UDP-Glucuronosyltransferase, the Role of the Amino Terminus in Dimerization*

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UDP-glucuronosyltransferases (UGTs) comprise an important enzyme system in mammals that is involved in detoxification of a variety of small hydrophobic compounds of both endogenous and exogenous origin. Some evidence suggests that these enzymes may function as oligomers; however, little is known about the domain of interaction or the mechanism of oligomerization. In this work, evidence for a functional dimerization between UGTs is provided by studies on mutated forms of UGT2B1. When two inactive forms of UGT2B1 were co-expressed in cell culture, catalytic activity was restored, indicating that UGT2B1 forms functional dimers. To delineate the dimerization domain, inactive fusion proteins containing the amino- or carboxyl-terminal domains of UGT2B1 were generated and expressed with active UGT2B1. Expression of a fusion protein containing only the amino-terminal half of UGT2B1 with active UGT2B1 caused a reduction in UGT2B1 catalytic activity. This reduction in activity was not observed when UGT2B1 was co-expressed with a fusion protein containing only the carboxyl-terminal half of UGT2B1, strongly suggesting that the amino-terminal domain is involved in dimerization. Truncation of the immediate amino terminus of UGT2B1 abolished UGT2B1 activity and dimer formation. Activity was also abolished by an L4R substitution in this region of the mature protein, which is highly conserved in the UGT family. These results indicate that UGTs can interact through their amino-terminal domains to form catalytically active dimers. Possible mechanisms resulting in the formation and stabilization of the UGT2B1 dimer are discussed.

The UDG-glucuronosyltransferases (UGTs)1 are members of a superfamily of glycosyltransferases that catalyze the covalent conjugation of a variety of aglycone substrates with the glucuronic acid moiety of UDP glucuronic acid. In mammals, these are important detoxifying enzymes that render lipophilic substrates, both endogenous and exogenous, more polar and thus more readily excreted in bile or urine (1–3). UGTs reside in the endoplasmic reticulum (ER) membrane and demonstrate type I topology such that the amino terminus and approximately 95% of the subsequent residues are located in the lumen, a 17-residue domain near the carboxyl terminus spans the membrane, and the carboxyl-terminal 19–24 residues are located in the cytoplasm.

The mammalian UGT isoforms have been separated into two families on the basis of sequence similarity and gene structure. The family 1 UGT isoforms (UGT1) are all encoded by one gene that has multiple unique exons located upstream of four common exons. The isoforms are generated by differential splicing of one unique exon 1 to the four common exons (exons 2–5). The first exon encodes roughly two-thirds of the luminal domain (approximately 290 amino acids), and exons 2–5 encode the remainder of the luminal domain, the transmembrane domain, and the cytosolic tail. As a consequence of the unusual gene structure and splicing mechanism, the UGT1 forms have variable amino-terminal halves and identical carboxyl-terminal halves (4). The UGT2 family differs in that each member is encoded by a separate gene comprised of six exons. However, the region of the protein encoded by exons 1 and 2 is equivalent to that encoded by the unique exons 1 of the UGT1 isoforms, and the subsequent intron/exon boundaries are in corresponding positions in both gene families. Within the UGT2 family, the amino-terminal half of the protein is most variable between isoforms while the carboxyl-terminal half of the protein demonstrates greater sequence conservation. A glycosyltransferase consensus region of approximately 29 amino acids has been defined in the conserved half of UGT and is thought to be involved in binding UDP, based on similarity in this region between UGTs and other enzymes that bind UDP-sugars (5). Studies with chimeric UGTs have demonstrated that the amino-terminal half of UGT determines substrate specificity and may therefore contain the aglycone binding site (6).

