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# A novel 57 kDa peroxisomal membrane polypeptide detected by monoclonal antibody (PXM1a/207B)

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**BALB/c mice were immunized with peroxisomal membranes prepared from rat liver. Spleen cells were fused with myeloma cells (P3/U1) and the hybridomas were selected using peroxisomal membranes. A monoclonal antibody (PXM1a/207B) which recognized peroxisomal membranes was selected. Using the antibody, a novel 57 kDa polypeptide was identified in the peroxisomal membrane fraction. Immunoblot analysis of the subcellular fractions demonstrated that the 57 kDa polypeptide was present exclusively in peroxisomal membranes. The 57 kDa polypeptide was partially digested by trypsin and chymotrypsin under conditions where peroxisomal particles remained intact, indicating that the polypeptide is exposed to the cytosolic face of the peroxisomal membrane. The amount of 57 kDa polypeptide increased in parallel with proliferation of peroxisomes by administration of clofibrate.**

## Introduction

Peroxisomes are bounded by a unit membrane with a diameter of 0.1–0.5 μm. The membrane incorporates a number of polypeptides not found in other organelles [1,2]. Recently polypeptides of 69/70 and 22 kDa were identified as major components, and polypeptides of 53, 35/36 and 26/27 kDa were identified as minor components of peroxisomal membranes in rat liver [3–5]. Van Veldhoven et al. [6] suggest that the 22 kDa polypeptide is involved with the nonspecific permeability of peroxisomal membranes to small molecules such as sucrose, lactate and amino acids. Kamijo et al. [7] suggest that the 69 kDa polypeptide has ATPase activity. Recent studies have also revealed that several other enzymes are located to peroxisomal membranes. For example, Hajra et al. [8] found dihydroxyacetonephosphate acyltransferase and alkyldihydroxyacetonephosphate synthetase in peroxisomal membranes, whilst Wanders et al. [9] and Singh and Poulos [10] found very long chain acyl-CoA synthetase. However, polypeptides which correspond to these enzymes have not yet been identified.

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Furthermore, peroxisomal membrane polypeptides seem to play an important role in the biogenesis of the organelle. Import of acyl-CoA oxidase into rat liver peroxisomes can be demonstrated in vitro in a reconstitution system [11], and evidence has also been presented that this import is mediated via a specific receptor in the peroxisomal membrane [12]. Abnormal peroxisomal membrane ghosts, which did not contain matrix proteins have also been found in fibroblasts of patients with Zellweger syndrome [13,14], suggesting that the ghosts lack a component necessary for the assembly of the organelle. Since the ghosts have a number of the integral membrane polypeptides, such as 69, 53, 26 and 22 kDa [13–16], other polypeptides on the membrane might be responsible for the assembly of the organelle. However, these polypeptides which may be involved in the assembly of peroxisomes, have also not yet been identified.

In these circumstances, identification of novel peroxisomal membrane polypeptides may be important in understanding the assembly and functions of the organelle. In this study, we have prepared monoclonal antibody (PXM1a/207B) which recognizes peroxisomal membranes. With this monoclonal antibody, we have found a novel 57kDa polypeptide which is orientated to cytosolic face of the peroxisomal membrane. Induction of the 57 kDa polypeptide by clofibrate has also been investigated.

## Material and Methods

*Materials*

$^{125}$I-labeled protein A (2.60–3.70 TBq/g) was purchased from ICN Biomedicals Inc. (Irvine, CA, U.S.A.). Nycodenz was obtained from Nyegaard Co. (Oslo, Norway). Alkaline phosphatase conjugated goat anti-mouse IgG + IgM was from Tago Inc. (Burlingame, CA, U.S.A.). Rabbit anti-mouse IgG + IgA + IgM was from Zymed (San Francisco, CA, U.S.A.). Rabbit anti-mouse serum for determination of subclass of monoclonal antibodies was from Miles Lab. (Elkhart, ID, U.S.A.). Proteinase inhibitors such as leupeptin, antipain, chymostatin, pepstatin, phenylmethylsulfonyl fluoride and trypsin inhibitor were obtained from Sigma (St. Louis, MO, U.S.A.). Protein standards for molecular weight determination were from Bio-Rad (Richmond, CA, U.S.A.). All other reagents were of analytical grade.

