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Comparison of Glucuronidating Activity of Two Human cDNAs, UDPGTh1 and UDPGTh2

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Two human liver UDP-glucuronosyltransferase cDNA clones, HLUG25 and UDPGTh2 were previously shown to encode isozymes active in the glucuronidation of hyodeoxycholic acid (HDCA) and certain estrogen derivatives (e.g., estriol and 3,4-catechol estrogens), respectively. In this study we have found that the UDPGTh2-encoded isoform (UDPGTh2) and HLUG25-encoded isoform (UDPGTh1) have parallel aglycone specificities. When expressed in COS 1 cells, each isoform metabolized three types of dihydroxy- or trihydroxy-substituted ring structures, including the 3,4-catechol estrogen (4-hydroxyestrone), estriol, 17-epiestriol, and HDCA, but the UDPGTh2 isozyme was 100-fold more efficient than UDPGTh1. UDPGTh1 and UDPGTh2 were 86% identical overall (76 differences out of 528 amino acids), including 55 differences in the first 300 amino acids of the amino terminus, a domain which conferred the substrate specificity. The data indicated that a high level of conservation in the amino terminus was not required for the preservation of substrate selectivity. Analysis of glucuronidation activity encoded by UDPGTh1/UDPGTh2 chimeric cDNA constructed at their common restriction sites, *Sac* I (codon 297), *Nco* I (codon 385), and *Hha* I (codon 469), showed that nine amino acids between residues 385 and 469 were important for catalytic efficiency, suggesting that this region represented a domain which was critical for the catalysis but distinct from that responsible for aglycone selection. These data indicate that UDPGTh2 is a primary isoform responsible for the detoxification of the bile salt intermediate as well as the active estrogen intermediates.

Key words : UDP-Glucuronosyltransferase cDNA, UDPGTh1, UDPGTh2, HLUG25, Chimeric cDNA

INTRODUCTION

UDP-glucuronosyltransferase (UDPGT) is a family of integral membrane enzymes that catalyze the glucuronidation of many potentially toxic xenobiotics and endogenous compounds. An undetermined number of isoforms catalyze the transfer of glucuronic acid from UDP-glucuronic acid to a large number of lipophilic acceptor substrates to generate water-soluble glucuronides, thereby enhancing the rate of excretion from cells (Dutton, 1980). Due to the difficulties associated with the purification of this labile and membrane-bound class of proteins (Burchell, 1981; Lilienblum *et al.*, 1985; Chowdhury *et al.*, 1986), very little information is available in the literature regarding purification of human transferases. Complementary DNAs isolated from rat (Irani, 1980; Iyanagi *et al.*, 1986), mouse (Kimura and Owens, 1987) and human (Jackson *et al.*, 1987; Harding *et al.*, 1988) were reported which encoded transferases that glucuronidate primary estrogens, or exogenous compounds such as p-nitrophenol (Jackson *et al.*, 1987), 4-methylumbelliferone (Kimura and Owens, 1987). Fournel-Gigleux *et al.* (1989) reported that a human liver UDP-glucuronosyltransferase cDNA (HLUG 25) encoded a 6α-hydroxylated bile acid (hyodeoxycholic acid, HDCA) transferase isoform, a finding confirmed in our laboratory upon expression of an identical coding cDNA, UDPGTh1. Even though the liver and kidney are the known sites of HDCA glucuronidation, little or no UDPGTh1 mRNA has been detectable in kidney by Northern analysis, suggesting that an additional isoform (s) is (are) involved. In order to understand the transferase isozyme responsible for detoxifying the critical endogenous aglycone, we examined the suitability of a series of bile acid derivatives as substrates for cloned human transferase isoformes iso-

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lated in the laboratory.

MATERIALS AND METHODS

Materials

UDP-glucuronic acid, cholic acid, hyocholic acid, and all steroid derivatives were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HDCA was from Aldrich Chemical Co. (Milwaukee, WI, USA). [^{35}S] methionine was purchased from ICN Biomedical, Inc. (Costa Mesa, CA USA). [^{14}C]UDP-glucuronic acid was product of Amersham (Arlington Heights, IL, USA). Restriction enzymes and other reagents used in molecular biology techniques were supplied from New England Biolabs (Beverly, MA, USA), Pharmacia (Piscataway, NJ, USA), and Bethesda Research Laboratories (Bethesda, MD, USA). The pSVL vector was product of Pharmacia (Piscataway, NJ, USA) and COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The Bluescript plasmids and XL-1 Blue cells were from Stratagene (La Jolla, CA, USA). Reagents used for cell culture studies were purchased from Gibco (Grand Island, NY, USA) or Mediatech (Washington DC, USA).

