Expression Cloning and Characterization of Oxidative 17β- and 3α-Hydroxysteroid Dehydrogenases from Rat and Human Prostate

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Intracellular levels of active steroid hormones are determined by their relative rates of synthesis and breakdown. In the case of the potent androgen dihydrotestosterone, synthesis from the precursor testosterone is mediated by steroid 5α-reductase, whereas breakdown to the inactive androgens 5α-androstan-3α,17β-diol (3α-adiol), and androsterone is mediated by reductive 3α-hydroxysteroid dehydrogenases (3α-HSD) and oxidative 17β-hydroxysteroid dehydrogenases (17β-HSD), respectively. We report the isolation by expression cloning of a cDNA encoding a 17β-HSD6 isozyme that oxidizes 3α-adiol to androsterone. 17β-HSD6 is a member of the short chain dehydrogenase/reductase family and shares 65% sequence identity with retinol dehydrogenase 1 (RoDH), which catalyzes the oxidation of retinol to retinal. Expression of rat and human RoDH cDNAs in mammalian cells is associated with the oxidative conversion of 3α-adiol to dihydrotestosterone. Thus, 17β-HSD6 and RoDH play opposing roles in androgen action; 17β-HSD6 inactivates 3α-adiol by conversion to androsterone and RoDH activates 3α-adiol by conversion to dihydrotestosterone. The synthesis of an active steroid hormone by back conversion of an inactive metabolite represents a potentially important mechanism by which the steady state level of a transcriptional effector can be regulated.

The formation and growth of the prostate as well as other male reproductive organs is dependent on androgens, a class of C19 steroids that act through the androgen receptor to regulate gene expression. The two predominant androgens in this regard are testosterone, 1 which is formed in the testes from androstenedione by the action of a reductive 17β-hydroxysteroid dehydrogenase (17β-HSD3) enzyme (1), and dihydrotestosterone, which is formed from testosterone by steroid 5α-reductase isozymes and is the active androgen in the prostate (2). 5α-Reduction of testosterone is an irreversible reaction. Androgen action is terminated when these steroids are converted to metabolites that have little or no affinity for the androgen receptor. A major catabolic pathway involves the sequential conversion of dihydrotestosterone to 5α-androstan-3α,17β-diol (3α-adiol) and thereafter to androsterone (3). Both of these reactions are potentially reversible (see Fig. 1).

Under normal conditions, an equilibrium exists between androgen synthesis and breakdown and a steady state level of active hormone is maintained in target tissues. An imbalance between input and output can lead to androgen excess and contribute to certain disease states, especially benign prostatic hyperplasia and prostate cancer. Two mechanisms are postulated to produce excess dihydrotestosterone in the prostate. In one, an elevated expression of steroid 5α-reductase leads to increased synthesis of the hormone. In the other, a decrease in the breakdown of dihydrotestosterone occurs as a consequence of either reduced expression of a catabolic enzyme or by a change in the direction of the reaction. The latter mechanism is illustrated by the differential metabolism of 3α-adiol (Fig. 1). If a reductive 3α-hydroxysteroid dehydrogenase (3α-HSD) activity predominates in the prostate, then dihydrotestosterone is converted into 3α-adiol and thereafter into androsterone, which is readily glucuronidated (4) and excreted from the tissue. However, if an oxidative 3α-HSD activity predominates, then 3α-adiol arising within the tissue or from exogenous sources is converted back into the active androgen dihydrotestosterone (Fig. 1).

Evidence supporting that androgen excess can be caused by an increase in steroid 5α-reductase activity has been obtained from enzyme measurements and immunohistochemical analysis of benign prostatic hyperplastic and prostate cancer tissue (5, 6). The levels of steroid 5α-reductase activity and protein are increased in these tissues. Dihydrotestosterone can also be synthesized from 3α-adiol. When radiolabeled 3α-adiol is administered to castrated and functionally hepatectomized rats, it is converted into dihydrotestosterone and to a lesser extent into androsterone in the ventral prostate (7). The mechanism responsible for this conversion has not been elucidated, in part due to a paucity of information regarding androgen catabolizing enzymes in the prostate. Two closely related reductive 3α-HSD isozymes are expressed in the human prostate (8), and an oxidative 17β-HSD type 2 isozyme and mRNA are present in benign and malignant human prostate tissue (9) but the roles of these enzymes in the metabolism of dihydrotestosterone are not known.

