Cloning and characterization of a simian UDP-glucuronosyltransferase enzyme UGT2B20, a novel C₁₉ steroid-conjugating protein

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Steroid glucuronidation by UDP-glucuronosyltransferase (UGT) enzymes is a mechanism leading to catabolism and elimination of steroid hormones. To establish an animal model to investigate the conjugation of steroids by UGT enzymes, previous results revealed that simian and human are unique in having high levels of circulating androsterone glucuronide and androstane-3 α ,17 β -diol (3d-Diol) glucuronide. A cDNA, UGT2B20, was isolated from cynomolgus monkey liver and prostate libraries. The cDNA was 2075 bp in length and contained an open reading frame of 1590 bp, encoding a protein of 530 amino acid residues. The UGT2B20 clone was transfected and stably expressed in the human embryo kidney HK293 cell line, and the transferase activity of UGT2B20 was tested with 73 compounds. This enzyme was shown to be active with androgens, such as testosterone, dihydrotestosterone (DHT) and 3 α -Diol, and on catecholestrogens including 1,3,5,10-oestratriene-3,4-diol-17-one. Kinetic analysis performed with intact cells yielded apparent K_m

values of 1.1, 2.3 and 4.6 μ M for 3 α -Diol, DHT and testosterone respectively. Reverse transcriptase-PCR analysis demonstrated that UGT2B20 transcript is expressed in several tissues including the liver, prostate, kidney, epididymis and adrenal of the cynomolgus monkey. Amino acid sequence alignment shows that the UGT2B20 protein is 92% identical with UGT2B15. Both enzymes have similar apparent K_m values for DHT and 3 α -Diol, and demonstrate similar transcript tissue distribution. The characterization of simian UGT2B20 as a structural and functional homologue of human UGT2B15 further demonstrates the similarities of steroid glucuronidation in these two species, and indicates the relevance of using the monkey as an animal model to study and understand steroid glucuronidation in extrahepatic-steroid target tissues.

Key words: androgen metabolism, glucuronidation, steroid catabolism, glycosyltransferase.

INTRODUCTION

UDP-glucuronosyltransferase (UGT) enzymes are important phase-II detoxifying enzymes that conjugate xenobiotics [1] as well as endogenous compounds, such as bilirubin, bile acids, thyroxine, biogenic amines, fat-soluble vitamins and steroids, which have functional groups of oxygen, nitrogen, sulphur or carbon [2–5]. Glucuronidated compounds are more polar, less toxic and are generally eliminated from the body through the bile or urine.

Mammalian UGT enzymes are categorized into two families, UGT1 or UGT2, based on similarity of their primary structures [2,6]. Proteins of the UGT2B subfamily catalyse the glucuronidation of several classes of endogenous substrate, including bile acids, fatty acids and steroid hormones [7,8]. Although the human UGT2B proteins demonstrate an overlap of substrate specificities, they are specific for different classes of steroids, which are glucuronidated at their hydroxyl groups. It is widely accepted that the liver is a major site of glucuronidation; however, it is now clear that other human tissues, such as the prostate, testis, kidney and breast, also express steroid-conjugating UGT enzymes [8–10].

To obtain an appropriate animal model to study the role of steroid glucuronidation in humans, a comparison of the circulating levels of 5 α -reduced C₁₉ steroid glucuronides among mammalian species showed that human and monkey are unique in having high levels of circulating androsterone (ADT) glucuronide and androstane-3 α ,17 β -diol (3 α -Diol) glucuronide [11]. In a separate study, humans and simians were found to be unique in

having adrenals that secrete large amounts of the precursor steroids dehydroepiandrosterone and dehydroepiandrosterone sulphate, which are converted into potent androgens and/or oestrogens in peripheral tissues [12]. As found in humans, extrahepatic steroid target tissues, which include the prostate, testis, skin and breast, of the monkey also express steroid-conjugating UGT transcripts. All these data indicate that the monkey is the most appropriate animal model for studying the role of steroid glucuronidation in extrahepatic tissues.

The similarities of steroid glucuronidation between the human and monkey are supported by the high sequence conservation and similar characteristics of three novel UGT2B enzymes isolated from the cynomolgus monkey (UGT2B9, UGT2B18 [7,13] and UGT2B19) (G. Bélanger, O. Barbier, D. W. Hum and A. Bélanger, unpublished work). The transcripts encoding the three simian proteins are expressed in a wide range of extrahepatic steroid target tissues. Two of the enzymes, UGT2B9 and UGT2B19, are active on several classes of steroid, whereas UGT2B18 glucuronidates only C₁₉ steroids containing a hydroxyl group at position 3 α of the molecule. In the present study, we report the isolation and characterization of a novel simian UGT enzyme, UGT2B20, that glucuronidates C₁₈ and C₁₉ steroids.