*Purification of peroxisomes and preparation of the membranes*

Peroxisomes were purified from rat liver by differential centrifugation in sucrose followed by isopycnic centrifugation in Nycodenz [11] with some modifications. About 2.0 ml of a light mitochondrial fraction was layered onto a 20 ml linear Nycodenz gradient (density span from 1.15–1.27 g/ml) in a Hitachi RP 50–2 rotor (Hitachi, Tokyo, Japan). The gradient rested on a 2.0 ml cushion of 1.32 g/ml Nycodenz and the sample was overlaid with 1.0 ml of 0.25 M sucrose containing 1 mM EDTA, 0.1% (v/v) ethanol and 5 mM Hepes-KOH (pH 7.4). Centrifugation was carried out at 50000 rpm (193000 × $g_{av}$) for 90 min at 4°C with slow acceleration and deceleration. The value of total $g \times$ min was chosen to obtain the same effective centrifugation as used by Leighton et al. [17], which is sufficient for the peroxisomes to reach their equilibrium density. The homogenization medium (0.25 M sucrose containing 1 mM EDTA and 0.1% (v/v) ethanol, pH 7.4) contained leupeptin, chymostatin, antipain and pepstatin (10 μg/ml of each). These protease inhibitors were also added to the light mitochondrial fraction to a final concentration of 10 μg/ml of each. The purity of peroxisomes was essentially the same as that previous reported [11] which was about 95% calculated from the activity of catalase. The peroxisomes were well separated from mitochondria, microsomes and lysosomes. Peroxisomal membranes were prepared by the sodium carbonate procedure [18].

*Preparation of mitochondria, microsomes and their membranes*

Mitochondria were prepared by sucrose density gradient centrifugation as described in [17] except that a Hitachi RP 50-2 rotor was used instead of a Beaufay zonal rotor. Microsomes were prepared by differential centrifugation by the method of De Duve et al. [19]. Membrane fractions from both mitochondria and microsomes were prepared by the sodium carbonate procedure [18].

*Immunization and preparation of monoclonal antibodies against peroxisomal membranes*

Female BALB/c mice were injected with the peroxisomal membrane fraction (120 μg protein) four times every alternate week. The membranes were suspended in 100 μl of 20 mM Tris-HCl (pH 7.4), containing 150 mM NaCl and SDS (0, 0.1 or 1.0% (w/v)). The first sample was emulsified with complete Freund's adjuvant in a total volume 0.2 ml and immunized subcutaneously. The second and third immunizations were carried out intraperitoneally, the samples being emulsified with incomplete Freund's adjuvant. A final intraperitoneal boost contained no SDS. Spleen cells removed 4 days after the final injection were fused with myeloma P3/U1 cells and cultured in HAT (hypoxanthine, aminopterin and thymidine) medium. Hybridomas were selected by ELISA using the peroxisomal membrane fraction. For the ELISA tests, the peroxisomal membranes were suspended in 20 mM Tris-HCl (pH 8.6) by sonication. The membranes (4.0 μg/well) were coated on the immunoplates (Falcon 3912 Micro Test Flexible Assay Plate, Becton Dickinson, Oxnard, CA, U.S.A), incubated with hybridoma medium (PBS/0.05% Tween 20) and detected with alkaline phosphatase conjugated goat anti-mouse IgG + IgM using *p*-nitrophenyl phosphate as substrate [20]. Cells recovered from positive wells were cloned twice by limiting dilution.

*Immunoblotting*

For immunoblot analysis, samples were run through 7–15% gradient polyacrylamide gel in the presence of 0.1% (w/v) SDS and transferred to nitrocellulose sheets (0.1 μm pore size, Schleicher and Schuell, Dassel, F.R.G.) by the method of Small et al. [21]. The sheets were incubated with monoclonal antibodies and the antigenic material detected with alkaline phosphatase conjugated anti-mouse IgG + IgM using 5-bromo-4-chloro-3-indolyl phosphate as substrate [22]. For the quantitative immunoblot of 57 kDa polypeptide, rabbit anti-mouse immunoglobulins was used as the second antibody and the immuno complex was detected by $^{125}$I-labeled protein A. Fluorography and densitometric scanning were carried out by the method described previously [11]. Proteins on nitrocellulose sheets were stained with AuroDye (Janssen Life Sciences, Olen, Belgium).

