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Characterization of the HSD17B4 gene: D-specific multifunctional protein 2/17β-hydroxysteroid dehydrogenase IV ☆

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Abstract

The *HSD17B4* gene codes for a 80 kDa multifunctional enzyme containing three distinct functional domains and is localized in peroxisomes. The N-terminal part exhibits 3-hydroxyacyl-CoA dehydrogenase and 17β-hydroxysteroid dehydrogenase activity whereas the central part shows enoyl-CoA hydratase activity. The carboxy-terminal part of the protein has sterol-carrier-protein activity. The protein is widely expressed, however in several tissues like brain, uterus and lung its expression is limited to specific cells like Purkinje cells or luminal epithelium. The *HSD17B4* gene consist of 24 exons and 23 introns with classical intron-exon junctions spanning more than 100 kbp. The importance of the HSD17B4 protein is stressed by the identification of patients with severe clinical abnormalities due to mutations in the *HSD17B4* gene. We have now checked the consequences of one frequent mutation, G16 S, which results in inactivation of the enzyme due to loss of interaction with NAD+.

1. Introduction

Several 17β-hydroxysteroid dehydrogenases (17β-HSDs) catalyze the oxidation/reduction reactions at position C17 of the steroid molecules at the final steps in androgen and estrogen biosynthesis thereby modulating the biological function of the steroid hormones. Until now, four different human 17β-HSDs have been characterized [1]. 17β-HSD I and III participate in the synthesis, whereas 17β-HSD II and IV are involved in the inactivation of steroids. Further orthologs of the 17β-HSDs, types V, VI and VII have been described in human, rat and mice [2–5] and await further characterization.

We have recently identified the cDNA coding for the human 17β-HSD IV (gene name *HSD17B4*) which catalyzes the oxidation of the steroid hormones Δ5-androstene-3β,17β-diol and 17β-estradiol [6]. The product of the *HSD17B4* gene is an 80 kDa three domain multifunctional enzyme and belongs to the SDR gene family [7]. Ortholog proteins are also cloned from rat, mice, pig, chicken and guinea pig [6,8–13]. Using immunogold electron microscopy we have shown that the 80 kDa protein is localized in peroxisomes [14]. The N-terminal part has activities of 17β-estradiol de-

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hydrogenase type IV and D-specific 3-hydroxyacyl-CoA dehydrogenase, the central part has D-specific 2-enoyl-CoA hydratase activity reactive with straight and 2-methylbranched 2-enoyl-CoAs such as pristanoyl-CoA and the enoyl-CoAs of di- and trihydroxycholestanoic acid [8,15–17]. The *HSD17B4* gene product is known as: D-specific bifunctional protein, multifunctional enzyme 2, multifunctional protein 2, 17β-hydroxysteroid dehydrogenase IV [8, 11, 17–19]. For clarity we will use the abbreviation 17β-HSD IV in this work.

Although the 17β-HSD IV is ubiquitously expressed, in some tissues like brain, lung and uterus there is a distinct pattern of cell-specific expression, as we show in this paper. Recent research on peroxisomal disorders revealed that this enzyme is deficient in Zellweger syndrome [18,20,21]. This motivated us to identify and analyze the structure of the HSD17B4 gene and study the effect of a mutation in the gene on the enzymatic activity.

2. Materials and methods

2.1. Northern blot

Two human poly(A^+) RNA blots were obtained from Clontech (Palo Alto, CA). The membranes were hybridized with [α-^{32}P]-labeled EcoRI fragment corresponding to nucleotides 302 to 1753 of the human 17β-HSD IV [6] as described previously [22].

2.2. Immunohistochemistry

Porcine tissues were fixed and processed for paraffin embedding. Rehydrated 3 μm sections were incubated with a F1 monoclonal antibody conjugated with peroxidase and color reaction was developed with diaminobenzidine/H_2O_2. In some cases a secondary anti-mouse antibody labeled with fluorescent dye Cy3 was used to visualize the F1 binding on sections [23].

