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Subcellular localisation and processing of non-specific lipid transfer protein are not aberrant in Rhizomelic Chondrodysplasia Punctata fibroblasts

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The import into peroxisomes and maturation of peroxisomal 3-oxoacyl-CoA thiolase are impaired in patients with the Rhizomelic form of Chondrodysplasia Punctata (RCDP). Here we show by means of immunoblotting and subcellular fractionation that non-specific lipid transfer protein (nsLTP), another peroxisomal protein synthesised as a larger precursor, is localised in peroxisomes and is present as the mature protein in RCDP fibroblasts. Thus the component of the import machinery defective in RCDP is not required for the import of nsLTP into peroxisomes.

Peroxisome; Rhizomelic Chondrodysplasia Punctata; Non-specific lipid transfer protein; Sterol carrier protein 2; Peroxisomal 3-oxoacyl-CoA thiolase

1. INTRODUCTION

Peroxisomes, which are subcellular organelles first characterised biochemically in the 1960's by De Duve and co-workers [1], contain enzymes involved in a number of important metabolic processes such as the β-oxidation of very-long-chain fatty acids and the biosynthesis of ether phospholipids (reviewed in [2]). In recent years various inherited diseases in man have been described in which one or several peroxisomal functions are impaired [3–8]. In one category of such diseases, exemplified by the cerebro-hepato-renal (Zellweger) syndrome (ZS), morphologically distinguishable peroxisomes are decreased in number or even absent and there is a generalised loss of peroxisomal functions [3–6]. The genetic defect in such disorders is thought to involve the import system for a variety of peroxisomal proteins [6]. In a second category of peroxisomal diseases there is a deficiency of a single peroxisomal enzyme, presumably due to a mutation in the structural gene for that enzyme.

A third category of peroxisomal diseases comprises the Rhizomelic form of Chondrodysplasia Punctata (RCDP). This disease is characterised by an impairment in ether phospholipid biosynthesis [9–12], a decreased capacity to catabolise phytanic acid [9–12], and a defect in the import into peroxisomes and subsequent maturation of peroxisomal 3-oxoacyl-CoA thiolase [13–16]. In contrast to most peroxisomal proteins, 3-oxoacyl-CoA thiolase is synthesised as a larger precursor [17,18] that is cleaved after association of the enzyme with peroxisomes [19]. The C-terminal tripeptide sequence, -(Ser/Ala/Cys)-(Lys/Arg/His)-Leu, which functions as a peroxisome-targeting signal for many peroxisomal proteins [20–23], is absent in 3-oxoacyl-CoA thiolase [24–26]. Swinkels et al. [27] have recently shown that the signal for directing peroxisomal 3-oxoacyl-CoA thiolase to the peroxisomes resides in the N-terminal presequence. It is conceivable that the primary defect in RCDP is a mutation in a gene encoding a protein required for the import into peroxisomes of 3-oxoacyl-CoA thiolase and a few other proteins.

Another peroxisomal protein synthesised as a larger precursor with a cleavable N-terminal presequence is non-specific lipid transfer protein (nsLTP), also known as sterol carrier protein 2 [28,29]. In cells lacking functional peroxisomes, nsLTP is deficient [30–32] and the processing of the precursor of nsLTP is impaired [31]. In both these respects the behaviour of nsLTP resembles that of 3-oxoacyl-CoA thiolase. Since there is a striking homology in the amino acid sequence upstream

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of the processing site in the two proteins (see [2,33]), a single peroxisomal protease may be responsible for the processing of both proteins. Furthermore, these facts suggest that the protease is deficient in cells lacking peroxisomes. From cDNA analysis it is evident that nsLTP has been highly conserved between mammalian species and that, in contrast to peroxisomal 3-oxoacyl-CoA thiolase [34], it contains the C-terminal tripeptide sequence, -Ala-Lys-Leu, which could function as a peroxisomal targeting signal [33,35–37]. It was therefore of interest to investigate whether or not nsLTP shows the same abnormalities as peroxisomal 3- oxoacyl-CoA thiolase in fibroblasts from RCDP patients. This paper describes the results of the study.