Isolation of UDPGTh1 and UDPGTh2 from a human liver cDNA library

The two human liver transferase cDNAs, UDPGTh1 and UDPGTh2, were isolated from a λgt 11 cDNA library by hybridization to the mouse transferase clone ^{32}P-UDPGTm1 (Kimura & Owens, 1987) which was labeled by the oligo-primed technique using a kit (Pharmacia), [α-^{32}P]deoxycytidine triphosphate, and the supplier's protocol. Each clone was sequenced by the direct plasmid dideoxy protocol. The protein encoded by the clone, HLUG25, which is identical to UDPGTh1, was characterized biochemically by Fournel-Gigleux *et al.* (1987).

Expression of transferase proteins encoded by pUDPGTh1 and pUDPGTh2.

UDPGTh 1 and UDPGTh2 were subcloned into the pSVL plasmid to produce the expression units pUDPGTh 1 and pUDPGTh2, with the sense strand downstream of the promoter element in the vector. Each recombinant plasmid was transfected into COS 1 cells. In order to ensure that the comparisons of activities were based on equivalent amounts of specific protein, we established the relative level of synthesis of each radiolabeled protein. Cells were transfected with pUDPGTh 1 or pUDPGTh2 and incubated for 68 hours in regular medium, for 1 hour in methionine-free medium, and finally, for 4 hours in the presence of [^{35}S]methionine (100 μCi/mL). The [^{35}S]methionine-labeled transferases were solubilized, immunocomplexed with either control IgG or antimouse transferase IgG, and detergent washed. The immunocomplexes were detached and analyzed on a 7.5% polyacrylamide gel and processed for autoradiography.

Glucuronidation assay using [^{14}C]UDP-glucuronic acid

Preliminary data indicated that UDPGTh1 and UDPGTh2 glucuronidated similar substrates. In order to determine the relative amounts of glucuronide generated by control and either UDPGTh1 or UDPGTh2 transfected COS 1 cell homogenates were assayed in a total volume of 0.075-mL reaction mixture containing 10 or 100 μM aglycone, 50 μM [^{14}C]UDP-glucuronic acid (33.3 Ci/mmole), 50 mM Tris-HCl, pH 7. 7, 4 mM MgCl, 1 mM ascorbic acid, 1 mM phenylmethanesulfonyl fluoride, and 1.0 g/mL leupeptin. Reactions were incubated for 16 hours at 25°C and analyzed by thin-layer chromatography (TLC). The same amount of specific protein (based on the ^{35}S-labeled immunoprotein study above) was added to each reaction mixture. The products were quantified by scanning the plates on an Ambis radioanalytical system Mark II (Ambis, San Diego, CA, USA). The minimal amount of glucuronide product detected under the assay conditions used was approximately 1.7 pmol (with signal/background ratio of 2) above a background of 50 cpm. Plates were exposed to X-ray film to obtain autoradiograms.

Construction of chimeric cDNAs between UDPGTh1 and UDPGTh2

Chimeras between UDPGTh1 and UDPGTh2 were constructed in Bluescript by using the common restriction enzyme sites *Sac* I, *Nco* I, or *Hha* I to create exchanges at codons 297, 385, and 469, respectively. The regions surrounding the endonuclease sites involved in joining the two fragments were sequenced by the dideoxy protocol as described above. The chimeras were then subcloned into pSVL and expressed in COS 1 cells.

RESULTS AND DISCUSSION

Deduced amino acid differences between UDPGTh1 and UDPGTh2

The two human liver UDP-glucuronosyltransferase clones, UDPGTh1 and UDPGTh2, were originally identified as encoding isozymes active in the glucuronidation of HDCA (Fournel-Gigleux *et al.*, 1989) and estriol and 3,4-catechol estrogens (Ahn *et al.*, 1997). The two encoded proteins have 76 different amino acids overall; there are 55 in the 300 amino-terminal residues and 21 in the 228 carboxyl-terminal residues

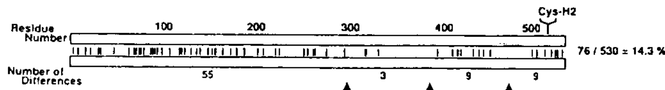

Fig. 1. Amino acid differences between the UDPGTh1 and UDPGTh2 isozymes. The vertical lines between the two clear strips represent amino acid differences found residues between 1 and 528. The number of differences between the restriction enzyme sites was shown below the diagram. Specific restriction enzyme sites which were used in the creation of chimeras were indicated by arrows. Cys-h2 refers to a cysteine residue in UDPGTh2 which is absent in UDPGTh1.

