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Molecular Cloning of a Novel Type of Rat Cytoplasmic 17β-Hydroxysteroid Dehydrogenase Distinct from the Type 5 Isozyme

Shuhei Ishikura[1,*], Kengo Matsumoto[1], Masaharu Sanai[1], Kenji Horie[1], Toshiyuki Matsunaga[1], Kazuo Tajima[2], Ossama El-Kabbani[3] and Akira Hara[1,†]

[1]Laboratory of Biochemistry, Gifu Pharmaceutical University, Gifu 502-8585; [2]Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa 920-1181; and [3]Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University, Parkville, Victoria 3052, Australia

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Rat liver contains two cytosolic enzymes (TBER1 and TBER2) that reduce 6-*tert*-butyl-2,3-epoxy-5-cyclohexene-1,4-dione into its 4*R*- and 4*S*-hydroxy metabolites. In this study, we cloned the cDNA for TBER1 and examined endogenous substrates using the homogenous recombinant enzyme. The cDNA encoded a protein composed of 323 amino acids belonging to the aldo-keto reductase family. The recombinant TBER1 efficiently oxidized 17β-hydroxysteroids and xenobiotic alicyclic alcohols using NAD^+ as the preferred coenzyme at pH 7.4, and showed low activity towards 20α- and 3α-hydroxysteroids, and 9-hydroxyprostaglandins. The enzyme was potently inhibited by diethylstilbestrol, hexestrol and zearalenone. The coenzyme specificity, broad substrate specificity and inhibitor sensitivity of the enzyme differed from those of rat NADPH-dependent 17β-hydroxysteroid dehydrogenase type 5, which was cloned from the liver and characterized using the recombinant enzyme. The mRNA for TBER1 was highly expressed in rat liver, gastrointestinal tract and ovary, in contrast to specific expression of 17β-hydroxysteroid dehydrogenase type 5 mRNA in the liver and kidney. Thus, TBER1 represents a novel type of 17β-hydroxysteroid dehydrogenase with unique catalytic properties and tissue distribution. In addition, TBER2 was identified as 3α-hydroxysteroid dehydrogenase on chromatographic analysis of the enzyme activities in rat liver cytosol and characterization of the recombinant 3α-hydroxysteroid dehydrogenase.

Key words: aldo-keto reductase family, 9-hydroxyprostaglandin, 3α-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, 20α-hydroxysteroid dehydrogenase, xenobiotic ketone reductase.

Abbreviations: AKR, aldo-keto reductase; HSD, hydroxysteroid dehydrogenase; 17HSD5, 17β-HSD type 5; PG, prostaglandin; rHSD, protein encoded in the gene for rat 17HSD5; RT, reverse transcription; SDR, short-chain dehydrogenase/reductase; TBE, 6-*tert*-butyl-2,3-epoxy-5-cyclohexene-1,4-dione; TBEH; 6-*tert*-butyl-2,3-epoxy-4-hydroxy-5-cyclohexen-1-one; TBER, TBE reductase.

A variety of carbonyl compounds are present in foods, environmental pollutants, and drugs, and are formed through the biological transformation of endogenous components and xenobiotics that are ingested. The xenobiotic and endogenous carbonyl compounds are metabolized into the corresponding alcohols by many mammalian NAD(P)H-dependent reductases (*1*, *2*). According to the accumulated knowledge on the functions and structures of these reductases, most carbonyl-reducing enzymes have been grouped into two distinct protein families, the short-chain dehydrogenase/reductase (SDR) and aldo-keto reductase (AKR) superfamilies (*3*, *4*). However, there are several carbonyl-reducing enzymes whose physiological roles and structures remain unknown. One such enzyme is a rat liver reductase for 6-*tert*-butyl-2,3-epoxy-5-cyclohexene-1,4-dione (TBE), a metabolite of 3-*tert*-butyl-4-hydroxyanisole, which is a phenolic antioxidant used as a food preservative (*5*). The enzyme exists in two forms, TBER1 and TBER2, in rat liver cytosol. They are monomeric proteins with similar molecular masses of around 36 kDa, but differ in the stereoselective reduction of TBE, coenzyme specificity and substrate specificity for steroids. TBER2 reduces TBE into 6-*tert*-butyl-2,3-epoxy-4(*S*)-hydroxy-5-cyclohexen-1-one (4*S*-TBEH), and exhibits high NADP(H)-linked 3α-hydroxysteroid dehydrogenase (HSD) activity, suggesting its identity with the 3α-HSD that is named AKR1C9 in the AKR superfamily (*6*). On the other hand, TBER1 utilizes NADPH and NADH equally as the coenzyme and preferentially catalyzes the reduction of TBE to 6-*tert*-butyl-2,3-epoxy-4(*R*)-hydroxy-5-cyclohexen-1-one (4*R*-TBEH). Furthermore, TBER1 reduces 5β-androstan-3α-ol-17-one and prostaglandin (PG) E_2, and oxidizes 17β-estradiol with both $NADP^+$ and NAD^+ as the coenzymes. Thus, it is likely that TBER1 is related to 17β-HSD or PG-metabolizing enzymes.

Eleven distinct types of 17β-HSD have been found in mammals, differing in substrate specificity, tissue, developmental and subcellular distribution patterns, and/or the

[†]To whom correspondence should be addressed. Phone/Fax: +81-58-237-8586, E-mail: hara@gifu-pu.ac.jp
[*]Present address: The Hospital for Sick Children, 555 University Ave, Toronto, Ontario M5G 1X8, Canada.

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preferred reaction direction (*7–9*). Structurally, 17β-HSD type 5 (17HSD5) belongs to the AKR family, and the other types are members of the SDR family. Although the human and mouse 17HSD5s (AKR1C3 and AKR1C6, respectively) have been characterized through their cDNA cloning and functional analyses (*10–14*), they differ from the other types of 17β-HSD in their cytoplasmic localization, monomeric nature, and ability to reduce xenobiotic carbonyl compounds. In addition, the human and mouse 17HSD5s differ in the specificity for substrates other than 17β-hydroxysteroids and in tissue distribution. The human enzyme exhibits 3α-HSD, 20α-HSD and PGF synthase activities, and its mRNA has been detected in many tissues (*10–12*). By contrast, the mouse enzyme shows only low 20α-HSD activity, and is predominantly expressed in the liver (*13*, *14*). Recent rat genomic analysis has predicted a gene for a protein similar to mouse 17HSD5, but the enzyme's properties and tissue distribution remain unknown.

In this study, we cloned the cDNAs for TBER1 and rat 17HSD5, and compared the properties and tissue distribution of the enzymes, in order to elucidate their physiological roles, and their relationship to other dehydrogenases and reductases for xenobiotic compounds. In addition, the identity of TBER2 with 3α-HSD was examined by chromatographic analysis of the two enzymes in rat liver cytosol, using the recombinant 3α-HSD.

