PHOTOAFFINITY LABELING STUDIES OF THE HUMAN RECOMBINANT UDP-GLUCURONOSYLTRANSFERASE, UGT1*6, WITH 5- AZIDO-UDP-GLUCURONIC ACID

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(Received July 19, 1996; accepted January 7, 1997)

ABSTRACT:

Recombinant human liver UDP-glucuronosyltransferase (UGT), UGT1*6, which catalyzes the glucuronidation of small phenols, previously expressed in a V79 cell line (1) was photolabeled with [β-32P]5N UDP-glucuronic acid ([β-32P]5N UDP-GlcUA). Two polypeptides with an approximate molecular weight of 54 kDa were extensively photolabeled in the recombinant cell line while the nontransfected cell line showed no photoincorporation in this area. The identity of the two polypeptides as UGTs, which correspond to two different glycosylation forms of the same enzyme, was confirmed by Western blot using a polyclonal monospecific antibody directed against the 120 amino acids of the N-terminal end of UGT1*6. Preincubation with UDP-glucuronic acid (UDP-GlcUA) inhibited the photoincorporation of the probe into the polypeptides indicating competition of both the photoprobe and the nucleotide-sugar for the same binding site. It was further shown that photoincorporation of [β-32P]5N UDP-GlcUA into the UDP-GlcUA-binding site was saturable. The lack of photoincorporation of a related photoprobe, [β-32P]5N UDP-glucose ([β-32P]5N UDP-Glc), into UGT1*6 demonstrated specificity of this enzyme for UDP-GlcUA. In enzymatic assays, unlabeled 5N UDP-GlcUA was shown to be an effective cosubstrate of the glucuronidation of 4-nitrophenol catalyzed by UGT1*6. The studies were further extended by demonstrating that photolabeling of UGT1*6 was inhibited by several active site-directed inhibitors. Finally, photoaffinity labeling was used in the purification of the labeled UGT1*6 using preparative gel electrophoresis. In conclusion, we have demonstrated that photoaffinity labeling with [β-32P]5N UDP-GlcUA is an effective tool for the characterization of enzymes such as recombinant UGTs that use UDP-GlcUA.

UGTs are a family of membrane-bound isoenzymes catalyzing the conjugation of glucuronic acid with various compounds of both exogenous and endogenous origin such as drugs, pollutants, bilirubin, steroid hormones, bile acids, and thyroid hormones (2). This biotransformation process results in the conversion of compounds from hydrophobic to more hydrophilic and facilitates their excretion in urine or bile. UDP-GlcUA1 is the essential cosubstrate of all UGTs; therefore, the synthesis and application of [β-32P]5N UDP-GlcUA (fig. 1) as a photoaffinity probe has become a powerful tool for the characterization of UGTs.

The basic principle of photoaffinity labeling is that the photoprobe, initially reversibly bound to a specific site, becomes covalently bound to amino acid residue(s) within its binding site upon irradiation with ultraviolet light. In the case of [β-32P]5N UDP-GlcUA, irradiation of the azido group in [β-32P]5N UDP-GlcUA generates a reactive nitrene which covalently binds to the UDP-GlcUA binding site of specific proteins (3). This technique facilitates the photo labeling of proteins of interest and their characterization in crude membrane preparations without purification to homogeneity. The advantage of this approach is that the protein of interest can be extensively investigated in its natural protein-phospholipid environment. As applied to the study of UGTs, investigations with this compound have been done to confirm the lumenal orientation of the UDP-GlcUA binding site (4) and to characterize various UGT inducers (5), and inhibitors (6).
Additionally, the photolabeling of proteins in intact endoplasmic reticulum provided evidence for the presence of carrier-mediated UDP-GlcUA transport (7).

In this paper, we describe the application of photoaffinity labeling to the characterization in eukaryotic V79 cells of recombinant human UGT1*6 which catalyzed the glucuronidation of small and planar phenols. The availability of a single recombinant protein allowed the study of the unique features of an individual UGT without interference from multiple isoforms present in microsomal preparations.