*Other methods*

The preparation of antiserum against peroxisomal membranes has been described elsewhere [13]. In order

to initiate peroxisome proliferation, rats were fed chow containing 0.5% (w/w) clofibrate for 2 weeks [23]. Protein was measured with the protein assay kit (Bio-Rad, Richmond, CA, U.S.A.) with bovine γ-globulin as standard [24]. Catalase was assayed according to the method of Baudhuin et al [25]. *N*-Acetyl-β-D-glucosaminidase was assayed according to Findlay et al [26]. Cytochrome-*c* oxidase and esterase were assayed as previously described [27]. The density of each subcellular fractions was calculated from the refractive index.

## Results

### *Preparation of monoclonal antibodies against peroxisomal membranes*

BALB/c mice were immunized with the peroxisomal membrane fraction either with or without SDS, as described in Material and Methods. The optimal titer of antiserum against peroxisomal membrane polypeptides was obtained where the membranes were immunized with 0.1% (w/v) SDS. The spleen cells were subsequently fused with myeloma cells. The hybridomas were screened by ELISA to determine which produced antibodies recognizing peroxisomal membranes, but not mitochondrial and microsomal membranes. Fifteen clones were obtained after 2 times limiting dilution. As shown in the immunoblot in Fig. 1, a single clone (PXM1a/207B) reacted with a novel 57 kDa polypeptide. The subclass of the monoclonal antibodies was shown to be IgM by Ouchterlony analysis.

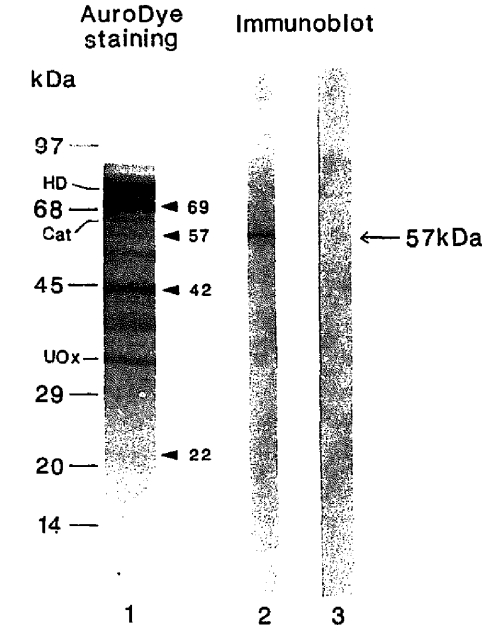


Fig. 1. Reactivity of monoclonal antibody PXM1a/207B with peroxisomal membrane polypeptides as determined by immunoblotting. A peroxisomal membrane fraction prepared by sodium carbonate procedure was submitted to SDS-PAGE and transferred to nitrocellulose membranes. Lane 1; peroxisomal membrane polypeptides stained with AuroDye. Major peroxisomal integral membrane polypeptide were 69, 42 and 22 kDa. Some major matrix proteins such as HD(hydratase-dehydrogenase), Cat(catalase) and UOx(urate oxidase) are also present in the membrane fraction. Lanes 2 and 3; Immunoblot analysis of peroxisomal membrane polypeptides. Lane 2; monoclonal antibody PXM1a/207B. Lane 3; hybridoma culture medium as control. The arrow indicates the position of 57 kDa polypeptide. The molecular weight standards are indicated to the left of lane 1. The peroxisomal membrane fraction of each lane was prepared from 50 μg of peroxisomes.