2. MATERIALS AND METHODS

2.1. *Cell lines and culture conditions*

Cultured skin fibroblasts used in these studies were obtained from control subjects, from patients with clinical and biochemical manifestations characteristic of RDCP (cell lines MCHE85AD and GJOLD90AD; see [14] for details) and from a patient with ZS (cell line GRO87AD; see [38] for details). The cells were cultured in a one-to-one mixture of Ham F10 (Gibco, Glasgow, UK) and Dulbecco's modified Eagle medium (Gibco) supplemented with 10% (by vol.) foetal calf serum (Gibco), under 5% CO_2.

2.2. *Subcellular fractionation of cultured skin fibroblasts on continuous Nycodenz gradients*

Fibroblasts were homogenised and fractionated on linear Nycodenz gradients (10–40%) exactly as described by Wanders et al. [39].

2.3. *Biochemical assays*

The activity of glutamate dehydrogenase, lactate dehydrogenase, catalase and β-hexosaminidase was measured as described previously [40].

2.4. *Immunoblot analysis*

Samples were subjected to electrophoresis on 10–18% polyacrylamide gradient gels essentially as described by Laemmli [41]. Immunoblot analysis was performed as described previously [14]. Nitrocellulose sheets were incubated overnight with affinity-purified antibodies directed against rat-liver nsLTP prepared as described by Teerlink et al. [42] or with antibodies directed against 3-oxoacyl-CoA thiolase as described previously [14]. Antigen–antibody complexes were visualised by incubation with goat anti-rabbit Ig conjugated to peroxidase, followed by colour development using tetramethylbenzidine (Sigma, St. Louis, MO, USA).

nsLTP from rat liver was isolated as described in [43]. Rat-liver pre-nsLTP was overexpressed in *Escherichia coli* and purified from the bacterial lysate as described in [44].

3. RESULTS

3.1. *Immunoblot analysis of the nsLTP in cultured skin fibroblasts*

An immunoblotting experiment was carried out using antibodies directed against nsLTP. The results are shown in Fig. 1. In fibroblasts from control subjects the cross-reactive immunological material present corresponded to the 14 kDa mature form of nsLTP. In ZS fibroblasts the amount of cross-reactive material was below the limit of detection. These findings are in agreement with earlier results obtained with liver homogenates and fibroblasts from control subjects and ZS patients [30,31].

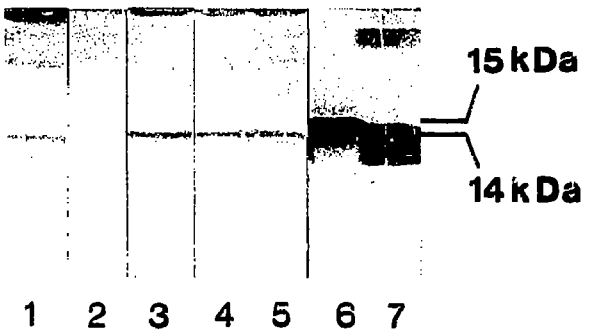

Fig. 1. Immunoblot analysis of fibroblasts from control subjects, a ZS patient and RCDP patients using antibodies directed against rat-liver non-specific lipid transfer protein (nsLTP). For experimental details see Materials and Methods. (1) Control fibroblasts (100 μg); (2) ZS fibroblasts (cell line GRO87AD, 100 μg); (3) RCDP fibroblasts (cell line MCHE85AD, 100 μg); (4) control fibroblasts (100 μg); (5) RCDP fibroblasts (cell line GJOLD90AD, 100 μg); (6) pre-nsLTP from rat liver; (7) 100,000 × g rat-liver supernatant.

In RCDP fibroblasts the molecular weight of nsLTP was identical to that of the mature protein seen in control fibroblasts, indicating that the processing of nsLTP is not impaired. Furthermore, the amount of cross-reactive material was comparable to that in control fibroblasts.

