Porcine 80-kDa Protein Reveals Intrinsic 17β-Hydroxysteroid Dehydrogenase, Fatty Acyl-CoA-hydratase/Dehydrogenase, and Sterol Transfer Activities*

Frauke Leenderst, J acob G. Tesdorpf, Monika Markus, Thomas Engel, Udo Seedorf, and Jerzy Adamski

From the Max-Planck-Institut für experimentelle Endokrinologie, Postfach 610309, 30603 Hannover, Germany and the Institut für Arterioskleroseforschung, Universität Münster, 48149 Münster, Germany

Four types of 17β-hydroxysteroid dehydrogenases have been identified so far. The porcine peroxisomal 17β-hydroxysteroid dehydrogenase type IV catalyzes the oxidation of estradiol with high preference over the reduction of estrone. A 2.9-kilobase mRNA codes for an 80-kDa (737 amino acids) protein featuring domains which are not present in the other 17β-hydroxysteroid dehydrogenases. The 80-kDa protein is N terminally cleaved to a 32-kDa fragment with 17β-hydroxysteroid dehydrogenase activity. Here we show for the first time that both the 80-kDa and the terminal 32-kDa (amino acids 1–323) peptides are able to perform the dehydrogenase reaction not only with steroids at the C17 position but also with 3-hydroxyacyl-CoA. The central part of the 80-kDa protein (amino acids 324–596) catalyzes the 2-enoyl-acyl-CoA hydratase reaction with high efficiency. The C-terminal part of the 80-kDa protein (amino acids 597–737) is similar to sterol carrier protein 2 and facilitates the transfer of 7-dehydrocholesterol and phosphatidylcholine between membranes in vitro. The unique multidomain structure of the 80-kDa protein allows for the catalysis of several reactions so far thought to be performed by complexes of different enzymes.

The redox reactions at position C17 of the steroid molecule are catalyzed by a number of different 17β-hydroxysteroid dehydrogenases (17β-HSD) (1–3). Until now, four human 17β-HSDDs were characterized. The soluble 17β-HSD type I consisting of 327 amino acids (aa) was cloned from human placenta and performs the oxidation of estradiol at the same efficiency as the reduction of estrone (4–6). The 17β-HSD type II is a microsomal enzyme of 387 aa that slightly prefers the oxidation over the reduction of estrogens and androgens (7, 8). The testes predominantly express the microsomal 17β-HSD type III consisting of 310 aa and is responsible for the reduction of estrogens and androgens (9). The porcine 17β-HSD type IV inactivates hormones very efficiently because of its 360-fold preference for steroid oxidation (10, 11) and is the first steroid metabolizing enzyme localized in peroxisomes (12). The enzyme is primarily translated as an 80-kDa protein from a 2.9-kilobase message (13). The post-translational modifications include an N-terminal cleavage leading to a 32-kDa peptide (10). A fraction of the 32-kDa peptide is covalently linked to actin through an e(γ-glutamyl)-lysine bond (14). Recently, cloning of the human and mouse 80-kDa 17β-HSD type IV showed a close relationship revealing 85% amino acid similarity, the same multidomain structure, and identical kinetic parameters of the 17β-HSD IV (15, 16). In contrast, the overall similarity between sequences of four human 17β-HSD type I-IV is less than 25%.

The amino acid sequence comparison with the Swissprot and EMBL data bases (17) revealed several interesting features of the type IV enzyme (Fig. 1). The N-terminal part shows homologies to the family of short chain alcohol dehydrogenases (18–20), especially to the two short chain alcohol dehydrogenases domains of the multifunctional (hydratase-dehydrogenase) enzymes of peroxisomal β-oxidation of fatty acids in Saccharomyces cerevisiae (21) and Candida tropicalis (22). The central domain of the 17β-HSD type IV is 40 and 38% identical (Fig. 1) with the C-terminal parts of the S. cerevisiae and C. tropicalis multidomain proteins, respectively. The C-terminal extension of the 80-kDa protein shows an intriguing similarity to the sterol carrier protein 2 (SCP2) which is assumed to participate in the intracellular transport of steroids and lipids (23–27). Although the SCP2 was first identified as a 13-kDa protein it is, however, as well part of a 60-kDa fusion protein between SCP2 and a peroxisomal 3-oxoacyl-CoA thiolase named SCPx (28–30). Recently, it was demonstrated that SCP2 and SCPx are expressed from a single gene via alternative transcription initiation from two distinct promoters (31, 32).