(Fig. 1). The observation that the amino termini contained the greatest number of differences between these two clones was consistent with the location of the most variable region between other transferase isoforms (Iyanagi *et al.*, 1986). In a study with chimeric cDNA constructs (Jackson, 1987) it was shown that the selection of the acceptor substrate was controlled by the amino terminus (~300 residues). In the case of other transferase isoforms, two human bilirubin and one phenol-metabolizing isoforms contained unique amino termini with 41-48% identity, whereas the 244-amino acid carboxyl termini of the isoforms were identical. The conservation in the carboxy domain suggests that this region is involved in a function common to all isozymes, such as the transfer of glucuronic acid from UDP-glucuronic acid.

Relative levels of synthesis of UDPGTh1 and UDPGTh2 in transfected COS1 cells

In order to ensure that equal amounts of specific proteins were being compared, the levels of synthesis were assessed at 72 hours after transfection of the individual expression units, pUDPGTh1 and pUDPGTh 2 into COS 1 cells. The results with [^{35}S]-labeled protein showed that equal amounts of UDPGTh1 and UDPGTh2 were immunocomplexed when similar amounts of cell homogenate were used.

Comparison of glucuronidating activity of the UDPGTh1 and UDPGTh2 isozymes

While we were carrying out a more detailed analysis of the substrate selectivity of UDPGTh1 and UDPGTh2 and their chimeras, it became apparent that the two parental forms were the same series of acceptor substrates. Both UDPGTh1 and UDPGTh2 had been screened for glucuronidating activity. UDPGTh1 was examined for acceptor substrate activity upon expression in yeast, as well as in COS 1 cells. The results confirmed the findings in a published study (Fournel-Gigleux *et al.*, 1989) which showed that the HLUG25-encoded protein glucuronidates HDCA at an apparently low rate. Comparisons of activity showed that the