Experimental evidence suggests that UGT may form a dimer or tetramer in vivo. Peters et al. (7) indicated that UGT1A1 may act as both a dimer and a tetramer based on radiation inactivation analysis of the enzyme. Gschaidmeier and Bock (8) have also examined radiation inactivation of microsomal UGTs and suggested that monoglucuronidation of phenols may be catalyzed by a dimeric form of UGT while diglucuronidation is catalyzed by a tetramer. Matern et al. (9), analyzed UGT by gel filtration, which also indicated that the enzyme exists in oligomeric forms. However, gel filtration of membrane-bound proteins can give unreliable estimates of molecular weight. Recently, Koivwai et al. (10) demonstrated that an inactive mutant form of UGT1A1 could act as a dominant negative mutant in vitro, reducing bilirubin activity, presumably by dimerizing with the active form of the enzyme. The region of the UGT protein that is involved in dimerization is unknown. The aim of this study was to demonstrate functional dimerization in UGT2B1 and to determine whether dimerization occurs in the conserved or variable domain of the protein.

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1 The abbreviations used are: UGT, UDP-glucuronosyltransferase; ER, endoplasmic reticulum; HRP, horseradish peroxidase; EGT, ecdsysteroid glucosyltransferase; PAGE, polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Antibodies—Goat anti-mouse UGT antiserum, which recognizes all UGT isoforms, has been previously described (11). Rabbit anti-goat antibody and anti-goat horseradish peroxidase (HRP)-goat HRP conjugate were purchased from Dako Corp. (Carpintera, CA). The mouse anti-MRGS-6-his antibody and the anti-mouse-HRP conjugate were purchased from Qiagen GmbH (Hilden, Germany) and Sigma, respectively.

UDP-Glycosyltransferase cDNAs—The UGT2B1 cDNA in the Okyama-Berg expression vector (pCD) has been previously described (12). A fragment of the Autographa californica nuclear polyhedrosis virus genome containing the egt gene in the pUC19 vector was kindly provided by Dr. L. Miller, Department of Entomology, University of Georgia, Athens, GA.

Mutagenesis—Mutagenesis was performed by two methods. The UGT2B1 cDNA was either subjected to oligonucleotide-mediated site-directed mutagenesis using the Transformer site-directed mutagenesis kit (CLONTECH), or alternatively, mutations were generated by polymerase chain reaction. The oligonucleotides used in both methods were obtained from Life Technologies, Inc. (Melbourne, Vic., Australia). Mutations were confirmed by sequencing using the dideoxy chain-termination method (13). Plasmid DNA was prepared for transfection by the double CsCl gradient method (14).

Chimeric molecules were constructed between UGT2B1 and ecysyo-teroid glucosyltransferase (EGT) by introducing XhoI restriction sites into the UGT2B1 cDNA at the exon 2/3 boundary and into the egt gene at a corresponding position based on global sequence alignment. The 5’ and 3’ DNA fragments were exchanged, and the resultant chimeric DNAs were subcloned into the pCMV5 expression vector (Dr. M. Stinsky, University of Iowa, IA) for expression in COS7 cells.

The signal peptide domain of UGT2B1 was altered by introducing the 6-histidine tag sequence from the pQE vector series (Qiagen GmbH) into the cDNA by restriction fragment exchange. The histidine peptide replaced most of the hydrophobic core of the leader peptide, leaving the initiation methionine and signal peptide cleavage site intact. Signal peptide cleavage during expression in COS7 cells was verified by immunoblotting with an antibody directed toward the 6-His tag sequence.

Cell Culture and Transfection—COS7 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), pH 7.0–7.2, supplemented with 10 mM HEPES, 10% new born calf serum, and 80 mg/liter gentamycin at 37 °C and 10% CO2. Cells were transfected at approximately 80% confluency by a modification of the DEAE-dextran method (15) with 15 μg of plasmid DNA per 175 mm2 flask. Cells were harvested at approximately 48 h post-transfection with a rubber scraper and washed in 1 × phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4, pH 7.4).

