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The Human UDP Glucuronosyltransferase, UGT1A10, Glucuronidates Mycophenolic Acid

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The cDNA encoding the UDP glucuronosyltransferase, UGT1A10, has been cloned from human colon. The deduced amino acid sequence of the cDNA is 90% similar in sequence to that of a previously characterized form, UGT1A9 (Hlug P4), and contains a signal peptide and carboxyl-terminal hydrophobic domain characteristic of all UDP glucuronosyltransferases isolated to date. The enzyme synthesized in UGT1A10 cDNA-transfected COS-7 cells has a relative molecular mass of 56 kDa and is very active in the glucuronidation of mycophenolic acid (apparent Km of 34 μM and Vmax of 0.6 nmoles/min/mg protein). Other UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10 and 2B11) synthesized in COS cells had relatively little activity towards mycophenolic acid, suggesting that UGT1A10 may have a significant role in the elimination of this antineoplastic and immunosuppressive agent in vivo.

Mycophenolic acid is a potent and specific inhibitor of mammalian inosine-monophosphate dehydrogenase, a key enzyme in the de novo biosynthesis of purines (1). This property of mycophenolic acid is the principal reason for its cytostatic effects on lymphocytes and other cell types and has been exploited to develop the prodrug, mycophenolate mofetil, the 2-4-morpholino ethyl ester of mycophenolic acid, as an immunosuppressant and antineoplastic agent. Mycophenolate mofetil is the first drug in 10 years to be approved for the treatment of allograft rejection (1). Its clinical usefulness as a chemotherapeutic agent, however, remains to be evaluated, although there is evidence that it can decrease the risk of lymphoma development. Studies *in vivo* demonstrate that the efficacy of mycophenolic acid is limited by its rapid conversion to the biologically inactive 7-O-glucuronide (2, 3). The UDP-glucuronosyltransferase(s) (UGT)[2] involved in this process has not been identified.

To date, 12 forms of UGT have been identified in humans. These forms catalyze the glucuronidation of a wide range of toxins, therapeutic drugs and endogenous substances, including steroid hormones, fatty acids, bile acids and biogenic amines, to water soluble products that are readily excreted. The UGTs are located in the endoplasmic reticulum of cells and are widely distributed in the liver, kidney, gastrointestinal tract, lung, skin, testis and brain. The UGTs have been divided into 2 families, UGT1 and UGT2, based on their amino acid sequence similarities (4-9).

The cDNAs encoding five human family 1 forms, UGT1A1 (HUG-Br1)(6), 1A3 (10), 1A4 (HUG-Br2)(6), 1A6 (Hlug-P1)(7), and 1A9 (Hlug-P4)(8) have been expressed in cell culture and the substrate specificities of the corresponding proteins analyzed. These forms all have identical carboxy terminal domains of 245 residues that are encoded by exons 2-5 of the UGT1 locus, but have unique amino terminal domains of 280-290 residues that are encoded by one of a series of exon 1 sequences upstream from exons 2-5 (11). Analysis of the UGT1 locus has revealed the present of several additional exons 1 with the potential to encode new family 1 forms. One of these exons 1, 1j (GenBank accession number U39550), was identified from the sequencing of genomic clones. This exon is unusual as it does not appear to encode a putative signal peptide, a characteristic of all other UGTs identified to date. In this work, we have cloned the cDNA corresponding to the transcript containing exon 1j and expressed it in cell culture. The activity of the encoded protein, designated UGT1A10, was assessed with mycophenolic acid.

MATERIALS AND METHODS

Isolation of UGT cDNA and expression in transfected COS cells. Human liver and colon samples were obtained as previously de-

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Abbreviations are: UGT, UDP glucuronosyltransferase; PCR, polymerase chain reaction; RT, reverse transcription.

[2] The names of UDP glucuronosyltransferase forms are those recommended by a subcommittee of the IUBMB-IUPHAR Nomenclature Committee and supported by participants of the 8th International Workshop on Glucuronidation and the UDP Glucuronosyltransferases held in Iowa City, Iowa, May, 1996.