EXPERIMENTAL

Materials

UDP-glucuronic acid (UDPGA) and all aglycones were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and ICN Pharmaceuticals Inc. (Quebec, Canada). Radioinert steroids were

Abbreviations used: UGT, UDP-glucuronosyltransferase; DHT, dihydrotestosterone; ADT, androsterone; 3 α -Diol, androstane-3 α ,17 β -diol; RT, reverse transcriptase; UDPGA, UDP-glucuronic acid.

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purchased from Steraloids Inc. (Wilton, NH, U.S.A.). [^{14}C]-UDPGA (285 mCi/mmol) was obtained from NEN Dupont (Boston, MA, U.S.A.) and [α - ^{32}P]dCTP (3000 Ci/mmol) was from Amersham (Ontario, Canada). Geneticin (G418) and Lipofectin were obtained from Gibco BRL (Ontario, Canada). Protein-assay reagents were obtained from Bio-Rad (Richmond, CA, U.S.A.). Restriction enzymes and other molecular biology reagents were from Pharmacia LKB Biotechnology Inc. (Milwaukee, WI, U.S.A.), Gibco BRL, Stratagene (La Jolla, CA, U.S.A.) and Boehringer Mannheim (Indianapolis, IN, U.S.A.). AmpliTaq DNA polymerase was from Perkin-Elmer Cetus (Branchburg, NJ, U.S.A.). Human embryonic kidney (HK293) cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

Monkey RNA isolation

Total RNA was isolated from monkey liver, pancreas, colon, intestine, bladder, gall bladder, adrenal, kidney, prostate and epididymis, mammary gland, heart, spleen, vagina, ovary, thyroid and stomach, and from HK293 cells, according to the Tri reagent acid phenol protocol as specified by the supplier (Molecular Research Center Inc., Cincinnati, OH, U.S.A.). The mRNAs from monkey liver and prostate tissues were obtained by affinity chromatography through oligo(dT)-cellulose (Pharmacia).

cDNA isolation

Affinity-purified liver and prostate mRNAs was used to construct cDNA libraries in the λ ZAP expression vector as specified by the manufacturer's instructions (Stratagene). The library was not amplified for screening as previously described [9]; and the UGT2B20 cDNA clone was excised from the pBK-CMV vector using a helper phage as instructed by the supplier (Stratagene). The UGT2B20 cDNA clone was sequenced in both directions by dideoxy sequencing using specific oligonucleotides [9].

Transcription/translation *in vitro* of the UGT2B20 cDNA

The entire UGT2B20 cDNA in the pBK-CMV vector was transcribed and translated using T3 RNA polymerase in the transcription/translation-coupled rabbit reticulocyte lysate system from Promega (Madison, WI, U.S.A.) in the presence of [^{35}S]methionine. The protein product was separated on a 10% SDS/polyacrylamide gel and exposed on Hyperfilm-MP for 1 h.

Stable expression of UGT2B20

HK293 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 10 mM Hepes, 110 $\mu\text{g}/\text{ml}$ sodium pyruvate, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 10% fetal bovine serum in a humidified incubator, with an atmosphere of 5% CO_2 , at 37 °C. pBK-CMV-UGT2B20 (5 μg) was used to transfect HK293 cells using Lipofectin and a stable transfectant was selected in media containing 1 mg/ml G418 as previously described [9].

Microsome isolation

HK293 cells (8×10^6) were homogenized in 5 ml of homogenization buffer [13] and centrifuged at 12000 g for 20 min at 4 °C. The supernatant was recentrifuged at 105000 g for 1 h at 4 °C. The resulting microsome pellet was resuspended in 0.5 ml of homogenization buffer, aliquoted and stored at -80 °C. For the tunicamycin treatment, HK293 cells stably expressing UGT2B15 and UGT2B20 were incubated in the presence of 1 $\mu\text{g}/\text{ml}$ tunicamycin for 16 h [14], prior to the isolation of microsomes.

Western blotting using human anti-UGT2B17 (EL-93) antibody

Microsomal proteins from untransfected HK293 cells and from HK293 cells stably expressing UGT2B20 and UGT2B15 treated or not with tunicamycin were separated by SDS/PAGE (10% gel). The proteins were transferred on to a nitrocellulose membrane and probed with the EL-93 anti-UGT2B antisera (1:1000 dilution) as reported [15]. An anti-rabbit IgG horse antibody conjugated with horseradish peroxidase (Amersham) was used as the second antibody, and the resulting immunocomplex was visualized using an enhanced chemiluminescence kit (Renaissance, Quebec, Canada) and exposed on hyperfilm for 5 min (Kodak Corp., Rochester, NY, U.S.A.).