**Materials and Methods**

UDP-GlcUA (ammonium salt), N-butyric acid (sodium salt), [14C]4-nitrophenol (11.7 mCi/mmol), prestained triose isomerase from rabbit muscle and prestained pyruvate kinase from chicken muscle were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade. The inhibitors (fig. 2) PP36 (DHPGAdU), PP37P (DHPAdU), PP37 (DHPASU), and PP55 (DPASU) were synthesized as described in (9). The first step of the synthesis consisted in the incubation of azido-UTP, sucrose, and [32P]Pi (ICN) with sucrose polymerized just before loading the samples. Recombinant UGT membrane fractions (3 mg) were incubated with 40 μM [β-32P]5N3-UDP-GlcUA (final concentration 2.5 μCi/mmol, 0.5 mM in dimethyl sulfoxide); 0.1 mM of either UDP-GlcUA or 5N3-UDP-GlcUA, in a final volume of 50 μl. Incubation was performed at 37°C for 20 min and the glucuronidation reaction was stopped by addition of 20 μl ice-cold ethanol to the tubes followed by centrifugation at 5,000 x g for 5 min. Sixty μl of the resulting supernatant was applied to preadsorbent layer TLC plates (J.T. Baker, Inc, Phillipsburg, NJ) and the radiolabeled reactants were then scraped into vials and vortexed in a solution of 0.5 ml methanol and 5 ml Ecoscint A (National Diagnostics, Atlanta, GA) and counted for radioactivity in a LKB 1214 Rackbeta liquid scintillation counter.

**Preparation of Specific Anti-UGT1*6 Antibodies.** Monospecific polyclonal antibodies raised in sheep against the 120 N-terminal amino-acids of UGT1*6 were used for identification of this protein in recombinant V79 cells (11). 

**Cell Culture and Preparation of Membrane Fractions.** Stable expression of human enzyme UGT1*6 in a eukaryotic V79 cell line as described in (1) was modified (12). An enriched membrane fraction was prepared from cells treated with 2 mM N-butyric acid (sodium salt) 24 hr before harvesting the cells. The cell pellets, obtained from one hundred 100 x 20-mm dishes (about 100 mg protein) were homogenized in 0.25 M sucrose, 5 mM Hepes (pH 7.4), sonicated and centrifuged as previously described (12) to obtain membrane fractions of the recombinant cell line. The method described by Bradford (13) was used to evaluate the protein concentration using bovine serum albumin (Sigma Chemical Co.) as a standard.

**Enzymatic Assay.** Glucuronidation of [14C] 4-nitrophenol by UGT1*6 was evaluated according to a method derived from a previously described glucuronidation assay (14). The following incubation medium was used: 50 μM, lanes 2, 4, 6, 8 and 300 μM, lanes 3, 5, 7, 9. Lane 1, no inhibitor.

**Effect of uridinyl-derived inhibitors on the photoaffinity labeling of human recombinant UGT1*6 with [β-32P]5N3-UDP-GlcUA.**

Experiments were performed as described in Materials and Methods and carried out with 50 μg of enriched membrane fractions of recombinant V79 cells expressing the human UGT1*6 and a final concentration of 40 μM [β-32P]5N3-UDP-GlcUA. Two concentrations of the inhibitors were used (150 μM, lanes 2, 4, 6, 8 and 300 μM, lanes 3, 5, 7, 9, Lane 1, no inhibitor).

**Preparative Gel Electrophoresis.** The Bio-Rad Prep Cell apparatus model 407 was used according to the manufacturer’s recommendations. A 12% SDS polyacrylamide running gel, pH 8.8 (approximately 6 x 3 cm) was polymerized just before loading the samples. Recombinant UGT membrane fractions (3 mg) were incubated with 40 μM [β-32P]5N3-UDP-GlcUA (2.5 μCi/mmol), equilibrated for 1 min at room temperature, followed by irradiation.
tion with a handheld UV lamp for 90 sec. The final volume of the photolabeled fractions was 100 μl. Proteins were precipitated by addition of 300 μl of 10% trichloroacetic acid (TCA) and mixed with 7 mg of TCA-precipitated membrane fractions (not photolabeled), prestandard triose phosphate isomerase from rabbit muscle (35.2 kDa), and prestandard pyruvate kinase from chicken muscle (75.2 kDa) in a denaturing buffer (3.6 M urea, 20 mM Tris, 0.14 M dithiothreitol, 5% SDS (w/v), bromophenol blue, pH 8.0). Electrophoretic separation was performed as previously described (10). Selected fractions containing photolabeled protein were subjected to analytical electrophoresis (10% polyacrylamide) followed by Western blot as previously described (11).