MATERIALS AND METHODS

cDNA Cloning—To design primers for PCR-based cloning of the cDNA for TBER1, we determined the partial sequence of the enzyme that was purified from livers of Wistar rats (*5*). The protein sequence determination, including the reductive pyridylethylation of the protein, its BrCN-cleavage, digestion with lysylendopeptidase, isolation of peptides and sequencing by automated Edman degradation, was performed as described previously (*15*). An initial cDNA fragment (528 bp) for AKR1C24 was amplified by PCR against a λgt11 rat liver cDNA library (Stratagene) using a sense primer (5′-CTTCAGCTGGATTATGTTGATCTTCTC) and an antisense primer (5′-CAAATTCTCTCTCATCTCATTCTC), which correspond to positions 106–113 and 274–281, respectively, of the amino acid sequence of TBER1 (Fig. 1). PCR involving KOD-Plus DNA polymerase (Toyobo) was performed for 2 min at 94°C, followed by 35 cycles of 94°C for 15s,

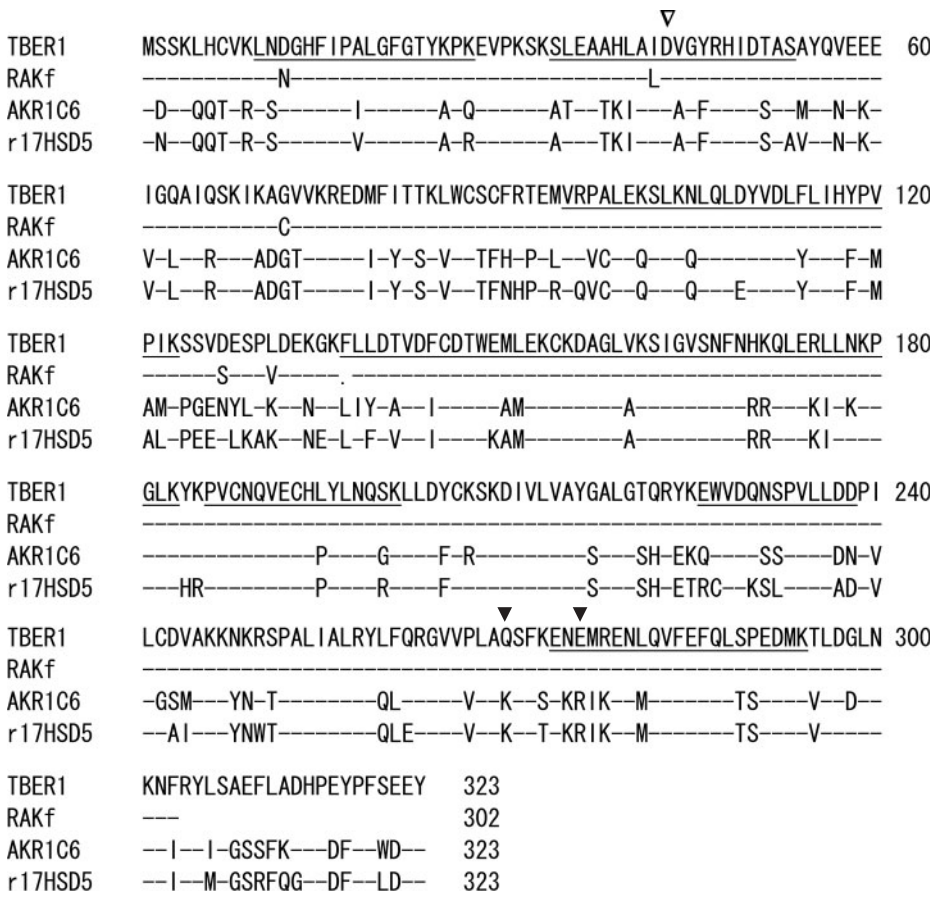

Fig. 1. **Alignments of deduced amino acid sequences of TBER1, RAKf, mouse 17HSD5 (AKR1C6), and rat 17HSD5 (r17HSD5).** The sequences of the peptides derived from the purified rat liver TBER1 are underlined. Identical amino acids are indicated by dashes, and a period in the RAKf sequence delineates a gap that is inserted to preserve the optimal similarity with TBER1. The sequence (from 1 to 303) deduced from the cDNA for rat liver-regeneration protein is the same as that of TBER1, except that Asp43, indicated by an open arrowhead, is replaced by Gly. Black arrowheads indicate the positions of the basic residues that interact with the 2′-phosphate of NADP(H) in the NADP(H)-dependent enzymes in the AKR superfamily. The accession numbers of TBER1, its C-terminal truncated form and r17HSD5 are AB221345, AB221346 and XM_225538, respectively.

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55°C for 30 s and 68°C for 1 min. Subsequently, the cDNA fragment was labeled using a DIG DNA labeling and detection kit (Roche), and used for plaque hybridization screening of the full-length TBER1 cDNA against the λgt11 rat liver cDNA library. Three cDNA clones (1,932 bp) were obtained and sequenced with a CEQ2000XL DNA sequencer (Beckman Coulter). The sequences of the three cDNAs were identical, but the predicted open reading frame contained an additional stop codon at position 910, which is not the expected codon at position 970. Therefore, reverse transcription (RT)-PCR was performed against total RNA samples prepared from the livers of four 14-week-old male and female rats. In the PCR, *Pfu* DNA polymerase was used together with a sense primer (tber-N: 5′-ATGAGCTCCAAACTGCACTGTG) and an antisense primer (tber-C: 5′-CAGTCCCCATGCTTAATATTCCTC), which anneal to positions 1–22 and 961–984, respectively, of the sequence of the cDNA. The PCR was performed for 5 min at 94°C, followed by 29 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min. The amplified products (984 bp) were subcloned into pCR T7/CT-TOPO vectors (Invitrogen), and the expression constructs were transfected into *Escherichia coli* BL21 (DE3) pLysS according to the protocol described by the manufacturer. Thirty-three of the obtained 34 cDNA clones showed the same sequence, but differed from the above cDNA and the remaining cDNA clone by one nucleotide at position 910: C (for the former cDNAs) versus T (which causes a stop codon in the latter cDNA). The former and latter cDNAs were designated as R1-L and R1-S, respectively.

The expression of the two mRNAs corresponding to R1-S and R1-L in rat liver was also examined by means of diagnostic restriction with *Taq*I. Because the nucleotide difference at position 910 bears the *Taq*I site only in R1-L, cDNA fragments (nucleotides 369–984) were amplified by RT-PCR from total RNA samples from the livers, digested by *Taq*I (4 units) at 65°C for 3 h in the buffer supplied by the manufacturer (Stratagene), and then analyzed by electrophoresis on a 1% agarose gel. The PCR was performed for 5 min at 94°C, followed by 29 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, using a sense primer (tber-N-1: 5′-GTCAAGTGTTGATGAATC) and antisense primer tber-C. On agarose gel electrophoresis, two DNA fragments of 542-bp and 74-bp derived from the R1-L cDNA were detected, in contrast to the non-digested R1-S cDNA (616 bp). Furthermore, we carried out RT-PCR to detect the mRNA corresponding to R1-S. The PCR was performed for 5 min at 94°C, followed by 29 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, using sense primer tber-N-1 and an antisense primer, 5′-CTCTGCTGAAAGATATCA, which anneals to positions 910–927 of R1-S. Under these conditions a DNA fragment (559 bp) was amplified from the full-length R1-S cDNA, but not from the full-length R1-L cDNA.

The cDNA for rat 17HSD5 was isolated from the total RNA of rat liver by RT-PCR using *Pfu* DNA polymerase and the following primers. A sense primer (r17-N: 5′-ATGAATTCTAAGCAG) and an antisense primer (r17-C: 5′-AAAGCATAACACAGACCT) were designed based on the mRNA sequence (accession no. XM_225538) predicted from a gene, Akr1c6_predicted, in the rat genome database of NCBI. The products that were amplified under the same conditions as those for the PCR for TBER1 cDNA were subcloned into the pCR T7/CT-TOPO vectors, and the sequences of the inserts were verified by DNA sequencing. The above DNA and RNA techniques were performed as described by Sambrook *et al.* (*16*), and as previously reported (*17*, *18*).