Microsome Preparation—COS7 cells were harvested, washed twice with 1 × phosphate-buffered saline, resuspended in 0.4 ml HB (0.5 M sucrose, 10 mM Tris·Cl, 1 mM EDTA, pH 7.5), and lysed by sonication using a Heat Systems-Ultrasoundsonicator on ice. The samples were then diluted to 0.25 M sucrose with one volume of TE (10 mM Tris·Cl, 1 mM EDTA, pH 7.5) and centrifuged in an Eppendorf centrifuge at 300,000 × g pellet unbroken cells and nuclei. The resulting post-nuclear supernatant was layered over a 0.1-ml cushion of HB and centrifuged at 10 × 106 × g for 2 h at 4 °C. The supernatant was removed, and the microsomal pellet was resuspended in sterile distilled water and stored frozen at −70 °C.

Endoglycosidase H Treatment and Immunoblotting—Microsomes from transfected COS7 cells were diluted to approximately 2 μg of protein/μl in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) and heated to 95 °C for 5 min. The samples were then diluted into 50 mM citrate buffer, pH 5.5, and incubated overnight at 37 °C with 200 units of Endoglycosidase H (New England Biolabs, Labrador, Qld, Australia). The samples were then combined with one-third volume of TE (10 mM Tris·Cl, 1 mM EDTA, pH 7.5) and centrifuged in an Eppendorf centrifuge at 5000 × g for 2 h at 4 °C. The supernatant was removed, and the microsomal pellet was resuspended in sterile distilled water and stored frozen at −70 °C.

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used as the substrate for chromogenic detection of protein bands that were quantified using a scanning laser densitometer (LKB).

Assay—UGT activity in transfected COS7 cell microsomes was assayed using 60 μM (0.24 μCi) [14C]-testosterone (NEN Life Science Products) and 2 mM UDPGA, 50 mM Tris-Cl, pH 7.5, 2 mM MgCl₂ in a 60-μl reaction for 16 h at 37 °C. Duplicate assays were performed. The relative activity of UGT2B1 was calculated by dividing the averaged activity by the amount of UGT2B1 protein expressed. The latter was determined by densitometry as described above.

RESULTS

High Molecular Weight UGT2B1-associated Proteins—When UGT2B1 was expressed in COS7 cells, the expected 52-kDa protein was detected by immunoblotting with an anti-UGT antiserum. In addition, a high molecular weight band was consistently observed. This band had an apparent molecular mass of approximately 110 kDa and was sometimes accompanied by a slightly smaller band of approximately 100 kDa. The two bands were not endogenous COS7 cell proteins as they did not appear in mock transfected cells (Fig. 1). Aberrations in heterologous expression, which could lead to the production of large UGT-fusion proteins, were considered but discounted. The possibility of premature initiation of transcription from a cryptic promoter upstream of the cDNA or stop codon read-through was excluded by expressing the UGT2B1 CDNA from two different expression vectors, which contain different backbone plasmid sequences, and by introducing additional stop codons downstream of the native stop codon (data not shown). It is likely, therefore, that these bands are not expression artifacts but represent highly stable associations between UGT2B1 and either another protein (hetero-oligomer) or itself ( homo-oligomer). The 100-kDa band, which was less intense than the 110-kDa band and less consistent in its expression, does not represent an unglycosylated form of the upper band as both bands remained apparent after deglycosylation with endoglycosidase H (not shown). Both bands demonstrated a mobility shift indicating that they are glycoproteins (or glycoprotein complexes). The lower band could be a moderately detergent stable complex that forms between UGT2B1 and some unknown accessory molecule. It was destabilized by truncation of the cytosolic tail and transmembrane domain (UGT2B1.493), suggesting that the interaction involves these domains of UGT (Fig. 1). The highly stable 110-kDa band was clearly formed by a different mechanism as it was unaffected by truncation of the carboxyl terminus; however, it was abolished by modification of the amino terminus (Fig. 1; 6-His-UGT2B1). Amino-terminal modification of UGT2B1 was achieved by altering the signal peptide with a hydrophilic histidine tag (6-His-UGT2B1). The expressed protein was not recognized by antibodies to the 6-His-tag, indicating that signal peptide cleavage had occurred (not shown). However, the protein was inactive and did not form the 110-kDa complex. The most likely explanation for this functional change in the mature UGT protein is aberrant signal peptide processing as has been reported for signal peptide mutations in other proteins (16, 17) (Fig. 1). The proposed cleavage of UGT2B1 downstream of the normal cleavage site must therefore remove residues that are critical both for activity and for formation of the high molecular weight complex. The 6-His-UGT protein is not discernibly smaller than wild-type UGT2B1, thus the amino-terminal truncation must involve only a few amino acids. The first seven amino acids of the mature UGT are highly conserved, comprising the motif GKhkhWP (where h is a hydrophobic residue, either Val, Leu, or Ile) in almost all isoforms. We therefore investigated the role of this conserved domain by site-directed mutagenesis (Fig. 2A). Substitution of the lysine residue with alanine (UGT2B1.K25A) did not affect activity; however, substitution of the second hydrophobic position with the charged