3.2. *Subcellular localisation of the nsLTP*

To study the subcellular localisation of nsLTP in RCDP, cultured skin fibroblasts were fractionated on continuous Nycodenz gradients as described in Materials and Methods. The results are shown in Fig. 2. As judged by the distribution of the activities of marker enzymes, the peroxisomes, mitochondria and cytosol were well resolved from each other in these gradients. The distribution of the marker enzymes was similar in gradients of RCDP fibroblasts and control cells.

Immunoblot analysis revealed that nsLTP was present in peroxisomal fractions from RCDP fibroblasts and that the amount of antigen was comparable to that in the peroxisomal fractions of control cells (Fig. 3). In contrast, peroxisomal 3-oxoacyl-CoA thiolase was not present in peroxisomal fractions from RCDP fibroblasts (Fig. 3), as described previously [13,14,16].

4. DISCUSSION

Immunoblotting of cultured skin fibroblasts revealed that nsLTP, which is synthesized as a precursor protein with an N-terminal peptide extension, is processed to its mature form in fibroblasts from RCDP patients, as is the case in cells from control subjects. Subcellular fractionation studies revealed that the mature form of nsLTP was located in the peroxisomal fractions of both

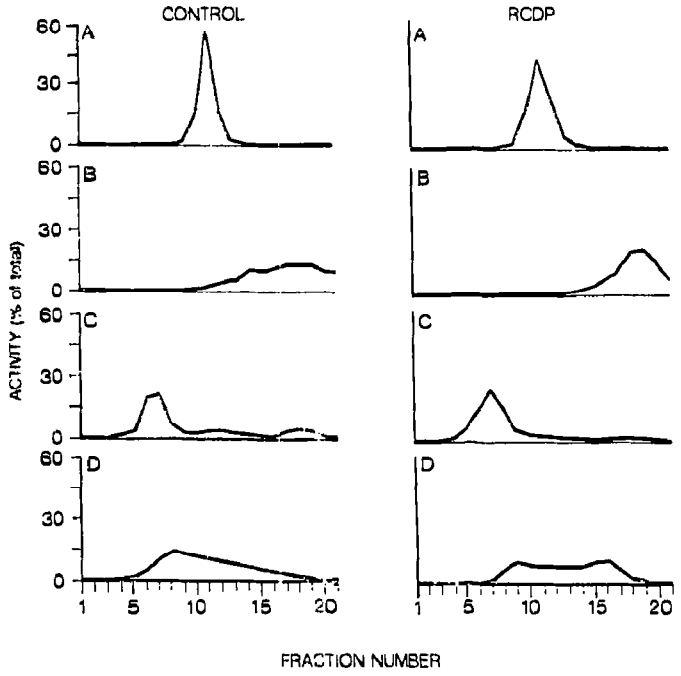

Fig. 2. Subcellular fractionation of fibroblasts from a control subject and an RCDP patient on Nycodenz gradients. Fibroblast homogenates from a control subject and an RCDP patient were prepared and fractionated as described in Materials and Methods. Fractions were collected starting from the bottom of the tube and analysed for the activity of the marker enzymes; (A) glutamate dehydrogenase (mitochondria); (B) lactate dehydrogenase (cytosol); (C) catalase (peroxisomes); and (D) β-hexosaminidase (lysosomes). Activities are expressed as percentages of total activity across the gradient.

control and RCDP fibroblasts. We therefore conclude that the post-translational import of nsLTP into peroxisomes is not impaired in RCDP fibroblasts, in contrast to the import of 3-oxoacyl-CoA thiolase. This indicates that at least one component of the import machinery is not common to nsLTP and 3-oxoacyl-CoA thiolase.