2.3. Screening and characterization of 17β-HSD IV genomic clones

A [α-^{32}P]-dCTP (Amersham, Little Chalfont, UK) labeled full-length 17β-HSD IV cDNA probe [6] was used to screen two different genomic λ dash DNA libraries from human NB4 lymphoblastic cells and from human normal blood. In parallel a 1.4 kb EcoRI fragment of the human 17β-HSD IV cDNA was used to screen a P1 (male ICRFP 700) and a PAC (female RPCI6 709) library (Resource Center, Primary Database of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics, Berlin, Germany). To obtain the promoter region we screened with a 200 bp fragment (located in the most 5′region of the human cDNA) the human genomic NB4 library. Positive clones were isolated, the inserts excised and subcloned into the polylinkers of pBluescript SKII+, KSII+(Stratagene, La Jolla, CA), PCRII (Invitrogen, San Diego, CA) and pGem-3Zf (Promega, Charbonnières, France) and sequenced as described [24]. The intron sizes were determined either by sequencing or by long range PCR using the Elongase system (Gibco, Eggenstein, Germany).

2.4. Protein expression and analysis

Full length cDNA (wild type and mutant) was subcloned into pEL vector and transformed in yeast (*Saccharomyces cerevisiae*). The expression of the protein was induced by transfer of yeasts into oleic acid rich medium [21]. The suspension of yeasts was used for the activity measurements. The N-terminal domain of the 17β-HSD IV (amino acids 1-323) was expressed in *E. coli* as a fusion protein with glutathione-S-transferase [15]. The forward mutagenesis primers 5′-TTTT-GGATCCATGGGCTCACCGCTGAGGTTCGACG-GGCGGGTGGTACTGGTCACCAGCGCGGGGG-CAGG-3′ and 5′-TTTTGGATCCATGGGCTCAC-CGCTGAGGTTCGACGGGCGGGTGGTACTGG-TCACCGCCGCGGGGGCAGG-3 introduced the BamHI site and mutations G16 S or G16A, respectively. Reverse primer ′5′-TTTTGGTACCTTATGT-TGCTGTAGACGTTGCAC-3 introduced a KpnI site. The PCR-amplified DNA was digested with BamHI and KpnI and cloned into the pGex 2 T PL2 vector, a modified pGex 2 T vector (Pharmacia, Freiburg, Germany), with an additional KpnI site [15]. The constructs were verified by restriction analysis and full length sequencing. *E. coli* (strain JM107) were grown in LB media containing 50 μg/ml Ampicillin. The expression was induced by 0.2 mM isopropyl-D-thiogalactopyranoside. Cell extract preparation, the purification of the glutathione-S-transferase fusion proteins and the cleavage with thrombin, were performed as described [15]. Pure N-terminal domain of the 17β-HSD IV was used for further studies. Enzymic activities of the 17β-HSD IV were measured with either (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA, acetoacetyl-CoA or with 17β-estradiol as described [15,21,25].

3. Results and discussion

3.1. Expression of HSD17B4 gene

Human 17β-HSD IV is widely distributed throughout the organism and has high levels of expression in liver and kidney followed by heart and gonads (Fig.