Glucuronidation assay using microsome preparations

To screen for substrates that react with UGT2B20, assays were performed using 6 μM [^{14}C]UDPGA, 94 μM unlabelled UDPGA, 200 μM aglycone and 25 μg of proteins from microsome preparations in 50 mM Tris/HCl (pH 7.5)/10 mM MgCl_2 /100 $\mu\text{g}/\text{ml}$ phosphatidylcholine/8.5 mM saccharolactone in a final volume of 100 μl . Assays were performed for 16 h at 35 °C, and were terminated by adding 100 μl of methanol. Chromatography analysis was performed and formation of glucuronide determined as previously described [9]. Compounds that demonstrated reactivity with UGT2B20 in this screening assay were subsequently reassayed to assess activity in the same buffer, containing 6 μM [^{14}C]UDPGA, 494 μM unlabelled UDPGA and 200 μM aglycone, for 15 min at 35 °C. Under these saturating conditions, the enzyme reaction was linear for 30 min.

pH and temperature effects on UGT2B20 activity

The effect of pH on eugenol glucuronide formation was ascertained by assay conditions used for assessing enzyme activity in the presence of 50 mM Tris/HCl, with pH varying from 5.5 to 9.5 at 35 °C. The effect of temperature was determined by incubation at temperatures from 25 to 40 °C. The assays were always performed with 200 μM eugenol, in the presence of 25 μg of microsomal proteins.

Protein-stability analysis in intact cells

HK293 cells stably expressing UGT2B20 and UGT2B15 were plated at a density of 2×10^5 cells/well in 12-well plastic plates and treated with 20 $\mu\text{g}/\text{ml}$ cycloheximide for 2, 6 and 12 h. After treatment, the glucuronidation activity was assessed by incubating the cells with 5 μM radioinert 3 α -Diol and 20 nM [^3H]3 α -Diol as substrate, in the presence of 20 $\mu\text{g}/\text{ml}$ cycloheximide. The cells expressing UGT2B15 were assayed for 5 h, and the cells expressing UGT2B20 for 12 h. Due to the low stability of UGT2B20 activity, the enzyme was reassayed after cycloheximide treatment for 15, 30, 60, 120, 240 and 360 min, to determine the protein-activity half-life.

To determine enzyme activity, the amount of glucuronidated radiolabelled product in the medium was measured following organic extraction and scintillation counting as previously described [16]. The results were normalized by measuring DNA content, quantified by fluorometric assay with 3,5-diaminobenzoic acid [17,18].

Apparent K_m determination in intact HK293 cells

Apparent K_m [$K_{m(\text{app})}$] determinations were performed by incubating intact HK293 cells with radiolabelled steroid substrates as previously described [13]. Cells were incubated for 12 h at 37 °C with 0.5, 1, 2.5, 5, 10 or 20 μM radioinert 3 α -Diol with

UGT2B20	<u>MSLKWTSVFLLLQLSCYFSSGSCGKVLVWPTEYSHWINMKTILEELVRRRHEVTVLTS</u> <u>SA</u>	60
UGT2B15	I.....Q.G.....	
UGT2B17	M.....Q.G.....I.....	
UGT2B20	<u>STFV</u> <u>ND</u> <u>SKSSAIKFEVYPTSLTKNDMEDSLMKLLDIWTYSISNSTFLSYFSKLQELCWEY</u>	120
UGT2B15	..L..A.....L.....L....L..I..R..I..GV.KN..W....Q.....	
UGT2B17	..IL..A.....L.....L..FF..MF.R.....KN..W....Q.....	
UGT2B20	YYYSEKLCDAVLNKKLMTKLKETFVDVILADALNPCGELLAELFNIPFVYSLRFTVGYT	180
UGT2B15	.D..N.....M..Q.S.....L.....S....	
UGT2B17	SD.NI...E.....R..Q.S....L....V.....L....L.....S....	
UGT2B20	FEKNGGGFLFPSPYVPVMSSELSQMTFTERIKNMIHKLYDFWFQIHDIKWDQFYSEV	240
UGT2B15	I.M.....M.....Y.L.....	
UGT2B17	V.....I.M.....YM.....AY.L.....	
UGT2B20	LGRPTTLFETMRKAEMWLIRTYWDFEFPFPLPNVDFVGGLHCKPAKPLPKEMEETFVQSS	300
UGT2B15	G.....	
UGT2B17	G.....	
UGT2B20	GENGVVVFSLGSMISNMSEERANMIASALAIQPKVLWKFDGKKPNTLGSNTRLYKWLPO	360
UGT2B15	I.....S.....R.....	
UGT2B17	I.....S.....R.....	
UGT2B20	NDLLGHPKTKAFITHGGTNGIYEAIYHGIPMVGIPLFADQHDNIVHMKVKAALSVDIRT	420
UGT2B15	A..A.....	
UGT2B17	A..A.....	
UGT2B20	MSSRDLLNALKSVINEPIYKENAMKLSRIHHDQPMKPLDRAVFWIEFVMRHKGAKHLRVA	480
UGT2B15	D.V....V.....	
UGT2B17	D....I.....V.....	
UGT2B20	AHNLTWIQYHSLDVIAFLACVAIVIFIITKCCFLFCFRKLAKTGKKKKWD	530
UGT2B15	T.....F.....R.	
UGT2B17	TM..M.....R.	