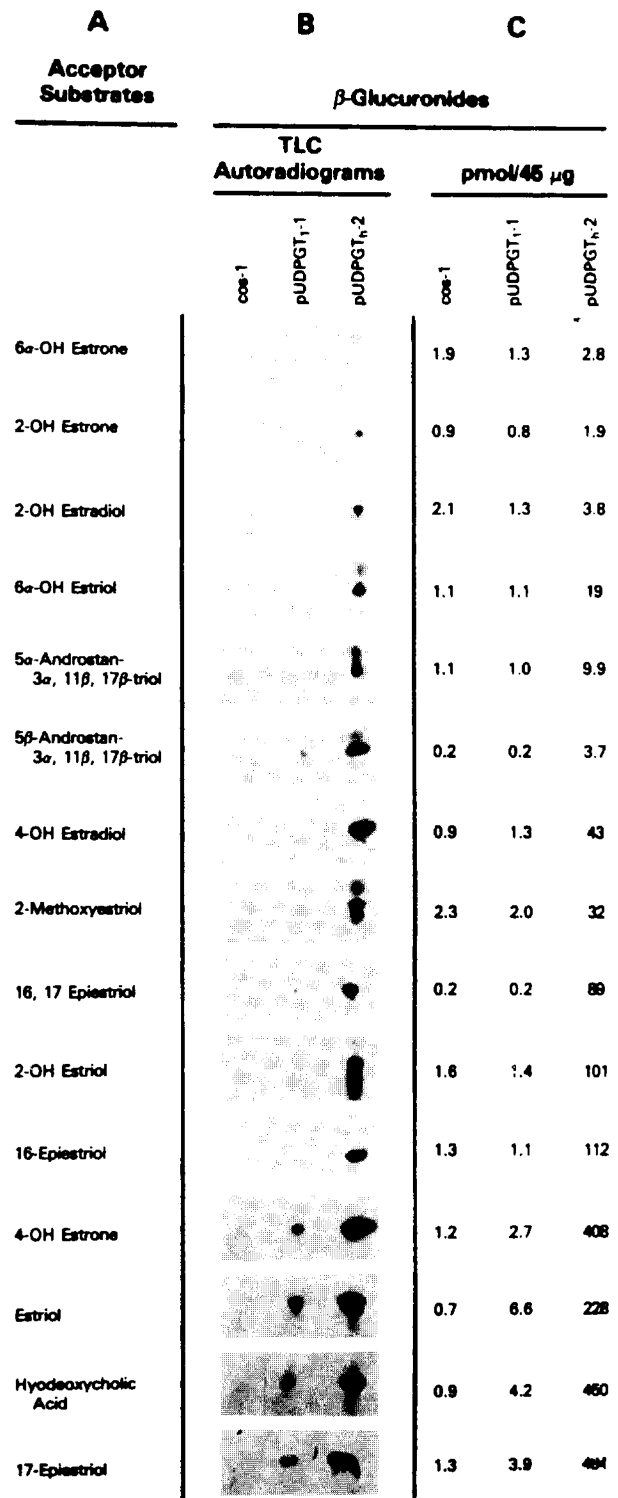

Fig. 2. Comparison of the relative catalytic activity of UDPGTh1 and UDPGTh2 in trasfected COS 1 cells. Transferase activities in either pUDPGTh1 or pUDPGTh2 transfected or control cells were assayed for glucuronidation of acceptor substrates listed. The assays were carried out with 10 or 100 μM substrate concentrations using conditions described under Materials and Methods. The level of glucuronidation (panel C) was quantitated as indicated under Materials and Methods.

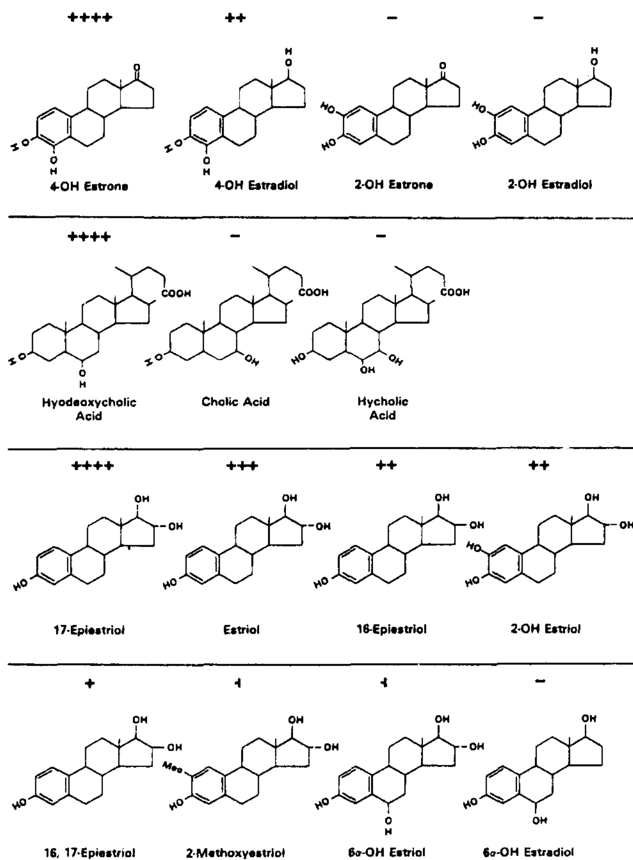

Fig. 3. Structures of the acceptor substrates for UDPGTh1 and UDPGTh2. The relative substrate specificity of UDPGTh 1 and UDPGTh2 and catalytic efficiencies indicated that three categories of chemical structures, 3,4-catechol estrogens (line 1), HDCA (line 2), and estriols (line 3 and line 4), were preferred and were equally effective. For the results with UDPGTh2 the relative effectiveness of structures in each category was shown by the number of plus signs (+) above compound.

