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Ultrastructural localization of a peroxisomal protein in rat liver using the specific antibody against the non-specific lipid transfer protein (sterol carrier protein 2)

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An antibody against the non-specific lipid transfer protein from rat liver was purified by immunoabsorbent affinity chromatography. This antibody in conjunction with protein A-colloidal gold was used to localize the transfer protein in rat liver by electron microscopy. Labeling by this immunocytochemical technique was found to be mainly restricted to the peroxisomes; low labeling was observed in the cytoplasm. Subsequent analysis of isolated peroxisomes by immunoblotting indicated that the non-specific lipid transfer protein (mol. wt. 14800) was absent from this organelle and that a protein of molecular weight 58000 was responsible for the immunological response. Immunoblotting of the membrane-free cytosol showed the presence of both proteins. It remains to be established to what extent the non-specific lipid transfer protein in the cytosol and the high-molecular weight protein in the peroxisomes are related.

Introduction

The rat liver soluble fraction contains proteins that stimulate the microsomal conversion of sterol precursors in cholesterol biosynthesis [1–4]. Recent studies have firmly established that major proteins, active in this stimulation are the fatty acid binding protein [5,6] and the non-specific lipid transfer protein [7]. The latter protein has been purified from rat [8,9] and bovine liver [10] and appears identical to sterol carrier protein 2 [7,11]. Inherent to its ability to transfer cholesterol to membranes, the non-specific lipid transfer protein stimulates the microsomal acyl-CoA : cholesterol acyltransferase activity [7,12,13] and pregnenolone synthesis in adrenal mitochondria [14]. However, its physiological function remains to be established as this protein also transfers phospholipids and glycosphingolipids between membranes [8,15]. Recently, a non-specific lipid transfer protein has also been purified from maize seedlings [16].

In a recent study we have demonstrated by use of an enzyme immunoassay that the non-specific lipid transfer protein is present in a great variety of rat tissues with the highest levels in liver and intestinal mucosa [17]. Here we report on an attempt to localize this protein in rat liver by an electron microscopic immunocytochemical technique, using the specific IgG against non-specific lipid transfer protein and protein A-colloidal gold.

Materials and Methods

Materials

Polyvinylchloride microtiter plates were ob-

tained form Flow Laboratories (McLean, VA, U.S.A.); *o*-phenylenediamine from Sigma (St. Louis, MO, U.S.A.); goat anti-rabbit IgG conjugated to horse-radish peroxidase from Nordic immunological Laboratories (Tilburg, The Netherlands); bovine serum albumin from Calbiochem (San Diego, CA, U.S.A.); amido black from Serva (Heidelberg, G.F.R.); protein molecular weight marker kit from Pharmacia (Uppsala, Sweden); sodium dodecyl sulfate from Pierce (Rockford, IL, U.S.A.); acrylamide and bis-acrylamide from UCB (Brussels, Belgium) and nitrocellulose sheets and HRP colour development reagent from Biorad (Richmond, CA, U.S.A.).

Methods

Electron microscopic immunocytochemical detection. Livers from 6-month-old male Wistar rats were fixed in situ by means of the perfusion-fixation method described by Wisse [18]. The animals were anaesthesized with chloroform. The fixative consisted of 0.5% glutaraldehyde, 2% paraformaldehyde in 0.01 M phosphate-buffered saline (buffer 1) (pH 7.2). Perfusion was started with buffer 1 at 25°C for 20 s, immediately followed by the fixative. Per 100 g animal weight 5 ml of fixative per min were applied. Total perfusion time was 7 min. The liver was excised and the perilobular parts were removed and cut into blocks of about 1 mm^3 with a razor blade in fresh fixative. Fixation was continued for 30 min, whereafter the tissue blocks were rinsed in buffer 1/glycine (50 mM). The blocks were soaked in 2.3 M sucrose in buffer 1 for at least 45 min at 4°C.