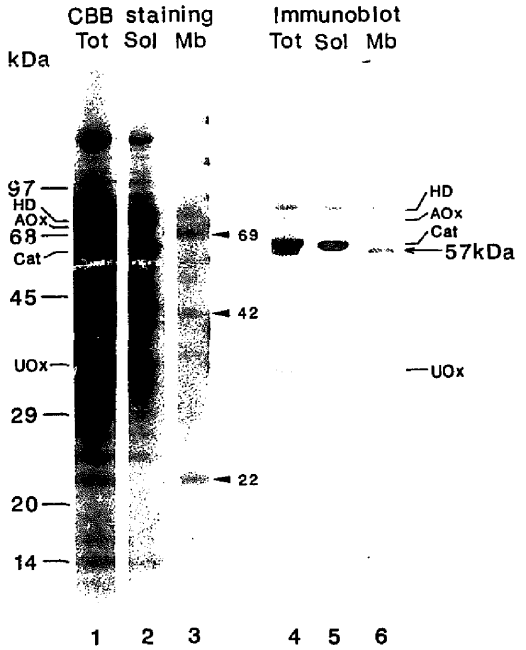


Fig. 2. Immunoblot analysis with monoclonal antibody PXM1a/207B of subfraction of peroxisomes. Organelle proteins stained with Coomassie brilliant blue (lanes 1–3). Total peroxisome proteins (Tot) were 50 μg . Soluble proteins (Sol) and membrane proteins (Mb) were derived from 50 μg of peroxisomal proteins. Immunoblot analysis with PXM1a/207B (lanes 4–6). Tot were 10 μg protein, Sol and Mb were derived from 10 ng of peroxisomal proteins. AOx, acyl-CoA oxidase; Cat, catalase; HD, hydratase-dehydrogenase; UOx, urate oxidase.

### *Localization of the 57 kDa polypeptide in peroxisomal membranes*

We examined the localization of the 57 kDa polypeptide in purified peroxisomes. The sodium carbonate procedure was used for rapid and quantitative separation of the membrane fraction. Integral membrane polypeptides, such as 69, 42 and 22 kDa, were detected in the membrane fraction (Fig. 2, lane 3). Most of the

matrix proteins (hydratase-dehydrogenase, acyl-CoA oxidase, catalase and urate oxidase) were solubilized (Fig. 2, lane 2). Under these conditions, the 57 kDa polypeptide was detected in the membrane fraction, but was not seen in the supernatant fraction by immunoblotting using monoclonal antibody PXM1a/207B (Fig. 2, lanes 5 and 6). The monoclonal antibody reacted slightly with some major matrix proteins, such as hydratase-dehydrogenase, acyl-CoA oxidase and urate oxidase. The antibody also reacted with catalase (Fig. 2, lanes 4 and 5). The localization of these matrix proteins in peroxisomes, however, was quite different from that of the 57 kDa polypeptide (Fig. 2, lanes 5 vs. 6).

The localization of the 57 kDa polypeptide in peroxisomes was further analyzed by Nycodenz gradient fractionation (Fig. 3). The 57 kDa polypeptide was mainly recovered in fractions 8–11, which correspond to the peak of catalase activity existing in peroxisomal particles. The density of the peak fraction of both the 57 kDa polypeptide and catalase was 1.24 g/cm$^3$, which coincides with that of the peroxisomes in rat liver [17]. Furthermore, as shown in Fig. 4, the 57 kDa polypeptide was detected only in purified peroxisomal membrane fraction, and not in mitochondrial or microsomal membrane fractions. These results clearly show that the 57 kDa polypeptide is an integral polypeptide of the peroxisomal membrane.

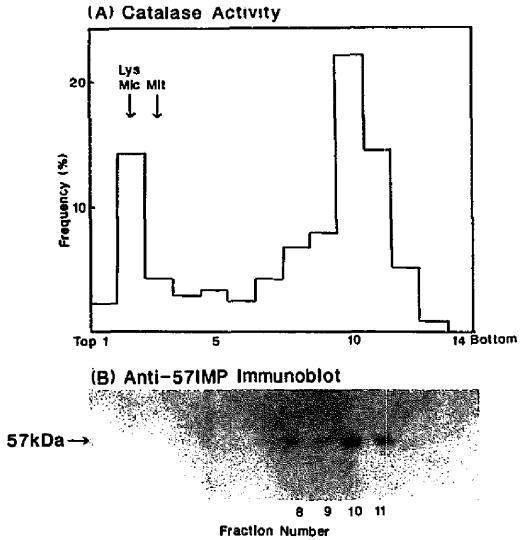


Fig. 3. Subcellular fractionation by Nycodenz gradient. (A) Catalase, cytochrome-*c* oxidase, esterase and *N*-acetyl-β-D-glucosaminidase were measured as marker enzymes of peroxisomes, mitochondria (Mit), microsomes (Mic) and lysosomes (Lys) respectively. Distribution of catalase is shown by solid line. Peak fractions of other marker enzymes are indicated by arrows. (B) Immunoblot analysis with PXM1a/207B. Membranes were prepared from 3.5 μl of each fraction from top (No. 1) to bottom (No. 14) of the gradient. The protein contents in the 3.5 μl aliquots of fractions 2–5 and 8–11 were 56, 138, 61, 22 and 7.7, 8.5, 16, 13 μg, respectively. Arrows shows the position of 57 kDa polypeptide.

