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Peroxisomal protein import

In vivo evidence for a novel translocation competent compartment

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In homogenates of isolated hepatocytes separated by Nycodenz density gradient centrifugation, two peroxisomal populations are identified that differ in buoyant density. Organelles present in a high density fraction (1.22–1.23 g/cm³) represent mature peroxisomes. Vesicles of intermediate density (1.16–1.17 g/cm³) represent mature peroxisomes. Vesicles of intermediate density (1.16–1.17 g/cm³) are present in much lower concentration and seem to play a particular role in the import and distribution of newly synthesized peroxisomal proteins. In a typical pulse-chase experiment with a 7.5 min pulse, peroxisomal acyl-CoA oxidase is first imported into the peroxisomal fraction of intermediate density. After a chase of up to 30 min, the enzyme is found in mature peroxisomes.

Hepatocyte; Peroxisome; Peroxisomal protein import; Acyl-CoA oxidase

1. INTRODUCTION

During the last few years considerable progress has been made in the understanding of the peroxisome biogenesis (for reviews see [1,2]). However, our knowledge on the components participating in peroxisomal protein translocation and on the mechanism of this process is still poor. New information may be gained from studies on yeast [3,4] or CHO-mutant cells [5] as well as on cells from patients with peroxisomal disorders [6,7] that reveal gene products obligatory for correct peroxisome assembly. A more direct approach is to study perixosomal protein import in vitro. Thus far only few data were obtained using in vitro systems [8–10] and some of these experiments have been difficult to reproduce. In this paper, therefore, we would like to report on in vivo results which demonstrate that acyl-CoA oxidase, used as a peroxisomal marker, in isolated hepatocytes is first imported into a novel translocation-competent peroxisomal fraction of intermediate equilibrium density before it appears in the bulk of 'normal' peroxisomes which are defined by their high equilibrium density.

2. MATERIALS AND METHODS

2.1. *Animals*

Male rats of the Wistar strain weighing 130–150 g were used for the experiments. The animals were treated with the hypolipidemic drug clofibrate (Serva, Heidelberg) for 10–14 days by adding the drug at a concentration of 0.25% to the pulverized rat chow. The animals had free access to water and this chow.

Abbreviations: SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PMP, peroxisomal membrane polypeptide.

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2.2. *Pulse-chase experiments*

Hepatocytes were isolated by the collagenase procedure as described previously [11]. The isolated cells of which 90–95% were viable finally were resuspended in bicarbonate and methionine-deficient RPMI- 1640 medium (Sigma, Munich) to which was added bicarbonate (21 mM) and HEPES (25 mM, pH 7.4). Cells (50 mg w.wt.) were incubated in the presence of 600 μCi [^{35}S]methionine (Amersham, Braunschweig, spec. act. >1.000 Ci/mmol) in a total volume of 0.75 ml at 37°C for 7.5 min. The synthesis of radioactively labeled protein was inhibited by the addition of unlabeled methionine (2 mM) and the cells were incubated for an additional 15 and 30 min. After this dilution of the specific radioactivity of [^{35}S]methionine, no further increase in the amount of trichloroacetic acid precipitable radioactivity was detected. Pelleted cells were resuspended in 0.25 M sucrose, 10 mM glycylglycine pH 7.4, 1 mM EDTA, 0.1% ethanol, 3% dextrane T10 (Pharmacia, Freiburg) and 0.5 mM digitonin (Merck, Darmstadt) and homogenized by 10 strokes in a 2 ml glass-Teflon homogenizer at 850 rpm. Unbroken cells and nuclei were removed by centrifugation and the supernatants separated on 14.5–45% w/v Nycodenz (Immuno, Heidelberg) density gradients (5 ml total volume) containing an inverse sucrose gradient [12]. Centrifugation was performed in a TV 865 rotor (Sorvall, Du Point, Bad Nauheim) at 80,000 × *g* for 2 h. Ten 0.5 ml fractions were collected from the bottom to the top. For protease treatment they were diluted with 250 μl of 0.25 M sucrose, 100 mM Tris-HCl pH 7.4 and incubated at 0°C for 30 min in the presence of either 500 μg subtilisin (Boehringer, Mannheim) per mg of protein or subtilisin plus 0.25% each of Triton X-100 and deoxycholate. The trichloroacetic acid-precipitated proteins were separated by SDS-PAGE and visualized by fluorography. Fluorographs obtained after short exposure times of 48 h were quantitated by densitometric scanning using a Chromoscan 3 (Joyce-Loeble, Gateshead) densitometer. Protein was determined by the Lowry method as described [12].

