0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 7
Printed in U.S.A.

Characterization of Molecular and Catalytic Properties of Intact and Truncated Human 17β-Hydroxysteroid Dehydrogenase Type 2 Enzymes: Intracellular Localization of the Wild-Type Enzyme in the Endoplasmic Reticulum*

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ABSTRACT

Human 17β-hydroxysteroid dehydrogenase (17HSD) type 2 is a widely distributed enzyme that primarily converts the highly active 17β-hydroxysteroids to their inactive keto forms. In the present study, full-length human 17HSD type 2 was localized in the endoplasmic reticulum using a double immunofluorescence labeling technique. As a consequence of its strong membrane interaction, full-length human 17HSD type 2 could not be solubilized as a biologically active form *in vitro*. However, by deleting the first 29 amino acids from the N-terminus, we were able to purify a catalytically active enzyme from the cytosolic fraction of Sf9 insect cells. Biochemical and catalytic properties of the purified truncated human 17HSD type 2 protein confirm its suitability for structure-function analyses of the enzyme. Both intact and truncated 17HSD type 2 enzymes efficiently catalyzed the oxidation of estradiol, testosterone, dihydrotestosterone, androstenediol, and 20α-dihydroprogesterone. The oxidation of estradiol brought about by human 17HSD type 2 was effectively inhibited by several other steroidal compounds, such as 2-hydroxyestradiol, 5β-androstan-3α,17β-diol, 5α-androstan-3α,17β-diol, and 5α-androstan-3β,17β-diol. The broad substrate specificity of human 17HSD type 2 together with its predominant oxidative activity and intracellular location, as observed in this study, indicate the physiological role of the enzyme to be primarily an inactivator of highly active 17β-hydroxysteroids. (*Endocrinology* **140:** 3334–3341, 1999)

THE INTERCONVERSIONS between neutral and phenolic 17-oxo- and 17β-hydroxysteroids are catalyzed by a number of 17β-hydroxysteroid dehydrogenases (17HSDs) that differ in their tissue distribution, substrate and cofactor specificities, and subcellular localization (1–7). Each of the 17HSD enzymes possesses almost unidirectional activity; types 1 and 3 catalyze reductive reactions of sex steroids, whereas types 2, 4, 5, and 6 are responsible for oxidative pathways. Human 17HSD type 2 converts the highly active 17β-hydroxysteroids, such as estradiol (E_2), testosterone (T), and dihydrotestosterone (DHT), to their inactive keto forms (3, 8, 9). Furthermore, studies carried out *in vitro* indicate that 17HSD type 2 is able to use C_{20}-steroids as substrates, namely by catalyzing the oxidation of 20α-dihydroprogesterone (20α-P) to progesterone (P) (3). The expression of human 17HSD type 2 messenger RNA (mRNA) has been detected in a large variety of tissues. Its 1.5-kb mRNA is strongly expressed in the endometrium, placenta, liver, and small intestine, and in smaller amounts in the pancreas, colon, kidney, and prostate (8–10). 17HSD type 2 mRNA has also been found to be present in human breast, endometrial and prostate cancer cell lines (8, 9). The broad tissue distribution together with the predominant oxidative activity of 17HSD type 2 suggest that the enzyme may play an essential role in the inactivation of highly active 17β-hydroxysteroids from the blood circulation, thus diminishing sex hormone action in target tissues.

Although there are several reports concerning 17HSD type 2 mRNA and the protein translated *in vitro*, studies involving purified type 2 protein have not been reported. The primary structure of human 17HSD type 2 possesses two putative signal sequences and a putative transmembrane region consisting of 30 hydrophobic amino acids close to the N-terminus (3). The enzyme is, therefore, thought to be associated with the endoplasm reticulum (ER). In the present study, precise intracellular localization of human 17HSD type 2 was

Received October 26, 1998.
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* This work was supported by the Research Council for Health of the Academy of Finland (Projects 3314 and 30099), the Technology Development Center of Finland (TEKES, Project 4476), and Dr. Saal van Zwanenbergstichting, Organon (Oss, The Netherlands). The WHO Collaborating Centre for Research on Reproductive Health is supported by the Ministries of Education, Social Affairs and Health, and Foreign Affairs of Finland.
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determined using a double immunofluorescence labeling technique. The substrate specificity of the enzyme was also analyzed in some detail. In addition, by studying the structural elements needed for both membrane interaction and enzyme activity, we were able to characterize the biochemical properties of an active truncated form of human 17HSD type 2 that lacks strong association within the membrane.

Materials and Methods

Construction of human 17HSD type 2 expression vectors

The *Not*I-*Sal*I fragment of full-length human 17HSD type 2 complementary DNA (cDNA) (3) was amplified by PCR using the flanking primers 5′-ATATAGCGGCCGCATATGAGCACTTTCTTCTCG-3′ and 5′-ATATAGTCGACTAGGTGGCCTTTTTCTT-3′. To generate a set of cDNA constructs of human 17HSD type 2 with N- or C-terminal deletions of varying lengths (Fig. 1) by PCR, intact cDNA was used as a template. All synthesized PCR products were then cloned into the *Not*I and *Sal*I sites of the pK503–9 vector (11, 12), which is a FLAG sequence containing transfer vector of the Bac-to-Bac Expression System (Life Technologies, Grand Island, NY).

To express intact and truncated forms of human 17HSD type 2 enzymes in a mammalian cell system, a pcDNA3 vector (Invitrogen, San Diego, CA) was modified by inserting the FLAG-coding sequence immediately after the ATG translation initiation codon. The construction strategy, described in detail in Fig. 2, resulted in a modified pcDNA3 vector that allowed the cloning of *Not*I-*Sal*I human 17HSD type 2 cDNA fragments into the *Not*I-*Xho*I sites of the vector to obtain amino-terminal FLAG fusion proteins. All of the expression constructs generated were confirmed by sequencing (13).