Results

Photoaffinity Labeling and Identification of UGT1*6 Protein by Western Blot Analysis. The photoaffinity probe [β-32P]5N3UDP-GlcUA, previously used for the labeling of the microsomal UGTs (3, 4), was tested on recombinant human liver UGT1*6 expressed in V79 cells (fig. 4A) (1, 12). Fig. 4B shows the Western blot analysis with anti N-terminal UGT1*6 monospecific polyclonal antibodies of membrane fractions of nontransfected (lanes 1 and 2) and transfected cells (lanes 3, 4) labeled by [β-32P]5N3UDP-GlcUA as revealed by autoradiography. The nontransfected V79 cells showed no photoincorporation of [β-32P]5N3UDP-GlcUA in the area of the molecular mass corresponding to UGT1*6 (54 kDa), nor any visible immunoreactive band of UGT protein (fig. 4, lanes 1 and 2). In contrast, two protein bands, in the area of 54 kDa, were photolabeled with the probe in transfected cells (fig. 4A). The photoaffinity labeling was fully protected by 0.3 mM UDP-GlcUA (fig. 4A, lane 4) and the two radioactive bands corresponded to immunoreactive proteins revealed by Western blot (fig. 4B, lanes 3 and 4). No incorporation of radioactivity into the UGT1*6 polypeptides was observed when the photoprobe was incubated with the membrane fractions in absence of irradiation (results not shown). GPDS, with a molecular weight of about 37 kDa (10, 15), was also extensively labeled by the same photoaffinity probe, but the polypeptide was not recognized by the antibodies.

Characterization of Photoaffinity Labeling of UGT1*6. Specificity of UGT1*6 photoaffinity labeling by [β-32P]5N3UDP-GlcUA was studied by varying the concentration of the photoprobe from 0 to 120 μM. Fig. 5 shows that photoincorporation of the label was saturable at a concentration of [β-32P]5N3UDP-GlcUA above 90 μM. Apparent half saturation of photoincorporation was reached at 40.3 ± 6.1 μM.

Evidence that 5N3UDP-GlcUA is a Cosubstrate for UGT1*6. An important criterion for the specificity of the photoprobe towards the UDP-GlcUA binding site of the recombinant enzyme is whether it functions as cosubstrate for the UGT1*6 catalyzed glucuronidation reaction. To evaluate this aspect, an aglycone substrate of UGT1*6 enzyme, [14C]-4-nitrophenol (0.5 mM), was incubated with 5N3UDP-GlcUA and enriched membrane fractions (50 μg protein) of the recombinant cell line. As shown in fig. 6, [14C]-4-nitrophenol-glucuronides were detected after incubation with the photoprobe. The relative specific activity found under these experimental conditions was of 0.96 ± 0.17 nmoles/min/mg protein (lane 3) compared with 1.99 ± 0.06 nmoles/min/mg protein (lane 2) when UDP-GlcUA was used as a substrate at the same concentrations as 5N3UDP-GlcUA (0.1 mM).

Photoaffinity Labeling in the Presence of Inhibitors. A series of uridine derivative inhibitors of recombinant human liver UGT1*6 has been previously characterized by us (16). The structure of these compounds as well as the nomenclature used in this study is detailed in fig. 2. Photoaffinity experiments with [β-32P]5N3UDP-GlcUA were performed on enriched membrane fractions of transfected cells in the presence of 150 and 300 μM inhibitor and 40 μM photoprobe (fig. 3). PP55B (D-DPMSU) and PP36 (DHPGAdU) did not show any significant dose-dependent modification of photoincorporation of the probe in the area of UGT1*6, even at the highest concentration (84 and 99% of control incorporation, respectively). All other compounds produced a dose-dependent decrease of photoincorporation. PP37 (DHPASU) had the most potent protective effect, producing a 84% decrease in the incorporation of radioactivity at 150 μM and a 87% decrease at 300 μM. PP37P (DHPASU), the isopropylideneuridine form of PP37 (DHPASU), inhibited labeling by 23% at a concentration of 150 μM, and by 69% at 300 μM. With PP50 (DAPSiU) protection against photoincorporation of the probe at a concentration of 300 μM was observed; however, no detectable difference in the inhibitory effect of the A and B stereoisomers was noticed (83% for both). The inhibitors PP55A (L-DPMSU) and PP55B (D-DPMSU) (the uridine forms of PP50 (DAPSiU)) were slightly less effective than PP37 (DHPASU), and PP50 (DAPSiU) at the same concentration (300 μM), with 71% and 82% inhibition, respectively.
UDP-Sugar Specificity of Photolabeling of UGT1*6. To determine whether UGT1*6 had a specific requirement for UDP-GlcUA, photoaffinity studies were conducted using [$\beta$-$32$P]5N$_3$ UDP-Glc as the photoprobe. Membrane proteins (50 $\mu$g) were preincubated with [$\beta$-$32$P]5N$_3$ UDP-Glc and photolabeled using the same conditions described for [$\beta$-$32$P]5N$_3$ UDP-GlcUA. Neither the control cells, nor cells transfected with UGT1*6 showed any photoincorporation of [$\beta$-$32$P]5N$_3$ UDP-Glc (data not shown).