2.3. *Immunoprecipitation, SDS-PAGE and electrophoretic protein transfer*

The trichloroacetic acid-precipitated proteins were dissolved in 4%

SDS and diluted to 0.1% SDS with a solution of 140 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 2 mM methionine and 1% Triton X-100. To the solution anti-acyl-CoA oxidase antibody, preadsorbed to protein A-Sepharose was added and antigen-antibody complexes were allowed to form for 2 h. After several washings of the protein A-Sepharose beads antigen and antibodies were dissolved in 20 μl sample buffer and subjected to SDS-PAGE [13]. For Western analysis, proteins were transferred onto nitrocellulose sheets (Schleicher and Schuell, Dassel) using Tris–glycine buffer, pH 8.1 in a tank system. Antigen–antibody complexes were visualized by horse radish peroxidase-coupled second antibody and diaminobenzidine as the chromogenic substrate [14].

3. RESULTS

A post-nuclear supernatant obtained from isolated hepatocytes was separated by isopycnic centrifugation. The peroxisomal membrane protein with apparent molecular weight 22,000 (PMP 22) was used as a marker to analyze the distribution of peroxisomes along the gradients. As shown in Fig. 1, the marker appears at two locations: the major portion is confined to the 'normal' peroxisome population of high equilibrium density. In addition, a smaller portion is consistently found in gradient fractions of intermediate density. The bulk of peroxisomal proteins in these gradients is clearly confined to fractions of high equilibrium density (fractions 2–4) which contain most of the hepatic peroxisomes as confirmed by electrophoretic protein analysis of the gradient fractions (Fig. 1). The dual distribution of PMPs is not only observed in isolated hepatocytes but also in post-nuclear supernatants of rat liver homogenates (not shown). The two peroxisome populations were studied with respect to their competence to translocate peroxisomal matrix proteins. Acyl-CoA oxidase was chosen as the candidate protein, because it is expressed at a high rate by clofibrate-treatment of the animals [15,16], it is known to carry the SKL-targeting sequence and therefore represents one protein of a family of proteins with the same targeting signal [17], and a polyclonal anti-acyl-CoA oxidase antiserum with high specificity was available. In a typical pulse-chase experiment isolated hepatocytes were incubated in methionine-free tissue culture medium and pulsed with [^{35}S]methionine for 7.5 min. After this time incorporation of [^{35}S]methionine was largely prevented by the addition of unlabeled methionine and the incubation was continued for additional 15 and 30 min. Post-nuclear supernatants were prepared and separated on Nycodenz density gradients. Each gradient fraction was treated with protease to reveal the portion of the enzyme imported, and with protease-plus-detergent to demonstrate susceptibility to the protease as a control.