Expression and purification of human 17HSD type 2 protein

Recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPVs) for pK503–9 constructs coding for intact human 17HSD type 2 or enzymes lacking the first 29 and the first 49 amino acids of the N-terminus (N-29 17HSD type 2 and N-49 17HSD type 2) were generated using the Bac-to-Bac Expression System in *Spodoptera frugiperda* (Sf9) cells. The recombinant proteins were produced by infecting exponentially growing Sf9 cells with 17HSD type 2 AcNPVs at a multiplicity of infection of 1 in 1000-ml Spinner flasks (Techne, Cambridge, UK) as described previously (14, 15).

Production of the truncated N-29 17HSD type 2 enzyme was scaled up to a 30-liter bioreactor (Biostat UD 30, B. Braun International, Melsungen, Germany). The optimal level of expression was reached at about 72 h postinfection, whereafter the Sf9 cells were harvested by centrifugation at 1,000 × *g* for 10 min and stored at −70 C. To purify the fusion protein, harvested cells were suspended in buffer A [50 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 0.5 mM phenylmethylsulfonylfluoride, 0.02% NaN_3, 20% glycerol, 14 mM 2-mercaptoethanol, and 0.5% *n*-octylglucoside] and then disrupted in an ice bath by sonication (four times, 20 sec each time, 0.5-min intervals). The protein was then immunoprecipitated from the 100,000 × *g* fraction of the cell lysate using an anti-FLAG M1 affinity chromatography column (Eastman Kodak Co., New Haven, CT), according to the manufacturer's instructions. The fractions containing the highest 17HSD type 2 concentrations, detected by SDS-

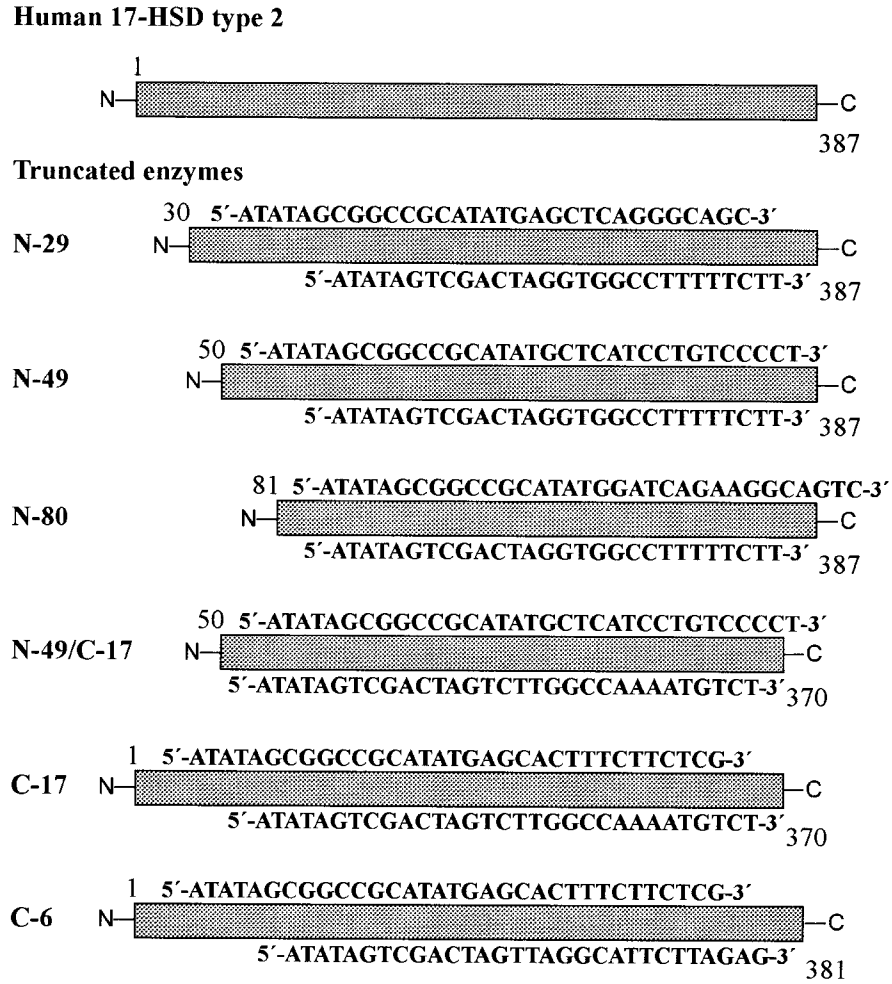

FIG. 1. Structures of the truncated 17HSD type 2 enzymes constructed and flanking primers used in PCR. N-29, N-49, and N-80 consisted of deletions of 29, 49, and 80 N-terminal amino acids of human 17HSD type 2, respectively. N-49/C-17 truncated enzyme lacked the first 49 N-terminal residues together with the last 17 amino acids from the carboxyl-terminal region. C_{17} and C_6 truncated enzymes were constructed by deleting the last 17 and 6 C-terminal amino acids, respectively.