The 80-kDa protein reveals a complex structure which was unknown among other 17β-hydroxysteroid dehydrogenases and enzymes of peroxisomal β-oxidation of fatty acids. To evaluate the activities suggested by the amino acid similarities, the functionalities of the purified porcine 17β-hydroxysteroid dehydrogenase as well as the expressed recombinant single domains were assayed.

MATERIALS AND METHODS

Expression of 80-kDa 17β-Hydroxysteroid Dehydrogenase Type IV and 32-kDa Peptide in the Human Embryonal Kidney Cell Line 293 (HEK 293)—DNA fragments containing the coding sequence for the amino acids 1–737 (entire 80-kDa coding region, abbreviated as p80) and aa 1–323 (N-terminal 32-kDa fragment, p32) of the porcine 17β-estradiol dehydrogenase were obtained from the cDNA (13) by polymerase chain reaction amplification using appropriate oligonucleotide...
Multifunctional 80-kDa Protein

expression was induced by 0.2 mM (final) isopropyl-D-

NADH activity was assayed spectrophotometrically at 340 nm by following the hydration of the trans-double bond using crotonyl-CoA as substrate. Products of the reaction were separated on a reversed phase HPLC column. All other materials were from Sigma, Deisenhofen, Germany.

RESULTS

Expression of the 80-kDa and the 32-kDa Forms of the 17β-

Hydroxysteroid Dehydrogenase in HEK 293 Cells—Sterol and phospholipid transfer activities were measured by monitoring the net transfer of 7-dehydrocholesterol (7-DHC) and phosphatidylcholine (PC) from small unilamellar vesicles to E. coli protoplasts (25). Briefly, small unilamellar vesicles containing egg yolk PC/7-DHC (65:35 mol%) were incubated with B. megaterium protoplasts at 37°C for 30 min. The assays contained 2 mg of liposomal lipid, 2.5 mg of protoplat, and 1 nmol of protein in a total volume of 500 µl of SPA buffer (300 mM sucrose, 0.3% (v/v) NaNO₃, 60 mM potassium phosphate, pH 6.2). Proteoliposomes were then separated from small unilamellar vesicles by centrifugation in an Eppendorf centrifuge for 4 min at 8,000 rpm. The proteoliposomes were washed with SPA buffer, resuspended in the same buffer, and lysed with an equal volume of 15% ethanolic KOH. The 7-DHC was extracted with 1.2 ml of n-hexane and quantified by recording a UV spectrum between 320 and 250 nm (molecular absorption coefficient at 294 nm 7,200 M⁻¹ cm⁻¹). Determination of the PC transfer was performed enzymatically as described earlier (25). Incubations using human SCP2, SCPx, or SCP2-glutathione S-transferase fusion proteins were used as positive controls, negative controls contained bovine serum albumin. Human proteins were expressed and purified as described (25). All other materials were from Sigma, Deisenhofen, Germany.

Expression of the 80-kDa and the 32-kDa Forms of the 17β-

Hydroxysteroid Dehydrogenase in HEK 293 Cells—Plasmids containing the inserts coding for the full-length 80 kDa (pREP10-p80) or only the N-terminal 32-kDa peptide (pREP10-p32) were used to transfect HEK 293 cells. The capability to convert estradiol to estrone is only observed in the cells that were transfected with the expression vector containing the DNA coding for the 80-kDa translation product or the 32-kDa peptide, but not the control cells which are not transfected or transfected with the vector only. The ability to convert 17β-estradiol (E₂) to estrone (E₁) increased with time after transfection with pREP10-p80 (Fig. 2) or pREP10-p32 (not shown). After 72 h the ability to oxidize E₂ reached a plateau. Typically, the cells transfected with either pREP10-p32 or pREP10-p80 released about 25-fold higher specific 17β-HSD IV activity.