To gain further insight into androgen catabolism in the prostate, we took an expression cloning approach to identify gene products capable of metabolizing 3α-adiol. These experiments identified an oxidative 17β-HSD isozyme that converts 3α-adiol to androsterone and whose mRNA is expressed in the rat ventral prostate and liver. The encoded protein, referred to as 17β-HSD6, shares extensive sequence homology with retinol dehydrogenase 1 (RoDH1) (10), which led us to test the hypothesis that RoDH1 might also metabolize steroid hormones. Ex-
pression studies with rat and human RoDH cDNAs show that RoDH can also serve as an oxidative 3α-HSD that efficiently catalyzes the back reaction of 3α-adiol to dihydrotestosterone.

EXPERIMENTAL PROCEDURES

Expression Cloning—Oligo(dT)-primed cDNA was synthesized from rat ventral prostate poly(A)⁺-enriched RNA (5 μg) using a SuperScript cDNA synthesis kit (Life Technologies, Inc., Bethesda, MD). cDNAs were ligated to SalI adaptors, size-fractionated by gel filtration, and unidirectionally ligated into SalI-NotI digested pCMV6 vector (11). A 20-μl ligation mixture was divided into 0.1-μl aliquots, which were individually transformed into 100 μl of MAX Efficiency DH5α competent cells (Life Technologies, Inc.), yielding pools of 5,000 independent recombinants per transformation. The transformation mixture (1 ml) was diluted by the addition of 3 ml of Luria-Bertani medium supplemented with ampicillin (100 μg/ml). After an overnight incubation at 37 °C, plasmid DNA was isolated from the bacterial cells by column purification (Promega Mini-Prep, Madison, WI) and transfected into mammalian cells.

Pools of cDNAs were transfected into human embryonic kidney 293 cells (ATCC CRL number 1573) by the calcium phosphate method using an MBS Transfection kit (Stratagene, La Jolla, CA). The 293 cells were grown in monolayer at 37 °C in an atmosphere of 8–9% CO₂ and maintained in medium A (Dulbecco’s modified Eagle’s medium containing 100 units/ml of penicillin and 100 μg/ml streptomycin sulfate and supplemented with 10% (v/v) fetal calf serum). On day 0 of a transfection experiment, 700,000 cells were plated in medium A per 50-mm dish. On day 2, cells were cotransfected with 4.5 μg of plasmid cDNA and 0.5 μg of pVA1, a plasmid encoding adenovirus VA1 RNA, which enhances expression of transfected cDNAs via a translational mechanism (12). After the transfection procedure, which involved a 3-h incubation at 35 °C in a 3% CO₂ atmosphere, the cells were washed once with phosphate-buffered saline and then placed under 3 ml of medium A. At 16 h, medium A was replaced with medium A containing 50 mM [13H]3α-adiol substrate (9-αH-3α-adiol, specific activity 49.0 Ci/mmol), and the cells were incubated at 37 °C for an additional 3 h. Thereafter, 1 ml of medium was removed from each dish and subjected to extraction with 7 volumes of methylene chloride. Separation of steroids was performed as outlined below.

In an initial screen of 36 pools of 5,000 cDNAs each, 13 pools showed conversion of 3α-adiol into androsterone over background levels obtained in mock-transfected cells. The DNAs from one of several positive pools (number 12, chosen at random), were retransformed into Escherichia coli DH5α cells to generate pools of approximately 1,000 independent transformants each. These plasmid DNAs were transfected into 293 cells to identify 6 positive pools. Further subfractionation into pools of 200, 50, 10, and individual cDNA-containing plasmids yielded a final positive clone.

Enzyme Assays—For assay of enzyme activity in transfected cell lysates, cells were harvested in ice-cold phosphate-buffered saline from dishes 30 h post-transfection using a rubber policeman pelleted by centrifugation, and either frozen in liquid N₂ for storage at −80 °C, or lysed and assayed immediately. Cell pellets were homogenized in 10 mM potassium phosphate, pH 7.0, 150 mM KCl, 1 mM EDTA with three short pulses of a Brinkmann Polytron. Enzyme assays were performed in 0.1 M sodium phosphate buffer at pH 7.5. Incubations were performed at 37 °C without shaking for 5–15 min and contained a final cofactor concentration of 1 mM. The concentration of protein in cell extracts was determined by the method of Bradford using a kit (Bio-Rad Protein Assay) and bovine serum albumin as a standard. Assay mixtures (500 μl) were extracted with 14 volumes of methylene chloride. Following isolation and evaporation of the organic phase, steroids were dissolved in 35 μl of chloroform/methanol (2:1), spotted on thin layer chromatography plates (LK5D Silica Gel, Whatman Lab Sales) and resolved by development in chloroform/ethyl acetate (3:1). Reaction products were visualized by exposure to PhosphorImaging plates and analyzed on a BAS 1000 PhosphorImager (Fuji, Stamford, CT).