Tissue blocks were placed on Reichert specimen stubs and quenched in liquid nitrogen. Frozen specimens were transferred to a Reichert FC-4/OMU-4 cryo-ultramicrotome and sectioned at −90°C. The temperature setting was the same for the specimen, the knife and the plate for ambient temperature regulation. The sectioning speed was 1.0 mm/s. Section thickness control was set at 0.1 μM, resulting in over 85% section yield. Sectioning was performed with a dry glass knife, according to Tokuyasu [19] with a scoring angle of 42°C and a clearance angle of 3°. Specimens were trimmed to a rectangle of approx. 0.3 mm × 0.2 mm. Sections were collected from the knife and transferred to bioform-covered Nickelgrids according to Tokuyasu [19].

Grids with sections were stored overnight on 2% gelatin in buffer 1. Immediately before labeling the gelatin was liquified at 37°C. Grids were rinsed on buffer 1/50 mM glycine, buffer 1 containing 0.1% gelatin (Merck 4070), 0.5% bovine serum albumin (Sigma A 9647) (buffer 2) for 5 min each. All the immuno-incubation as well as the washing steps were done using buffer 2. Incubation with the anti-non-specific lipid transfer protein IgG (diluted 1 : 50) lasted for 1 h, followed by six washing steps for a total time of 30 min. Marking was done using protein A-gold (5 nm) complexes diluted in buffer 2. The marking incubation lasted 1 h and was followed by seven washing steps on buffer 2 for a total time of 30 min, two washing steps on buffer 1/50 mM glycine and four steps on distilled water also for a total time of 30 min.

In control experiments, the specific IgG was omitted, or replaced by adsorption-chromatography cleared serum. Sections were contrasted and embedded according to Tokuyasu [20] using 1.1% tylose (Fluka), 0.05% uranylacetate, and observed and photographed using a Philips EM 420 with an objective of 25 μm at 80 kV

Preparation of antibodies. Non-specific lipid transfer protein was purified from rat liver and used for the immunization of rabbits, as described previously [9,17]. Total IgG was isolated from the serum and specific IgG was obtained by affinity chromatography using non-specific lipid transfer protein coupled to Sepharose 4B [17].

Preparation of subcellular fractions Liver homogenates, subcellular membrane fractions and 105 000 × *g* supernatant fractions were prepared from Wistar rats as described before [21]. For the isolation of peroxisomes a 30% (w/v) liver homogenate in 0.25 M sucrose/0.1% (w/v) ethanol was prepared by two strokes of a Potter-Elvehjem homogeniser. A 'large-granule fraction' was isolated essentially as described by Baudhuin [22]. The homogenate (20 ml) was centrifuged (10 min at 1000 × *g*) and the resulting pellet resuspended in 20 ml 0.25 M sucrose/0.1% (w/v) ethanol with a Pasteur pipette. After another centrifugation (10 min at 1000 × *g*) the supernatants were combined and centrifuged for 12.5 min at 20 000 × *g*. The pellet was washed once by resuspension in 10 ml 0.25 M sucrose/0.1% (w/v) ethanol. The ensuing pellet, representing the 'large-granule fraction' was

resuspended in 5 ml of 0.25 M sucrose/0.1% (w/v) ethanol. An aliquot (2.2 ml) of this fraction was loaded on top of a discontinuous sucrose gradient, consisting of layers of 55%, 52%, 48.2%, 41%, and 33% (w/w) sucrose (6 ml each). Centrifugation was carried out in a Beckman SW 27 rotor for 3 h at 26000 rpm. Peroxisomes collected at the interface between 52 and 48.2% (w/w) sucrose. After addition of four volumes of water the peroxisomes were sedimented by centrifugation (30 min at $25000 \times g$). In this preparation catalase activity, as measured according to Holmes and Masters [23], was enriched about 5-fold relative to the 'large-granule fraction'. This preparation was further characterized by electron microscopy indicating a high content of intact peroxisomes.

Protein was determined according to Lowry et al. [24].

Enzyme immunoassay. Levels of non-specific lipid transfer protein were determined by an enzyme immunoassay as described previously [17].

