Baumgart, E., K. Stegmeier, F. H. Schmidt, and H. D. Fahimi. 1987. Lab. Invest. 56:554–564). We have used ultrastructural cytochemistry in conjunction with immunoblotting and immunoelectron microscopy to investigate the effects of this drug on peroxisomal membranes. Highly purified peroxisomal fractions were obtained by Metrizamide gradient centrifugation from control and treated rats. Immunoblots prepared from such peroxisomal fractions incubated with antibodies to 22-, 26-, and 70-kD peroxisomal membrane proteins revealed that the treatment with BM 15766 induced only the 70-kD protein. In sections of normal liver embedded in Lowicryl K4M, all three membrane proteins of peroxisomes could be localized by the postembedding technique. The strongest labeling was obtained with the 22-kD antibody followed by the 70-kD and 26-kD antibodies. In treated animals, double-membraned loops with negative catalase reaction in their lumen, resembling smooth endoplasmic reticulum segments as well as myelin-like figures, were noted in the proximity of some peroxisomes. Serial sectioning revealed that the loops seen at some distance from peroxisomes in the cytoplasm were always continuous with the peroxisomal membranes. The double-membraned loops were consistently negative for glucose-6-phosphatase, a marker for endoplasmic reticulum, but were distinctly labeled with antibodies to peroxisomal membrane proteins. Our observations indicate that these membranous structures are part of the peroxisomal membrane system. They could provide a membrane reservoir for the proliferation of peroxisomes and the expansion of this intracellular compartment.

The biogenesis of peroxisomes (POs) has been the subject of intensive research in recent years (for review see references 14, 21, 41). All PO proteins, including PO membrane proteins (PMP), are synthesized on free ribosomes and probably transported posttranslationally into the organelle (24, 25, 40, 47, 53, 55, 64).

Recently, it has been shown that the targeting signal for the import of proteins into POs may be located not only at the carboxy-terminal end of the PO proteins (28, 29) but also in the amino-terminal part or even in the midportion (63), and it has been suggested that the proteins may be bound to a receptor before the ATP-dependent translocation over the PO membrane (38). Nevertheless, it remains unclear whether there are specialized regions in the PO membrane which preferentially import PO proteins from the cytoplasm.

The early observations of the close morphological association of POs with the smooth ER (SER) (51) led to the suggestion that POs may arise by budding from the ER (19, 52). Although this concept has been abandoned in the mean time, the exact nature and function of membranous structures seen occasionally attached to or associated with the PO membranes remains to be elucidated. For example, in proliferating POs, induced by partial hepatectomy or by treatment with hypolipidemic drugs, tail-like extensions or by treatment with hypolipidemic drugs, tail-like extensions or by treatment with hypolipidemic drugs, tail-like extensions as well as myelin-like figures were noted in the proximity of some peroxisomes. Serial sectioning revealed that the loops seen at some distance from peroxisomes in the cytoplasm were always continuous with the peroxisomal membranes. The double-membraned loops were consistently negative for glucose-6-phosphatase, a marker for endoplasmic reticulum, but were distinctly labeled with antibodies to peroxisomal membrane proteins. Our observations indicate that these membranous structures are part of the peroxisomal membrane system. They could provide a membrane reservoir for the proliferation of peroxisomes and the expansion of this intracellular compartment.

1. Abbreviations used in this paper: DAB, 3,3'-diaminobenzidine; PAG, protein A-gold; PMP, peroxisomal membrane protein; POs, peroxisomes; SER, smooth endoplasmic reticulum.
proliferation of POs induced by hypolipidemic drugs is a useful model for the investigation of peroxisomal biogenesis. In this study we have used a new hypocholesteremic drug BM 15766, which has a well-defined target in the pathway of cholesterol biosynthesis (3, 4). We showed recently that this drug induces marked proliferation of POs in perivenous hepatocytes (5) causing distinct alterations of the PO membrane. From the several PMPs (23, 32, 33), we have used monospecific antibodies against those with M, of 22, 26, and 70 kD in conjunction with immunoblotting and immunoelectron microscopy, to characterize the membranous structures associated with proliferating POs. In addition, ultrastructural cytochemistry for marker enzymes of POs (catalase) and ER (glucose-6-phosphatase) have been applied to assess the relationship of the two organelles.

The results indicate that those membranous structures which are associated with proliferating POs are heavily labeled with the antibody against 70-kD PMP, indicating that they belong to the PO membrane system.