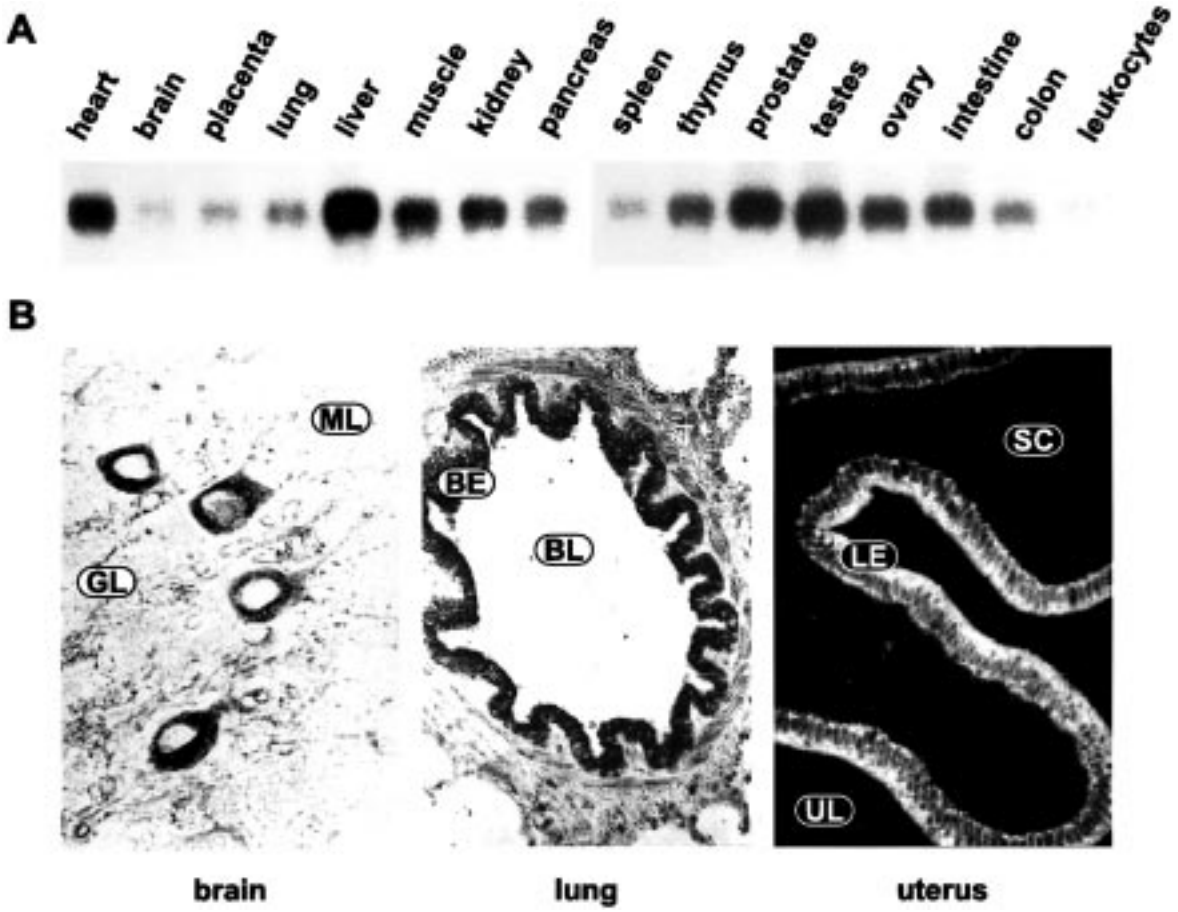

Fig. 1. Expression of the *HSD17B4* gene (A) mRNA expression in human tissues. The membranes were hybridized with [α-^{32}P]-labeled EcoRI fragment corresponding to nucleotides 302 to 1753 of human 17β-HSD IV [6]. A single band of 3 kb is observed in all tissues tested. (B) Protein expression in porcine tissues. Sections were stained with anti-17β-HSD IV monoclonal antibody F1 and visualised by color reaction developed with peroxidase (brain and lung) or with a secondary fluorescent labeled (Cy3) antibody (uterus). GL, granular layer; ML, molecular layer; BE, bronchial epithelium; BL, bronchial lumen; UL, uterus lumen; LE, lumenal epithelium; SC, stroma cells.

1A). However, immunohistochemical analysis of tissues showing low 17β-HSD IV mRNA expression, like brain, lung and uterus, reveals that the enzyme is present in specific cells within these organs. In the brain the 17β-HSD IV is confined to Purkinje cells (Fig. 1B) and the anterior pituitary (not shown). In the lung the bronchial epithelium expresses high levels of the 17β-HSD IV and in the uterus the protein is present in lumenal and glandular epithelium and not in stromal cells. Taking into account the steroid inactivating properties of the enzyme, it may play a role in the regulation of local concentrations of steroid hormones in the sense of intracrinology [26].

3.2. Determination of the intron-exon structure of the HSD17B4 gene

The resolution of the genomic organization became of special interest after the observation that deficiencies in the human *HSD17B4* gene lead to a severe peroxisomal disease [18,20,21]. Peroxisomal organelle deficiency results in disorders of lipid, fatty acid and sterol metabolism such as Zellweger syndrome, adrenoleukodystrophy, infantile refsum disease and hyperpipecolic acidemia [27,28]. Patients with peroxisomal deficiency reveal high plasma concentrations of long chain fatty acids, bile acids and deficient synthesis of plasmalogens. This exerts pleiotropic influence of renal function impairment and neuronal development (neuronal migration defects and degeneration). To understand the molecular basis of this inherited disease we have recently mapped the *HSD17B4* gene to chromosome 5q2 [29] and identified its genomic organization (Fig. 2) [24].