Figure 1 Amino acid sequence of UGT2B20 and alignment with UGT2B15 and UGT2B17

The putative membrane signal sequence is denoted by the overbar and the sequence of the putative membrane-anchoring domain is underlined. Potential asparagine-linked [N(X/S/T)] glycosylation sites are indicated by bold overbars, and the broken overbar indicates the consensus 'signature sequence' of UGT enzymes.

20 nM radiolabelled [^3H]3 α -Diol; or with 0.5, 1, 2.5, 5, 10 or 20 μM radioinert dihydrotestosterone (DHT) with 20 nM radiolabelled [^3H]DHT; or with 0.5, 1, 2, 2.5, 5 or 10 μM of radioinert testosterone with 20 nM radiolabelled [^3H]testosterone. The level of product formation was determined by measuring the level of glucuronidated radiolabelled product in the medium [16,17]. The reactions were confirmed to be linear throughout the course of the assays, and less than 5% of the substrates were converted.

Reverse transcriptase (RT)-PCR analysis

The tissue distribution of UGT2B20 was achieved using an RT-PCR technique as previously reported [9], using 5 μg of total RNA from cynomolgus monkey tissues and HK293 cells. Reverse-transcriptase reactions were performed using 500 pmol of oligo(dT) in the presence of 200 units of SuperScript II reverse transcriptase according to the manufacturer's instructions (Gibco BRL). The PCR reactions were performed with one tenth of the

cRNA product and 80 ng of the specific sense primer, 5'-AATGATATGGAAGATTCTCTTATGAAACTGC-3', and 80 ng of the specific antisense primer, 3'-CTGTAAACTGGTCCCACTTCTTTATATCATG-5', using AmpliTaq DNA polymerase. The PCR was performed for 30 cycles (1 min at 94 $^{\circ}\text{C}$, 1 min at 67 $^{\circ}\text{C}$, 1 min at 72 $^{\circ}\text{C}$) after which one-fifth of the PCR product was electrophoresed on an ethidium bromide-stained 1.5% agarose gel. All PCR reactions were controlled using the sense primer, 5'-TGGGTGTGAACCATGAG-3', and antisense primer, 5'-CCCAGCGTCAAAGGTGG-3', for glyceraldehyde-3-phosphate dehydrogenase. The identity of all PCR products was verified by direct sequencing [19].

RESULTS

Isolation of the UGT2B20 cDNA

To isolate simian UGT2B cDNA clones, liver and prostate cDNA libraries were made with mRNA isolated from the cynomolgus monkey [13], and were screened with a combination

Table 1 Similarity between the primary structures of UGT2B20 and other UGT2B isoenzymes

The sequence identity and the number of amino acids different in the N-terminal domain from residues 1 to 290, in the C-terminal domain from residues 291 to 529, and in the entire protein is as indicated. The steroid specificity of each of the enzymes is shown. No activity was detected with UGT2B10. Abbreviations: AA, amino acids; E₂, oestradiol; E₃, oestriol; Etio, etiocholanolone; 4-OHE₁, 1,3,5,10-oestratriene-3,4-diol-17-one; HDCA, hydoxycholeic acid. UGT2B15 and UGT2B17, which share the most sequence identity with UGT2B20, are shown in **bold**.

Source	Enzyme	Substrate specificity	UGT2B20 amino acid identity					
			N-terminal domain		C-terminal domain		Overall	
			AA different	% Identity	AA different	% Identity	AA different	% Identity
Rat	UGT2B1	Testosterone, DHT, E ₂	105	64	57	76	162	69
	UGT2B2	ADT, Etio	120	59	64	73	184	65
Human	UGT2B4	HDCA, 4-OHE ₁ , 3 α -Diol	88	69	41	82	129	75
	UGT2B7	4-OHE ₁ , E ₃	96	67	39	83	136	75
	UGT2B10	None detected	88	69	38	83	126	76
	UGT2B15	3α-Diol, DHT, testosterone	30	90	11	95	41	92
	UGT2B17	DHT, ADT, 3α-Diol, testosterone	42	86	12	95	54	90
Simian	UGT2B9	4-OHE ₁ , DHT, ADT	89	69	40	83	129	75
	UGT2B18	ADT, 3 α -Diol, Etio	92	68	39	83	131	75
	UGT2B19	Testosterone, Etio, 4-OHE ₁	96	67	40	83	136	74

of human probes synthesized from the full-length cDNAs of UGT2B7, UGT2B10 and UGT2B15 [7,9,13]. Twenty positive cDNAs were isolated, and nucleotide-sequence analysis revealed five novel clones, including UGT2B9, UGT2B18, UGT2B19 and UGT2B20. The cDNA of UGT2B20 was 2075 bp in length, and contained an open reading frame of 1590 bp, a 5'-untranslated region of 13 bp and a 3'-untranslated region of 472 bp. The protein primary structure of 530 amino acids, which was deduced from the nucleotide sequence, revealed the presence of several conserved sequences found in UGT2B enzymes [2] (Figure 1). The UGT2B20 protein contains a characteristic hydrophobic signal peptide from residues 1 to 23, and a hydrophobic transmembrane region between amino acids 494 and 510. Whereas most UGT proteins have two or three potential asparagine-linked glycosylation sites [NX(S/T)], UGT2B20 contains four sites, present at positions 65, 104, 316 and 486.