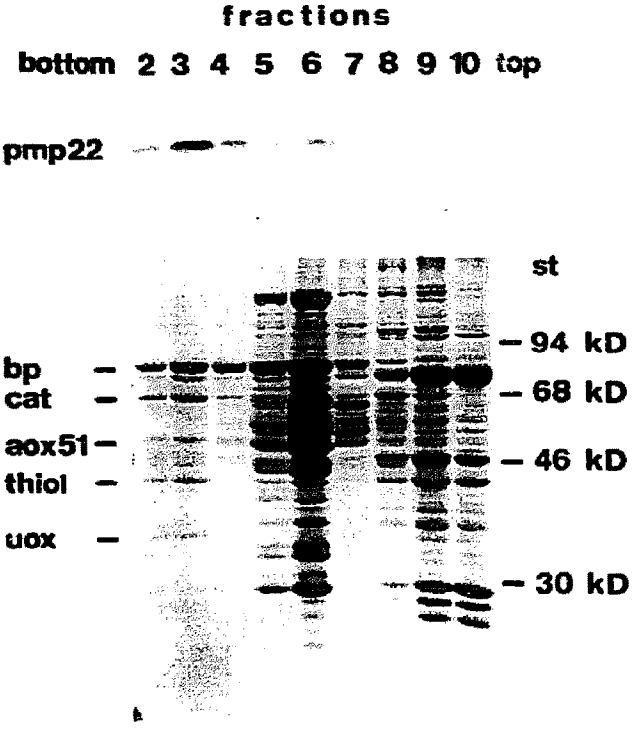

Fig. 1. Distribution of PMP 22 in peroxisomal fractions of isolated hepatocytes. Post-nuclear supernatants of isolated hepatocytes were separated on Nycodenz density gradients and the gradient fractions analyzed by immunoblotting for PMP 22 (upper panel) and by SDS-PAGE and Coomassie-staining for the total protein composition (lower panel). The bulk of peroxisomal proteins is localized in fractions 2–4 as revealed by the marker proteins catalase (cat), the 51 kDa subunit of acyl-CoA oxidase (aox51), 3-ketoacyl-CoA thiolase (thiol), and urate oxidase (uox). Note that portions of peroxisomal matrix proteins are released from peroxisomes damaged by homogenization and unspecifically stick to cellular constituents, e.g. enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, bifunctional protein (bp). This is not the case with the integral PMP 22 which is found in lower concentrations also in fractions 5–7, indicating a second peroxisomal population.

Table I

Distribution of newly synthesized acyl-CoA oxidase (71 kDa subunit) in density gradient fractions of pulse-labeled hepatocytes

Time	% Radioactivity in fraction									
	1	2	3	4	5	6	7	8	9	10
7.5 min-pulse	–	6.9	23.5	7.0	11.9	41.7	8.9	–	–	–
15 min-chase	–	4.3	25.1	24.1	8.8	15.4	15.8	6.6	–	–
30 min-chase	–	19.2	36.8	12.5	13.3	12.0	5.8	0.4	–	–

The data from the pulse chase experiment shown in Fig. 2 were quantitated by densitometric analysis of fluorographs obtained after exposing the gels to the X-ray films for 48 h. The determination of radioactivity in aliquots of immunoprecipitates subjected to SDS-PAGE practically demonstrated the same result.

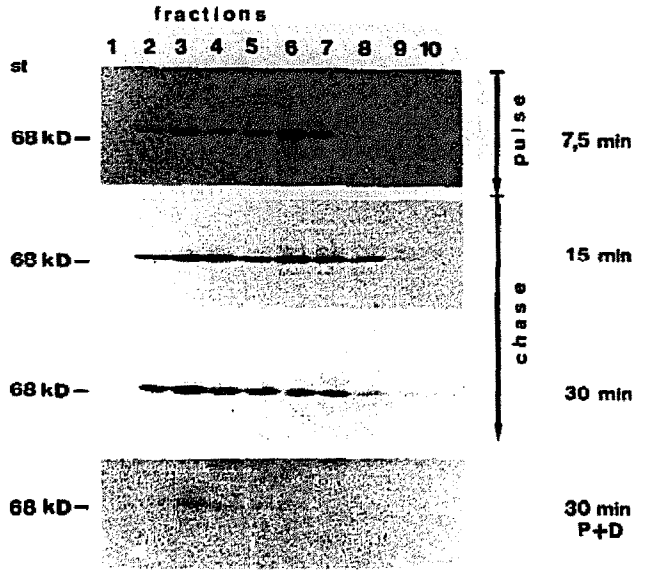

Fig. 2. Import and intracellular transport of the 71 kDa subunit of peroxisomal acyl-CoA oxidase in isolated hepatocytes. From isolated hepatocytes which had incorporated [^{35}S]methionine for 7.5 min (pulse), post-nuclear supernatants were fractionated on Nycodenz density gradients, and the distribution of newly synthesized acyl-CoA oxidase was established by immunoprecipitation, SDS-PAGE and fluorography. The same analysis was performed with cells which, after the pulse, were incubated in the presence of 2 mM unlabeled methionine for 15 and 30 min (chase). Before immunoprecipitation each sample was treated with subtilisin (500 μg/mg of protein) to digest nonimported acyl-CoA oxidase. Gradient fractions of hepatocytes chased for 30 min were also treated with the same concentration of protease plus Triton X-100 and deoxycholate (0.25% each; 30 min P+D) to demonstrate that the activity of subtilisin is sufficient to digest all the acyl-CoA oxidase present provided that the membrane barrier is abolished by the detergent. Note that in this kind of experiment with post-translational import, translocation still continues during a certain chase time due to the existence of a distinct cytosolic pool of radiolabeled precursor.

Immunological detection of acyl-CoA oxidase along the gradient is shown in Fig. 2. These fluorographic results were quantitated by densitometric scanning (Table I). During the pulse time acyl-CoA oxidase is synthesized and imported predominantly into peroxisomes of intermediate density (fractions 5–7) with approximately half the amount present in the high density bulk peroxisomes (fractions 2–4). After chase times of 15 and 30 min, the localization of newly synthesized and imported enzyme changed considerably: the maximum of protease-resistant acyl-CoA oxidase is now found in fractions of high density.