The carboxy-terminal SKL tripeptide sequence, -(Ser/Ala/Cys)-(Lys/Arg/His)-Leu, which has been identified as a peroxisomal targeting signal by Subramani and co-workers [23] and others [21,22], is present in nsLTP [33,35–37], but not in 3-oxoacyl-CoA thiolase [24–26]. In patients with RCDP the machinery used for the import of proteins which contain this targeting signal seems to be intact. Thus, nsLTP could be imported into the peroxisomes of RCDP patients by use of its C-terminal SKL tripeptide. However, it can not be excluded that in control cells nsLTP is imported into the peroxisomes by the machinery which is involved in the import of 3-oxoacyl-CoA thiolase.

The fact that nsLTP is processed to a 14 kDa mature form indicates that the putative peroxisomal protease responsible for its processing is normally active in RCDP fibroblasts. Sequence comparison revealed a striking homology between the amino acid sequence upstream of the processing site of nsLTP and 3-oxoacyl-CoA thiolase [2,33], suggesting that a single peroxisomal protease is responsible for the peroxisomal processing of both proteins. If this is indeed the case, it can be excluded that a mutation in a gene encoding a peroxisomal protease is responsible for the deficiency in the import of 3-oxoacyl-CoA thiolase in RCDP patients. Most likely the RCDP phenotype is caused by a mutation in a gene which encodes a receptor or another component of the import machinery which is in-

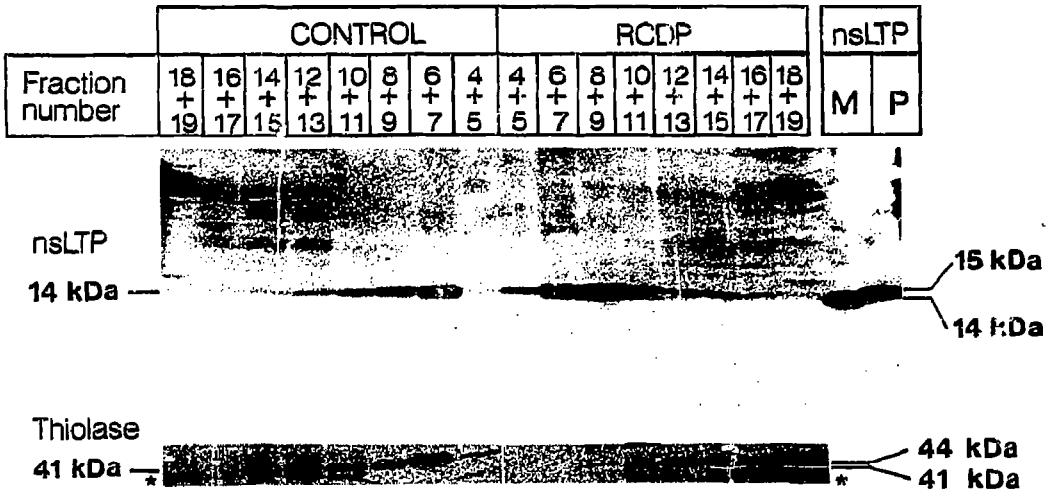

Fig. 3. Immunoblot analysis of non-specific lipid transfer protein (nsLTP) and 3-oxoacyl-CoA thiolase in fractions obtained after Nycodenz fractionation of fibroblast homogenates. Cultured skin fibroblasts from an RCDP patient and from a control subject were fractionated as in Fig. 2. Aliquots of two fractions were pooled and subjected to immunoblot analysis by use of affinity-purified antibodies directed against rat liver nsLTP or antibodies directed against 3-oxoacyl-CoA thiolase as described in Materials and Methods. The first 8 lanes are fractions from the gradient of control fibroblasts; fractions 6 + 7 are the peroxisomal peak fractions. The next 8 lanes are fractions from the gradient of RCDP fibroblasts; fractions 8 + 9 are the peroxisomal peak fractions. M, mature form of nsLTP from rat liver; P, precursor form of nsLTP from rat liver; *a-specific protein bands.

volved in the import of a specific group of peroxisomal proteins, including 3-oxoacyl-CoA thiolase.

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