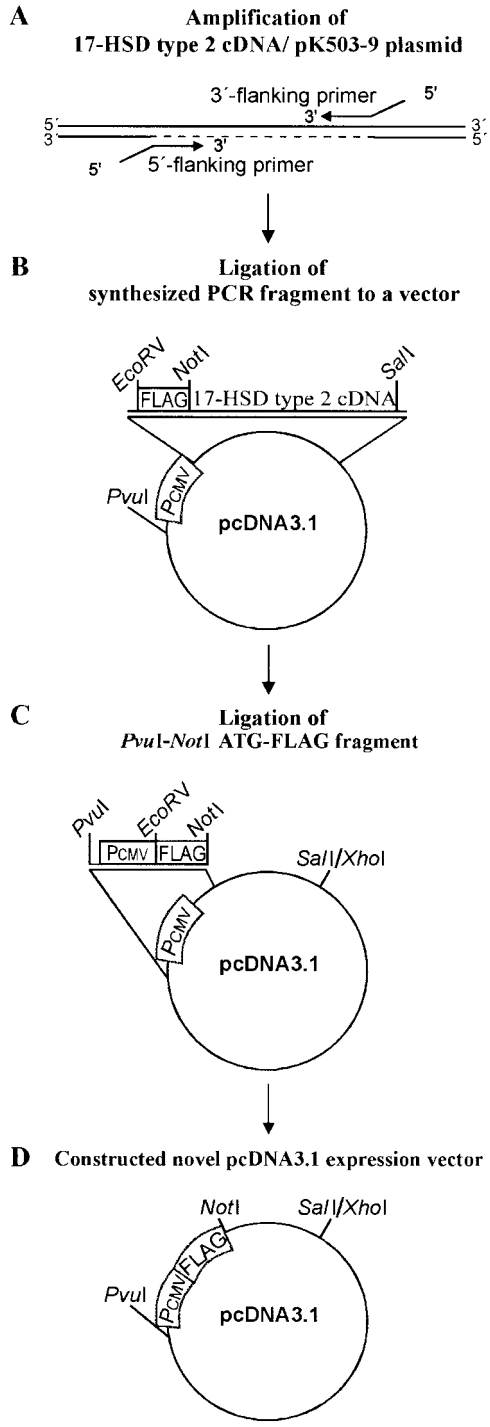

FIG. 2. Strategy for the construction of the FLAG sequence-containing pcDNA3.1 expression vector. A, FLAG-17HSD type 2 cDNA was generated by PCR with flanking primers (5′-ATATAGCTAGCGATATCATGGACTACAAGGACGACGA-3′ and 5′-ATATAGTCGACTAGGTGGCCTTTTCTT-3′), using full-length human 17HSD type 2/pK503–9 plasmid as a template. B, The amplified PCR fragment with *Eco*RV and *Sal*I ends was then ligated to the pcDNA3 vector. C, The ATG-FLAG coding sequence from the constructed plasmid was further cloned into *Pvu*I-*Not*I sites of a novel pcDNA3 expression vector. D, The *Not*I-*Xho*I sites of the constructed pcDNA3 vector could be used as a cloning site for the *Not*I-*Sal*I human 17HSD type 2 cDNA fragments to express amino-terminal FLAG fusion proteins in eukaryotic cells.

PAGE, were pooled, concentrated, and loaded onto a Superose 12 gel filtration column (1.0 × 30 cm; 0.2 ml/min) connected to a fast protein liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden). N-29 17HSD type 2 protein was eluted with 50 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl, 0.02% NaN_3, 20% glycerol, and 14 mM 2-mercaptoethanol. The pure protein peak was collected and stored at −70 C.

The protein concentration of the purified N-29 17HSD type 2 was measured by the method of Bradford *et al.* (16). The purified N-29 protein was further characterized by 11% SDS-PAGE carried out on a Mini-PROTEAN II apparatus (Bio-Rad Laboratories, Inc., Richmond, CA). The proteins were visualized in the electrophoresis gels by 0.1% Coomassie blue staining.

Measurement of 17HSD type 2 activity in vitro and in cultured cells

The activity of 17HSD was measured *in vitro* as previously described by Puranen *et al.* (12, 28) with minor modifications. Protein samples diluted in 10 mM potassium phosphate, pH 7.5, containing 0.01% BSA and 14 mM 2-mercaptoethanol were mixed with ^{3}H-labeled E_2, estrone (E_1), T, androstenedione (A-dione), DHT, 5α-androstenediol (A-diol), dehydroepiandrosterone (DHEA), 20α-P, or P to a final concentration of 0.5–4 μmol/liter. The reactions were initiated by adding NAD^+/NADH (1.0 μmol/liter) as a cofactor, followed by incubation for 1 min at 37 C. After incubation, the reactions were stopped by immediate freezing in an ethanol-dry ice bath, and the steroids were extracted into diethyl ether-ethyl acetate (9:1). Substrates and reaction products were separated using an acetonitrile-water (48:52, vol/vol) solution as a mobile phase in a Symmetry C_{18} reverse phase chromatography column (3.9 × 150 mm) connected to a HPLC system (Waters Corp., Milford, MA), as previously described (15, 17). K_m and k_{cat} values of 17HSD type 2 were calculated using a GraFit program (Erithacus Software Ltd., Staines, UK). The kinetic values shown represent the average ± SD of at least three independent experiments carried out in triplicate. One micromole of product formed per min was defined as representing 1 U enzyme activity.

For comparing the catalytic properties of the truncated 17HSD type 2 enzymes with those of the wild-type enzyme, activity measurements in cultured human embryonic kidney 293 cells were carried out by plating the cells at a density of 1.2×10^6 cells/10-cm diameter petri dish in DMEM (Life Technologies) supplemented with 10% FCS. The cells were allowed to attach overnight and were then transfected with the intact and truncated forms of 17HSD type 2 cDNA constructs (5 μg DNA/1.2×10^6 cells) using a lipofection-based transfection method (DOTAP, Boehringer Mannheim, Mannheim, Germany). Transfection was carried out for 18 h, 10 ml fresh medium were added, and incubation was continued for an additional 48 h. Transfected cells were then plated into 6-well plates at a density of 200,000 cells/well and allowed to attach for 24 h. Activity measurements of the intact and truncated human 17HSD type 2 enzymes were performed by applying 2 ml serum-free medium containing 0.2 μM ^{3}H-labeled E_2 (0.4 nmol/well) to the cells. Oxidative 17HSD activity, from E_2 to E_1, was measured at four different time points (0.5–4 h). After incubation, the media were collected, frozen in dry ice, and kept at −20 C before extracting and separating the steroids (see above).