residue arginine (UGT2B1.L27R) abolished activity (Fig. 2B). The 110-kDa complex was formed with both mutants. Thus the hydrophobic motif appears to be critical for UGT2B1 function but is not essential for dimer formation.

The nature of the extremely strong interaction that stabilizes the 110-kDa complex is unknown. However, the complex was not recognized by an antibody that was directed specifically to the amino-terminal half of UGT2B1 (not shown), suggesting that epitopes in the amino-terminal half of the protein are masked. Most non-covalent protein interactions are abolished during SDS-PAGE resolution. Thus, a likely explanation for the appearance of this highly stable band is that inadvertent covalent cross-linking occurs during expression that stabilizes a naturally occurring UGT2B1 oligomer, probably a dimer, based on the molecular weight. To further investigate the possibility that UGT may homo-oligomerize in the amino-terminal domain, a functional assay was developed.

Functional Compensation of UGT Defects—To demonstrate that UGT2B1 acts as a homo-oligomer, two inactive forms of UGT2B1 with different functional defects were examined. UGT2B1.493 is a truncated form of UGT2B1 from which the cytosolic tail and transmembrane domain were removed (18), whereas UGT2B1.L363P is a full-length protein with a point mutation in the UDP-glucuronosyltransferase consensuses region (5, 19) (Fig. 3A). Both proteins were inactive when expressed individually in COS cells. In contrast, testosterone glucuronidating activity was detected in COS cells that co-expressed both proteins (Fig. 3B). Thus a functional interaction must occur between the two proteins that permits partial compensation of each mutation and restoration of activity. These results also indicate that this interaction does not occur between the cytosolic or transmembrane domains of the monomers. Further
more, UGT2B1.493 forms only the 110-kDa band and not the weaker 100-kDa band, supporting the view that the former band represents a UGT2B1 homo-oligomer.

Dominant Negative UGT Chimeras—The hypothesis that homo-oligomerization of UGT2B1 is mediated by the amino-terminal half of the molecule was confirmed by analysis of chimeric UGT2B1 fusion proteins. UGT2B1/EGT contains the amino-terminal half of UGT2B1 (exons 1 and 2) fused in frame to the carboxyl-terminal half of the related glycosyltransferase, EGT. Conversely, EGT/UGT2B1 contains the amino-terminal half of EGT fused in frame to the carboxyl-terminal half of UGT2B1 (exons 3–6) (Fig. 4A). These chimeric molecules appeared to be correctly glycosylated when expressed in COS7 cells, indicating that they were translocated into the ER and remained resident in this organelle (Fig. 4B). The UGT2B1/EGT chimera formed a high molecular mass complex of greater than 120 kDa, which is presumed to be equivalent to the 110-kDa complex associated with UGT2B1 expression. In contrast, EGT/UGT2B1 formed no high molecular mass complexes (Fig. 4B). The chimeric proteins demonstrated no catalytic capacity, presumably due to incompatibility between the aglycone and UDP-sugar binding domains. Co-transfection of COS7 cells was performed with equimolar amounts of UGT2B1 cDNA and the chimeric expression constructs, and the catalytic activity of UGT2B1 was measured in each transfectant. As the molecular weights of UGT2B1, UGT2B1/EGT, and EGT/UGT2B1 differed substantially, the level of expression of each protein was readily quantitated by densitometric scanning of Western blots. This allowed the relative activity of UGT2B1 to be calculated and permitted the ratio of wild-type to chimeric protein to be determined for each transfectant. Co-transfection of COS7 cells was performed with equimolar amounts of UGT2B1 cDNA and the chimeric expression constructs, and the catalytic activity of UGT2B1 was measured in each transfectant. The ratio of expression of wild-type to chimeric protein was approximately 4:3, and when this factor is incorporated into our calculation, the expected reduction in activity is approximately 38%, which is in good agreement with the observed value. Thus the co-expression study supports the dimer model for UGT2B1.