Kinetic constants for steroid substrates and cofactors were measured by conventional Lineweaver-Burk analyses. All assays were repeated in triplicate using cell lysates from different transfection experiments. Ten concentrations of substrate between 0.01 and 5 μM were used for each steroid. For the determination of apparent cofactor Kᵣ values, five concentrations between 1 and 16 μM were used for NAD⁺ and five concentrations between 1 and 10 mM were used for NADP⁺, both with [13H]3α-adiol at 2.0 μM.

13-cis-Retinoic acid (13-cis-RA) and 9-cis-retinoic acid (9-cis-RA) were dissolved in ethanol to a final concentration of 10 mM and stored under nitrogen at −20 °C in amber vials. Enzyme assay solutions were prepared by diluting [13H]3α-adiol substrate to concentrations of 0.05–0.5 μM with extraction reagents.
described above were supplemented with these solutions to achieve final retinoid concentrations between 0.001 and 100 μM and incubations were performed under low light conditions. Inhibition constants (apparent Ki values) were obtained by Dixon plot analysis of transfected cell lysate activity in the presence of inhibitory concentrations of 13-cis-RA and 9-cis-RA with two different [3H]3α-adiol substrate concentrations (15 and 50 nM).

RoDH1 cDNA Cloning—A directional rat liver cDNA library was constructed in LambdaZaplox (Life Technologies, Inc.) using a superscript cDNA synthesis kit (Life Technologies, Inc.). Oligo(dT)-primed cDNA was prepared from 5 μg of mRNA, cDNAs were unidirectionally ligated into the LambdaZaplox vector and packaged with a MaxPlax Lambda Packaging extract (Epicentre Technologies, Madison, WI). This library was screened with a probe generated by radiolabeling with random hexamers (Mega Prime Labeling kit, Amersham) from the 17b-HSD6 cDNA shown in Fig. 3. Out of 100,000 recombinants screened, 18 hybridization positive plaques were identified, two of which were purified through additional rounds of screening, and subjected to DNA sequence analysis using SP6 and T7 primers. Another 100,000 recombinants were screened with a 39-untranslated region probe to produce an additional seven hybridization positive plaques, of which four were analyzed by DNA sequencing.

Human RoDH cDNAs were isolated from a prostate cDNA library prepared as described above. Approximately 105 recombinants were screened with a radiolabeled probe corresponding to nucleotides 1–966 of the rat RoDH1 cDNA (10). After hybridization in 50% formamide at 42 °C, filters were washed under reduced stringency conditions (0.3M NaCl, 0.03 M sodium citrate, 0.2% (w/v) SDS at 55 °C for 1 h). Two identical hybridization positives were isolated and subjected to DNA sequence analysis.

RNA Blotting—Total cellular RNA was isolated by guanidine thiocyanate extraction using RNA STAT 60 (TEL-TEST "B", Inc., Friendswood, TX). Poly(A)†-enriched RNA was isolated with a mRNA Purification Kit (Pharmacia Biotech Inc.). RNA was size-fractionated by electrophoresis through a 1.4% (w/v) agarose gel, transferred to nylon filters (Biotrans, ICN, Costa Mesa, CA) by capillary blotting, and hybridized and washed as described (13). Human Multiple Tissue RNA blots were purchased from CLONTECH (Palo Alto, CA). All RNA blots were probed with 39-untranslated region probes, which were generated by amplification via the polymerase chain reaction, gel purification, and random hexamer radiolabeling. The individual probes were: 17b-HSD6, nt 1246–1521 of Fig. 3 (GenBank accession number U89280); rat RoDH1, nt 1252–1347 (GenBank accession number U18762) described in Ref. 10; and human RoDH, nt 1226–1513 (GenBank accession number U89281), this study.

Prostate Regulation Experiments—The effects of androgens on the expression of 17b-HSD6 mRNA in the rat ventral prostate were determined in normal (N), castrate (C), and androgen-supplemented castrates (C1T) as described (14). Briefly, groups of sexually mature Sprague-Dawley rats (Harlan, Inc., Indianapolis, IN) (n = 4–7) were either castrated via a scrotal route or sham operated on day 0 of an experiment. On days 7–10, the N and C groups received daily injections (0.2 ml) of vehicle (sesame oil) alone, and the C1T group received 0.2-ml injections containing 2 mg of testosterone acetate. Animals were killed on the morning of day 10, ventral prostates were dissected free of connective tissue and fat, and total RNA was isolated by extraction with acidified phenol/guanidinium isothyocyanate. Hybridization signals were quantified by densitometry, normalized to the level of β-actin mRNA, and expressed as a percentage of signal measured in sham operated animals.