By screening the human λ dash, P1 and PAC genomic libraries we obtained a set of clones representing the full contig of the *HSD17B4* gene. Primers derived from the cDNA [6] were used to elucidate the intron-exon borders of the gene. No differences were found between the cDNA sequence and genomic exon sequences. The sequences of intron-exon borders showed the classical AG and GT sequences of splice

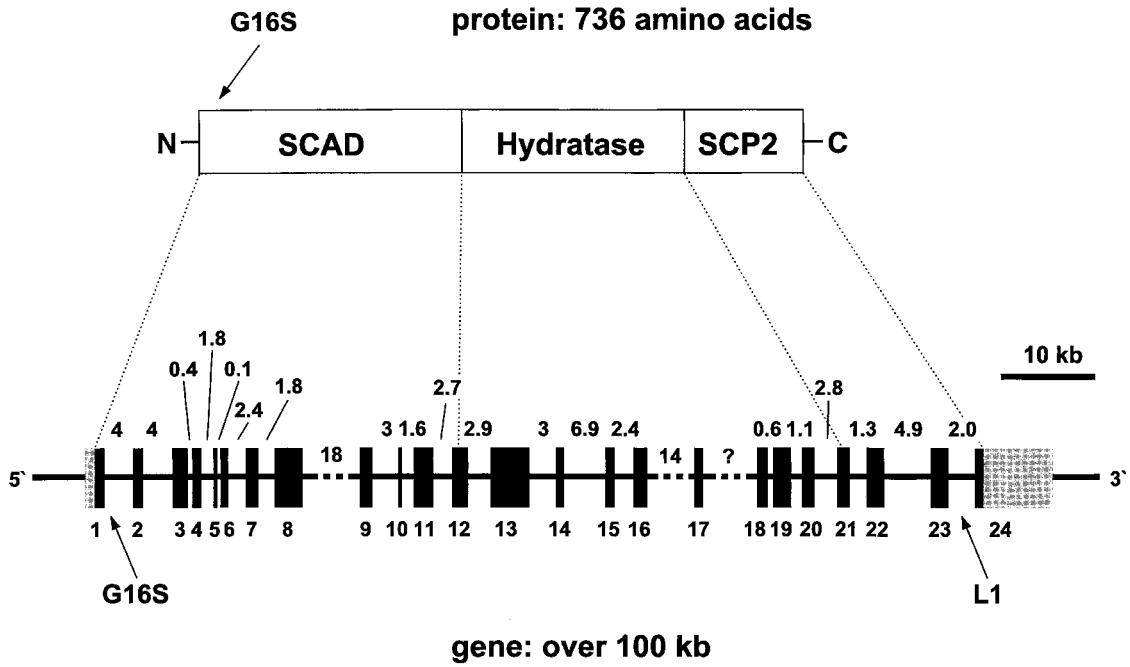

Fig. 2. Structure of the *HSD17B4* gene. A schematic structure of the 17β-HSD IV enzyme is given above that of the gene. Arrows points to the position of the G16 S mutation and an L1 element. The black boxes representing exons are drawn to scale according to the indicated 10 kb marker. The sizes of introns are given above; introns smaller than 7 kb are drawn to scale; larger introns are represented by broken line. The numbers of the exons are below the boxes. Exons 1 and 24 consist of translated (black box) and untranslated regions (grey box).

donor and acceptor sites. Our analysis of the genomic structure of the human *HSD17B4* indicates that the gene includes 24 exons that span approximately 100 kb as demonstrated by PCR on genomic DNA. The size of the exons ranges from 21 bp (exon 5) to 286 bp (exon 13). The intron between exon 23 and 24 contains a L1 element 3280 bases upstream from the 5′-boundary of exon 24. The nucleotide sequences (about 30 kbp) of the *HSD17B4* gene have been submitted to the GenBank Data Bank (accession numbers AF057720 to AF057740).