Isolation of Photolabeled UGT1*6 Polypeptides by Preparative Gel Electrophoresis. Fig. 7 shows a SDS-PAGE separation of a pool of fractions of UGT1*6 polypeptides purified to apparent homogeneity by preparative electrophoresis. Lane 1 shows the Coomassie blue staining of the combined fractions containing the UGT1*6 polypeptides. Final confirmation of the polypeptides as UGTs was obtained through Western blot analysis using specific anti-N-terminal UGT1*6 (data not shown). Lane 2 is the autoradiograph of the photolabeled and purified UGT1*6 enzyme.

Discussion
Development of photoaffinity analogs has provided a powerful tool for the investigation of different aspects of enzyme-substrate or enzyme-inhibitor interactions. Since UGTs are closely related membrane-bound isoenzymes, photoaffinity experiments previously published were performed on microsomal membrane fractions containing the entire range of UGT isoforms with overlapping substrate specificity (3, 4, 7, 10, 15). Development of specific UGT cDNAs and their expression in various cellular systems allows characterization to be carried out with individual UGTs in isolation from other isoforms.

The work presented here demonstrates that [$\beta$-$32$P]5N$_3$ UDP-GlUCUA is a specific photoaffinity probe for recombinant human liver UGT1*6. Photoaffinity labeling followed the criteria established by Haley for active site photolabeling (17): First, proteins which specifically bind UDP-GlUCUA are photolabeled. As shown in fig. 4, two
polypeptides in the molecular weight range of UGTs are photolabeled in membrane fractions of the recombinant cell line. These polypeptides were identified as UGT1*6 by comparison of the photolabeling pattern in non-transfected V79 cells and the recombinant cell line, in conjunction with Western blot analysis with specific antibodies directed against 120-N terminal amino acids of the enzyme. The same observation has been reported for UGT1*6 expressed in COS-7 cells (18) and for other recombinant UGTs (19). We have shown previously (3, 4, 7, 10, 15) that microsomal UGT1*6 enzyme purified to homogeneity by preparative electrophoresis after photolabeling.

Photoaffinity labeling of membrane fractions of a recombinant cell line expressing the human liver UGT1*6 with [β-32P]SN,UDP-GlcUA and separation of the radiolabeled proteins on Bio-Rad Prep Cell apparatus was performed as described in Materials and Methods. Pooled and concentrated fractions containing UGT1*6 were analyzed by SDS-PAGE on a 10% gel. Lane 1 shows proteins stained with Coomassie blue. Lane 2 is an autoradiograph of the separation shown in Lane 1. Fragments were identified as UGT1*6 polypeptides by Western blot analysis with a specific antibody directed against 120-N terminal amino acids of the enzyme.

The detection of two bands of apparent molecular mass 54–56 kDa was dependent on UV-irradiation (data not shown). This excludes any unspecific enzymatic phosphorylation process and strongly indicates that only specific photolabeling of the proteins occurs. This study has shown that SN,UDP-GlcUA is also a cosubstrate of the glucuronidation reaction catalyzed by this enzyme (fig. 6). Therefore, the binding of this photoprobe to the enzyme’s active site could be anticipated to be similar to that of the cosubstrate, UDP-GlcUA. The other photolabeled protein in V79 cells, UDP-glucose: dolichylphosphate glycosyltransferase (Glc-P-Dol synthase, GPDS) was also extensively protected by UDP-GlcUA (fig. 4A), as shown previously with microsomal preparations (3, 4, 7, 10, 15). The two radiolabeled polypeptides with an apparent molecular mass of 54–56 kDa were identified as UGT1*6 using Western blot analysis. These two bands are thought to correspond to different levels of glycosylation of the same polypeptide based on endoglycosidase treatment which produces a single polypeptide band of apparent molecular mass 50–52 kDa. The same observation has been reported for UGT1*6 expressed in COS-7 cells (18) and for other recombinant UGTs (19).

Fig. 7. Radiolabeled UGT1*6 enzyme purified to homogeneity by preparative electrophoresis after photolabeling.

2 T. Pillot, S. Fournel-Gigleux, and J. Magdalou, unpublished observations.
photoaffinity labeling, such as cosubstrate specificity studies, identification and selection of inhibitors, and as an aid in protein purification. However, it is anticipated that the most significant application of this technique will be the identification of UGTs with unknown substrate specificity, isolation of peptides of the active site, and for characterization of UGTs for which antibodies are not available.

Acknowledgments. We wish to thank Prof. B. Burchell for kindly providing the cDNA used to express UGT1*6 and J. Little and G. Zawada for their critical evaluation of this manuscript.

References