Expression and Purification of Recombinant Proteins—*E. coli* cells transfected with the expression plasmids harboring the above cDNAs were cultured in LB medium containing ampicillin (50 μg/ml) at 37°C until the absorbance at 600 nm reached 0.5. Then isopropyl-1-thio-β-D-galactopyranoside (1 mM) was added, and the culture was continued for 24 h at 20°C. The cells were collected and an extract was prepared as described previously (*17*). The proteins encoded in the two cDNAs were examined by Western blotting using anti-TBER1 antibodies raised in rabbits, after injection with the rat liver TBER1. The antibodies in the antiserum were partially purified by $(NH_4)_2SO_4$ fractionation and anion-exchange chromatography on a DEAE-Sephacel column (*16*). The polyclonal antibodies reacted with the two products derived from the two cDNAs, but did not cross-react with rat 17HSD5 or 3α-HSD.

Recombinant TBER1 expressed from R1-L was purified by the methods for the purification of the respective hepatic enzymes (*5*), except that the buffers were supplemented with 20% (v/v) glycerol to stabilize the enzyme. Recombinant rat 17HSD5 was purified from an *E. coli* extract by $(NH_4)_2SO_4$ fractionation and three subsequent column chromatography steps. The enzyme fraction, precipitated between 35 and 75% $(NH_4)_2SO_4$ saturation, was dialyzed against Buffer A (10 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 1 mM EDTA and 20% glycerol), and then applied to a Sephadex G-100 column (3 × 70 cm) equilibrated with Buffer A. The enzyme fraction was applied to a Q-Sepharose column (2 × 20 cm) equilibrated with Buffer A. The enzyme appeared in the non-adsorbed fractions, which were then applied to a Red-Sepharose column (1.5 × 5 cm) equilibrated with Buffer A. The column was washed with Buffer A containing 0.1 M NaCl, and the enzyme was eluted with Buffer A containing 0.5 mM $NADP^+$ and 0.1 M NaCl. The recombinant protein expressed from R1-S was partially purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 column chromatography by detecting the 36-kDa protein by both SDS-PAGE and Western blot analyses. The recombinant rat 3α-HSD was expressed and purified as described (*19*).

Assay of Enzyme Activity—The reductase and dehydrogenase activities of the recombinant enzymes were assayed by measuring the rate of change in NAD(P)H absorbance (at 340 nm) and its fluorescence (at 455 nm with an excitation wavelength of 340 nm), respectively. The standard reaction mixture for the reductase activity consisted of 0.1 M potassium phosphate, pH 7.4, 0.1 mM NAD(P)H, substrate and enzyme, in a total volume of 2.0 ml. The dehydrogenase activity was determined with 1 mM NAD^+ or 0.25 mM $NADP^+$ as the coenzyme in the standard reaction mixture. To detect low enzyme activities in the cell extracts and enzyme preparations during the purification, 0.1 M potassium phosphate, pH 6.0, and 0.1 M glycine-NaOH, pH 9.5, were employed instead of the phosphate buffer, pH 7.4. The representative substrates used for the assay of activities of TBER1, 17HSD5 and 3α-HSD were 1 mM TBE, 0.1 mM testosterone and 50 μM androsterone,

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respectively. α- and β-3-hydroxyhexobarbitals were gifts from Dr. R. Takenoshita (University of Fukuoka, Japan), and *trans*-benzene dihydrodiol and 21-dehydrocortisol were synthesized as described previously (*20*, *21*). TBE, 4*R*-TBEH and 4*S*-TBEH were prepared by the methods described previously (*22*, *23*). One unit (U) of enzyme activity was defined as the amount that catalyzed the reduction or formation of 1 μmol NAD(P)H per minute at 25°C. The apparent K_m and k_{cat} values were determined over a range of five substrate concentrations at a saturating concentration of the coenzyme by fitting the initial velocities to the Michaelis-Menten equation. The kinetic constants and IC_{50} values are expressed as the means of two determinations. Protein concentration was determined by the method of Bradford (*24*) using bovine serum albumin as the standard.

Product Identification—To identify reaction products, reduction was conducted in a 2.0 ml system comprising 0.1 mM NAD(P)H, substrate (10–50 μM), enzyme (3 μg), and 0.1 M potassium phosphate, pH 6.5 (for TBER1) or pH 7.4 (for rat 17HSD5). The substrate and products were extracted into 6 ml ethyl acetate at 30 min after the reaction had been started at 37°C, and then analyzed by liquid chromatography/mass spectrometry and TLC as described previously (*18*). The products of TBE reduction by the enzymes were analyzed by the HPLC method (*22*).

Tissue Distribution—Tissues were excised from 12–14-week-old male and female Wistar rats. For analysis of the expression of mRNAs for TBER1 and rat 17HSD5, total RNA samples were prepared from the tissues, and then RT-PCR was carried out using *Taq* DNA polymerase (Takara) and gene-specific primer pairs, tber-N and tber-C (for TBER1 cDNA), and r17-N and r17-C (for rat 17HSD5 cDNA), as described above.

To determine whether TBER1 and 17HSD5 are cytosolic NAD^+- and $NADP^+$-dependent 17β-HSDs, respectively, in rat tissues, cytosolic fractions of the homogenates of the livers (12 g) and stomachs (9 g) were prepared (*5*), and the enzymes were partially purified by $(NH_4)_2SO_4$ fractionation, and two subsequent chromatography steps on Sephadex G-100 and Q-Sepharose columns, as described above.

RESULTS

Cloning of cDNA for TBER1—The sequences of the peptides, except for the two lysylendopeptidase-digested peptides, derived from the purified rat liver TBER1 perfectly matched regions of the amino acid sequence (positions 106–280 in Fig. 1) deduced from the 528-bp cDNA fragment, which had been amplified through the initial PCR with the rat liver cDNA library. Subsequent screening of 2×10^5 plaques of the rat liver cDNA library with the cDNA fragment probe gave three positive clones. The inserts (1,960 bp) of the three clones had the same sequence, which had a 740-bp noncoding region, a 912-bp open reading frame, starting from an ATG codon and terminating at a TGA stop codon, and a 308-bp 3′-noncoding region, including a polyadenylation hexamer (AATAAA at positions 1,169–1,174) and a subsequent poly(A) tail. The 303-amino acid sequence encoded in the cDNA perfectly matched the sequences of all the peptides derived from the rat liver TBER1. The sequence (nucleotides from –29 to 1184) of the cDNA was identical to that of a transcript (accession no. NM_001014240) of a gene predicted on recent rat genomic analysis, except that the three nucleotides at positions 135, 229 and 910 are A, G and C, respectively, in the transcript of the gene. The substitutions at positions 135 and 229 do not affect the 303-amino acid sequence deduced from the cDNA, but the T-to-C substitution (at position 910) changes the stop codon to an Arg codon (Fig. 1). Since the sequences of the cDNA clone and the genomic transcript are different, the sequence (nucleotides 1–984) of the cDNA for TBER1 was re-examined by RT-PCR with total RNA samples prepared from four rat livers. Of the 34 cDNA clones isolated, only one was the same as the above cDNA, the others having a 972-bp open-reading frame due to the T-to-C substitution at position 910. The former and latter cDNAs were designated as R1-S and R1-L, respectively. Expression of the mRNAs corresponding to the two cDNAs in ten other rat liver samples was also examined by means of a combination of RT-PCR and *Taq*I digestion. This method discriminates the two cDNAs, because the endonuclease site is present in R1-L, but not in R1-S. The amplified cDNAs (nucleotides 369–984) derived from the liver samples were all digested into two fragments of 542 bp and 74 bp (data not shown). In addition, no DNA fragment was amplified on RT-PCR analysis of another eleven rat liver samples using the primers that specifically anneal to R1-S.