3.3. Effect of G16 S mutation on the activity of 17β-HSD IV

The molecular basis of the recently identified D-specific bifunctional enzyme deficiency is the mutation G16 S [21]. This mutation, is localized in the first exon (Fig. 2). The sequence 15 T*G*AGA**GL**G**R**A24 is mutated to 15 TSAGA**GL**G**R**A24 (conserved glycines of SDR gene family are given in bold face) [21]. When expressed in yeast the G16 S mutant is not able to convert (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA (Table 1). In control experiments the wild type protein has enoyl-CoA hydratase activity and D-hydroxyacyl-CoA dehydrogenase activity [21]. We have further explored if the loss of function is due to replacement of G16 by any amino acid other than glycine or due to insertion of a polar group. N-terminal domains of wild type protein and two mutant proteins G16 S and G16A were expressed in *E. coli* and purified to homogeneity (Fig. 3). In vitro both mutants were inactive with acetoacetyl-CoA and 17β-estradiol, whereas wild type converted the substrates with velocities shown in Table. 1. The activities of the purified N-terminal domain of 17β-HSD IV are close to that

Table 1
Enzymatic activities of wild type and mutant 17β-HSD IV (measurements were performed as described in materials and methods; —not performed)

	Yeast suspension activity in nmol/min/mg prot		Purified from *E. coli* acivity in nmol/min/mg prot	
Protein	Enoyl-CoA hydratase	D-hydroxyacyl-CoA dehydrogenase	17β-hydroxysteroid dehydrogenase	D-hydroxyacyl-CoA dehydrogenase
Wild type	6.29	8.49	0.14	2.01
G16 S	Not detectable	Not detectable	Not detectable	Not detectable
G16A	—	—	Not detectable	Not detectable

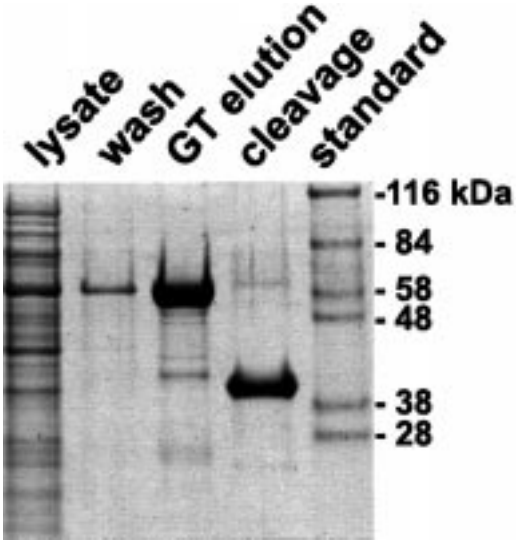

Fig. 3. Expression of the N-terminal domain of 17β-HSD IV. *E. coli* was transfected with pGex vectors coding for N-terminal domain of 17β-HSD IV. Bacterial lysates were subjected to affinity purification on glutathione-agarose. The affinity matrix was washed as described [15]. A part of the matrix, to control the size of the fusion protein, was treated with glutathione. The rest of agarose was incubated with thrombin which resulted in a release of N-terminal domain only from the matrix. The results are shown for wild type protein. Mutated proteins had the same molecular mass and are not shown. SDS-PAGE and Coommassie staining.

known for fatty acyl-CoA metabolizing enzymes [16,30,31]. The velocity of the 17β-estradiol oxidation is several fold lower than that of fatty acyl-CoA. However, all other known 17β-HSDs have the same conversion rates for steroids of 0.1 to 0.25 nmol/min/mg prot. [5]. Because the enzymatic parameters (V_{max}, K_m) of the 17β-HSD IV for both fatty acyl-CoA and steroids are within that known for other enzymes of SDR gene family it remains to be settled which substrates are physiological.

The G16 S mutation in the 17β-HSD IV protein disturbs the conformation of the Rossman fold required for cofactor (NAD^+) binding. The mutation is lethal, most probably because the D-specific pathways of pristanic acid and di/tri-hydroxycholestanoic acid metabolism are disrupted. Another closely related enzyme, the multifunctional protein 1 [32], is L-specific and can not substitute the deficient D-pathway [17]. Because the 17β-HSD IV has ubiquitous distribution any mutation would affect the whole organism. The lack of observation of any steroid hormone related phenotype might be due to compensation by other 17β-hydroxysteroid dehydrogenases.

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