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scribed (10). A λgt10 human colon cDNA library (7.2×10^5 plaques) was screened with a ^{32}P-end labeled 29-mer oligonucleotide, 5′-GCC-TCTAGAAATGCTTCTGGAGAACATGG (incorporating nucleotides 874-893 of UGT1 exon 2). This oligonucleotide was designed to recognize only members of family 1 under the hybridization conditions used in the screening process as previously described (10). A total of 165 positive clones were plaque purified and the sizes of their inserts determined on agarose gels after EcoR1 digestion. The larger inserts were sequenced to identify 22 clones encoding UGT1A9-like sequences, including several clones encoding UGT1A10. These clones were all truncated at either the 5′ or 3′ ends and did not contain full coding regions. Based on the sequences of these UGT1A10 clones, primers encompassing the initiation (5′-TCCAAGCTTCTGGCA-TGGCTCGCGCAGGG) codon and following the termination codon (5′-GACAAGCTTATGGTTCAAAATTTTACCTTATTTCCC) were synthesized and used to amplify UGT1A10 cDNA from a human colon mRNA sample. These primers contained Hind III restriction sites to facilitate subcloning. The first cDNA strand was synthesized from mRNA using the reverse primer and reverse transcriptase at 42°C. The amplification was performed at 94°C, 1 min, 55°C, 1 min and 72°C, 2 min for 30 cycles. The PCR product of about 1.5 Kbp was isolated and cloned into the Hind III restriction site of the mammalian expression vector pCMV5 (12). The resultant recombinants were transfected into COS-7 cells using diethylaminoethyldextran and subsequent chloroquine treatment. The transfected cells were harvested after 48-60 hours and the microsomal fraction prepared and stored at −70°C until used in assays and Western blots. Microsomal proteins were separated by electrophoresis on SDS-polyacrylamide gels. The separated proteins were electrotransfered to nitrocellulose membranes and probed with UGT-specific antibody (10). Two UGT1A10 cDNAs that expressed active enzyme in COS cells were fully sequenced with a series of oligonucleotide primers spanning the coding region. Bases that differed to the published sequence were verified on both strands in three independent clones.

The cDNAs encoding UGT1A4 and UGT1A9 were isolated by the cloning of PCR products synthesized with family 1 specific primers (forward primer, 5′-CTGGAATTCGCTTCTGCTGAGATGGCCACA, for UGT1A4; forward primer, 5′-CCCAAGCTTCTGGGCTGAAGT-TCTCTGATG, for UGT1A9 and the reverse primer for both as used to synthesize UGT1A10). The identity of the cDNAs was verified by restriction mapping and sequencing. The cDNAs were expressed in COS-7 cells in the pCMV5 vector as described above. The expression vectors encoding UGT1A6, UGT2B7, UGT2B10 and UGT2B11 were prepared as previously described (13-15).

Enzyme assays. The glucuronidating activities of microsomal preparations from human livers and cDNA transfected COS-7 cells were measured by the thin layer chromatographic method as described previously (10). Briefly, incubations (75 μl total volume) were carried out at 37°C for 30 min and contained 0.1mM mycophenolic acid, 1 mM UDP glucuronic acid, ^{14}C-UDP-GlcUA (0.2 mCi), 20 μg of microsomal protein, and 8 mM $MgCl_2$ in 100 mM Tris-HCl buffer, pH 7.5. In some experiments, mycophenolic acid glucuronidation was assayed in 100 mM Tris-HCl buffer, pH 6.8. Reaction rates were linear under all conditions.

The reaction was terminated with 150 ul of ethanol and the glucuronides were analyzed by TLC in chloroform/methanol/water/acetic acid (65:25:4:2 by volume). In some experiments, the assay mixture was treated with alkali to hydrolyze acyl-glucuronides before chromatography (16). Incubations containing mock transfected COS cells and incubations without added aglycone served as controls.