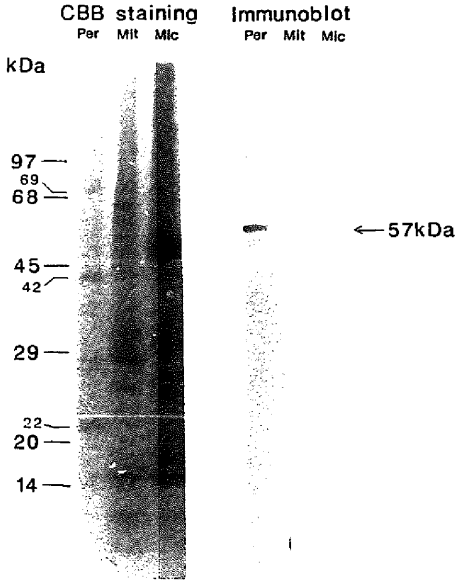


Fig. 4. Localization of the 57 kDa polypeptide in peroxisomal membranes by immunoblotting with PXM1a/207B. Gel stained with Coomassie brilliant blue (lanes 1–3). Immunoblot analysis with PXM1a/207B (lanes 4–6). Lanes 1 and 4, peroxisomal membranes (Per); lanes 2 and 5, mitochondrial membranes (Mit); Lanes 3 and 6, microsomal membranes (Mic). Organelle membranes were prepared from 50 μg of organelle protein for Coomassie brilliant blue staining and from 10 μg for immunoblotting.

*Orientation of the 57 kDa polypeptide on peroxisomal membranes*

Purified peroxisomes were treated with trypsin and chymotrypsin to determine the orientation of the 57 kDa polypeptide on the peroxisomal membrane. The 57 kDa polypeptide band detected by immunoblotting disappeared after mild proteolysis (Fig. 5B). This is similar behaviour to that shown by the 22 kDa integral membrane polypeptide which was visible in Fig. 5A among the total proteins and was also partially degraded (Fig. 5A, lanes 1 and 2). Under these conditions, the latency of catalase remained unchanged (about 80%) by the treatment of trypsin and chymotrypsin. Furthermore, many matrix proteins inside the peroxisomes such as hydratase-dehydrogenase, acyl-CoA oxidase, catalase and urate oxidase were not degraded, as judged by Coomassie brilliant blue staining (Fig. 5A). These results demonstrate, therefore, that the 57 kDa protein is partially exposed on the cytosolic face of the peroxisomal membrane.