4. DISCUSSION

We present two lines of evidence for a novel translocation-competent peroxisomal population: first, in homogenates prepared from isolated hepatocytes of clofibrate-treated animals, two peroxisome populations are consistently found which are distinguished by their difference in equilibrium density in Nycodenz gradients. The high density fractions (1.22–1.23 g/cm^3) contain the well-known bulk of mature peroxisomes [12] whereas a different population is present in fractions of intermediate density (1.16–1.17 g/cm^3). The organelles present in these fractions are defined as peroxisomes by the presence of specific peroxisomal membrane markers [13,18]. By electron microscopic immunocytochemistry these peroxisomes were identified as small vesicles having diameters between 50 and 100 nm (Heinemann et al., submitted). Second, newly synthesized peroxisomal acyl-CoA oxidase, after a short pulse of 7.5 min, is found predominantly in the intermediate-density compartment and after a chase is distributed preferentially to high-density peroxisomes. These observations are novel, and provide evidence that the translocation of a peroxisomal matrix protein is confined to a particular import competent peroxisome population, and are compatible with the transport of a newly synthesized protein from one peroxisomal compartment to another. The question has been addressed in the past by several authors [19,20] as to by which way newly synthesized proteins reach the compartment of mature peroxisomes. Experimental evidence has favoured a model of biochemical homogeneity of peroxisomes [20]. However, the present data indicate that peroxisomes may represent a physically and functionally heterogeneous population.

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REFERENCES

[1] Lazarow, P.B. (1988) in: The Liver: Biology and Pathology (Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D. and Shafritz, D.A. eds.) pp. 241–254, Raven Press, New York.
[2] Borst, P. (1989) Biochim. Biophys. Acta 1008, 1–13.
[3] Erdmann, R., Veenhuis, M., Mertens, D. [illegible] Kunau, W.-H. (1989) Proc. Natl. Acad. Sci. USA 86, 5419–5423.
[4] Höhfeld, J., Veenhuis, M., Kunau, W.-H. (1991) J. Cell Biol. 114, 1167–1178.
[5] Tsukamoto, T., Miura, S., Fujiki, Y. (1991) Nature 350, 77–81.
[6] Brul, S., Westerveld, A., Strijland, A., Wanders, R.J., Schram, A.W., Heymans, H.S., Schutgens, R.B., van den Bosch, H. and Tager, J.M. (1988) J. Clin. Invest. 81, 1710–1715.
[7] Roscher, A.A., Hoefler, S., Hoefler, G., Paschke, E., Paltauf, F., Moser, A. and Moser, H. (1989) Pediatr. Res. 26, 67–72.
[8] Fujiki, Y. and Lazarow, P.B. (1985) J. Biol. Chem. 260, 5603–5609.
[9] Imanaka, T., Small, G.M. and Lazarow, P.B. (1987) J. Cell Biol. 105, 2915–2922.
[10] Thieringer, R., Suto, H., Han, Y., Cohen, G. and Lazarow, P.B. (1991) Molec. Cell Biol. 11, 510–522.
[11] Just, W.W. and Schimassek, H. (1980) Eur. J. Cell Biol. 22, 649–653.
[12] Hartl, F.-U., Just, W.W., Koster, A. and Schimassek, H. (1985) Arch. Biochem. Biophys. 237, 124–134.
[13] Hartl, F.-U. and Just, W.W. (1987) Arch. Biochem. Biophys. 255, 109–119.

[14] Blum, H., Beier, H. and Gross, H.J. (1987) Electrophoresis 8, 93–99.
[15] Lazarow, P.B., Fujiki, Y., Mortensen, R. and Hashimoto, T. (1982) FEBS Lett. 150, 307–310.
[16] Miyazawa, S., Hayashi, H., Hijikata, M., Ishii, N., Furuta, S., Kagamiyama, H., Osumi, T. and Hashimoto, T. (1987) J. Biol. Chem. 262, 8131–8137.
[17] Gould, S.J., Keller, G.-A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) J. Cell Biol. 108, 1657–1664.
[18] Hashimoto, T., Kuwabara, T., Usuda, N. and Nagata, T. (1986) J. Biochem. 100, 301–310.
[19] Poole, B., Higashi, T. and de Duve, C. (1970) J. Cell Biol. 45, 408–415.
[20] Lazarow, P.B., Shio, H. and Robbi, M. (1980) In 31st Mosbach Colloq. Biol. Chem. Organelle Formation, (Bucher, T., Sebald, W. and Weiss, H., eds.) pp. 187–206, Springer-Verlag, New York.