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MARAGWTSPVPLCVCLLLTCGFAEAGKLLVVPMDGSHWFTMQSVVEKLILRGHEVVVVMP   60
EVSWQLERSLNCTVKTYSTSYTLEDQNREFMVFAHAQWKAQASQSIFSLLMSSSSGFLDLF  120
FSHCRSLFNDRKLVEYLKESSFDAVFLDPFDTCGLIVAKYFSLPSVVFTRGIFCHHLEEG  180
AQCPAPLSYVPNDLLGFSDAMTFKERVWNHIVHLEDHLFCQYLFRNALEIASEILQTPVT  240
AYDLYSHTSIWLLRTDFVLDYPKPVMPNMIFIGGINCHQGKPLPMEIEAYINASGEHGIV  300
VFSLGSMVSEIPEKKAMAIADALGKIPQTVLWRYTGTRPSNLANNTILVKWLPQNDLLGH  360
PMTRAFITHAGSHGVYESICNGVPMVMMPLFGDQMDNAKRMETKGAGVTLNVLEMTSEDL  420
ENALKAVINDKSYKENIMRLSSLHKDRPVEPLDLAVFWVEFVMRHKGAPHLRPAAHDLTW  480
YQYHSLDVIGFLLAVVLTVAFITFKCCAYGYRKCLGKKGRVKKAHKSKTH            530
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FIG. 1. The deduced amino acid sequence of UGT1A10. The putative signal peptide and transmembrane regions are underlined. Potential glycosylation sites (NXS/T) are indicated by arrows.

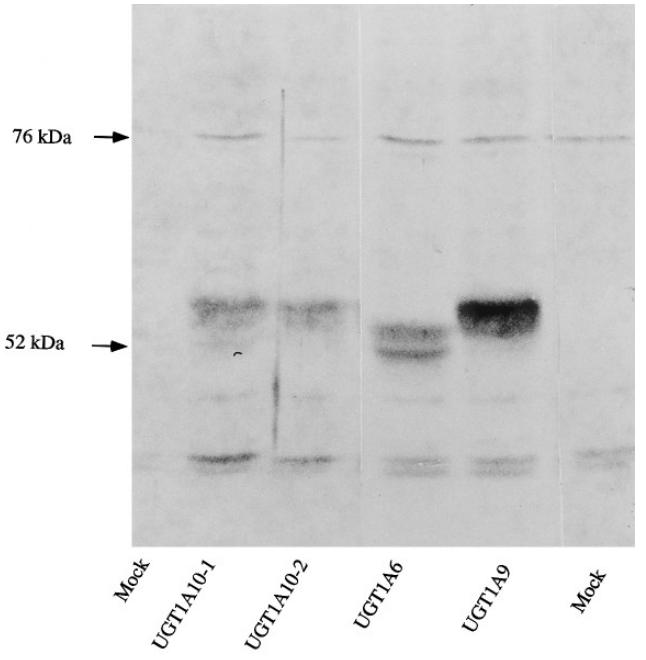

FIG. 2. Immunoblot analysis of UGT1A10 Expressed in COS-7 Cells. Microsomal proteins (25 μg) from COS cells (lanes 1–6) were separated by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate, transfered to a nitrocellulose membrane and probed with UGT-specific antibody. COS cell extracts were from untransfected cells (Lanes 1 and 6) and cells transfected with UGT1A10, clones 1 and 2 (lanes 2 and 3), UGT1A6 (lane 4) and UGT1A9 (lane 5) cDNAs. The positions of the standard proteins, ovalbumin (52 kDa) and bovine serum albumin (76 kDa) are indicated by arrows.