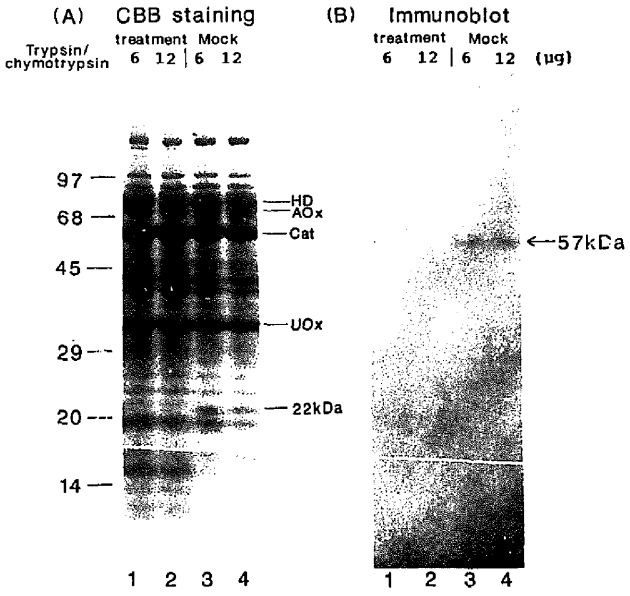


Fig. 5. Orientation of the 57 kDa polypeptide on peroxisomal membranes. Peroxisomes (600 μg) were treated with trypsin and chymotrypsin (6 μg or 12 μg each) in 100 μl of 0.25 M sucrose, 1 mM EDTA, 0.1%(v/v) ethanol containing 5 mM Hepes-KOH, pH 7.4 at 0°C for 20 min. After incubation, proteinase inhibitors (30 μg of soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM of leupeptin, chymostatin, antipain and pepstatin) were added to the reaction mixture. For the mock treatment (Mock), incubations were carried out in the presence of above proteinase inhibitors. (A) The peroxisomes were suspended in SDS buffer and were subjected to SDS-PAGE. Organelle proteins (60 μg each) were stained with Coomassie brilliant blue. (B) Immunoblot analysis with PXM1a/207B. Membranes were prepared by the sodium carbonate method, starting with 10 μg of organelle protein and subjected to SDS-PAGE and immunoblotting.

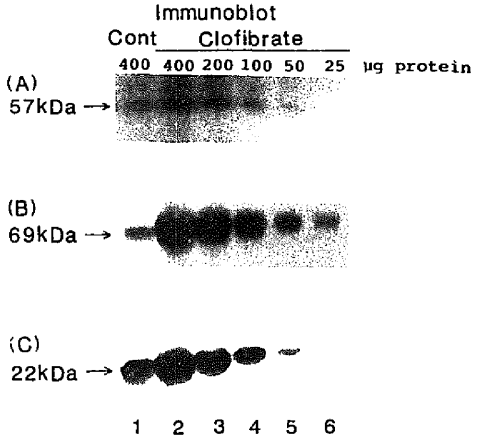


Fig. 6. Effect of clofibrate on induction of the 57 kDa polypeptide. Total cell membranes were prepared from liver homogenate of normal or clofibrate treated rats. The membrane pellets were subjected to SDS-PAGE and immunoblotted with (A) PXM1a/207B or (B and C) with antiserum against peroxisomal membranes which reacted with 69 and 22 kDa polypeptides. Applied sample of each lane was derived from 25 to 400 μg of liver homogenate.

*Induction of the 57 kDa polypeptide associated with peroxisomal proliferation*

Hepatic peroxisomes proliferate markedly when clofibrate is administered in rats [23]. Inducibility of 57 kDa polypeptide was investigated in parallel with major integral membrane polypeptides such as 69 and 22 kDa. After treatment with clofibrate, the 57 kDa polypeptide increased about 4-fold and 69 kDa increased about 12-fold, whereas the 22 kDa only increased about 2-fold in liver (Fig. 6).

## Discussion

Knowledge of peroxisomal membrane polypeptides is essential to the understanding of the functions and assembly of peroxisomes. During assembly of the peroxisomes, it is well accepted that constituent proteins are posttranslationally imported into the organelle [28]. Recently, Gould et al. [29,30] and Miyazawa et al. [31] found that the COOH-terminal three amino acids, such as Ser-Lys-Leu are a peroxisomal targeting signal for

several peroxisomal matrix proteins. These results, together with our finding that proteinase pretreatment of peroxisomes diminished import of acyl-CoA oxidase into peroxisomes [12], suggest that there is a polypeptide on the peroxisomal membrane which mediates the import of peroxisomal matrix proteins. This is supported by the fact that matrix proteins are not imported into peroxisomal membrane ghosts from fibroblasts of patients with Zellweger syndrome [13,14]. To elucidate functions and assembly of peroxisomes, further information about peroxisomal membrane polypeptides is necessary.