When the recombinant proteins were expressed from the two cDNAs, R1-S and R1-L, in *E. coli* cells, high TBE reductase activity (0.38 U/mg) was observed in the extract of the cells, which expressed a 37-kDa recombinant protein from R1-L (Fig. 2A). In the case of the expression of the recombinant protein from R1-S, a 35-kDa protein immunoreactive with the anti-TBER1 antibodies was detected in the *E. coli* extract, but the activity (0.006 U/mg) was as low as that (0.010 U/mg) of the extract of *E. coli* cells transfected with the vector alone. The recombinant protein expressed from R1-L was purified by assaying the TBE reductase activity. The enzyme was eluted at approximately 36 kDa

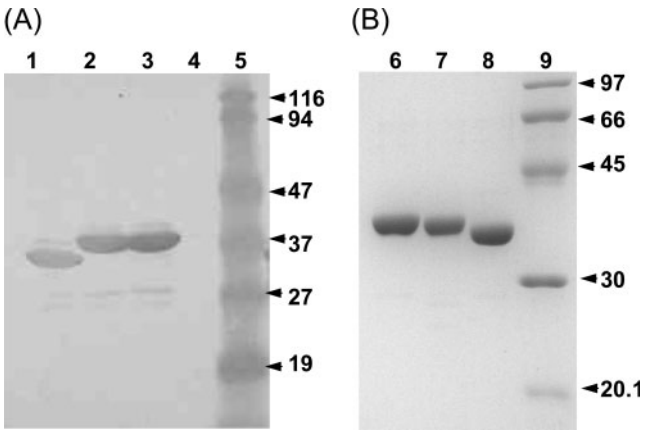

Fig. 2. **Western blot and SDS-PAGE analyses of recombinant proteins.** A: Western blotting using anti-TBER1 antibodies. Extracts (each 10 μg) of *E. coli* cells transfected with the expression vectors harboring R1-S (lane 1), R1-L (lanes 2 and 3), and the expression vector alone (lane 4) were analyzed. B: SDS-PAGE. The purified TBER1, rat 17HSD5 and 3α-HSD (each 2 μg) were run in lanes 6, 7 and 8, respectively, and stained with 0.2% Coomassie Brilliant Blue. The positions of molecular mass markers are indicated in kDa (lanes 5 and 9).

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in the Sephadex G-100 filtration step, suggesting its monomeric nature. The final preparation was more than 99% pure, as judged on SDS-PAGE (Fig. 2B), and the purification yield was 28% (17 mg/liter of culture). The enzyme exhibited NADPH-linked reductase activities towards 1 mM TBE (2.0 U/mg) and 1 mM 4-chlorobenzaldehyde (0.6 U/mg) at a pH optimum of 6.0, and the corresponding K_m values were 0.30 and 0.11 mM, respectively. The ratio of 4*R*-TBEH to 4*S*-TBEH produced on the reduction of TBE was 1.6:1. The molecular weight, specific activities, pH optimum, K_m values and stereospecific reduction of TBE are essentially identical to those of the native TBER1 purified from rat liver (*5*). Thus, R1-L was the cDNA for TBER1, and the C-terminal truncated protein encoded in R1-S was inactive towards TBE.

Specificity for Coenzymes and Substrates of TBER1— The purified recombinant TBER1 reduced 4-chlorobenzaldehyde and oxidized (*S*)-(+)-1,2,3,4-tetrahydro-1-naphthol (*S*-tetralol) in the presence of both NADP(H) and NAD(H) as the coenzymes. The pH optima of the NADH- and NADPH-linked reductase activities were both around 6.0, and those of the NAD^+- and $NADP^+$-linked dehydrogenase activities were 10.5 and 9.0, respectively. When the kinetic constants for the coenzymes were determined under different pH conditions, the K_m values for NADP(H) significantly increased from pH 6.0 to pH 8.0 (Fig. 3A) with few changes in the k_{cat} values, resulting in decreases in the k_{cat}/K_m values (Fig. 3B). The pH-dependent changes in the kinetic constants for NAD(H) were small, except that the K_m values for the coenzymes were high at pH 6.0. At pH 7.4, the catalytic efficiency (k_{cat}/K_m) for NAD(H) was higher (>17 fold) than that for NADP(H). In addition, the NADPH-linked reductase activity was highly inhibited by NAD^+ (IC_{50} = 0.09 mM), which was much less inhibitory to the NADH-linked activity (IC_{50} = 1.0 mM). $NADP^+$ (1 mM) did not have a significant influence on either the NADPH- or NADH-linked activity. Even on the oxidation of *S*-tetralol with 0.1 mM oxidized coenzymes, NADH showed higher inhibition than NADPH. The IC_{50} values for NADH in the $NADP^+$- and NAD^+-linked activities were 10 and 110 μM, respectively, whereas the respective values for NADPH were 90 and 180 μM. Furthermore, the K_m values for 4-chlorobenzaldehyde (0.12 mM) and *S*-tetralol (0.53 mM) in the NADP(H)-linked reactions were higher than those in the NAD(H)-linked ones (Table 1). The results indicated that TBER1 utilizes NAD(H) as the preferred coenzyme at the physiological pH of 7.4.

Under the assay conditions with the preferred coenzyme, NAD^+, at pH 7.4, the recombinant TBER1 highly oxidized several xenobiotic alicyclic alcohols (Table 1), which are substrates for most of the enzymes in the AKR1C subfamily (*3*, *10–14*). The enzyme also exhibited low dehydrogenase activities toward 9α,11β-PGF_2 and 9α,11α-PGF_2, but did not oxidize 9β,11α-PGF_2. In the reverse reaction, the enzyme efficiently reduced α-dicarbonyl compounds with aromatic or alicyclic ring(s) as well as known substrates, TBE, 9,10-phenanthrenequinone and aromatic aldehydes (*5*). No significant reductase activity towards monoketones, such as 4-nitroacetophenone, acetylpyridine, 4-benzoylpyridine, acetoin and PGD_2, was observed.