RESULTS

An expression cloning approach in mammalian cells was used to identify rat prostatic gene products that metabolize 3α-adiol. A cDNA library comprised of approximately one million independent clones was first prepared in a pCMV vector using rat ventral prostate mRNA as a template. Transformants
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### TABLE I

Kinetic constants of steroid metabolizing enzymes

| Enzyme          | Substrate | Product          | Activity | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
|-----------------|-----------|------------------|----------|-------|----------|---------------|
| 17β-HSD6        | 3α-Adiol  | Androsterone     | Ox$^*$ 17β-HSD | 0.1   | 2.5      | 25            |
|                 | Androsterone | Androstenedione | Ox 3α-HSD     | 0.2   | 0.1      | 0.5           |
|                 | Dihydrotestosterone | Androstenedione | Ox 17β-HSD | 0.5   | 0.2      | 0.4           |
|                 | Testosterone | Androstenedione | Ox 17β-HSD   | 1.1   | 0.2      | 0.2           |
|                 | Estradiol$^*$ | Estrone          | Ox 17β-HSD   | 0.8   | 2.1      | 2.6           |
| Rat RoDH1       | 3α-Adiol  | Dihydrotestosterone | Ox 3α-HSD | 0.1   | 0.5      | 5.0           |
|                 | Androsterone | Androstenedione | Ox 3α-HSD     | 0.1   | 0.2      | 2.0           |
|                 | Dihydrotestosterone | Androstenedione | Ox 17β-HSD | ND$^*$ | ND       |               |
|                 | Testosterone | Androstenedione | Ox 17β-HSD | 2.8   | 0.01     | 0.003         |
|                 | Estradiol | Estrone          | Ox 17β-HSD   | ND    | ND       |               |
| Human RoDH      | 3α-Adiol  | Dihydrotestosterone | Ox 3α-HSD | 0.1   | 1.1      | 11            |
|                 | Androsterone | Androstenedione | Ox 3α-HSD     | 0.1   | 0.8      | 8.0           |
|                 | Dihydrotestosterone | 3β-Adiol | Ox 3α-HSD | 0.1   | 0.1      | 1.0           |
|                 | Testosterone | Androstenedione | Ox 17β-HSD | 3.3   | 0.1      | 0.03          |
|                 | Estradiol | Estrone          | Ox 17β-HSD   | ND    | ND       |               |

$^*$ Ox, oxidative; Red, reductive.

$^b$ Experiments utilizing estradiol as substrate were carried out with transfected cell lysates that were different from those that used androgens as substrates. Thus, no direct comparisons can be made between the $V_{max}$ values reported here for these two classes of substrate.

$^c$ ND, not detected.

With the exception of reactions employing estradiol as substrate (see footnote b), kinetic constants were determined in the same experiment using 293 cell lysates transfected with cDNAs encoding the indicated enzyme. Raw data were analyzed with the Kcat' Program to calculate apparent $K_m$ and $V_{max}$ values.

from the library were divided into pools of 5,000 colonies, and plasmid DNA prepared from each pool was transfected into cultured embryonic kidney 293 cells. After 16–20 h, [3H]3α-adiol was added to the cell medium at a concentration of 50 nM and the incubation was continued for an additional 3 h. Thereafter steroids were extracted from the medium and separated by thin layer chromatography on silica gel plates. An initial screen of 36 pools revealed several that contained a cDNA encoding an enzyme that converted 3α-adiol into androsterone (Fig. 2A). No pools were identified that contained an activity capable of converting 3α-adiol to dihydrotestosterone. One of the pools (number 12) that contained a 3α-adiol → androsterone activity was subdivided into progressively smaller pools by transfection screening until a single cDNA that encoded this activity was identified (Fig. 2, B–F).

The nucleotide sequence of the rat cDNA insert harbored by the active clone is shown in Fig. 3. Analysis of the DNA sequence revealed a 5′-untranslated region of 231 nucleotides (nt), a potential coding region of 981 nt that could specify a protein of 327 amino acids, and a 3′-untranslated region of 318 nt followed by a polyadenylate tract. An in-frame stop codon was located 15 nucleotides upstream of the methionine codon that initiated the longest translational reading frame. The sequence around this methionine codon demonstrated a poor match (7/13 nt) with the Kozak consensus (GCCGCG/GAGGC-CAUGG, Ref. 15). A second methionine codon within a similar context (7/13 nt matches) was located at amino acid 11 of the deduced protein sequence (Fig. 3). This arrangement of methionine codons and their contexts’ precluded an unambiguous identification of the true amino terminus and complicated calculation of the molecular weight of the encoded protein. If the first methionine marks the amino terminus, then the predicted molecular weight of the enzyme is 37,100, whereas if the second methionine fills this role then the molecular weight is 36,100.