Materials and Methods

Animals

16 male Sprague-Dawley rats weighing 140-200 g and kept on a normal laboratory diet and water ad libitum were used. Half of them received 75 mg/kg body weight of BM 15766 suspended (75 mg/10 ml) in methylcellulose (tylose) for 14 d via a gastric tube. Corresponding controls were administered the same amount of tylose suspension. All animals were fasted for 16 h before death.

Drug

BM 15766, Cl\text{CH=CH-CH\text{\textsubscript{2}}-N-S-CH\text{\textsubscript{2}}-CH\text{\textsubscript{2}}-COOH}, was obtained through the courtesy of Boehringer-Mannheim GmbH (Mannheim, FRG). The drug inhibits 7-dehydrocholesterol-\Delta7-reductase, the enzyme catalyzing the last step of the cholesterol synthesis (4).

Isolation of Peroxisomes

Hepatic POs from controls and BM 15766-treated animals were isolated as described before (72). Briefly, crude PO fractions were obtained by differential centrifugation of the total homogenates prepared in a homogenization buffer (250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% ethanol, pH 7.4) containing in addition 0.2 mM PMSE, 1 mM \textalpha-aminocaproic acid, and 0.2 mM DTT. The crude PO preparations were subjected to density-gradient centrifugation (61). The complex (diluted 1:50) exhibited an optical density of 0.45 at 528 nm and was stored in 25% glycerol at -20°C.

Preparation of Antibodies

Monospecific antibodies against 22-, 26-, and 70-kD PMP were raised in rabbits as described previously (33). The specificity of the antibodies was assessed by immunoblotting. The antibodies (40 mg/ml) were diluted 1:1,000 or 1:2,000 before immunocytochemical incubation.

Preparation of Protein A-Gold (PAG) Complex

Gold soles with a particle size of 12 nm were conjugated to protein A (Pharmacia Fine Chemicals, Upsala, Sweden) at pH 5.9 using the method of Slot and Geuze (62) and the PAG complex was isolated by sucrose density-gradient centrifugation (6). The complex (diluted 1:50) exhibited an optical density of 0.45 at 528 nm and was stored in 25% glycerol at -20°C.

Immunoblotting

SDS-PAGE was performed using a microslab electrophoresis apparatus (KS 8010 MSE; Marysol Industry Co., Ltd., Tokyo). Samples containing 4.2 \mu g/lane PO proteins were applied to gels (9 × 5 × 0.1 cm, 10-12.5% resolving, 3% stacking gel), stacked at 15 mA and resolved at 25 mA (total time ∼1 h). Electrotacthaz onto nitrocellulose sheets was accomplished at 30 V for 60 min.

For protein staining, the blots were washed for 1 h with 0.15 M PBS containing 0.05% Tween 20 and stained overnight with "Auro Dye" solution (49) according to the manufacturer's specifications (Janssen Pharmaceutica, Beerse, Belgium).

For immunoblots, the nonspecific binding sites were blocked with 0.15 M 10% newborn calf serum/PBS, pH 7.2, containing 0.05% Tween 20 (NCS/PBS/T) for 1 h with gentle rocking, changing the medium three times. Nitrocellulose sheets were incubated overnight with the appropriate antibodies diluted 200 times with NCS/PBS/T, followed by a fourfold wash. Antigen--antibody complexes were visualized by PAG (15) and the signals thus obtained were amplified by an anti-protein A step (8). The immunoblots were quantitated using a computer-controlled densitometer system (Texture Analysis System; E. Leitz, Inc., Wetzlar, FRG) as described recently (6). The optical extinction of each band was multiplied with its surface area and the results for each membrane protein from PO preparations of control and treated rats were compared.

Morphology

The livers of all animals were fixed for 5 min by perfusion with 0.25% purified glutaraldehyde (Serva Feinbiochemica GmbH, Heidelberg, FRG) in 0.1 M Pipes buffer, pH 7.4, via the portal vein. Tissue sections, 50-100 μm thick, were cut with a "microslicer" (Dosaka Electron Microscopy Company, Kyoto, Japan), postfixed with the ferrocyanide-reduced OsO4 (39), and embedded in Epon 812.

Cytochemistry

For the cytochemical localization of catalase the tissue sections were incubated for 1 h with 5 mM 3,3'-diaminobenzidine (DAB) and 0.15% H2O2 in 0.01 M Teorell-Stenhagen buffer, pH 10.5, at 37°C (2). Glucose-6-phosphatase was localized using 2 mM disodium-glucose-6-phosphate as substrate dissolved in 0.1 M Tris-maleate buffer, pH 6.5, containing 3 mM CeCl3 (57). The preincubation was carried out without substrate at 37°C for 30 min, followed by incubation with substrate for 1 h.