The recombinant TBER1 also exhibited moderate 17β-HSD activity, and its K_m and k_{cat}/K_m values for

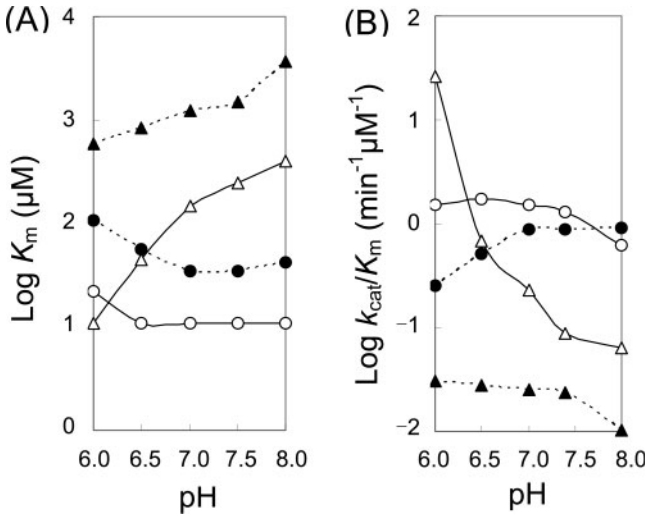

Fig. 3. **Effect of pH on kinetic constants for coenzymes in the NAD(H)- and NADP(H)-linked oxidoreduction catalyzed by TBER1.** A: Log K_m (μM) *vs.* pH. B, Log k_{cat}/K_m (min^{-1} μM^{-1}) *vs.* pH. The reductase and dehydrogenase activities were determined with 0.5 mM 4-chlorobenzaldehyde and 1 mM *S*-tetralol, respectively, as the substrate at the indicated pHs. Coenzymes: NADH (open circles), NAD^+ (solid circles), NADPH (open triangles), and $NADP^+$ (solid triangles).

most 17β-hydroxysteroids were lower and higher, respectively, than those for non-steroidal alcohols (Table 2). It also oxidized 20α- and 3α-hydroxysteroids at low rates, and the oxidized products of 4-pregnene-17α,20α-diol-3-one, 4-pregnen-20α-ol-3-one and 4-androsten-3α-ol-17-one were identified as 17α-hydroxyprogesterone, progesterone and 4-androstene-3,17-dione, respectively. In the reverse reaction, the enzyme reduced 17-, 20- and 3-ketosteroids, although the catalytic efficiency was lower than that in the oxidation direction. The reduced products of 5α-androstan-3α-ol-17-one, estrone and 17α-hydroxyprogesterone by the enzyme were identified as 5α-androstane-3α,17β-diol, 17β-estradiol and 4-pregnene-17α,20α-diol-3-one, respectively. On reduction of 5β-androstane-3,17-dione by the enzyme, three products, 5β-androstan-17β-ol-3-one, 5β-androstane-3α,17β-diol and 5β-androstan-3α-ol-17-one, were formed, supporting the dual 17β- and 3α-HSD activity of TBER1. The enzyme showed high activity toward 16-ketoestrone and 21-dehydrocortisol, which have the α-dicarbonyl structure. The reduced product of 21-dehydrocortisol was identified as cortisol, indicating that TBER1 also catalyzes the reduction of the 21-aldehyde group in the steroid substrate.

Although most of the above substrates were not tested in the previous study (*5*), they were similarly oxidized or reduced by TBER1 purified from rat liver in the presence of the preferred coenzyme, NAD(H), at pH 7.4. The K_m values for representative substrates were 102 μM (for *S*-tetralol), 215 μM (for *trans*-benzene dihydrodiol), 1130 μM (for 2-cyclohexen-1-ol), 2.0 μM (for isatin), 10 μM (for *S*-camphorquinone), 10 μM (for 5α-dihydrotestosterone), 27 μM (for testosterone), 34 μM (for 17β-estradiol), 14 μM (for 4-pregenene-17α,20α-diol-3-one), and 17 μM (for 4-androsten-3α-ol -17-one). These values were comparable with those of the recombinant enzyme shown in Tables 1 and 2. On the other

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Table 1. **Substrate specificity for non-steroidal substrates in the oxidation and reduction reactions catalyzed by TBER1 and rat 17HSD5.**

Substrate	TBER1			Rat 17HSD5	
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
Oxidation						
S-Tetralol	168	45	0.27		(0.5)	
S-Indan-1-ol	134	25	0.19	3,080	6.8	0.002
trans-Benzene dihydrodiol	215	38	0.18	2,300	4.1	0.002
α-3-Hydroxyhexobarbital	420	17	0.04	1,100	2.3	0.002
2-Cyclohexen-1-ol	1,500	26	0.02	7,000	3.5	0.0005
β-3-Hydroxyhexobarbital	1,180	7.2	0.006	840	3.5	0.004
9α,11β-PGF_2	24	0.15	0.006	161	1.8	0.01
9α,11α-PGF_2	72	0.17	0.002	54	0.51	0.01
Reduction						
9,10-Phenanthrenequinone	0.4	157	393	0.5	18	36
Isatin	2.2	105	48	2.1	11	5.2
S-Camphorquinone	9.9	113	11	5.7	12	2.1
R-Camphorquinone	33	78	2.4	1.6	11	6.9
TBE	180	75	0.42	16	9.2	0.58
4-Chlorobenzaldehyde	28	13	0.46	263	6.5	0.02
4-Nitrobenzaldehyde	96	18	0.20	11	7.9	0.72
2,3-Pentanedione	385	29	0.08	57	8.8	0.15
2,3-Butanedione	4,800	100	0.02	126	16	0.13
4-Nitroacetophenone		NS		84	8.3	0.10
Acetylpyridine		NS		266	4.5	0.02

K_m (μM), k_{cat} (min^{-1}), and k_{cat}/K_m (min^{-1} μM^{-1}) values were determined at pH 7.4 with NAD(H) for TBER1 and with NADP(H) for rat 17HSD5 as the coenzyme. The value in parenthesis was calculated from the specific activities with 1 mM *S*-tetralol. NS, no significant activity with 1 mM substrate.

Table 2. **Substrate specificity for steroids in the oxidation and reduction reactions catalyzed by TBER1 and rat 17HSD5.**

Substrate	TBER1			Rat 17HSD5	
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
(A) 5β-Androstane-3α,17β-diol	3.5	28	8.1	10	7.8	0.78
5α-Dihydrotestosterone	9.1	12	1.30	28	18	0.65
5β-Androstan-17β-ol-3-one	9.1	14	1.20	31	22	0.71
Testosterone	25	16	0.62	21	21	1.0
17β-Estradiol	33	9.0	0.27	14	25	1.8
5α-Androstane-3α,17β-diol	17	4.2	0.25	19	8.4	0.44
(B) 4-Pregenene-17α,20α-diol-3-one	12	7.9	0.66		(0.05)	
5α-Pregnan-20α-ol-3-one	5.1	2.4	0.47		NS	
4-Pregenen-20α-ol-3-one	8.1	3.2	0.39	24	8.5	0.35
4-Androsten-3α-ol-17-one	14	3.6	0.25		NS	
5β-Pregnan-3α-ol-20-one	8.4	0.83	0.10		NS	
(C) 5β-Androstan-3α-ol-17-one	9.2	0.36	0.04		NS	
5β-Androstane-3,17-dione	19	3.2	0.17	6.2	1.6	0.25
5β-Androstan-3α-ol-17-one	42	6.6	0.16	6.7	1.3	0.20
4-Androsten-3α-ol-17-one	13	1.3	0.10	8.5	1.9	0.22
5α-Androstane-3,17-dione	18	1.3	0.07	2.4	2.5	1.1
4-Androstene-3,17-dione	17	1.0	0.06	1.6	2.0	1.3
Estrone		(0.3)		11	2.7	0.25
Dehydroepiandrosterone		(0.06)		11	5.0	0.45
(D) 17α-Hydroxyprogesterone	2.5	2.5	1.0	50	6.2	0.12
5β-Pregnane-3,20-dione	1.6	0.78	0.49		(0.38)	
5β-Pregnan-20α-ol-3-one	19	1.0	0.05		NS	
(E) 16-Ketoestrone	0.4	113	283	0.7	5.2	7.5
21-Dehydrocortisol	4.5	23	5.1	2.8	7.2	2.6

K_m (μM), k_{cat} (min^{-1}), and k_{cat}/K_m (min^{-1} μM^{-1}) values were determined in the oxidation of (A) 17β-hydroxysteroids and (B) 3α- and 20α-hydroxysteroids, and in the reduction of (C) 3- and 17-ketosteroids, (D) 3- and 20-ketosteroids, and (E) steroids with dicarbonyl structures, using the coenzymes listed in Table 1. The values in parentheses were calculated from the specific activities with 20 μM substrates. NS, no significant activity was detected with 20 μM substrate.