Blotting and immunological detection of proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the procedure of Laemmli [25]. The slab gels contained 12.5% acrylamide. The samples used were lyophilized from distilled water and dissolved in the sample buffer containing 50 mM dithiothreitol. After electrophoresis the proteins were electrophoretically transferred from the gel to nitrocellulose sheets exactly as described by Burnette [26]. The blots were stained for protein with amidoblack (0.1%, w/v) in methanol/water/acetic acid (45 : 45 : 10, v/v). After 5 min the blot was destained with methanol/acetic acid/water (7 : 7 : 86, v/v) [27]. The immunological detection of the proteins was performed by a modification of the procedure of Towbin et al. [28]. The blots were washed with 20 mM Tris-HCl (pH 7.5)/500 mM NaCl (buffer 3) containing 0.05% (w/v) Tween-20. The blots were then immersed in 100 ml of buffer 3 containing 5% (w/v) bovine serum albumin and incubated at 37°C for 1 h on a rocking platform. Next, the blot was transferred to a solution of buffer 3 (100 ml) containing 3% (w/v) bovine serum albumin, 0.05% (w/v) Tween-20 and 12 μg/ml specific anti-transfer protein IgG and incubated overnight at room temperature. After a thorough washing with buffer 3 containing 0.05% (w/v) Tween-20, the blot was transferred to buffer 3 (100 ml) containing 3% (w/v) bovine serum albumin, 0.05% (w/v) Tween-20 and goat anti-rabbit IgG conjugated to horseradisch peroxidase (1 : 2000 dilution) and incubated for 2 h at room temperature. After washing with buffer 3/Tween-20 the peroxidase activity was visualized by transferring the blot to 120 ml of a solution containing 0.5 mg/ml HRP Colour Development Reagent (Bio-Rad), 16.7% (v/v) methanol and 0.015% (v/v) H_2O_2. After 20 min the reaction was stopped by washing the blot in distilled water.

Results and Discussion

Characterization of antibody

The antibody used in this study was isolated from a total IgG fraction by immunoabsorbent affinity chromatography, using the non-specific lipid transfer protein as ligand. This antibody amounts to 1–2% of the IgG applied to the column and inhibited the phosphatidylethanolamine transfer activity characteristic for the non-specific lipid transfer protein [17]. Recently we have developed an enzyme immunoassay for the quantitation of non-specific lipid transfer protein using the specific IgG [17]. In this assay, the antibody displayed the same affinity for our protein samples as for a preparation of pure sterol carrier protein 2 confirming the identity of both proteins. (The sample of sterol carrier protein 2 was kindly provided by Dr. T.J. Scallen).

Immunocytochemical localization in liver

Ultrathin cryosections of rat liver were incubated with the specific antibody whereupon the antigen-antibody complexes were labeled with a protein A-colloidal gold complex. The subcellular localization of the gold labeling in parenchymal cells was visualized by electron microscopy. As is shown in Fig. 1, gold particles were clearly concentrated over an electron-dense organelle which has the morphological characteristics of a peroxisome. Typical for a peroxisome, the crystalloid assembly of catalase can be seen [29]. In addition, a fair number of particles could be detected in the cytoplasmic matrix; virtually no particles were found to coincide with other subcellular organelles (e.g., mitochondria, endoplasmic reticulum and