The reaction catalyzed by the cDNA-encoded enzyme involves the oxidation of an alcohol substituent at carbon 17 of the 3α-adiol substrate to a 17-oxo group in the androsterone product (Fig. 1). Enzymes that catalyze this reaction are referred to as 17β-hydroxysteroid dehydrogenases (17β-HSD). Five 17β-HSD enzymes have previously been identified (17β-HSD1 through 17β-HSD5, see “Discussion”) and for this reason, the protein encoded by the cDNA was termed 17β-HSD6.

The kinetic constants that describe the conversion of 3α-adiol to androsterone by rat 17β-HSD6 were determined in cell lysates prepared from transfected 293 cells. An apparent $K_m$ of 0.1 μM and a $V_{max}$ of 2.5 nmol/min/mg cell lysate were measured for this enzyme (Table I). The expressed enzyme exhibited lower affinity and activity with dihydrotestosterone, testosterone, and estradiol. Additional experiments revealed that the 17β-HSD6 enzyme possessed a weak oxidative 3α-HSD activity, converting androsterone into 5α-androstenedione with an apparent $K_m$ of 0.2 μM and $V_{max}$ of 0.1 nmol/min/mg cell lysate protein. No oxidative 3α-HSD activity against 3α-adiol was detected in these experiments. The 17β-HSD6 enzyme preferred NAD+ (apparent $K_m = 3$ μM) over NADP+ (apparent $K_m = 1$ μM) as a cofactor when 3α-adiol was used as substrate. A comparison of the relative efficiencies (determined as $V_{max}/K_m$) of the reactions catalyzed by 17β-HSD6 showed that the enzyme was most active as an oxidative 17β-hydroxysteroid dehydrogenase against 3α-adiol (Table I).

The tissue distribution of rat 17β-HSD6 was assessed by RNA blotting. Poly(A$^+$)-enriched RNA was prepared from 10 tissues, transferred to a nylon filter, and probed with a radiolabeled DNA fragment. The probe was derived from the 3′-untranslated region of the rat 17β-HSD6 cDNA to reduce the possibility of cross-hybridization to homologous mRNAs (see below). A single mRNA of approximately 1.5–1.6 kb was detected in three tissues, including the ventral prostate, liver, and kidney (Fig. 4A). The levels of this mRNA were highest in the ventral prostate and liver, and much lower in the kidney. The abundance of 17β-HSD6 mRNA in the ventral prostate agrees well with the large number of positive pools identified in the initial expression cloning experiments (Fig. 2A) and the size of the rat mRNA detected by blotting (1.5–1.6 kb) is similar to that of the isolated cDNA (1.53 kb, Fig. 3).

To determine if circulating androgens influenced the expression of 17β-HSD6 in the ventral prostate, the level of this mRNA was compared in normal (lane 1), castrated (lane 2), and castrated plus androgen-supplemented (lane 3) rats (Fig. 4B). Castration was associated with a substantial decrease of 17β-HSD6 mRNA (to 13% of normal) in the ventral prostate. Administration of androgens to castrates for a 3-day period increased the level of 17β-HSD6 mRNA to 56% of normal levels.
The sequence conservation between 17\beta-HSD6, a steroid metabolizing enzyme, and RoDH, a retinoid metabolizing enzyme, raised the question of whether RoDH might act on certain steroid substrates. To test this idea, expression vectors containing either the rat or human RoDH cDNAs were constructed and transfected into cultured 293 cells. A vector containing a rat 17\beta-HSD6 cDNA served as a positive control in these experiments. Cell lysates were prepared from transfected cells, incubated with different radiolabeled steroid substrates, and the metabolism of these compounds was followed by thin layer chromatography. Recombinant rat and human RoDH actively catalyzed the oxidation of 3α-adiol to dihydrotestosterone (Fig. 6A), indicating that these enzymes were potent oxidative 3α-HSD (Fig. 1). For example, when 1 μg of cell lysate containing either recombinant human or rat RoDH was incubated with 3α-adiol, approximately half of the substrate was converted into a product (Fig. 6A, lanes 8 and 10) with a mobility identical to that of dihydrotestosterone standards (Fig. 6A, lanes 1 and 12). Increasing the amount of cell lysate containing human RoDH resulted in more conversion of substrate to product (lanes 4–7). NAD+ was the preferred cofactor in these experiments (apparent Km = 8–15 μM for rat and human isozymes) versus NADP+ (apparent Km = 7–10 μM).