For analyzing the substrate specificity of the wild-type 17HSD type 2 enzyme, full-length cDNA was transiently expressed in 293 cells under the cytomegalovirus promoter using pCMV6 vector (9). With E_2, E_1, T, A-dione, DHT, A-diol, DHEA, 20α-P, and P as substrates, 17HSD activity was measured using a 0.2-μM concentration of the ^{3}H-labeled substrate at three different time points (1–4 h). Furthermore, the substrate specificities of the intact human 17HSD type 2 expressed in 293 cells and the purified recombinant N-29 17HSD type 2 were analyzed by studying the ability of several steroidal molecules (Steraloids, Inc., Wilton, NY) to compete with 0.2 and 0.5 μM [^{3}H]E_2, respectively. Steroidal compounds were added at final concentrations of 1 and 10 μmol/liter, using an incubation time of 2 h in the experiments of the intact 17HSD type 2 enzyme and 1 min in the assays of recombinant N-29 17HSD type 2 enzyme. The amount of converted E_2 in the experiments of the intact 17HSD type 2 was analyzed in triplicate samples in at least three independent experiments and in the assays of recombinant N-29 17HSD

type 2 enzyme in duplicate samples in two or three independent experiments.

Immunoblotting

The amounts of wild-type and truncated forms of human 17HSD type 2 protein expressed in 293 cells were detected by immunoblotting. The proteins from cell homogenates were separated by SDS-PAGE (Mini-PROTEANII, Bio-Rad Laboratories, Inc.). The proteins were then transferred to an Immobilon-P polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA) and immunostained using the enhanced chemiluminescence detection system (Amersham, Aylesbury, UK). Monoclonal M5 antibody raised against the sequence of Met-FLAG peptide (Eastman Kodak Co., Rochester, NY) was used as primary antibody.

The immunoblotting of the recombinant N-29 17HSD type 2 fractions during the purification process was carried out as described above. The immunostaining was performed with the mouse monoclonal M2 antibody raised against FLAG-peptide (Eastman Kodak Co.) and the ProtoBlot AP system (Promega Corp., Madison, WI).

Immunocytochemistry

For subcellular localization of wild-type human 17HSD type 2 by indirect immunofluorescence, NIH-3T3 cells were seeded in a 6-cm diameter plate, 0.25×10^6 cells/plate. After overnight incubation, the cells were transiently transfected with 17HSD type 2 cDNA-pcDNA3 plasmid (10 μg DNA/$\sim 0.5 \times 10^6$ cells) using SuperFect transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The transfected NIH-3T3 cells were then grown for 48 h before plating them at a density of 2.0×10^4 cells/chamber of an eight-chamber slide (Nalge Nunc International, Naperville, IL). The cells were allowed to attach overnight before staining.

For double immunofluorescence labeling, the cells were briefly washed with PBS and fixed for 5 min in 100% methanol and for 2 min in 100% acetone at −20 C. The cells were washed with PBS and then permeabilized in 1% Triton X-100-PBS solution for 2 min. After blocking the nonspecific binding sites with 50% goat serum for 4 h at room temperature, the cells were incubated with anti-FLAG M5 monoclonal antibody (27 μg/ml) for 3 h. To analyze whether the fusion protein was colocalized with ER membranes, the cells were further incubated with either 1:50 diluted polyclonal antisera specific for the carboxyl-terminus of rat Grp78 (BiP) or for the C-terminal region of canine calnexin (StressGen Biotechnologies Corp., Victoria, Canada) at 4 C overnight. The cells were then washed with PBS and incubated for 2 h at room temperature, first with rhodamine-labeled donkey antirabbit IgG antibodies (20 μg/ml) and then with fluorescein-labeled goat antimouse IgG antibodies (20 μg/ml). The cells were then washed with PBS and mounted on slides with Glycergel mounting medium (DAKO Corp., Glostrup, Denmark). The double immunofluorescence labeled cells were viewed using a confocal laser scanning microscope (CLSM, revision 4.0, Leica Corp. Lasertechnic, Heidelberg, Germany) as described previously (18, 19). The instrument is built around a Nikon Optiphot-2 epifluorescence microscope (Nikon, Tokyo, Japan).

Results

Catalytic properties of N- and C-terminal deletion constructs of human 17HSD type 2

We found that full-length human 17HSD type 2 is expressed in fairly low amounts in Sf9 insect cells. Furthermore, it could not be solubilized as a biologically active form *in vitro*, suggesting strong membrane interaction of the enzyme. To obtain an active soluble enzyme suitable for large scale protein production and purification, a set of cDNA constructs coding for 17HSD type 2 with N- and C-terminal deletions of varying lengths was generated. Activity measurement in cultured 293 cells showed that under the conditions used, wild-type 17HSD type 2 converted 46.5% of E_2 (200 nM) to E_1 in 2 h (Fig. 3). In similar conditions, the truncated N-49 enzyme catalyzed 51.4% conversion of E_2, indicating that deletion of the first 49 N-terminal amino acids did not affect the catalytic properties of the enzyme. Corresponding conversions brought about by the truncated type 2 enzymes lacking the first 29 (N-29) or last 6 carboxyl-terminal (C-6) amino acids were 29.4% and 26.5%, respectively. Thus, these enzymes catalyzed the oxidative reaction of E_2 at an approximately 0.6-fold lower rate than that found for the intact enzyme. However, deleting the first 80 amino-terminal residues (N-80) or the first 49 amino-terminal residues simultaneously with the last 17 amino acids from the C-terminus (N-49/C-17) resulted in totally inactive enzymes (Fig. 3). The C-17 truncated form converted 1.3% of E_2 in 2 h (Fig. 3), also indicating complete loss of activity. Conversions of E_2 to E_1 for the intact and truncated 17HSD type 2 enzymes were calculated according to the amount of protein expressed in transfected 293 cells measured by Western blotting (data not shown). The solubility of the catalytically active truncated (N-29, N-49, and C-6) 17HSD type 2 enzymes was then analyzed using several ionic and nonionic detergents. In optimal conditions, 50% of the N-29 truncated type 2 enzyme could be solubilized in the presence of 0.5% *n*-octylglucoside detergent.