Acid phosphatase was localized with 3 mM disodium-CMP in 0.04 M sodium-acetate buffer, pH 5, and 5% sucrose containing 3 mM CeCl3 (57). Preincubation and incubation conditions were similar to those for glucose-6-phosphatase.

Immunoelectron Microscopy

For the immunocytochemical localization of the 22-, 26-, and 70-kD PMP, the tissue sections were embedded either in Lowicryl K4M (1, 7, 58) or in Epon 812 (50). Ultrathin sections were soaked for 1 h on drops of 1% BSA in 20 mM Tris-HCl, pH 7.4, and incubated overnight with the appropriate antibodies in 0.1% BSA in TBS. After washing several times on drops of TBS, the grids were incubated for 90 min with PAG complex diluted 1:50 or 1:60 in TBS. In some experiments Triton X-100 (0.05%) was added to the incubation and washing media. Subsequently, the grids were washed with distilled water and air dried, followed by contrasting with uranyl acetate and lead citrate.

Results

General Effects of Treatment with BM 15766

The serum sterols and triglycerides were reduced and the livers of treated rats were enlarged as described previously (5). This implies that the drug at the dose used (75 mg/kg body weight) exerted its hypolipidemic effect.

Characterization of the Isolated PO Fractions from Treated and Control Rats

The specific activity of the reference enzyme, catalase, in PO
fractions from control and treated rats was 8.77 and 10.8 U/mg protein, respectively. This was ∼38-fold higher than in the original homogenates, corresponding to our earlier observations (72). Furthermore, SDS-PAGE preparations of PO fractions, blotted onto nitrocellulose sheets and stained with Auro Dye (Fig. 1), revealed a pattern similar to that reported for highly purified POs (23). Although some differences between the blots of treated animals and controls were seen, the alterations of 22-, 26-, and 70-kD PMP in treated rats could not be readily detected in such preparations.

**Localization of PMPs in Isolated Fractions by Immunoblotting**

The immunoblots prepared from isolated PO fractions and incubated with antibodies against 22-, 26-, and 70-kD PMP (Fig. 2) revealed for each protein a single band at the expected Mr range, thus confirming the monospecificity of the antibodies used. In preparations incubated with 70-kD antibody, a very faint band with an Mr of 68 kD was also observed, which has been suggested to be the product of proteolytic modification of the 70-kD protein (33). Quantification of the immunoblots from treated animals revealed an increase of the 70-kD PMP by 330%, while the 68-kD PMP remained unchanged. The 22-, and 26-kD PMP were only slightly reduced (10%) in comparison to controls.