RESULTS

Two cDNAs with complete UGT1A10 coding regions were synthesized from human colon mRNA by reverse transcription and DNA amplification. The DNA sequences of each cDNA were identical but differed to that of the published UGT1j sequence (exon 1 of UGT1A10) in four positions. When the first base of the initiation codon is designated as 1, these differences were; 7delC, 69GC→CCG, 84insC and 90delC and were verified by sequencing both strands of three independent clones. These changes altered the reading frame of the first 90 bps of the coding region, resulting in a deduced amino acid sequence of 530 residues that contained a putative signal peptide (Fig. 1). Western blot analysis of lysates of COS-7 cells transfected with

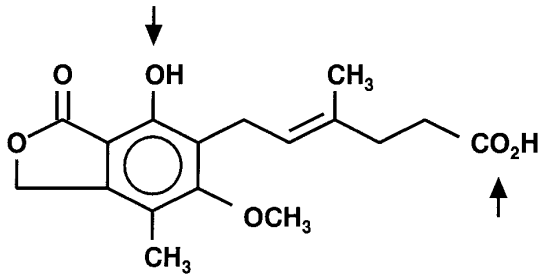

FIG. 3. Structure of mycophenolic acid. Potential sites of glucuronidation are indicated by arrows.

the UGT1A10 expression plasmids revealed the presence of a protein with a relative molecular mass of about 56 kDa (Fig. 2). This protein was not synthesized in mock transfected cells (Fig. 2). A faster migrating protein was also detected and is probably the result of incomplete glycosylation of the 3 potential glycosylation sites in UGT1A10 (Fig. 1). cDNA-expressed UGT1A10 is similar in size to the closely related UGT1A9 (HlugP4, 87% identical in sequence)(8) but larger than UGT1A6 (54 kDa, HlugP1, 64% identical in sequence)(7).

Microsomal preparations from COS-7 cells transfected with UGT1A10 cDNA were very active in the glucuronidation of mycophenolic acid. The glucuronidation of mycophenolic acid was not detected in COS-7 cells transfected with other human UGT cDNAs, including UGT1A1, 1A3, 1A4, 1A6 and 1A9 and UGT2B7, 2B10 and 2B11 under these assay conditions. The apparent Km and Vmax values for mycophenolic acid glucuronidation by cDNA-expressed UGT1A10 were 34 μM and 0.63 nmole/min/mg protein respectively.

Mycophenolic acid has two potential sites of glucuronidation; the phenolic 7-hydroxyl and the aliphatic carboxyl groups (Fig. 3). Previous reports have indicated that the main metabolite of mycophenolic acid is the 7-O-glucuronide (1). In our assay system, especially under conditions of prolonged incubation times and high concentrations of mycophenolic acid, a second product was detected (Fig. 4, M2). The ratio of this product to the 7-O-glucuronide was increased when the assay was performed in buffers favourable for acyl glucuronide formation ie pH 6.8 rather than pH 7.5. The product was also labile under alkaline conditions (16), indicating that it is likely to be the carboxy glucuronide of mycophenolic acid. A less abundant, faster migrating product (Fig. 4, M3) was also observed which may be the diglucuronide of mycophenolic acid.

DISCUSSION

The therapeutic effects and efficacy of many antitumour and immunosuppressive drugs are regulated by