Biochemical and catalytic properties of purified N-29 17HSD type 2

Two constructs coding for soluble and biologically active truncated forms of human 17HSD type 2 (N-29 and N-49 17HSD types) were expressed in the baculovirus expression system (14, 15). N-29 17HSD type 2 was produced in Sf9 cells in greater amounts than the N-49 truncated form; thus, N-29 17HSD type 2 was chosen for large scale protein production. The protein was purified from the cytosolic fraction of Sf9 cell

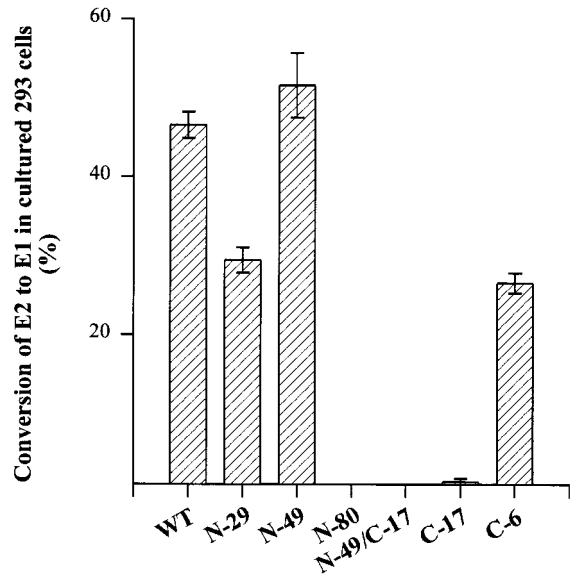

FIG. 3. Estrogenic activity of wild-type and truncated 17HSD type 2 enzymes expressed in 293 cells. Activity measurements of wild-type and deletion constructs of human 17HSD type 2 were performed using cultured 293 cells in six-well plates by adding 200 nM ^{3}H-labeled E_2 (0.4 nmol/well). After 2-h incubation, the amounts of E_1 formed were measured in triplicate specimens. Conversion of E_2 to E_1 was calculated according to the amount of enzyme expressed. Measurements were repeated at least three times, with similar results.

lysate in a two-step procedure. From the 30-liter batch culture of Sf9 cells, 15 mg pure N-29 17HSD type 2 protein were obtained. Analysis by SDS-PAGE revealed that the molecular mass of the monomers of the enzyme was approximately 40 kDa (Fig. 4), very close to that calculated (41.1 kDa). The catalytic properties of purified N-29 type 2 enzyme were characterized *in vitro* (Table 1). The K_m values were similar when using E_2, T, 20α-P, DHT, and A-diol as substrates (Table 1), indicating broad substrate specificity of the enzyme. The catalytic efficiencies (k_{cat}/K_m) indicated that the enzyme catalyzed the oxidative reactions of estrogens and androgens 10-fold more efficiently compared with the values measured for the corresponding reductive reactions *in vitro* (Table 1). Accordingly, the results of activity measurements performed in cultured 293 cells transiently transfected with intact 17HSD type 2 showed that the type 2 enzyme possesses exclusive oxidative activity by equally well converting E_2, T, DHT, and A-diol to their inactive counterparts (Fig. 5). However, the enzyme catalyzed the oxidative reaction from 20α-P to P approximately 2-fold less efficiently. This is in line with results obtained after activity measurement *in vitro*, which indicated that the catalytic efficiency of the purified N-29 enzyme with regard to oxidation of 20α-P was 16-fold lower compared with the corresponding estrogenic reaction (Table 1). We next carried out extensive analysis to characterize the

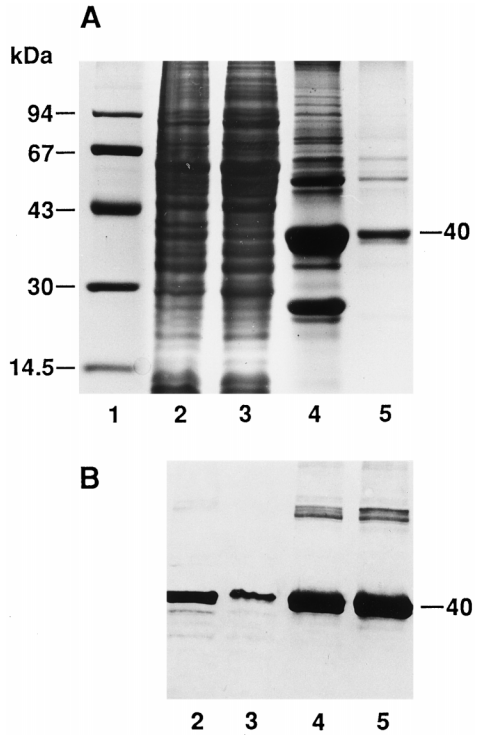

FIG. 4. Enrichment of N-29 17 HSD type 2 protein during purification process. After electrophoretic separation on 11% SDS-PAGE, the gel was stained with Coomassie blue (A) and immunostained with monoclonal M2 antibody raised against FLAG-peptide (B). Lane 1, Low mol wt marker; lane 2, Sf9 cell lysate; lane 3, 100,000 $\times g$ fraction of Sf9 cell lysate; lane 4, protein from anti-FLAG M1 affinity column; lane 5, N-29 17HSD type 2 from S12 gel filtration column.