UDPGTh1 isozyme metabolized each of the four best substrates seen for the UDPGTh2 isoform (Fig. 2). A substrate was included from each of the three categories seen for the more active isozyme: an estriol (containing three hydroxy groups), HDCA (3α,6α-dihydroxy bile salt derivative), and 4-hydroxyestrone (3,4-catechol estrogen). The greater activity associated with UDPGTh2 was also determined by the fact that related chemical structures were glucuronidated at a low rate. Conversion of these related compounds to glucuronides by UDPGTh1 was barely detectable with only 5β-androstan-3α,11β,17β-triol and 16,17-epiestriol (Fig. 3). On the basis of this study, it was concluded that excellent substrates for the UDPGTh2 protein, i.e., those that formed more than 200 pmol produced in 16 hours, could also serve as substates for UDPGTh 1. Regio- and stereospecificity of the hydroxy substituents were critical to these substate selections, on the basis of the ineffectiveness of 2,3-catechol estrogen and 3α,6β- and 3α,7α-dihydroxylithocholic acid derivatives and preference for 16α,17α-estriol over 16α, 17β- and 16α,17β-estriols (Fig. 2 and 3). The close proximity of dihydroxy substituent groups appears to also be critical requirement. The position of the glucuronide was not required to maintain the acceptor function. This point was further supported by the observation that 100% identity in the carboxyl termini of the two human bilirubin transferase isoforms with only 48% identity in the amino termini has maintained the substrate specificity. The basis for multiple estriol-glucuronidating enzymes may be related to important differences in kinetic parameters, overlapping substrate specificities, and responsiveness of either gene transcriptional unit to regulatory signals.

Relative activity of the chimeric transferase molecules

The chimeras with exchange at codon 469 did not

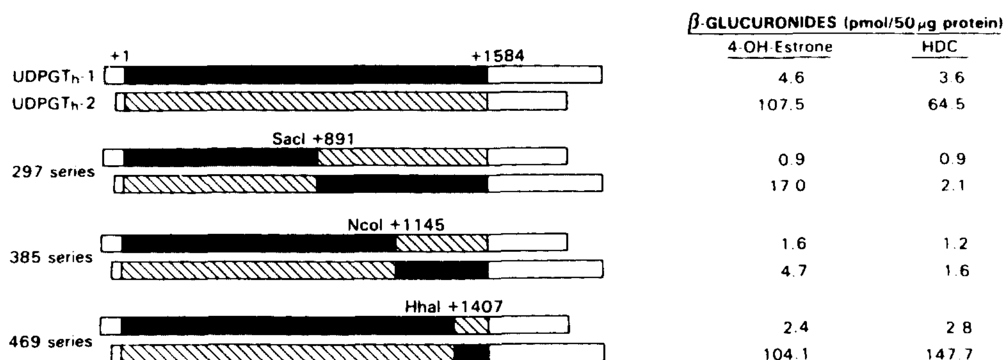

Fig. 4. Intact and chimeric constructs of UDPGTh1 and UDPGTh2 and the relative activity associated with each construct expressed in COS 1 cells. Chimeric constructs with exchanges at the common *Sac* I, *Nco* I, and *Hha* I sites in the cDNA generated exchanges at amino acids 297, 385, and 469, respectively. The intact and chimeras were subcloned into pSVL and transfected into COS 1 cells as indicated under Materials and Methods. The level of glucuronidation (right) by each encoded transferase (left) was determined after 8 hours of incubation as described under Materials and Methods.

change glucuronidation compared to the intact isoforms except that UDPGTh2 activity for HDCA appeared to increase (Fig. 4). There were nine amino acid differences between these carboxyl termini. Exchanges made at codons 297 and 385 completely abolished activity of UDPGTh2 without an appreciable effect on that of UDPGTh1. Between chimeras UDPGTh2/385 and UDPGTh2/469, there were also nine amino acid differences compared to this same region of UDPGTh1. This region between residues 385 and 469 excluded the unique amino acid terminus (~300 residues) and conserved the region between residues 481 and 499 seen in all characterized transferases. The fact that UDPGTh2/469 did not show a decrease in activity suggested that this conserved region was not a critical factor in the low turnover with UDPGTh1. On this basis of thses findings, it seems likely that the membrane-bound UDP-glucuronosyltransferases have many important domains not yet understood. The apparent equal preference of UDPGTh2 for HDCA, 17-epiestriol, and 3,4-catechol estrogen pointed the structure and configuration of an enzyme active site which conferred overlapping specificity due to chemical similarities. This conclusion was supported by the demonstration that the same specificity had been maintained in the low-activity UDPGTh1 isoform. Amino acid is necessary for this selectivity had been conserved in spite of the 82% difference in the amino acid terminus. Amino acids that affect the rate of product conversion have undoutedly undergone substitution in UDPGTh1. The relative effectiveness of the UDPGTh2 enzyme in conjugating three different metabolites remains unknown and needs further investigations.

ACKNOWLEDGEMENTS

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