Amino acid-sequence alignment shows that UGT2B20 is 92% and 90% identical with human UGT2B15 and UGT2B17, respectively (Table 1). Of the 41 amino acids that are different between UGT2B15 and UGT2B20 (Figure 1), 11 are found between residues 291 and 530 in the C-terminal domain, which has been proposed to bind UDPGA. It is interesting to note that UGT2B20 shows higher similarity with human isoforms UGT2B15 and UGT2B17 than with the monkey proteins UGT2B9, UGT2B18 and UGT2B19, which share 75%, 75% and 74% identity with UGT2B20, respectively. UGT2B20 is 65% identical to the rat UGT2B1 enzyme, which is principally active on testosterone and DHT.

To determine the ability of the cDNA to encode the predicted UGT2B20 protein, the cDNA in the pBK-CMV vector was transcribed by T3 RNA polymerase, and the resulting transcript was translated by rabbit reticulocyte lysates (Figure 2A). The protein product had an apparent molecular mass of 51 kDa, which is similar to other UGT2B proteins previously described [7,13].

Characterization of the UGT2B20 protein

To characterize the UGT2B20 protein, the cDNA was transfected into HK293 cells, and a stable cell line expressing UGT2B20 was established. Expression of the protein was demonstrated by the presence of a 55 kDa band in Western-blot analysis of micro-

Figure 2 Transcription/translation *in vitro* of UGT2B20 (A), and immunoblot analysis of HK293 cells stably expressing UGT2B20 and UGT2B15 (B)

(A) The transcription/translation product *in vitro* of the UGT2B20 cDNA was separated by SDS/10% PAGE. (B) Microsomal protein (25 µg) isolated from untransfected HK293 cells (lane 1), untreated HK293 cells stably expressing UGT2B20 (lane 2) or UGT2B15 (lane 4), and HK293 cells stably expressing UGT2B20 (lane 3) or UGT2B15 (lane 5) treated with 1 µg/ml tunicamycin, were chromatographed by SDS/PAGE (10% gel). The proteins were transferred by Western blot and probed with the EL-93 polyclonal antibody.

somal proteins extracted from the stable cell line (Figure 2B, lane 2). To ascertain if UGT2B20 is glycosylated, incubation of HK293-UGT2B20 cells with tunicamycin, which is an inhibitor of N-linked glycosylation, gave rise to the appearance of only a lower-molecular-mass 51 kDa immunoreactive protein (Figure 2B, lane 3). Similar results were obtained for UGT2B15 (Figure 2B, lanes 4 and 5), where the treatment of HK293-UGT2B15 cells with tunicamycin also gave rise to a faster-migrating protein; however, the presence of the higher-molecular-mass band also persisted.

To determine the substrate specificity of UGT2B20, a microsomal extract from the stably transfected HK293-UGT2B20 cell line was incubated with potential substrates, and conjugation was assessed by TLC. Of the 73 different steroids, bile acids, neuromodulators and exogenous compounds tested, only 15 were conjugated by UGT2B20 (Table 2). Glucuronidation of

Table 2 Reactivity of endogenous and exogenous compounds with UGT2B20 expressed in HK293 cells

Initial screening for reactive substrates was performed, where microsomes from HK293 cells stably expressing UGT2B20 were incubated with 6 μ M [14 C]UDPGA, and 94 μ M unlabelled UDPGA. + indicates a reactive substrate and — indicates no reactivity. Substrates that are conjugated by both UGT2B15 and UGT2B20 are underlined, and substrates that are only glucuronidated by UGT2B15 are in *italics*. The activity of UGT2B20 for the various substrates was determined by incubating microsomes with 6 μ M [14 C]UDPGA, 494 μ M unlabelled UDPGA and 200 μ M substrate. ND denotes not determined because the level of conjugation was too low to quantify. Values for glucuronide formation are the means $\pm$ S.D. of three independent experiments.