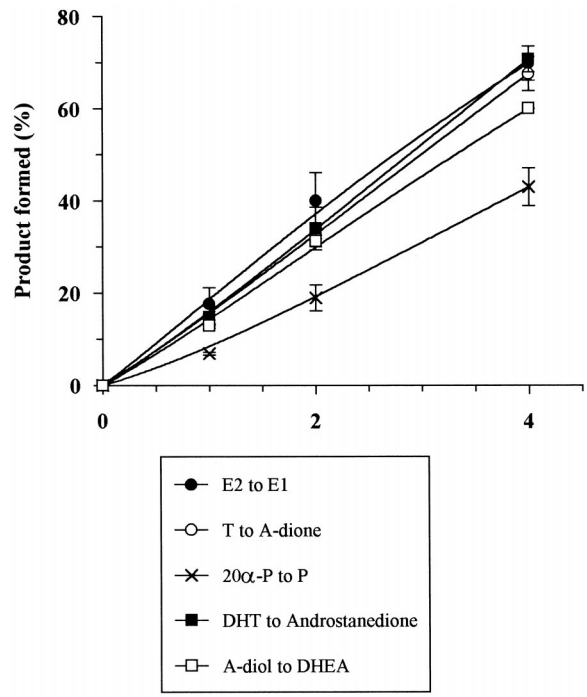

FIG. 5. Substrate specificity of human 17HSD type 2 expressed in 293 cells. E_2, E_1, T, A-dione, DHT, A-diol, DHEA, 20α-P, and P were used as substrates to analyze the substrate specificity of the human type 2 enzyme. To assess the linearity of the reactions, the activities were measured at three different time points (1, 2, and 4 h), using triplicate sampling. Measurements were repeated three times with similar results, and the activities presented represent typical reaction curves. Means and SDs are shown.

TABLE 1. Activity measurements of N-29 truncated 17-HSD type 2 enzyme *in vitro*

Substrate	Product	K_m (μM)	k_{cat} (U[a]/mg)	k_{cat}/K_m
Oxidative activity				
Estradiol	Estrone	0.35 ± 0.09	2.22 ± 0.59	6.4
Testosterone	Androstenedione	0.53 ± 0.19	1.52 ± 0.41	2.9
Dihydrotestosterone	Androstanedione	0.24 ± 0.03	1.50 ± 0.52	6.2
Androstenediol	Dehydroisoandrosterone	0.54 ± 0.11	1.37 ± 0.41	2.5
20α-Dihydroxyprogesterone	Progesterone	0.86 ± 0.26	0.34 ± 0.13	0.4
Reductive activity				
Estrone	Estradiol	0.51 ± 0.07	0.32 ± 0.05	0.6
Androstenedione	Testosterone	1.54 ± 0.15	0.50 ± 0.05	0.3
Dehydroisoandrosterone	Androstenediol	0.80 ± 0.19	0.20 ± 0.06	0.3
Progesterone	20α-Dihydroxyprogesterone	0.89 ± 0.26	0.04 ± 0.03	0.04

The concentration of the purified 17-HSD type 2 enzyme was measured by a protein assay from Bio-Rad Laboratories, Inc. The results are presented as the mean ± SD of at least three independent experiments measured in triplicate.

[a] One micromole of product per min was defined as 1 U enzyme activity.

substrate specificity of human 17HSD type 2, using a series of steroidal compounds that were tested for their ability to compete with E_2 as a substrate in 293 cells transfected with intact human enzyme and in assays with the purified recombinant N-29 17HSD type 2 (Table 2). Strong inhibition of type 2 enzyme activity was measured when using 2-hydroxyestradiol, 5β-androstan-3α,17β-diol, 5α-androstan-3α,17β-diol, and 5α-androstan-3β,17β-diol as competitive molecules (Table 2). These compounds inhibited the conversion of E_2 to E_1 to around the same degree as did T, A-diol, and 20α-P (Table 2), which are known to possess high specificity toward 17HSD type 2 (Fig. 5). In addition, 17α-estradiol, ethynyl estradiol, 17β-dihydroequilin, and coumestrol had significant inhibitory effects on the oxidation of E_2 catalyzed by the human type 2 enzyme. In contrast, no such inhibition was observed for glucocorticoids or progestins, except for 20α-P (Table 2).

TABLE 2. Inhibition by various steroidal compounds of oxidation of 0.2 μM estradiol brought about by 17-HSD type in cultured 293 cells (A) and 0.5 μM estradiol brought about by purified N-29 enzyme (B)

Steroid	Ic (μM)[a]	Inhibition (% ± SD)	
		A	B
17α-Estradiol	1	7.2 ± 1.9	25.2 ± 11.3
	10	31.1 ± 1.4	52.5 ± 7.5
Estriol	1	3.2 ± 14.0	11.5 ± 10.0
	10	20.3 ± 8.3	38.4 ± 13.8
Ethynylestradiol	1	19.1 ± 7.7	29.6[b]
	10	54.0 ± 4.2	65.9 ± 15.5
17β-Dihydroequilin	1	37.2 ± 9.8	ND
	10	88.4 ± 1.6	ND
2-Hydroxyestradiol	1	1.1 ± 6.1	14.4[b]
	10	66.9 ± 2.0	62.2[b]
Coumestrol	1	8.5 ± 18.9	14.7[b]
	10	49.7 ± 4.9	43.4[b]
Apigenin	1	—[d]	15.7[b]
	10	33.7 ± 8.1	66.6[b]
Testosterone	1	6.0 ± 8.3	24.1[b]
	10	69.6 ± 4.0	71.4[b]
Androstenediol	1	37.6 ± 4.8	40.2[b]
	10	82.6 ± 2.0	75.5[b]
5β-Androstan-3α, 17β-diol	1	25.8 ± 2.6	60.1 ± 4.7
	10	82.7 ± 2.3	91.6 ± 3.0
5α-Androstan-3α, 17β-diol	1	35.5 ± 8.8	59.7 ± 4.2
	10	87.1 ± 2.0	91.8 ± 4.1
5α-Androstan-3β,17β-diol	1	24.2 ± 11.8	57.0 ± 4.2
	10	54.6 ± 5.6	82.3 ± 1.1
Cortisol	1	—[d]	ND
	10	—[d]	ND
Cortisone	1	—[d]	ND
	10	—[d]	ND
Dexamethasone	1	—[d]	8.5 ± 9.1
	10	—[d]	11.3 ± 6.3
Prednisone	1	—[d]	9.8 ± 6.1
	10	—[d]	14.5 ± 7.8
Prednisolone	1	9.2 ± 8.3	5.2 ± 5.8
	10	11.2 ± 8.8	18.9 ± 2.3
20α-Dihydroxyprogesterone	1	13.6 ± 6.7	23.8 ± 5.8
	10	70.7 ± 1.5	72.6 ± 0.8
17-Hydroxypregnenolone	1	1.2 ± 0.9	5.7 ± 2.9
	10	—[d]	6.1 ± 1.1
Pregnenolone	1	4.2 ± 2.8	ND
	10	23.9 ± 1.5	ND
Medroxyprogesterone	1	1.7 ± 12.7	ND
	10	16.3 ± 6.4	ND