**Morphological and Cytochemical Observations**

The overall alterations of hepatic ultrastructure in rats treated with BM 15766 has been described in detail previously (5). In the present study, the main emphasis will be on the effects of treatment on POs with special attention on alterations of their membranes. In hepatocytes surrounding the terminal hepatic venules, marked proliferation of POs with formation of clusters was noted. These consisted of three to five POs which were separated only by a single cisterna of SER (Fig. 3 a). Membranous whorls resembling myelin-like figures were noted quite often in close proximity or in direct continuity with the limiting membranes of many POs (Figs. 3 b and 5 a). In addition, double-membraned loops, consisting of two parallel-running membranes separated by a distance of 40–60 nm, were seen in the close vicinity of many POs (Fig. 3 d). In contrast to the cisternae of SER, which are usually fenestrated in rat hepatocytes, the double-membraned loops did not exhibit any evidence of fenestration. Some double-membraned loops surrounding portions of the cytoplasm enclosed segments of SER, glycogen, and occasionally smaller POs (Figs. 3 d, 4, and 5 c). Some POs showed invaginations of their limiting membrane which appeared continuous with double-membraned loops extending into the cytoplasm. Some loops were also observed free in the cytoplasm at certain distance to POs (Fig. 5 b). Analysis of serial sections, however, revealed that such free loops were always continuous with the limiting membrane of a PO (Fig. 4). Some of the loops with cytoplasmic contents resembled autophagic vacuoles (Fig. 4, S1), although never any evidence of autophagic degradation of their contents was observed. The nonlysosomal nature of such structures was confirmed by acid phosphatase cytochemistry (CMPase), which was negative, while lysosomes and the trans-Golgi cisternae were positive (Fig. 5 d). In sections stained with DAB for catalase, similar double-membranous channels (without DAB reaction product in their lumen) interconnected occasionally two positively stained POs (Fig. 3 c). The absence of catalase reaction in the lumen of the double-membraned loops is shown also in Fig. 5 c. In spite of the direct continuity of the loop with the PO membrane, the DAB reaction product is confined to the matrix of the PO and does not extend into the double-membraned loop (Fig. 5 c). In sections incubated for glucose-6-phosphatase, the...
Figure 3. Electron micrographs of perivenous hepatocytes from treated animals. (a) Overview showing marked proliferation of POs (PO), which form large clusters consisting of three to five irregularly shaped particles. Note the close association of SER with the external surface of some POs. (b) A myelin-like figure (arrowheads) with apparent continuity with the PO membrane. (c) Section from material stained for catalase with DAB. A dumbbell-shaped PO (PO) is shown with two positively stained portions being interconnected by a catalase-negative double-membraned segment (arrowheads). (d) A distinct double-membraned loop (arrowheads) surrounding a small PO is shown next to a larger one (PO). The loop resembles somewhat the segments of SER, but in contrast to the latter, it is not fenestrated and shows a narrower lumen (40–60 nm in contrast to 70–90 nm for SER).
Figure 4. Serial ultrathin sections showing the relationship of a double-membraned loop (black arrowheads) to an adjacent PO (P) in the cytoplasm of a hepatocyte from an animal treated with BM 15766. In section $S_1$ to $S_9$, only the double-membraned loop is seen in association with glycogen particles (GLY) and some SER segments. Starting with section $S_4$, a PO becomes visible (white arrowheads), which in subsequent sections ($S_5$ and $S_6$) shows membrane continuity with the loop. Note also the close relationship of the loop with fenestrated segments of SER (particularly in $S_7$ to $S_9$). M, mitochondrion.
Figure 5. Electron micrographs from the livers of animals treated with BM 15766 showing alterations of POs. (a) Section incubated for glucose-6-phosphatase (G-6-Pase) showing a weak reaction in SER segments. A myelin-like figure, apparently surrounded by a double-membraned loop, is shown in close association with the membrane of a PO (PO). (b) Section incubated for the localization of glucose-6-phosphatase (G-6-Pase). Note the double-membraned loop (arrowheads) adjacent to a large PO (PO). Whereas, the loop is negative for glucose-6-phosphatase, all the SER cisternae are prominently stained. (c) Section incubated for catalase with DAB. Note the loop-like extension of the PO membrane (arrowheads). The catalase reaction product is confined to the matrix of the PO (PO) and does not extend into the loop. (d) Section incubated for acid phosphatase (Acid Pase) (CMPase). Note the positive staining in the trans-cisternae of the Golgi complex and in lysosomes (LYS). A PO (PO) with an altered limiting membrane (arrowheads) is free of reaction product. M, mitochondrion.
Figure 6. Sections of rat liver embedded in Lowicryl K4M and incubated with antibodies against PMP followed by PAG. (a) Localization of 70-kD PMP in a PO (PO) from a control animal. Note the distinct labeling of the PO membrane with gold particles, in contrast to a mitochondrion (MITO) and the SER membranes. (b) Localization of 70-kD PMP in the liver of a treated animal. The gold labeling is confined to the membranes of two POs (PO), while the mitochondria (MITO) and ER membranes are negative. The labeling density appears increased in comparison to the control rat (a). (c) Localization of 26-kD PMP in a control rat. The gold particles are noted on the limiting membrane of a PO (PO). This PMP showed the lowest labeling density in comparison to 22 and 70 kD. (d) Localization of 22-kD PMP in a control animal. Note the distinct labeling of the PO membrane. This PMP showed the highest labeling density in control rats in comparison to 26 and 70 kD.
double-membraned loops were negative while the cisternae of ER contained the electron-dense reaction product (Fig. 5 b). Based on the above observations, the double-membraned loops could be easily distinguished from segments of SER by (a) negative reaction for glucose-6-phosphatase; (b) the narrower lumen (40-60 nm); and (c) lack of fenestrations.

**Discussion**

**Alterations of the PO Membrane**

The treatment of rats with the hypocholesterolemic drug BM 15766 induced marked proliferation of POs in centrilobular hepatocytes. Such POs displayed distinct membrane alterations including tail- or loop-like extensions into the cytoplasm, association with myelin-like figures, and invaginations into the matrix. Moreover, double-membraned loops composed of two distinct membranes, separated by a distance of 40-60 nm, which were observed frequently in the cytoplasm of hepatocytes, were found by serial sectioning to be always associated with the PO membranes. Such loops exhibited a negative reaction for glucose-6-phosphatase, were not fenestrated and thus could be easily distinguished from segments of SER. Moreover, by immunoelectron microscopy, positive labeling for the 70-kD PMP was obtained not only in such loops, but also in myelin-like figures adjacent to some POs. These observations suggest strongly that loops and myelin-like figures belong to the PO membrane system and thus may provide a membrane reservoir for the proliferation of POs and for the expansion of this intracellular compartment. The existence of a distinct compartment consisting of POs (PO reticulum) was proposed by Lazarow et al. (42).
and such an interconnected network has been demonstrated since by reconstruction of serial ultrathin sections in mouse and rat hepatocytes (27, 74).