	Initial screening	Glucuronide formation (pmol/h per mg of protein)
Endogenous compounds		
C₁₉ steroids		
Testosterone	+	3.4 $\pm$ 0.1
DHT	+	ND
ADT	—	
Epiandrosterone	—	
Dehydroepiandrosterone	—	
Etiocolanolone	—	
Androst-5-ene-3 β ,17 β -diol	—	
5 α -Androstane-3 α ,17 β -diol	+	6.0 $\pm$ 0.3
5 α -Androstane-3 β ,17 β -diol	—	
5 β -Androstane-3 α ,17 β -diol	+	11.7 $\pm$ 1.4
5 β -Androstane-3 α ,11 β ,17 β -triol	—	
5 α -Androstane-3 α ,16 α ,17 β -triol	—	
5 β -Androstane-3 α ,11 α ,17 β -triol	—	
C₁₈ steroids		
Oestrone	—	
Oestradiol	—	
Oestriol	—	
1,3,5,10-Oestratriene-2,3-diol-17-one	+	ND
1,3,5,10-Oestratriene-3,4-diol-17-one	+	ND
1,3,5,10-Oestratriene-3,16 α -diol-17-one	—	
1,3,5,10-Oestratriene-2,3,17 β -triol	—	
1,3,5,10-Oestratriene-3,4,17 β -triol	—	
1,3,5,10-Oestratriene-2,3,16 α ,17 β -tetrol	+	ND
C₂₁ steroids		
Progesterone	—	
17-OH-progesterone	—	
Pregnenolone	—	
17-OH-Pregnenolone	—	
5 α -Pregnane-3 α -ol-20-one	—	
5 α -Pregnane-3 α ,20 α -diol	—	
5 α -Pregnane-3 α ,17 α -diol-20-one	—	
5 α -Pregnane-3 β ,17 α -diol-20-one	—	
5 α -Pregnane-3 β ,17 α -diol-11,20-dione	—	
5 β -Pregnane-3 α ,20 α -diol	—	
5 β -Pregnane-3 α ,17 α -diol-20-one	—	
5 β -Pregnane-3 α ,6 α ,17 α -triol-20-one	—	
Aldosterone	—	
Bile acids		
Chenodeoxycholic acid	—	
Cholic acid	—	
Lithocholic acid	—	
Hyodeoxycholic acid	—	
Cortisol and metabolites		
Cortisol	—	
11-Deoxycortisol	—	
Deoxycorticosterone	—	
Cortisone	—	
5 α -Tetrahydrocortisone	—	
Neuromodulators		
Dopamine	—	
5-Hydroxytryptamine	—	
Others		
Cholesterol	—	

Table 2 (contd.)

	Initial screening	Glucuronide formation (pmol/h per mg of protein)
Retinoic acid	—	
Tri-iodothyronine	—	
Thyroxine	—	
Vitamin D ₃	—	
Vitamin C	—	
Exogenous compounds		
Coumarins		
4-Methylumbelliferone	+	31.8 $\pm$ 3.8
Scopoletin	+	30.5 $\pm$ 2.4
Non-steroidal anti-inflammatory		
Ibuprofen	—	
Anthraquinones		
Anthraflavic acid	+	ND
Quinalizarin	—	
Flavonoids		
5,7-Dihydroxyflavone	—	
7-Hydroxyflavone	+	ND
Naringenin	—	
Opioids		
Codeine	—	
Morphine	—	
Naltrexone	—	
Other drugs		
4-Acetamidophenol	—	
Chloramphenicol	—	
Phenolic compounds		
<i>o,o'</i> -Biphenyl	—	
<i>p,p'</i> -Biphenyl	+	8.3 $\pm$ 0.4
Eugenol	+	21 $\pm$ 1.9
1-Naphthol	+	4.5 $\pm$ 0.1
<i>p</i> -Nitrophenol	+	23.6 $\pm$ 2.6
Phenolphthalein	—	
Phenol red	—	
Others		
Imipramine	—	

these compounds was not detected using control microsomal extracts from untransfected HK293 cells. The main endogenous substrates of UGT2B20 are 17 β -hydroxyandrogens; however, UGT2B20 does not glucuronidate neuromodulators and bile acids. The major exogenous substrate tested was 4-methylumbelliferone and, as found with most other UGT2B enzymes, UGT2B20 is active on planar phenols such as *p*-nitrophenol, eugenol and 1-naphthol.

The effects of pH and temperature on UGT2B20 enzyme activity were assessed using eugenol as substrate. Microsomal protein (25 μ g) from HK293-UGT2B20 cells was incubated in Tris/HCl buffer at varying pHs from 5.5 to 9.5, and at different temperatures from 25 to 40 °C. The optimal conditions for eugenol glucuronidation were pH 8 and 35 °C (results not shown). The enzyme activity was low at pHs under 7, and increased 10-fold at pH 8. However, under alkaline conditions higher than pH 8, the enzyme activity was significantly lower. The enzyme activity was also temperature dependent and was optimal at 35 °C, where eugenol glucuronidation was 2.5-fold higher than at 30 °C.