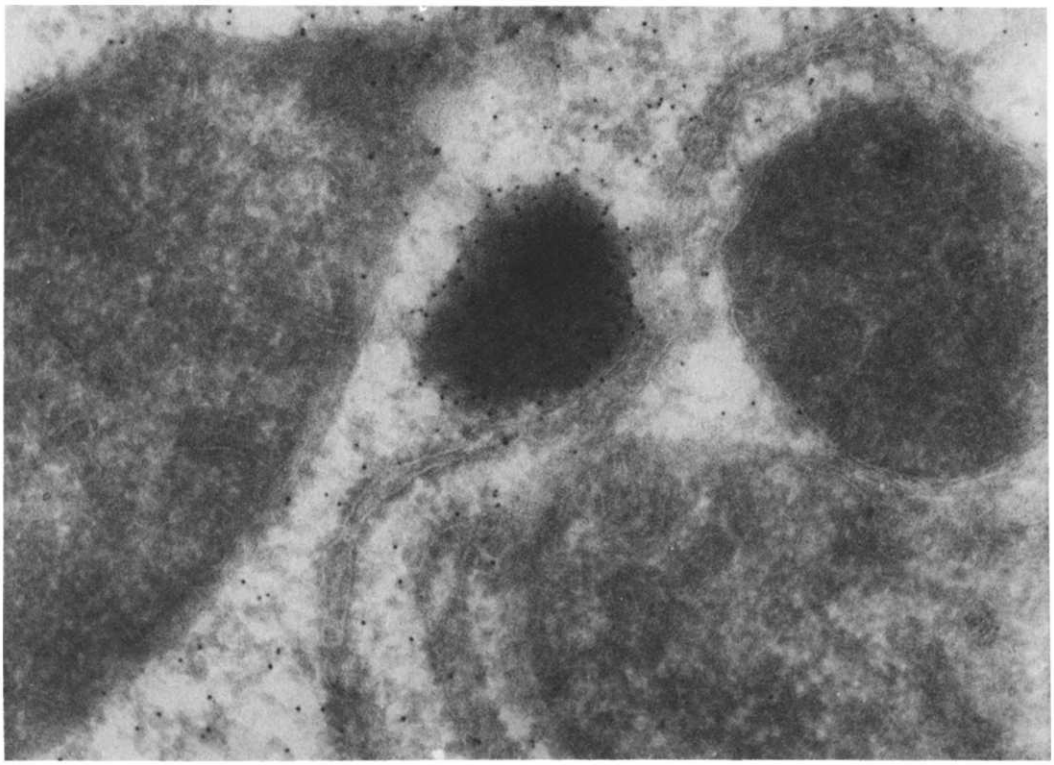

Fig. 1. Distribution of gold particles in a cryosection of rat liver Cryosections were first incubated with specific anti non-specific lipid transfer protein IgG and subsequently with a protein A-colloidal gold complex A peroxisome is visible at the centre of the micrograph. Magnification: approx. 50000×

lysosomes). Gold labeling was negligible when a control IgG was used or when the antibody was omitted.

Subcellular distribution

In order to find further proof for the presence of non-specific lipid transfer protein in the peroxisomes, rat liver homogenates were fractionated by differential centrifugation and the level of transfer protein determined by the enzyme immunoassay. A 'De Duve-plot' as shown in Fig. 2A indicates that relative to the membrane-free cytosol levels of transfer protein in the various membrane fractions were very low. A similar plot for the peroxisomal marker catalase shows that particularly the membrane fraction L was enriched in peroxisomes (Fig. 2b). As has been noted before, catalase is also prominently present in the supernatant fraction. In view of their fragility part of this catalase may originate from the peroxisomes. On the other hand, catalase is recognized as a constitutive protein of the cytoplasm [30]. Purification of the peroxisomes has also been achieved by applying a $20000 \times g$ membrane pellet on a discontinuous sucrose gradient (see Materials and Methods). The peroxisomal fraction which collected at the interface between 52 and 48.2% (w/w) sucrose was assayed for the non-specific lipid transfer protein by using the enzyme immunoassay. In agreement with the De Duve-plot (Fig. 2) the peroxisomal fraction lacked the transfer protein (data not shown).

Immunoblotting

The immunoreactivity of the peroxisomes as indicated by the immuno electron microscopic approach (Fig. 1) was further investigated by the immunoblotting procedure. To this end, purified peroxisomes (14 μg of protein), the $105000 \times g$ supernant protein (60 μg of protein) and the non-

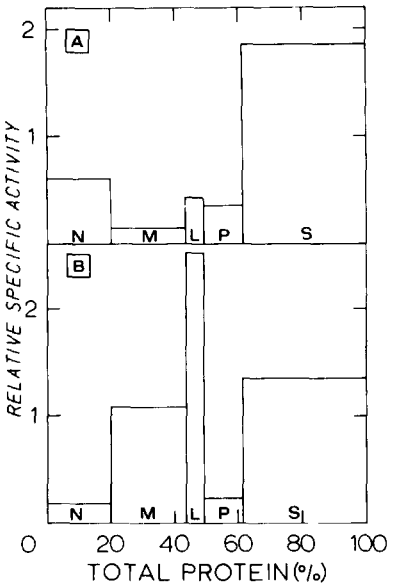

Fig 2 Subcellular distribution of non-specific lipid transfer protein (A) and catalase (B) in rat liver. On the ordinate each fraction is represented by its relative specific activity (i e., percentage of recovered transfer protein/percentage of total protein) On the abscissa the protein content of each fraction is expressed as percentage of total recovered protein. Fractions include nuclei and cell debris (N), heavy mitochondria (M), light mitochondria (L), microsomes (P) and supernatant (S).