To clarify if the processing of the 80-kDa protein to its N-terminal 32-kDa fragment is necessary for the activation of the 17β-hydroxysteroid dehydrogenase Western blot analysis was performed. HEK 293 cells transfected with plasmid coding for the full-length 80-kDa protein and harvested at time points after transfection as indicated. The 17β-HSD activity was assayed with 17β-estradiol in cell homogenates as described under "Materials and Methods." Open bars, control cells; dark bars, transfected cells.

Assay of in Vitro Sterol Carrier Activity and Phosphatidylcholine Transfer Activities—Sterol and phospholipid transfer activities were measured by monitoring the net transfer of 7-dehydrocholesterol (7-DHC) and phosphatidylcholine (PC) from small unilamellar vesicles to Bacillus megaterium protoplasts (25). Briefly, small unilamellar vesicles containing egg yolk PC/7-DHC (65:35 mol%) were incubated with B. megaterium protoplasts at 37°C for 30 min. The assays contained 2 mg of liposomal lipid, 2.5 mg of protoplat, and 1 nmol of protein in a total volume of 500 µl of SPA buffer (300 mM sucrose, 0.3% (v/v) NaNO₃, 60 mM potassium phosphate, pH 6.2). Proteoliposomes were then separated from small unilamellar vesicles by centrifugation in an Eppendorf centrifuge for 4 min at 8,000 rpm. The proteoliposomes were washed with SPA buffer, resuspended in the same buffer, and lysed with an equal volume of 15% ethanolic KOH. The 7-DHC was extracted with 1.2 ml of n-hexane and quantified by recording a UV spectrum between 320 and 250 nm (molecular absorption coefficient at 294 nm 7,200 M⁻¹ cm⁻¹). Determination of the PC transfer was performed enzymatically as described earlier (25). Incubations using human SCP2, SCPx, or SCP2-glutathione S-transferase fusion proteins were used as positive controls, negative controls contained bovine serum albumin. Human proteins were expressed and purified as described (25). All other materials were from Sigma, Deisenhofen, Germany.

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Fig. 2. Expression of 17β-hydroxysteroid dehydrogenase activity in HEK 293 cells transfected with pREP10-p80. HEK 293 cells were transfected with a pREP10-p80 vector coding for the full-length 80-kDa protein and harvested at time points after transfection as indicated. The 17β-HSD activity was assayed with 17β-estradiol in cell homogenates as described under "Materials and Methods." Open bars, control cells; dark bars, transfected cells.
Sterol and Lipid Transfer Activities—The ability to transfer 7-DHC and PC was first investigated in the VHF fraction of the porcine 17β-hydroxysteroid dehydrogenase purification (10). This fraction contains the 80-kDa protein (with its C-terminal SCP2-like domain) which under native conditions copurifies with 32-kDa enzyme, actin, and a covalent actin-32 kDa protein complex. High levels of transfer activities of both, 7-DHC and PC, are revealed by the VHF fraction. Under the assumption that these activities are due to the SCP2-like domain of the 80-kDa protein specific transfer activity for 7-DHC is 39% and for PC 44% of that evaluated for the human SCP2 (Table II). The specific transfer activities of the 80-kDa protein are close to those of SCpx.

More direct evidence on the involvement of the SCP2-related domain in the sterol and lipid transfer was obtained with the porcine recombinant peptide. Both the GST-SCP2 fusion product and the SCP2-like protein stimulate the transfer of 7-DHC and PC from donors to acceptors. The purified porcine SCP2-like peptide increases the transfer of 7-DHC and PC 147- and 158-fold over the control levels, respectively (Table II).