[a] Inhibitor concentration used in the experiments.
[b] Average from two independent experiments with duplicate samples.
[c] ND, Not detected.
[d] No inhibitory effect.

Immunocytochemical localization of human 17HSD type 2

NIH-3T3 cells transiently transfected with FLAG-tagged human 17HSD type 2 cDNA were used in double immunofluorescence labeling experiments to demonstrate the subcellular distribution of 17HSD type 2 in relation to the ER. For this purpose, cells were treated with anti-FLAG M5 monoclonal antibodies and then with ER-specific polyclonal antibodies raised against the carboxyl-terminus of either rat Grp78 (BiP) or canine calnexin. Confocal microscopic analyses of the double immunofluorescence labeled samples revealed precise colocalization of human 17HSD type 2 with ER (Fig. 6, A–C). The results were identical regardless of which of the two ER-specific antisera (BiP or calnexin) were used for the experiments. Interestingly, colocalization was observed all over the cytoplasm, although the most intense staining pattern was found in ER located next to the cell nuclei. To control the specificity of the immunoreactions, primary antibodies were used to stain nontransfected NIH-3T3 cells (Fig. 6, D–F), or they were omitted from some experiments (data not shown). In both cases, no visible staining pattern was observed, suggesting that the primary antibodies recognize their targets specifically.

Discussion

The primary structure of human 17HSD type 2 shows that it possesses 33 nonpolar amino acids close to its N-terminus that may act as a putative transmembrane region (3). The present results show that human type 2 protein could not be solubilized as a biologically active form despite the use of several detergents tested, which further supports the hypothesis that human 17HSD type 2 is an integral membrane protein. Previous results have also suggested that the N-terminal KYKK sequence at positions 26–29 in intact human 17HSD type 2 serves as a putative membrane insertion sequence, resulting in an N_{cyto}/C_{lumen} orientation of the protein, whereas the putative ER retention signal at the extreme C-terminus (amino acids 383–387) might be important for holding the enzyme in the ER (3, 20). The data presented here show that after deleting the first 29 amino acids from the N-terminus, the human enzyme still retains its catalytic activity. Furthermore, N-29 17HSD type 2 was also found to be more soluble than the full-length enzyme. This indicates that the first 29 amino-terminal residues are not functionally important, but facilitate membrane interaction of the enzyme. In this truncated protein, part of the putative transmembrane region is deleted. An enzyme lacking the first 80 residues of its N-terminus was, however, found to be totally inactive, suggesting that the last part of the transmembrane region (amino acids 50–80) is critical for the function of the enzyme. Our data also indicate that deletion of the hydrophilic lysine-rich retention motif (KKKAT) at the carboxyl-terminus did not have a significant effect on the catalytic properties of the enzyme. However, the amino acids between 371–381 seem to crucial for full activity of the enzyme. Interestingly, a PRALR

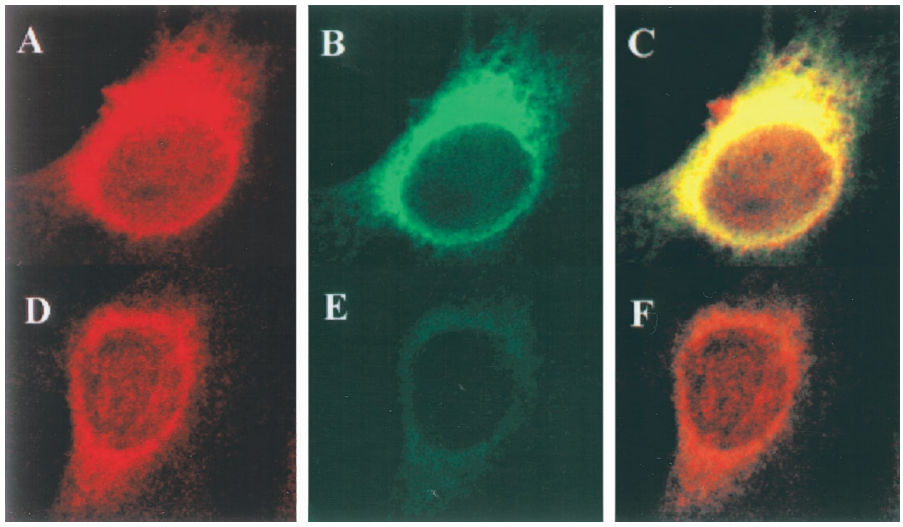

FIG. 6. Intracellular localization of human FLAG-tagged 17HSD type 2 in transiently transfected NIH-3T3 cell using confocal laser scanning microscope. A–C show the NIH-3T3 cell transiently transfected with FLAG-tagged human 17HSD type 2 cDNA; D–F show the nontransfected control cell. NIH-3T3 cells were double immunofluorescence-labeled using ER-specific polyclonal antisera against BiP protein followed by rhodamine-conjugated donkey antirabbit IgG (*left panels*, A and D) and with anti-FLAG M5 monoclonal antibody followed by FITC-conjugated goat antimouse IgG (*middle panels*, B and E). Panels on the *right* represent computer-generated combined optical sections of either transiently transfected (C, obtained by combining A and B) and nontransfected control NIH-3T3 cell (F, obtained from D and E). *Yellow color* demonstrates the colocalization of the two stainings.

sequence that is strictly conserved among the human, rat, and mouse 17HSD type 2 enzymes (3, 20, 21) is located in this region, suggesting that this motif may be functionally essential for the enzymes. On the other hand, it has been suggested that this conserved amino acid sequence could also have a role in translocation of the enzyme to the ER membrane (22).