The rat 17\β-HSD6 enzyme initially converts 3α-adiol to androsterone, whereas the RoDH enzymes convert 3α-adiol to dihydrotestosterone. Androsterone and dihydrotestosterone migrate with almost identical Rf values on silica gel plates (Fig. 6A), which makes unambiguous identification of the two compounds difficult. To resolve this issue, an experiment identical to that shown in Fig. 6A was carried out except that the steroids were chromatographed on aluminum oxide plates. Chromatography on this separation medium readily resolved androsterone and dihydrotestosterone (Fig. 6B), and confirmed the initial findings of oxidative 17β-hydroxysteroid dehydrogenase activity associated with 17β-HSD6 and of oxidative 3α-HSD activity associated with rat and human RoDH.

Several additional steroid products arising from the actions of rat and human RoDH were visualized in the experiments of Fig. 6, suggesting that like 17β-HSD6, the RoDH enzymes were multifunctional. The identities of the various products, their origins, and the kinetic characteristics of each reaction were determined by incubating cell lysates with appropriate steroid substrates. The results indicated that under the assay conditions employed, both rat and human RoDH enzymes were most efficient at converting 3α-adiol to dihydrotestosterone, but the human RoDH also possessed weak 3β-hydroxysteroid dehydrogenase activity and both enzymes manifest oxidative 17β-hydroxysteroid dehydrogenase activities when presented with appropriate substrates (Table I).

We next tested the ability of endogenous retinoids to act as inhibitors of the metabolism of 3α-adiol by 17β-HSD6 and RoDH. Using assay conditions described under “Experimental Procedures,” 13-cis-retinoic acid competitively inhibited 17β-HSD6 (apparent Ki = 4 μM), rat RoDH1 (Ki = 12 μM), and human RoDH (Ki = 30 μM) enzymes. In contrast, 9-cis-retinoic acid was an excellent competitive inhibitor of 17β-HSD6 (Ki = 0.4 μM) but was essentially inactive (Ki > 100 μM) against the rat and human RoDH enzymes. The ability of endogenous retinoids to inhibit RoDH at these concentrations is of potential pharmacological importance but the physiological significance of this inhibition is unknown since intracellular retinoid concentrations are not thought to exceed the low nanomolar range.

The finding that rat and human RoDH were capable of producing dihydrotestosterone from 3α-adiol suggested that these enzymes might play a physiologically important role in

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**Fig. 4. Tissue distribution and regulation of rat 17β-HSD6 mRNA.** A, poly(A)⁺-enriched RNA was isolated from the indicated tissues of male rats and aliquots (5 μg) were subjected to blot hybridization as described under “Experimental Procedures.” The radiolabeled probes used in the individual experiments and the exposure times for each are shown on the left of the autoradiograms. The positions to which size standards migrated are indicated on the right. B, poly(A)⁺-enriched RNA was isolated from the ventral prostates of sham-operated (N) rats, castrated (C) rats, or castrated rats readministered testosterone (C+T) as described under “Experimental Procedures,” and 2-μg aliquots were subjected to blot hybridization with a 17β-HSD6 probe (left panel). The hybridized blot was stripped of residual radioactivity and reprobed with a β-actin cDNA (right panel). Lanes are numbered below and the positions to which size standards migrated are shown on the left of the autoradiograms. Exposure times were 24 h for the left panel and 16 h for the right panel.

The amount of a control mRNA (β-actin) remained unchanged (right panel, Fig. 4B). These marked changes in 17β-HSD6 mRNA in response to androgen depletion and repletion could mean that the hormone is required for expression of 17β-HSD6 in the ventral prostate. Alternatively, the reduction in 17β-HSD6 mRNA levels after castration may be a secondary effect resulting from the marked decrease in the number of epithelial cells that occurs upon androgen withdrawal in this gland (16).