During enzyme assays using microsomes in the presence of saturating substrates, the activity of UGT2B20 was linear for

Figure 3 Stability of UGT2B20 and UGT2B15 enzyme activity

(A) Stable cell lines expressing UGT2B15 and UGT2B20 were treated with 20 $\mu\text{g}/\text{ml}$ cycloheximide for 2, 6 and 12 h, after which enzyme activity was determined by incubating the cells with radiolabelled 3 α -Diol in the presence of 20 $\mu\text{g}/\text{ml}$ cycloheximide. (B) Due to the instability of UGT2B20 activity, an additional study was performed where UGT2B20 activity was assayed after cycloheximide treatment for 15, 30, 60, 120 and 240 min. Approx. 50% of UGT2B20 activity was lost after 30 min. Results are expressed as percentage enzyme activity relative to the untreated control at time 0, and represent the means of two independent experiments, each performed in triplicate. (C) Effect of cycloheximide treatment on UGT2B20 expression level. HK293 cells stably expressing UGT2B20 were treated for 2, 4, 6 and 12 h with cycloheximide (20 $\mu\text{g}/\text{ml}$). After treatment, cells were homogenized and microsomes were isolated. Microsomal proteins (20 μg) from treated and untreated cells were then separated by SDS/PAGE (10% gel), were subsequently transferred on to nitrocellulose membrane and probed with the EL-93 antiserum, specific for UGT2B proteins. The results indicate no significant change in the level of UGT2B20 protein following inhibition of *de novo* protein synthesis by cycloheximide.

approx. 30 min. Even under the optimal conditions that were established, it was apparent that UGT2B20 was less stable than UGT2B15, which is the human enzyme with the highest sequence similarity and most similar substrate specificity. To determine if UGT2B20 is in fact less stable than UGT2B15, the HK293 cell lines stably expressing each of the enzymes were incubated with cycloheximide (20 $\mu\text{g}/\text{ml}$) for 2, 6 and 12 h to arrest protein synthesis; and the level of UGT activity was measured by incubation of intact cells with radiolabelled 3 α -Diol as substrate (Figure 3A). As previously described [18], the level of UGT2B15 activity was not affected after 12 h of treatment. However, the level of UGT2B20 activity was significantly decreased, and 50% of activity was lost after approximately 30 min, which indicates the relatively high lability of this UGT enzyme (Figure 3B). To ascertain if the loss of enzyme activity was due to protein degradation, Western-blot analysis demonstrated no significant change in the level of UGT2B20 protein during cycloheximide treatment from 0 to 12 h (Figure 3C). Thus the relatively rapid loss of UGT2B20 enzyme activity in microsome preparations was not due to protein turnover.

Kinetic analyses for UGT2B20 conjugation of 3 α -Diol, testosterone and DHT were performed using intact HK293 cells stably expressing UGT2B20 (Figure 4). The $K_{\text{m(app)}}$ values obtained for 3 α -Diol, DHT and testosterone were 1.1, 2.3 and 4.6 μM , respectively. However, the V_{max} values for testosterone were 6- and 2-fold higher than for 3 α -Diol and DHT, respectively. In our previous studies [7,9] on UGT2B enzymes, the $K_{\text{m(app)}}$ values obtained from stably transfected intact cells were shown to be similar to the values obtained from analysis performed with cell homogenates and microsome preparations. However, due to the instability of UGT2B20 activity in cell homogenates and microsome preparations, it was only possible to perform kinetic analysis using intact cells. Determination of K_{m} values using intact cells and cell homogenates both have the intrinsic limitation of potential interference by other cellular components. How-

Figure 4 Kinetic analysis of UGT2B20

The $K_{\text{m(app)}}$ and V_{max} values for UGT2B20 conjugation of 3 α -Diol, DHT and testosterone were determined by Lineweaver-Burk double-reciprocal plots. Experiments were performed using intact HK293 cells stably expressing UGT2B20. The results represent the means $\pm$ S.D. of two experiments each performed in triplicate.

Figure 5 Tissue distribution of UGT2B20 transcript

Total RNA isolated from cynomolgus monkey tissues and HK293 cells were analysed by specific RT-PCR analysis. One fifth of each RT-PCR product was separated on a 1.5% agarose gel. The 464 bp PCR product represents amplification of the UGT2B20 transcript. The specificity of the RT-PCR product was confirmed by direct sequencing. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

ever, incubation of HK293 cells with androsterone (ADT), DHT, 3 α -Diol and testosterone did not lead to conversion into other products, as demonstrated by HPLC (results not shown), and these substrates diffuse freely into the cells and therefore have equal access to the UGT2B enzymes in the endoplasmic reticulum.

Tissue distribution of UGT2B20 transcript

To ascertain the tissue distribution of the UGT2B20 transcript, RT-PCR analysis was performed with total RNA isolated from several monkey tissues. Reverse transcription was performed using an oligo(dT) primer, and the oligonucleotides used for PCR were specific for UGT2B20 and designed not to amplify the previously characterized monkey transcripts UGT2B9, UGT2B18 and UGT2B19. The 464 bp PCR product demonstrated UGT2B20 transcript expression in the monkey liver, prostate, epididymis, adrenal, vagina, ovary, mammary gland, small intestine and kidney. However, the heart, spleen, bladder, thyroid, colon, pancreas, gall bladder and stomach did not express detectable levels of this mRNA (Figure 5). The identity of the PCR product as an amplification of the UGT2B20 transcript was confirmed by direct sequencing, and the integrity of each RNA sample was verified by amplification of the glyceraldehyde 3-phosphate dehydrogenase transcript using specific oligonucleotides.