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hand, the partially purified protein encoded in the R1-S cDNA did not exhibit significant oxidoreductase activity towards any of the substrates listed in Tables 1 and 2, except that it exhibited low reductase activity (0.6 mU/mg) towards 20 μM 9,10-phenanthrenequinone.

Properties of Rat 17HSD5—The above results indicated that TBER1 is a NAD^+-dependent 17β-HSD distinct from the human and mouse $NADP^+$-dependent 17HSD5s, but it remains unknown whether 17HSD5 is present or not in rat tissues. Our database survey with the mouse 17HSD5 sequence against the recent rat genomic sequence revealed a gene that encodes a protein (rHSD) showing high sequence identity (86%) with the mouse enzyme (Fig. 1). Therefore, we isolated the cDNA for rHSD from rat liver by RT-PCR, and then expressed the recombinant enzyme. The *E. coli* cell extract expressing rHSD exhibited $NADP^+$-dependent testosterone dehydrogenase activity (0.05 U/mg). The enzyme was purified to homogeneity (Fig. 2B), with a yield of 46% (10 mg/liter of the culture). The gel filtration and SDS-PAGE analyses indicated that the enzyme was a 37-kDa monomeric protein. On oxidation of 0.1 mM testosterone, rHSD showed low K_m (1.5 μM) and high k_{cat}/K_m (15 min^{-1} μM^{-1}) values for $NADP^+$ compared to the respective values for NAD^+ (261 μM and 0.25 min^{-1} μM^{-1}). In the reverse reaction, NADPH (K_m = 0.5 μM and k_{cat}/K_m = 26 min^{-1} μM^{-1}) was also a better coenzyme than NADH (K_m = 26 μM and k_{cat}/K_m = 3.8 min^{-1} μM^{-1}). The values for the reduced coenzymes were determined with *R*-camphorquinone saturation (20 μM and 200 μM, respectively), because the K_m values for the substrate determined with 0.1 mM NADPH and NADH were 1.9 μM and 19 μM, respectively. The pH optima of the $NADP^+$-linked testosterone dehydrogenase and NADPH-linked *R*-camphorquinone reductase activities were 10.0 and 7.0, respectively. The coenzyme specificity and optimal pH of rHSD were distinct from those of TBER1.

rHSD was active towards the substrates of TBER1, but they differ from each other in the following points. (i) The catalytic efficiency of rHSD for the non-steroidal alcohols was lower than that of TBER1, whereas in the reverse reaction rHSD showed much lower K_m values for *R*-camphorquinone, TBE, 4-nirobenzaldehyde, 2,3-pentanedione and 2,3-butanedione, and uniquely reduced 4-nitroacetophenone and acetylpyridine (Table 1). On reduction of TBE, rHSD predominantly formed 4*S*-TBEH (the 4*R*/*4S* ratio = 1:34), which is in contrast to the 4*R*-TBEH selective reduction by TBER1. (ii) rHSD efficiently reduced a variety of 17-ketosteroids including estrone and dehydroepiandrosterone that were poor substrates for TBER1 (Table 2). (iii) rHSD exhibited 20α-HSD activity toward some steroids, but did not oxidize 3α-hydroxysteroids. The substrate specificity of rHSD is similar to that of mouse 17HSD5 (*13*, *14*). The results, together with the coenzyme specificity, indicated that rHSD was rat NADP(H)-dependent 17HSD5.

Rat 17HSD5 also differed from TBER1 in inhibitor sensitivity. It was more sensitive to inhibition by quercetin (IC_{50} = 1.0 μM), genistein (IC_{50} = 5.4 μM), and dicumarol (IC_{50} = 6.4 μM) than TBER1 (the respective IC_{50} values were 6.1, 26 and 38 μM). Conversely, the inhibitory potencies of diethylstilbestrol (IC_{50} = 7.5 μM), hexestrol (IC_{50} = 30 μM), and zearalenone (IC_{50} = 36 μM) for 17HSD5 were lower than those for TBER1 (the respective IC_{50} values were 1.7, 0.8 and 7.5 μM). The two enzymes were not inhibited by 10 μM medroxyprogesterone acetate, 50 μM betamethasone, 0.5 mM ketamine or 1.0 mM pyazole.

Expression in Tissues—The tissue distribution of the mRNAs for TBER1 and 17HSD5 in female and male rats was initially assessed by RT-PCR. As illustrated in Fig. 4A, the TBER1 transcript was detected in many tissues, of which the liver, kidney, stomach, small intestine and ovary showed high expression of the transcript. 17HSD5 mRNA was detected only in the liver and kidney. No apparent sex difference in the expression of the TBER1 mRNA was observed.

The expression of TBER1 and 17HSD5 was also analyzed by Q-Sepharose column chromatography of the cytosolic low-molecular weight (around 36 kDa) proteins of rat liver and stomach (Fig. 4B). The TBER activity was separated into two peaks, TBER1 and TBER2, which were coincident with a major peak of NAD^+-linked testosterone dehydrogenase activity and a $NADP^+$-linked androsterone dehydrogenase activity peak, respectively. The anti-TBER1 antibody reacted with the proteins in the TBER1 fraction from the liver and stomach, but not with those in the TBER2 fraction (data not shown). A major peak of $NADP^+$-linked testosterone dehydrogenase activity was observed for the non-adsorbed fraction on Q-Sepharose column chromatography of the liver cytosol, the elution pattern being similar to that on purification of the recombinant 17HSD5. Such $NADP^+$-linked testosterone dehydrogenase activity was not detected on chromatography of the stomach cytosol, coinciding with the lack of expression of the mRNA for 17HSD5 in the tissue. The peak of the NAD^+-linked testosterone dehydrogenase activity due to TBER1 was greater than the other minor peaks of the NAD^+- and $NADP^+$-linked activities.

Identity of TBER2 with 3α-HSD—The coelution of the TBER and androsterone dehydrogenase activities in the above chromatographic analyses suggested the identity of TBER2 with 3α-HSD. To confirm this, the recombinant rat 3α-HSD was purified, and its kinetic constants and stereoselective reduction on NADPH-linked reduction of TBE were determined. The purified recombinant enzyme showed $NADP^+$-linked androsterone dehydrogenase activity (1.29 U/mg), and efficiently reduced TBE (K_m = 23 μM, k_{cat} = 24 min^{-1}). The product ratio of 4*R*-TBEH to 4*S*-TBEH was 1:20. The results, together with the slightly faster migration of the recombinant 3α-HSD compared with TBER1 on SDS-PAGE (Fig. 2B), are almost identical to the properties of TBER2 purified from rat liver (*5*). In the reverse reaction at pH 7.4, the recombinant 3α-HSD oxidized *4S*-TBEH (K_m = 3.6 mM, k_{cat} = 6.1 min^{-1}), but not *4R*-TBEH. The enzyme also oxidized α-3-hydroxyhexobarbital (K_m = 1.6 mM, k_{cat} = 3.0 min^{-1}) and its β-isomer (K_m = 2.5 mM, k_{cat} = 4.7 min^{-1}), and *trans*-benzene dihydrodiol (K_m = 0.37 mM, k_{cat} = 3.6 min^{-1}).