A comparison of the predicted protein sequence of rat 17β-HSD6 to those in the data bases indicated that the enzyme shared sequence identity with numerous members of the short chain dehydrogenase/reductase family. Among these family members, the most closely related protein was rat RoDH1, an enzyme that catalyzes the conversion of retinol to retinal (10). An alignment of rat 17β-HSD6 and RoDH1 protein sequences is shown in Fig. 5 and revealed that the two proteins share 65% sequence identity over their entire lengths. The shared sequence identity began at the second methionine of the 17β-HSD6 sequence (see Fig. 3), which suggested that the second and not the first methionine may be the true initiator methionine codon. A cDNA clone encoding a human RoDH protein was subsequently isolated as described under “Experimental Procedures,” and a comparison of the deduced protein sequences of the rat 17β-HSD6, rat RoDH1, and human RoDH enzymes again revealed extensive sequence identities (Fig. 5).
androgen target tissues. To assess this potential role, RNA extracted from human and rat tissues was subjected to blot hybridization using probes derived from the 3′-untranslated regions of the respective cDNAs. The RoDH1 mRNA was detected in the liver of the rat (Fig. 7A). In contrast, a human RoDH mRNA of approximately 1.6 kb was detected in the liver and in lesser amounts in spleen, testis, and prostate (Fig. 7B). A human mRNA of a different size (approximately 0.8 kb) was detected at a moderate level in placenta. Whether this mRNA represents another gene product or arises from RNA processing of human RoDH transcripts was not determined. No cross-hybridizing mRNAs were detected when the filters containing human tissue RNAs were probed at low stringency with radio-labeled cDNA fragments derived from the 3′-untranslated region of the rat 17β-HSD6 clone or when these filters were probed at high stringency with near full-length rat 17β-HSD6 cDNA fragments (data not shown).

DISCUSSION

We report isolation of a cDNA encoding a rat 17β-hydroxysteroid dehydrogenase from ventral prostate. The 17β-HSD type 6 isozyme prefers to catalyze the oxidation of androgen substrates, particularly that of 3α-adiol to androsterone. The mRNA encoding this isozyme is present at high levels in rat liver and ventral prostate where the encoded protein presumably plays a largely catabolic role in degrading androgens. The 17β-HSD6 is a member of the short chain dehydrogenase/reductase family and shares 28% sequence identity with RoDH1. The RoDH mRNA is expressed at high levels in the rat and human liver and at lower levels in human spleen, testis, and prostate. Rat and human RoDH enzymes expressed in cultured cells actively convert 3α-adiol to dihydrotestosterone and thus exhibit strong oxidative 3α-HSD activities. The tissue distributions and preferred reaction directions of these enzymes suggest that RoDH may play an important anabolic role in androgen metabolism.

Six different enzymes that catalyze oxidation and/or reduction reactions at carbon 17 of steroid substrates have been isolated. Each of these 17β-HSD isozymes demonstrates a favored substrate and reaction direction, and they often have unique tissue distributions (17, 18). For example, the type 1 isozyme preferentially reduces estradiol to estrone and is abundant in the ovary and placenta (19). The 17β-HSD type 2 isozyme catalyzes the oxidation of androgens and is present in the endometrium and placenta (20, 21), whereas the type 3 isozyme is exclusively a reductive enzyme of the testis (1). The type 4 isozyme shows an oxidative preference with steroid substrates and the type 5 a reductive preference (17, 18). Although the different 17β-HSD isozymes act at the same carbon of a steroid substrate, pairwise comparisons between individual isozymes reveal less than 25% sequence identity, which suggests that their catalytic activity was acquired by convergent evolution (17, 18). This diversity in primary sequence is also characteristic of the 17β-HSD type 6 isozyme, which shares 28% sequence identity with the type 2 isozyme and lesser identities with the other five isozymes.

The 17β-HSD type 6 isozyme reported here appears to be largely an oxidative enzyme that is active against both androgens and estrogens when expressed in 293 cells (Table I). The mRNA encoding this isozyme is abundant in the rat liver and ventral prostate (Fig. 4). These properties predict that the physiological role of 17β-HSD6 is to inactivate androgens and estrogens in tissues and thereby to limit the extent of signaling activity mediated by these steroid hormones. Inactivation may be particularly important in the androgen-sensitive prostate where 3α-adiol can be converted to dihydrotestosterone, which in turn can stimulate growth of the gland. The presence of
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17β-HSD6 enzyme may thus exert a protective effect against unregulated benign or malignant prostate growth (22) by preventing the buildup of 3α-adiol and hence the synthesis of dihydrotestosterone via the back reaction.

The 17β-HSD6 isozyme shares signature sequence motifs with members of the short chain dehydrogenase/reductase family. Enzymes in this large family (>50 members, Ref. 23) are both soluble (3α/20β-hydroxysteroid dehydrogenase) and membrane bound (RoDH, Ref. 24). A hydropathy analysis of the 17β-HSD6 sequence did not reveal extended stretches of hydrophobic amino acids resembling classical transmembrane domains. However, the fact that 17β-HSD6 shares extensive sequence identity with the RoDH1 enzyme suggests that it may also be associated with the membrane compartment of the cell.