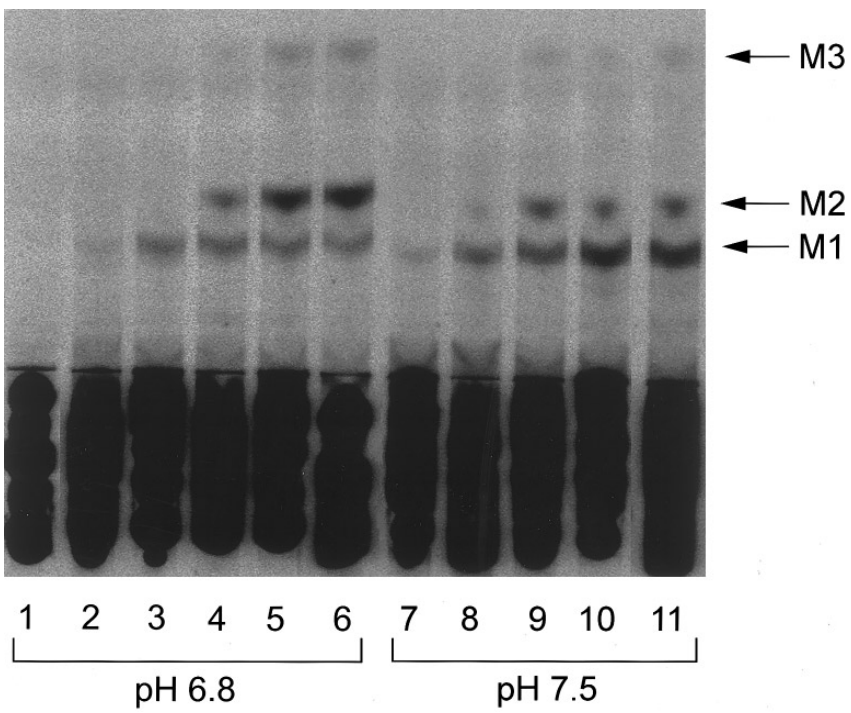

FIG. 4. Glucuronidation of mycophenolic acid in acidic and alkaline pH buffers. Incubations contained 0 (lane 1) or 1 μM (lanes 2 and 7), 5 μM (lanes 3 and 8), 25 μM (lanes 4 and 9), 125 μM (lanes 5 and 10) or 250 μM (lanes 6 and 11) mycophenolic acid and I mM ^{14}C-labeled UDP glucuronic acid in Tris HCl buffers pH 6.8 (lanes 1–6) or pH 7.5 (lanes 7–11). The reaction products were separated on a TLC plate and the plate was visualized by autoradiography. The position of the glucuronides (M1-3) are indicated.

metabolism. As glucuronidation is the prime pathway for the elimination of the antitumour and immunosuppressive antibiotic, mycophenolic acid, in humans (1), identification of the enzyme(s) responsible may lead to understanding and ultimately predicting factors likely to influence its elimination in individuals or defined patient groups. In this report, we have characterized the UGT form, UGT1A10 by cDNA cloning and expression and shown that it is active in the metabolism of mycophenolic acid. Although the levels of this form in the liver and extrahepatic tissues have yet to be determined, it may be that UGT1A10 plays a significant role in the elimination of mycophenolic acid *in vivo*. The inability of UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10, and 2B11 to glucuronide mycophenolic acid under the assay conditions used in this study, supports this possibility, although other, as yet unidentified, forms may be involved.

In this study, cDNA expressed UGT1A10 had the capacity to glucuronidate both the hydroxyl and carboxyl groups of mycophenolic acid in *in vitro* assays. However, as only the 7-hydroxyl-glucuronide has been detected to date (1-3), the formation of the acyl glucuronide may not be favoured *in vivo* (intracellular levels of mycophenolic acid are too low ie less than 5 μM and the pH is greater than 7) and hence may not be of physiological significance. The blood concentrations of mycophenolic acid which range from 0.01-0.1 μM after therapeutic doses of the prodrug mycophenolate mofetil, (about 4 g per day), are consistent with this suggestion (20).

Metabolic inactivation of drugs is an important mechanism that regulates the capacity of tumour cells to resist the toxic effects of chemotherapeutic agents. UGTs have been implicated in this drug resistance in many cultured cell lines. Daunorubicin resistance in the murine leukemic cell line, P338, has been attributed to enhanced glucuronidation of the active metabolite, daunorubicinol (21). In several human colorectal carcinoma cell lines, resistance to mycophenolic acid is mediated by a rapid conversion of mycophenolic acid to its inactive 7-O-glucuronide (3). Although, the specific UGT form(s) involved in this resistance has not been elucidated, our studies suggest that UGT1A10 may be a candidate enzyme, especially in cells of colorectal origin, as colon mRNA and a colon cDNA library were used as sources for the isolation of the UGT1A10 sequences.

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