DISCUSSION

Amino acid sequence comparisons suggest a relationship between the four human 17β-hydroxysteroid dehydrogenases and bacterial proteins involved in fatty acid metabolism (35). The high similarity of 17β-HSD IV to the C. tropicalis or S. cerevisiae enzymes participating in the peroxisomal β-oxidation of fatty acids even suggests a common ancestor (17, 35, 36). The porcine 17β-HSD IV is the first peroxisomal enzyme with proven dehydrogenase activity against steroids and fatty acids. The K_m values for 17β-estradiol (0.2–0.4 μM) and for crotonyl-CoA (31–35 μM) are compatible with the physiological concentrations of the substrates and are close to K_m observed in other dehydrogenases specialized in either substrate (1, 34, 37, 38). Recently, a rat homologue (85% amino acid identity) of the porcine protein has been purified (39) and cloned. The isolated rat enzyme shows activity of 3-hydroxyacyl-CoA dehydrogenase with fatty acids, 2-methyl-branched fatty acids, and bile acid intermediates (3-hydroxyacyl derivative of trihydroxyprostanic acid) (39). It remains to be settled which substances are the preferred in vivo substrates for the 80-kDa protein.

The domains of the multifunctional (fatty acids hydratase-dehydrogenase) FOX2 gene product of S. cerevisiae were studied by Hiltunen et al. (21). The deletion of the carboxyl-terminal domain (271 aa) resulted in a loss of hydratase activity (converting trans-2-enoyl CoA into Δ3 hydroxyacyl-CoA) but...
Porcine 80-kDa protein reveals activities of 17β-hydroxysteroid dehydrogenase, fatty acid-CoA dehydrogenase, and fatty acid-CoA hydratase. Kinetic parameters of 17β-hydroxysteroid dehydrogenase type IV were measured with 17β-estradiol, fatty acid-CoA hydratase with crotonyl-CoA, and fatty acid-CoA dehydrogenase with acetooxyl-CoA as described under "Materials and Methods." Recombinant 32-kDa (amino acids 1-323 representing 17β-hydroxysteroid dehydrogenase) and hydratase-like domains (amino acids 324-596) were expressed in E. coli using the pGex-p32 and pGex-hydratase vectors, respectively. They were characterized after purification on glutathione-agarose and thrombin cleavage. Recombinant 80-kDa protein was assayed in homogenates of HEK 293 cells transfected with pRep10-p80 vector and the activity was corrected for background conversion.

### Table I

| Sample                  | 17β-Hydroxysteroid dehydrogenase | Fatty acid-CoA dehydrogenase | Fatty acid-CoA hydratase |
|-------------------------|----------------------------------|-----------------------------|--------------------------|
|                         | $K_m\, \text{mmol} \times \text{mg}^{-1} \times \text{min}^{-1}$ | $V_{max}\, \text{mmol} \times \text{mg}^{-1} \times \text{min}^{-1}$ | $K_m\, \text{mmol} \times \text{mg}^{-1} \times \text{min}^{-1}$ | $V_{max}\, \text{mmol} \times \text{mg}^{-1} \times \text{min}^{-1}$ |
| N-terminal domain       | 0.3 0.14 31.1                    | 1.10 ND                     | ND ND                    |
| Hydratase domain        | ND ND ND                        | ND 34.7                    | 1.36 ND                  |
| Recombinant 80 kDa      | 0.4 0.11 35.3                    | 2.91 37.1                   | 3.92 34.0 ND             |
| VHF fraction            | 0.3 0.19 34.8                    | 3.31 ND                     | ND ND                    |

### Table II

| Sample                  | Transfer of 7-DHC | Transfer of PC |
|-------------------------|-------------------|----------------|
| Human SCP2a             | 54.3 ± 2.1        | 149.3 ± 4.1    |
| Human SCPx              | 22.4 ± 0.3        | 69.8 ± 1.8     |
| GST-Human SCP2           | 29.5 ± 0.7        | 72.4 ± 2.3     |
| VHF fractionb           | 21.4 ± 2.1        | 65.2 ± 0.9     |
| Porcine SCP2-like       | 12.6 ± 0.7        | 36.7 ± 1.3     |
| Porcine GST-SCP2-like   | 10.6 ± 0.1        | 21.7 ± 1.6     |
| Bovine serum albumin    | 0.8 ± 0.01        | 0.25 ± 0.07    |