DISCUSSION

The present cloning and expression of the cDNA for rat liver TBER1 indicated that the enzyme belongs to the AKR superfamily and is a novel type of 17β-HSD, *i.e.*, it is distinct from the human and mouse 17HSD5s (*10–14*) and rat 17HSD5. Of the two cDNAs isolated, the sequence of R1-S is highly similar to those of cDNAs for a rat liver

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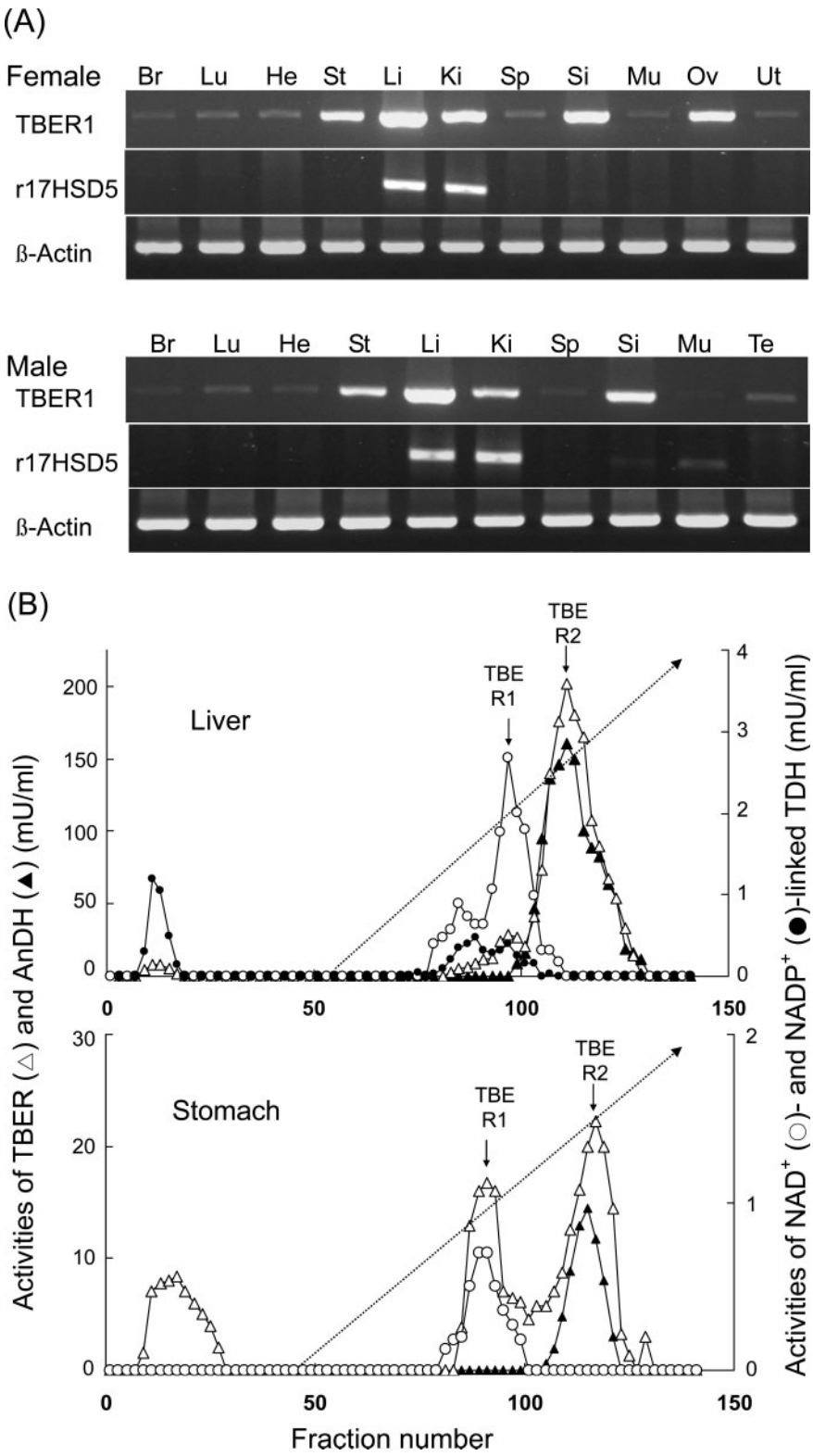

Fig. 4. **Tissue distribution of TBER1 and rat 17HSD5 (r17HSD5).** A: RT-PCR analysis of expression of the mRNAs for the two enzymes. Tissues: Br, brain; Lu, lung; He, heart; St, stomach; Li, liver; Ki, kidney; Sp, spleen; Si, small intestine; Mu, muscle; Ov, ovary; Ut, uterus; and Te, testis. The expression of the mRNA for β-actin was analyzed as a control. B: Elution patterns of the activities of NAD^+- and $NADP^+$-linked testosterone dehydrogenase (TDH), $NADP^+$-linked androsterone dehydrogenase (AnDH), and NADPH-linked TBE reductase (TBER) on Q-Sepharose chromatography of the cytosolic low molecular weight proteins from rat liver and stomach. The adsorbed enzymes were eluted with a linear gradient of 0–0.1 M NaCl (—). The dehydrogenase and reductase activities were assayed with 0.1 M glycine-NaOH, pH 9.5, and potassium phosphate buffer, pH 6.0, respectively. The peaks of TBER1 and TBER2 are indicated by arrows.

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regeneration-related protein (accession no. AY327508) and rat liver aldo-keto reductase f (RAKf) (*25*), which have stop codons at the same position as R1-S. However, RT-PCR, diagnostic restriction with *Taq*I and characterization of the recombinant enzymes indicated that another cDNA, R1-L, is the full-length one for the active TBER1. The identity of this cDNA clone was further supported by the almost same K_m values for representative substrates of the native hepatic TBER1 and the recombinant enzyme from R1-L under identical assay conditions. The sequence of R1-L is essentially identical to that of the transcript of the genomic gene (accession no. LOC364773), and no genes for the 303–amino acid polypeptide encoded by R1-S were identified on recent rat genomic analysis. R1-S is thus likely to represent an artifact mutation, *i.e.*, the C-to-T substitution at nucleotide 910 of the TBER1 mRNA, expressing during the cDNA isolation, although we can not rule out the possibility of the presence of a gene for the mRNA that has not been revealed by the draft sequence of the rat genome. R1-S encodes the C-terminal truncated 303–amino acid form of TBER1, which did not show any enzymatic activity towards TBER1 substrates, except for 9,10-phenanthrenequinone. This, in turn, implies experimental verification of the importance of the deleted C-terminal region in catalysis, probably substrate binding (*26*), for members of the AKR superfamily.