Members of the short chain dehydrogenase/reductase class share conserved primary and tertiary sequences and catalyze diverse biochemical reactions with different stereochemistries (23). For example, the 3α/20β-hydroxysteroid dehydrogenase enzyme will reduce both the 3-oxo and 20-oxo groups of progestagens (8) and the 17β-HSD2 enzyme will oxidize both 17- and 20α-alcohol substituents of steroids (17, 18). The 17β-HSD6 is also ambidextrous in its catalytic activities, oxidizing both carbon 17 and carbon 3 alcohol groups on certain steroid substrates (Table 1). An analysis of the kinetic constants associated with these two reactions suggests that oxidation at carbon 17 takes place more readily than does oxidation at carbon 3 (Table 1), which may suggest that the latter reaction only occurs in transfected cells at nonphysiological substrate and/or enzyme concentrations.

The high degree of sequence identity between rat 17β-HSD6 and rat RoDH1 led us to test whether RoDH1 was active against steroid substrates. This enzyme has been extensively characterized as a membrane-bound retinol dehydrogenase that participates in the conversion of retinol to retinal (10). Efficient oxidation of retinoids by RoDH1 is dependent on their prior association with the cellular retinol-binding protein (24). RoDH1 preferentially utilizes NADP+ as a cofactor with retinoid substrates (10), and like 17β-HSD6, exists in at least three isozymic forms (10, 25, 26). The observation reported here that rat RoDH1 and a human RoDH are oxidative 3α-HSDs with nanomolar affinities for 3α-adiol is important for several reasons. First, it confirms a hypothesis put forth in two recent reviews that RoDH enzymes might be active against both retinoids and steroids (27, 28). Second, although several reductive 3α-HSD isozymes have been isolated (reviewed in Ref. 8), an oxidative isozyme is a novelty. Third, and most importantly, RoDH isozymes may contribute to the synthesis of dihydrotestosterone, a powerful androgen that exerts both endocrine and autocrine effects in target tissues.

The rat RodH1 isozyme has been extensively characterized at both the biochemical (24) and molecular (10) levels. More recently, two additional rat RoDH isozymes, termed RodH2 and RodH3, have been defined at the molecular level (25, 26). The human RoDH cDNA isolated here shares 62% amino acid sequence identity with the rat RoDH1 isozyme and 61 and 63% identities with the RoDH2 and RoDH3 isozymes, respectively. We thus cannot state with certainty which of the three rat RoDH isozymes is orthologous to the human protein encoded by the cDNA characterized here. Additional studies on human RodH cDNAs and proteins will be required to determine the existence of isozymes and their relationships to the rat proteins. Despite this uncertainty in nomenclature, however, the current studies demonstrate that a known rat RoDH isozyme...
A majority of dihydrotestosterone is thought to be synthesized from testosterone by the actions of steroid 5α-reductase isozymes (2), and consistent with this biosynthetic origin, mutations that impair either steroid 5α-reductase type 1 or type 2 cause androgen insufficiency syndromes in males and females (29, 30). Nevertheless, several findings suggest that the so-called back reaction, in which 3α-adiol is converted into dihydrotestosterone (Fig. 1), may also serve as a physiologically important source of this androgen. Thus, 3α-adiol is a potent stimulator of in vivo prostate growth in the dog (31) and rat (7, 14, 32) and growth of the rat prostate in organ culture is also stimulated by this steroid (33). The identity of the RoDH isozyme(s) that catalyze this reaction in the rat ventral prostate remains to be determined, however, using reduced stringency hybridization conditions and coding region probes, RoDH transcripts were detected in this tissue and 13-cis-retinoic acid inhibited oxidative 3α-HSD enzyme activity in prostate extracts (data not shown).

Finally, inhibition of the major human prostatic steroid 5α-reductase isozyme decreases the concentration of dihydrotestosterone within the tissue to 90% of normal (34), but no further, suggesting that another biosynthetic source of this hormone exists. The observation that the mRNA for a RoDH isozyme is present in the human prostate (Fig. 7B) provides one possible explanation for this alternate biosynthetic source and confirms an earlier report of RoDH activity in prostatic microsomes (35). It will be of interest in the future to correlate the expression of this RoDH in diseased and normal prostate in an effort to understand the role of the enzyme in androgen-regulated growth of the gland and in prostatic retinoid metabolism.

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