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The specific 3-hydroxyacyl-CoA dehydrogenase activity was retained. This pointed indirectly to the localization of the dehydrogenase activity in the N-terminal part. In our report different enzymatic activities have been assigned unequivocally to the individual regions of the 80-kDa protein by analyses of the isolated domains expressed in E. coli. All the functionalities suggested by the amino acid similarities were observed in both the 80-kDa protein and in its isolated recombinant domains. This excludes the possibility that the processing into smaller peptides is a prerequisite for the release of the activities. However, at least the processing into a 32-kDa fragment was observed in porcine tissues. There are different cleavage efficiencies: high in hormone target organs like uterus and breast epithelium but low in non-target tissues such as kidney and liver (40). It is yet unclear if the separation of the 32-kDa 17β-HSD IV from the other parts is an advantage in hormone inactivation. Most probably the lack of the hydratase and SCP2 domains in the vicinity of the 17β-HSD IV could reduce the competition between steriods and fatty acids for the active center of 17β-HSD IV.

All amino acids which were shown to be essential for the lipid transfer activity of human SCP2 by site-directed mutagenesis are conserved in the porcine SCP2-like domain of the 80-kDa protein (17, 25). The relatively low activity of porcine SCP2 may be due to the fact that some amino acids, which may be required for the full activity are missing at the N-terminus. The high activity obtained with the purified native proteins supports the view that the separation between hydratase and SCP2 domains is not yet optimally set. As presumed earlier by Pfaff et al. (26) the SCP2 might have an important function in the beginning of steroidogenesis by stimulating the pregnenolone synthesis. The biological role of the C-terminal sterol transfer domain contained within the 80-kDa protein is not known at present. The results shown in Table I strongly suggest that the domain is not involved in the hydratase/dehydrogenase activity of the enzyme. Because the SCP2-like domain contains the peroxisomal targeting sequence AK1 (other domains are devoid of any targeting signals) one possibility would be to ensure proper peroxisomal localization of the whole protein. On the other hand this exclusive function does not explain the considerable degree of structural and functional conservation of the domain with SCP2. Therefore, an additional function appears to be likely. In vitro SCP2 seems not to facilitate the movement of most steroids (41) or fatty acids (42). However, we currently cannot exclude that 17β-HSD IV also utilizes other, more hydrophobic substrates in vivo which may require transfer from the peroxisomal membrane to the catalytically active site, located primarily in the peroxisomal matrix. Alternatively, at present it cannot be ruled out that the SCP2-like domain has no direct functional relevance with respect to the catalytic activity of the 80-kDa protein. Additional studies are clearly necessary in order to discriminate between these possibilities.

The advantages of the multidomain structure of porcine 80-kDa protein (17β-HSD IV + hydratase + SCP2) or of the SCPx (3-oxoacyl-CoA thiolase + SCP2) remain to be established. It permits the coordination of regulation of gene expression for functionally related but yet diverse enzymes. The composition of the 80-kDa protein allows for the catalysis of several processes of peroxisomal β-oxidation of fatty acids by a single macromolecule instead of a participation of several enzymes (21, 38, 39, 43). Such a concerted action might further be essential in the metabolism of sterols and steroids.

The porcine 17β-hydroxysteroid dehydrogenase IV is the first peroxisomal enzyme known to be stimulated by progesterone (40). The hormone is as well responsible for the regulation of other types of 17β-HSD (44, 45). The recently purified and cloned rat 80-kDa homologue responds as well to peroxisomal proliferators such as clofibrate and WY 14,643 (39, 46). The 80-kDa protein seems to be controlled by modulators of steroid and fatty acid metabolism. The high level of conservation of amino acid sequence (85% identity) between human, mouse, rat, and porcine 80-kDa proteins suggests an essential function of this type of protein.

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**Note:**
- [a] ND, activity not detected.
- [b] VHF, purified very hydrophobic fraction of the 17β-hydroxysteroid dehydrogenase type IV.