TBER1 has been described to utilize equally both NADPH and NADH based on the kinetic constants for the coenzymes determined at pH 6.0 (*5*). The significant pH-dependent changes in the K_m values for NADP(H) observed in this study clearly indicate that the enzyme exhibits a pronounced preference for NAD(H) at a physiological pH of 7.4. The pH-dependent changes in the kinetic constants can be explained by the ionization state of the acidic residue (Glu276). The crystal structures of NADP(H)-dependent human 20α-HSD (AKR1C1, *27*), 3α-HSD type 3 (AKR1C2, *28*), and rat 3α-HSD (*29*) in the AKR superfamily have shown that the specificity for NADP(H) is due to the interactions of basic residues at positions 270 and 276 with a charged 2′-phosphate of the coenzymes. The basic residues are conserved in the mouse and rat NADP(H)-dependent 17HSD5s, whereas the corresponding residues of TBER1 are Gln and Glu (Fig. 1), which are presumably located near the 2′-phosphate of NADPH in these enzymes. Of the two residues the side chain of Glu276 is fully ionized at pH > 7.0, and its carboxyl group may result in electrostatic repulsion of the negatively charged 2′-phosphate of NADP(H), leading to a decrease in affinity for the coenzymes. The ionization state of the residue does not have great effects on its interaction with the hydroxyl groups of adenine ribose of NAD(H), as shown by the crystal structures of NAD(H)-dependent enzymes (*30*). Under acidic pH conditions, the electrostatic repulsion is reduced by the decrease in the ionized Glu276 species. Although further site-directed mutagenesis studies are needed to confirm this hypothesis, Glu276 is conserved in other AKR1C subfamily members such as AKR1C19 (*31*), AKR1C22 (*32*), and rabbit and hamster morphine 6-dehydrogenases (*33*, *34*), which show the coenzyme preference for NAD(H) at pH ⩾ 7.4.

In the liver cytosol, and presumably in other tissues, the ratio of NAD^+ to NADH is about 1,000, whereas the $NADP^+$/NADPH ratio is about 0.01 (*35*). This suggests that the NAD(H)-preferring cytosolic TBER1 will function in the oxidation direction, which is also supported by the high inhibition of the NADPH-linked reductase activity by NAD^+. Although low oxidoreductase activity of hepatic TBER1 for some ketosteroids and 17β-estradiol was previously reported (*5*), its specificity and the kinetic constants for steroid substrates have not been determined. The present finding that the enzyme exhibits higher catalytic efficiency for hydroxysteroids than that for xenobiotic substrates suggests the involvement of the enzyme in steroid metabolism. Based on the substrate specificity for hydroxysteroids and tissue distribution, TBER1 may play a role in the inactivation of androgen and estrogen, and the production of progesterone from 4-pregnen-20α-ol-3-one in rat liver and stomach, where the enzyme is a major form of cytosolic low-molecular weight 17β-HSD. Among the known eleven types of 17β-HSD only the mRNA for the type 2 enzyme was identified in the gastrointestinal tracts of mice (*36*, *37*) and humans (*38*, *39*). 17β-HSD type 2 is suggested to be involved in the metabolism of orally ingested and environmental steroids, which is related to rapid cell proliferation in the gastroduodenal mucosa through inactivation of estrogens that regulate the growth, differentiation and functioning of various tissues including the gastrointestinal tract (*36*–*39*). TBER1, similar to 17β-HSD type 2, is an oxidative 17β-HSD, and its unique expression in the rat gastrointestinal tract suggests its physiological function is that proposed for 17β-HSD type 2.

TBER1 slowly reduces PGE_2 (*5*). The recombinant enzyme oxidized 9α,11β-PGF_2 and 9α,11α-PGF_2 ($PGF_{2\alpha}$), but did not show PGD_2 reductase or 9β,11α-PGF_2 dehydrogenase activity. This substrate specificity for PGs suggests that TBER1 acts as a 9-hydroxyprostaglandin dehydrogenase, but not a PGF synthase. Although the oxidized products of the two PGs were not identified in the present study, they must be PGD_2 and PGE_2, of which PGE_2 is well known to exhibit a protective effect on the gastric mucosa. While PGE_2 is metabolized to $PGF_{2\alpha}$ by several NADPH-dependent enzymes such as PGE_2 9-ketoreductase (*40*), 20α-HSD (*41*), and carbonyl reductase (*42*), the reverse reaction is catalyzed by only one enzyme, NAD^+-dependent 9-hydroxyprostaglandin dehydrogenase, which is distributed in rat kidney and liver (*43*). The kinetic constants for $PGF_{2\alpha}$ of TBER1 are comparable to those for PGs of the PGE_2-reducing enzymes, except that rat 9-hydroxyprostaglandin dehydrogenase shows a low K_m for $PGF_{2\alpha}$. Thus, TBER1 may play an additional role in the production of PGE_2 from $PGF_{2\alpha}$ in the stomach.

The substrate specificity of TBER1 for xenobiotic alcohols suggests that it acts as a 3-hydroxyhexobarbital dehydrogenase and a dihydrodiol dehydrogenase. An $NADP^+$-dependent 3-hydroxyhexobarbital dehydrogenase has been isolated from rat liver and is thought to be 3α-HSD (*44*). The identity of the two enzymes is supported by the present finding that the recombinant rat 3α-HSD oxidized α- and β-3-hydroxyhexobarbitals. In rat liver, 3α-HSD is also a major form of $NADP^+$-dependent dihydrodiol dehydrogenase that is involved in the metabolic activation of polycyclic aromatic hydrocarbons (*45*). TBER1 shows higher catalytic efficiency for 3-hydroxyhexobarbitals and *trans*-benzene dihydrodiol, a representative substrate of dihydrodiol dehydrogenase, than rat $NADP^+$-dependent 3-hydroxyhexobarbital dehydrogenase and 3α-HSD. With

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the high intracellular ratio of NAD^+/NADH and NADPH/$NADP^+$, the contribution of TBER1 to the xenobiotic metabolism may be greater than that of the known $NADP^+$-dependent enzymes.

In contrast to TBER1, rat 17HSD5 shows almost absolute coenzyme specificity for NADP(H), and will act as a reductase because of the high intracellular NADPH/$NADP^+$ ratio. The enzyme shows lower K_m values and higher catalytic efficiency for 17-ketosteroids than TBER1. Similar to the liver-specific expression of mouse 17HSD5 (*13*), the rat enzyme is specifically expressed in liver and kidney. Therefore, rat 17HSD5 contributes to the formation of active androgens and estrogen from circulating inactive 17-ketosteroids such as 5α-androstane-3,17-dione, 4-androstene-3,17-dione and estrone in the tissues. In addition, the broad substrate specificity for xenobiotic carbonyl compounds including aromatic ketones suggests that rat 17HSD5 functions as a xenobiotic carbonyl reductase. Since the enzyme reduced TBE and was coeluted with NADPH-linked TBER activity in the non-adsorbed fraction on Q-Sepharose chromatography, it probably acts as a minor TBER. Furthermore, the high catalytic efficiency for 21-dehydrocortisol suggests that rat 17HSD5 acts as a 21-HSD, which catalyzes the irreversible reduction of 21-dehydrocorticosteroids to corticoids (*21*, *46*). Rat liver contains cytosolic NADH- and NADPH-dependent forms of 21-HSD (*46*), of which the NADPH-dependent form is similar to 17HSD5 with respect to the high coenzyme specificity and molecular weight.

In summary, we have shown that, in addition to the identity of TBER2 with 3α-HSD, TBER1 is a novel type of 17β-HSD in the AKR superfamily, in which the enzyme has been assigned as AKR1C24. This, together with the identification of rat 17HSD5, indicates that the two types of cytoplasmic 17β-HSD, which differ in catalytic properties, are differently expressed in rat tissues. Based on the coenzyme preference of TBER1 for NAD^+ and its high expression in the gastrointestinal tract, we suggest that the novel 17β-HSD plays an important physiological role in the gastrointestinal tract through control of the concentrations of biologically active steroids and/or PGs, as well as xenobiotic metabolism.

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