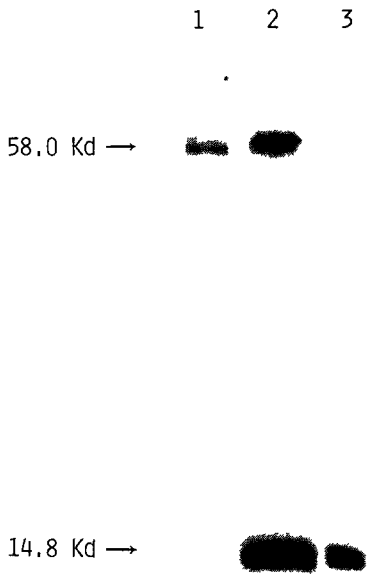

Fig. 3. Immunological detection of proteins in the 105000 × *g* supernatant and peroxisome fraction reactive with the specific anti non-specific lipid transfer protein IgG. Peroxisomal proteins (14 μg of protein, Slot 1), 105000 × *g* supernatant proteins (60 μg of protein, Slot 2) and pure non-specific lipid transfer protein (0 06 μg of protein, Slot 3) were resolved by electrophoresis on sodium dodecyl sulfate polyacrylamide gels (12 5%) After electrophoretic transfer from the gels to nitrocellulose sheets, the immunoreactive proteins were detected with the specific anti non-specific lipid transfer protein IgG followed by goat anti-rabbit IgG conjugated to horse radish peroxidase. The molecular weight of the immuno reactive proteins was estimated by comparison with the following marker proteins: phosphorylase *a* (94000); bovine serum albumin (67000); ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α-lactalbumin (14400)

specific lipid transfer protein (0.058 μg of protein) were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoretic transfer to nitrocellulose the blots were incubated with specific antibody. The antigen-antibody complexes were detected by second antibody conjugated to peroxidase (Fig. 3). The non-specific lipid transfer protein (Slot 3) was detected at the expected molecular weight (14800) and was also prominently present in the 105000 × *g* supernatant fraction (Slot 2), but completely lacking from the peroxisomes (Slot 1). However, the peroxisomal material does render a band at a molecular weight of 58000. The latter protein is also clearly seen in the 105000 × *g* supernatant. Antibodies from two different rabbits have an identical response. Similar experiments with a control IgG did not give any reaction.

In a previous study [17] we have fractionated the 105000 × *g* supernatant fraction by molecular sieve chromatography, and, in addition to the non-specific lipid transfer protein, have detected immunoreactive material in the corresponding high-molecular weight range. This protein fraction lacked phosphatidylethanolamine transfer activity and was no longer detectable by the antibody upon heating. The enzyme immunoassay developed for the quantitation of the non-specific lipid transfer protein routinely employed this heating step to remove this immunoreactive material from the sample to be assayed. This explains as to why the peroxisomes did not give a significant response

in the enzyme immunoassay (see Fig. 2). However, the immunoblotting (Fig. 3) strongly suggests that the immunoreactive protein (mol. wt. 58000) in the peroxisomes is similar to that observed in the 105000 × *g* supernatant.

Conclusion

Immunocytochemical labeling in conjunction with immunoblotting has demonstrated that the peroxisomes contain a protein of molecular weight 58000 which is immunologically related to the non-specific lipid transfer protein. This protein may also occur in the membrane-free cytoplasm but is not detectable in other subcellular organelles. As of now, it is believed that the non-specific lipid transfer protein plays a role in the intracellular cholesterol metabolism [7,11,13]. It remains to be established whether a functional relationship exists between the non-specific lipid transfer protein and the high molecular weight protein. In this respect it is to be noted that the peroxisomes are actively involved in lipid metabolism including synthesis of bile acids [31] and plasmalogens [32], and the β-oxidation of fatty acids [33,34].

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