It should be emphasized, however, that the loops and hook-shaped structures noted above are not unique features of the experimental model used in this study. Indeed, they have been observed in numerous previous reports and can be clearly identified in many published electron micrographs, although they were often mistaken as segments of SER. Thus, they were described in developing rat (70) and chicken hepatocytes (20), as well as in regenerating rat liver after partial heptectomy (51). Furthermore, they are particularly frequent in experimental conditions associated with PO proliferation (22, 35–37, 43, 56, 66–68, 75). Hruban et al. (36) designated such loops as “gastruloid cisternae,” which they considered as bridges between ER and POs. Finally, it should be mentioned that similar membranous structures in association with POs have also been described in cells other than mammalian hepatocytes (9, 10, 26, 30, 46, 69). Because of the negative DAB reaction for catalase in the lumen of double-membraned loops (Fig. 5 c) their identification as part of the PO membrane system has been extremely difficult. In this respect the immunocytochemical localization of PMP provides a unique approach for the proper identification of these membranous structures. Recently Santos et al. (59, 60), using mono- and polyspecific antibodies against the PMPs, detected by immunocytochemistry “ghost-like” structures in the cytoplasm of fibroblasts from Zellweger syndrome patients. These authors suggested that the ghosts represented PO membranes which could not import the matrical proteins because of defects in the translocation machinery. Moreover, long sinuous tubules with positive DAB content were described in the intestinal epithelial cells of a patient with neonatal adrenoleukodystrophy (11). The possible relationship of the ghost-like structures in fibroblasts and the sinuous tubules in intestinal epithelial cells with the double-membraned loops noted in the present study deserves further investigation.

Another important feature of proliferating POs in this study was their close association with myelin-like figures (Figs. 3 b and 5 a) which exhibited positive immunolabeling for the 70-kD PMP. Although myelin figures were considered for many years to be fixation artifacts, the systematic studies of Blanchette-Mackie and Scow (12, 13) have established that free ribosomes and probably transported posttranslationally to the organelle membrane (24, 40, 64).

The integral membrane proteins which are present exclusively in POs exhibit major bands of M. 70-, 68-, 26-, and 22-kD (23, 33, 40). The 68- and 70-kD proteins are closely related, the former being the product of proteolysis of the latter. Moreover, it has been suggested that PMPs of M. 42.5 and 28 kD are products of proteolysis of the 70-kD PMP (32). Treatment with PO-proliferating agents does not induce a uniform increase of all PMPs. Thus, the 70-kD integral membrane protein increases significantly after the treatment with clofibrate, thyroxine, Wy-14.643, and di(2-ethylhexyl)phthalate while the 68-kD (18) and the 22-kD PMP remain almost unchanged (18, 32, 33). Hashimoto et al. (33) and Crane et al. (18) additionally reported a slight induction of the 26-kD PMP.

In the present study a significant induction of 70-kD PMP with little alteration of the 68-, 26-, and 22-kD PMP was detected by immunoblotting. The augmentation of the 70-kD PMP was also noted in immunocytochemical preparations obtained by the postembedding technique (Fig. 6, a and b). Our observation of the localization of the 70-, 22-, and 26-kD PMP by the postembedding technique are essentially similar to those of Hashimoto et al. (33).

Little is known about the exact function of various PMPs. A pore-forming activity in the PO membrane has been described corresponding most closely to the presence of a protein with an M. between 22 and 28 kD, which may be responsible for the high permeability of POs (71). The fact that mainly the 70-kD PMP is reduced in the livers of patients with Zellweger syndrome (65), while it is significantly increased after the treatment with thyroxine and other PO-proliferating agents suggests that this protein could participate in the translocation of matrix proteins from the cytosol into the POs. Since the double-membranated loops were heavily labeled for the 70-kD PMP (Fig. 7 b), it would be tempting to speculate that those membranous structures are specialized regions of the PO membrane system which would preferentially import matrix proteins into the PO compartment.
References

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