In this study, we prepared monoclonal antibodies against peroxisomal membranes and found a novel 57 kDa polypeptide. Initially the subcellular localization and orientation of the polypeptide on the membrane were studied. The following evidence suggested that the 57 kDa polypeptide is an integral polypeptide of the peroxisomal membranes. (1) The 57 kDa polypeptide was detected only in the peroxisomal membrane fraction, in parallel with other integral membrane polypeptides such as 69, 42 and 22 kDa (Fig. 2). (2) The 57 kDa polypeptide was shown to be located to the peroxisomal fractions by Nycodenz gradient centrifugation (Fig. 3). (3) The 57 kDa polypeptide was detected in purified peroxisomal membrane fraction but not in mitochondrial nor in microsomal membrane fractions (Fig. 4). (4) The 57 kDa polypeptide together with the 69, 42 and 22 kDa was recovered in the detergent phase, but not in the aqueous phase by temperature-induced phase separation in Triton X-114 (data not shown).

Degradation of the polypeptide on exposure of peroxisomes to exogenous trypsin and chymotrypsin (Fig. 5B) suggest that the 57 kDa polypeptide is exposed at least partially to the cytosolic face of the membrane. As a reference polypeptide, 22 kDa was also partially degraded (Fig. 5A), a finding consistant with previous observations [3].

It is well known that peroxisomes proliferate on clofibrate treatment [23,32]. The 57 kDa polypeptide increased (about 4-fold) after clofibrate treatment, as did the 69 kDa [3,4]. However, the 22 kDa increased only 2-fold as a result of this treatment (Fig. 6). This suggest that the 57 kDa as well as the 69 kDa polypeptide, are closely coupled to peroxisomal proliferation.

It is possible that one of the known peroxisomal membrane enzymes is the 57 kDa polypeptide. In rat liver these include, for example, cytochrome-$b_5$ [33,34], cytochrome-$b_5$ reductase [34], glutathione *S*-transferase [35], ATPase [7,36], dihydroxyacetonephosphate acyltransferase [8], alkyldihydroxyacetonephosphate synthetase [8] and very long chain [9,10] and long chain acyl-CoA synthetases [37]. Among these enzymes, dihydroxyacetonephosphate acyltransferase, alkyldihydroxyacetonephosphate synthetase and ATPase catalyzed by 69 kDa polypeptide are known to be located exclusively in the peroxisomes. However, both alkyldihydroxyacetonephosphate synthetase and dihydroxyacetonephosphate acyltransferase seem to be different from the 57 kDa polypeptide, since they did not increase in rat liver following by clofibrate [38] and according to proteinase pretreatment [39] seem to be located to internal side of the peroxisomal membrane. Another candidate for the 57 kDa polypeptide is a component which connects with the import machinery for peroxisomal proteins. Several membrane polypeptides are known to play a role in the posttranslational import of matrix protein into mitochondria [40], chloroplasts [41] and nuclei [42]. A number of components may be involved in a similar mechanism of posttranslational import of the peroxisomal matrix into the peroxisomes. Further studies will be necessary to determine whether the 57 kDa polypeptide is involved with the import of peroxisomal matrix proteins.

The 53 kDa polypeptide identified by antiserum against peroxisomal membranes [13,14] and the 63 kDa polypeptide identified by phosphorylation of peroxisomal membranes in nafenopin treated rats [43] are other candidates for the 57 kDa polypeptide. However, both polypeptides are different from the 57 kDa polypeptide, since 53 and 63 kDa polypeptide were separated from 57 kDa polypeptide by SDS-PAGE (data not shown). In addition, 55–58 kDa polypeptide recognized by antibodies against sterol carrier protein-2 may be different from the 57 kDa polypeptide, since the 55–58 kDa polypeptide localized exclusively to the soluble fraction of the peroxisomes [44]. We conclude from this evidences that the 57 kDa polypeptide is a novel polypeptide on peroxisomal membranes.

The monoclonal antibody (PXM1a/207B) reacted with some major peroxisomal matrix proteins (Fig. 2, lanes 4 and 5). It is not yet known whether these proteins and the 57 kDa polypeptide share similar antigenic determinant or these abundant matrix proteins react with the antibody nonspecifically as described by Birk and Koepsell [45]. Further studies are required to elucidate the cross-reaction of the antibody with the major matrix proteins.

In this study, we identified a 57 kDa peroxisomal integral membrane polypeptide using a newly developed monoclonal antibody (PXM1a/207B). This 57 kDa polypeptide was shown to expose to the cytosolic face of the peroxisomal membrane and was shown to be involved in the proliferation of peroxisomes. The function of the 57 kDa polypeptide in peroxisomal membranes will be the subject of future research.

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