As antibodies to 17HSD type 2 were not available, cDNA constructs encoding a FLAG-tagged full-length human protein were transiently expressed in NIH-3T3 cells to allow localization of the enzyme by immunocytochemical techniques. The present results reveal that recombinant N-FLAG/17HSD type 2 is colocalized with Grp78 (BiP), a soluble protein present in the lumen of the ER (23, 24). This indicates that the human 17HSD type 2 protein, when expressed in NIH-3T3 cells, is localized in the ER, and that the N-terminal lysine-rich signal peptide is not cleaved out during the insertion process. This, together with the poor solubility of the enzyme, further suggest that human 17HSD type 2 is a transmembrane protein in the endoplasmic reticulum. Our finding is consistent with previous results reported by Wu *et al.* (3), who showed that expression of the enzyme *in vitro* was stimulated by microsomes, and that the translated protein cofractionated with the membrane pellet. However, the exact subcellular localization of the enzyme has remained unclear. The intracellular ER location of human 17HSD type 2 allows the enzyme to rapidly and efficiently inactivate high activity 17β-hydroxysteroids before they are able to occupy nuclear steroid receptors.

To avoid the difficulties in purification associated with intact 17HSD type 2, we produced a truncated form of the enzyme lacking the first 29 amino-terminal residues. Activity measurements of the purified N-29 enzyme carried out *in vitro* showed that it possesses high specificity toward C_{18}- and C_{19}-steroids, but it is also able to catalyze, although at a lower reaction rate, interconversion between C_{20}-steroids. Similarly, the intact type 2 enzyme catalyzes the oxidation of E_2, T, A-diol, DHT, and 20α-P in cultured cells. In addition to the broad substrate specificity, both truncated and intact 17HSD type 2 enzymes possess predominant oxidative activities, converting highly active 17β-hydroxysteroids into less potent ketosteroids. The results obtained in the activity measurements indicate identical catalytic properties of the truncated and intact forms of 17HSD type 2, and thus confirm the suitability of the constructed N-29 enzyme for structure-function analyses. Furthermore, the purification protocol described here results in a highly homogeneous protein, allowing us to scale up protein production, which is needed for extensive crystallization studies. As the structure of human 17HSD type 1 according to x-ray crystallography has been recently resolved (25–27), elucidation of the three-dimensional structure of the type 2 protein would be useful to study more closely the structural features of the enzymes that allow them to use different steroids as substrates and to catalyze opposite reactions.

Based on the present findings, human 17HSD type 2 shows a wider specificity for androgenic substrates than for estrogens or progestins. Our findings and those of others (3, 8, 28) indicate that by inactivating the most active prostate-specific androgens, T, DHT, and A-diol, 17HSD type 2 could play a central role in protecting prostatic cells from excessive androgen action. Furthermore, the present results reveal the effective inhibition of oxidation of E_2 by several other androgens present in the prostate and urine, such as 5β-androstan-3α,17β-diol, 5α-androstan-3α,17β-diol, and 5α-androstan-3β,17β-diol. This suggests that the enzyme could also diminish androgen action in the prostate by metabolizing intermediary steroids as well. The metabolic pathways of these compounds could also be physiologically important in epithelial cells of the gastrointestinal and urinary tracts,

where strong 17HSD type 2 mRNA expression has been detected (29). The results showed that 17β-dihydroequilin has a significant inhibitory effect on the activity of 17HSD type 2 when E_2 was used as a substrate. This compound is a metabolite of equilin, which is commonly used for estrogen replacement therapy and prevention of osteoporosis in postmenopausal women (30). Similarly, a contraceptive compound, ethynyl estradiol, is a potent substrate for 17HSD type 2. The data therefore indicate that the enzyme is capable of metabolizing several orally administered steroidal compounds possessing a tetracyclic ring and a hydroxy group at position C_{17}, at the epithelium of the intestine. This, in turn, is likely to be important with regard to the pharmacology of steroidal drugs. Human 17HSD type 2 can also use 2-hydroxyestradiol as a substrate, further suggesting that in addition to E_2, the enzyme could catabolize catechol estrogens in the liver. The recent finding that 17HSD type 2 mRNA is expressed in liver hepatocytes in both male and female mice (29) suggests that the enzyme has a role in the general hepatic inactivation of steroids. In conclusion, the predominant oxidative activity and broad substrate specificity of human 17HSD type 2 observed in this study indicate that this enzyme is primarily involved in diminishing the stimulatory effects of highly active 17β-hydroxysteroids, even in tissues not classified as traditional sex steroid target tissues. However, there is one exception, in that human 17HSD type 2 catalyzes the oxidation of 20α-P to a biologically more potent form (P), as also observed previously (3). Substrate preference of human 17HSD type 2 for several distinct steroidal molecules suggests easy accessibility and open conformation of the active site. The activity measurements showed that the recombinant N-29 17HSD type 2 has preserved the broad substrate specificity during the purification process. Based on the present findings, the purified N-29 truncated form of human 17HSD type 2 could be used for resolving the structure of the enzyme by x-ray crystallography, which would further allow detailed characterization of the structural motifs and amino acids related to the substrate specificity of the enzyme.

Acknowledgments

We thank Ms. Saara Korhonen, Ms. Pirkko Ruokojärvi, Ms. Marja-Liisa Norrena, and Ms. Marja-Riitta Hurnasti for their skillful technical assistance.

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