DISCUSSION

To obtain an *in vivo* system in which to study the glucuronidation of steroid hormones, it was previously ascertained that simians may be the most appropriate animal model [11]. Unlike some common laboratory mammals, such as the rat and mouse, which have different patterns of steroidogenesis to humans, simians express the same enzymes involved in steroid synthesis, and have similar patterns of tissue-specific steroidogenesis. The similarity between the human and monkey UGT2B proteins was demonstrated by Northern-blot analysis with human cDNA probes, which hybridized to simian transcripts of the appropriate size. Also, polyclonal antibodies raised against human UGT2B proteins recognized the monkey proteins in tissue extracts [13]. Characterization of the novel simian UGT2B20 cDNA clone further demonstrates the relatedness of the UGT2B proteins from these two species. Of all the UGT2B proteins that have

been characterized to date, UGT2B20 shares the highest identity with human UGT2B15 and UGT2B17. Comparisons of the N-terminal portion of the proteins, between residues 1 and 290, which have been proposed to contain the aglycone-binding domain, reveal that UGT2B20 is 90% identical to UGT2B15.

UGT2B20 is most similar to UGT2B15 with respect to specificity; they conjugate 40 substrates in common [20]. Both enzymes conjugate 3 α -Diol, DHT and testosterone, which have an OH group at the 17 β position. However, it is interesting that, unlike UGT2B17, UGT2B20 does not glucuronidate ADT, which has a 3 α -OH group, despite the high identity between these two enzymes. In fact, UGT2B17 and UGT2B20 have very similar substrate specificities except for their ability to conjugate ADT. Kinetic analysis of UGT2B20 and UGT2B15 [15] demonstrates that both enzymes conjugate 3 α -Diol, DHT and testosterone with similar $K_{m(app)}$ values in the micromolar range.

The substrate specificity of UGT2B20 shares some similarities with simian UGT2B9 and UGT2B19; however, both of these enzymes are active on C₂₁ steroids, which are not substrates for UGT2B20. In contrast, the specificity of simian UGT2B18 is considerably different, since it conjugates predominately C₁₉ and not C₁₈ steroid substrates. All four of the monkey enzymes characterized can glucuronidate 3 α -Diol, which is one of the major steroid glucuronides in the circulation. However, UGT2B19 and UGT2B20 do not conjugate ADT, which is the other major steroid glucuronide found in the plasma of primates. Based on sequence similarity, substrate specificity and kinetic analysis, it is apparent that of all the primate UGT2B proteins characterized thus far, UGT2B20 is the closest structural and functional simian homologue of the human UGT2B15 enzyme.

A potential mechanism by which UGT enzymes with overlapping substrate specificity can have distinct physiological roles is by tissue-specific expression of the different proteins. The UGT2B20 transcript was found expressed in several extrahepatic tissues, such as the epididymis, adrenals, prostate, breast and kidney. Expression of UGT2B transcripts in peripheral steroid target tissues indicates that these tissues are not only involved in the production of steroid hormones [21], but are also capable of conjugating and catabolizing steroid metabolites.

The main difference observed between the human UGT2B15 and simian UGT2B20 proteins is at the level of enzyme stability. The half-life of UGT2B20 activity was approximately 30 min, whereas the activity of UGT2B15 was unchanged after 24 h. Although UGT2B20 is glycosylated, as shown by the tunicamycin studies, it is unlikely that the presence of the additional fourth

potential asparagine-linked glycosylation site plays a role in protein lability. It has been reported that UGT enzyme stability was not affected by inhibiting protein glycosylation [14]. In addition, UGT2B17 and UGT2B15 have very different stabilities, despite containing conserved glycosylation sites. As shown with other proteins [22], it is possible that a few changes in amino acid composition are responsible for differences in enzyme stabilities.

Characterization of the UGT2B20 protein demonstrates its ability to conjugate steroids, and also demonstrates the usefulness of the monkey as an appropriate animal model in which to study steroid metabolism in extrahepatic steroid target tissues *in vivo*. The high lability of UGT2B20 suggests that it may play a role in regulating steroid levels in response to acute physiological conditions. We have previously demonstrated that expression of the labile UGT2B17 protein is regulated by cytokines and growth factors, whereas expression of the more stable UGT2B15 protein is not changed by these effectors [8,23,24], which is consistent with the level of the more labile protein being regulated and changed depending on physiological requirements. Characterization of the simian UGT2B cDNA clones and encoded proteins provides the tools required for studies *in vivo* to further understand the physiological role of steroid glucuronidation in extrahepatic steroid target tissues.

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