DISCUSSION

For some time it has been believed that UGT may act as an oligomer; however, little firm evidence has been published in support of this view. In this paper, we examined a stable high molecular mass complex that was associated with expression of UGT2B1 and devised functional assays to investigate the likely relationship between the high molecular mass complex and oligomerization. The complex of 110 kDa was always observed in microsomes of COS cells containing catalytically active UGT2B1, but was only sometimes observed in transfected COS cell microsomes devoid of activity. Furthermore, the functional compensation of two different, inactivating mutations in UGT2B1 by co-expression provides the strongest evidence to date that the active form of UGT is a homo-oligomer. This interaction was further defined by co-expression analysis of UGT2B1 and a chimeric protein that acted as a dominant negative mutant. This study demonstrated that UGT2B1 acts as a homodimer and also allowed the dimerization domain to be localized to the amino-terminal, variable half of the protein. The potential dimerization between two different UGT monomers within the aglycone recognition domain may allow formation of new substrate recognition sites, within such a heterodimer, that do not exist in the homodimeric forms of the enzymes. Recently, possible heterodimers of UGT2B1 and UGT1 isoforms were revealed by chemical cross-linking studies on liver microsomal preparations (20). Although substrate specificity was not assessed, the complex formation between UGT2B1 and these other UGTs was associated with alterations...
in the kinetic parameters of the glucuronidation of testosterone, a substrate of UGT2B1 (21).

The 110-kDa band that was associated with UGT2B1 expression is proposed to be a stable dimer of UGT2B1 monomers. The formation of this 110-kDa complex was inhibited by truncation of the immediate amino terminus, which also correlated with loss of enzymatic activity. Site-directed mutagenesis demonstrated that a conserved hydrophobic motif near the amino terminus is critical for activity. However, the precise role of this conserved region is unknown.

A high molecular weight band was also observed in a chimeric construct that contained the amino-terminal half of UGT2B1. Thus there is a correlation between dimerization of UDP Glucuronosyltransferase 2B1 with loss of enzymatic activity. Site-directed mutagenesis demonstrated that a conserved hydrophobic motif near the amino terminus is critical for activity. However, the precise role of this conserved region is unknown.

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In conclusion, our results provide compelling evidence that UGT2B1 monomers form catalytically active dimers via their amino termini.

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**Fig. 4. Co-expression of UGT2B1 with chimeric glycosyltransferases.** A, chimeric molecules constructed between UGT2B1 and the related soluble enzyme EGT. The UGT2B1 sequence is in **black** and the EGT sequence in **gray**. The shaded region represents the glycosyltransferase consensus region. B, deglycosylation of chimeric glycosyltransferases. Microsomes prepared from transfected COS7 cells were treated with endoglycosidase H as described under "Materials and Methods" and analyzed by SDS-PAGE and immunoblotting. C, UGT2B1 was co-expressed with the chimeric constructs UGT2B1/EGT and EGT/UGT2B1 in COS7 cells, and activity was measured as described in the legend to Fig. 2. Relative activity is expressed as a ratio of activity to UGT2B1 protein.