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

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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-GlcUA is the essential cosubstrate of all UGTs; there-fore, 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 photolabeling 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 luminal 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), peroxidase 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 (DHPA-SiU), PP37 (DHPASA), PP30 (DPASiU), and PP55 (DPASU) were synthesized as described in (8). The synthesis of PP56B (D-DFPSU) was described separately (6).

Synthesis of [β-32P]-5-Azido-UDP-GlucUA and [β-32P]-5-Azido-UDP-Glucose. [β-32P]-5N-UDP-Glc and [β-32P]-5N-UDP-Glucose were synthesized and purified as previously described (3, 9). The first step of the synthesis consisted of the incubation of azido-UDP, sucrose, and [32P]Pi (ICN) with sucrose containing 12.5 mM MgCl₂ for 5 min at room temperature. In some cases this incubation was done in the presence of 150 and 300 mM inhibitors (fig. 3).

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

Cell Culture and Preparation of Membrane Fractions. Stable expression of recombinant human enzyme UGT1*6 in a eukaryotic V79 cell line as described in (1) was modified (12). An enriched membrane fraction was incubated with 2 mM N-acetylcysteine (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 μg of membrane fraction protein; 0.1 M Tris-HCl (pH 7.4), 10 mM MgCl₂, [14C]4-nitrophenol (0.124 μCi/mmol, 0.5 mM in dimethyl sulfoxide); 0.1 mM of either UDP-GlcUA or 5N-3 UDP-GlucUA, 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 reagents were separated with chloroform/methanol/glacial acetic acid/water (65:25:2:4, v/v). [14C]-4-nitrophenol-β-D-glucuronide was identified by autoradiography of the TLC plates. The radiolabeled spots 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 some cases this incubation was done in the presence of 150 and 300 μM inhibitors (fig. 3).

Preparative Gel Electrophoresis. The Bio-Rad Prep Cell apparatus model 491 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]-5N-UDP-GlucUA (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), pre-stained trisio phosphate isomerase from rabbit muscle (35.2 kDa), and pre-stained 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% acrylamide) 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]5N3 UDP-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 non-transfected (lanes 1, 2) and transfected cells (lanes 3, 4) labeled by [β-32P]5N3 UDP-GlcUA as revealed by autoradiography. The non-transfected V79 cells showed no photo-incorporation of [β-32P]5N3 UDP-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]5N3 UDP-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]5N3 UDP-GlcUA above 90 μM. Apparent half saturation of photoincorporation was reached at 40.3 ± 6.1 μM.

Evidence that 5N3 UDP-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 5N3 UDP-GlcUA and enriched membrane fractions (50 μg protein) of the recombinant cell line. As shown in fig. 6, [14C]-4-nitrophenyl-glucuronides were detected after incubation with the photoprobe. The relative specific activity found under these experimental conditions was 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 5N3 UDP-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]5N3 UDP-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). PP56B (D-DPMSU) and PP36 (DHPGAdU) did not show any significant dose-dependent modification of photo-incorporation 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 photo-incorporation. 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 300 μM and a 87% decrease at 300 μM. PP50 (DPASiU), the isopropylideneuridine form of PP50 (DPASiU), inhibited labeling by 23% at a concentration of 300 μM and a 88% decrease at 300 μM. PP50 (DPASiU) 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-DPASU) and PP55B (D-DPASU) (the uridine forms of PP50 (DPASiU)) were slightly less effective than PP37 (DHPASU), and PP50 (DPASiU) at the same concentration (300 μM), with 71% and 82% inhibition, respectively.

Fig. 4. Western blot analysis of membrane fractions of nontransfected and transfected V79 cells after photoaffinity labeling with [β-32P]5N3 UDP-GlcUA. A, Autoradiograph of photolabeled proteins from nontransfected V79 cells and from cells expressing recombinant UGT1*6. B, Western blot with specific sheep anti N-terminal UGT1*6 antibody. 50 μg of human recombinant UGT1*6 membrane fractions were photolabeled with 40 μM [β-32P]5N3 UDP-GlcUA as described in Materials and Methods. For the protection experiments (lanes 2 and 4), samples were preincubated with 0.3 mM UDP-GlcUA for 10 min on ice before photolabeling. For both A and B, lanes 1 and 2 are V79 control membrane fractions, lanes 3 and 4 are membrane fractions of V79 cells expressing the human UGT1*6.
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 [β-32P]5N3 UDP-Glc as the photoprobe. Membrane proteins (50 μg) were preincubated with [β-32P]5N3 UDP-Glc and photolabeled using the same conditions described for [β-32P]5N3 UDP-GlcUA. Neither the control cells, nor cells transfected with UGT1*6 showed any photoincorporation of [β-32P]5N3 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 [β-32P]5N3 UDP-GlcUA 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-GlcUA are photolabeled. As shown in fig. 4, two
These two bands detected by UDP-GlcUA (fig. 4A), as shown previously with labeled protein in V79 cells, UDP-glucose: dolichylphosphate glycosyltransferase of this photoprobe to the enzyme’s active site could be anticipated to formation 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. Fractions were identified as UGT1′6 polypeptides by Western blot analysis with a specific antibody directed against 120-N terminal amino acids of the enzyme.

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 nontransfected V79 cells and the recombinant cell line, in conjunction with Western blot analysis with specific antibodies directed against this enzyme. Second, the photoinsertion of the probe into UGT1′6 was saturable. Third, protection against the photoinsertion of the probe was observed in the presence of UDP-GlcUA, and various active site-directed inhibitors (figs. 3 and 4). Finally, incorporation of radioactive to the UGT1′6 polypeptides was strictly 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).

We have shown previously (3, 4, 7, 10, 15) that microsomal proteins in the range of 52–56 kDa could be effectively photolabeled with [β-32P]SN,UDP-Glc. This observation resulted in the identification of a new class of human protein(s) conjugating hydrophobic moieties with glucose (14). The observation that [β-32P]SN,UDP-Glc, which is structurally related to [β-32P]SN,UDP-GlcUA, failed to label the UGT1′6 polypeptides (data not shown), confirmed the fact that UDP-Glc was not a cosubstrate of this enzyme (12). Structurally the only difference between UDP-Glc and UDP-GlcUA is the presence of an anionic carboxyl moiety in UDP-GlcUA. It has been shown that the presence of this moiety is essential for binding of UDP-GlcUA to a purified UGT from pig liver (20). The present photoaffinity and enzymatic assays fully confirm this for the recombinant human liver UGT1′6.

In addition, the potential inhibitory effect of UDP-GlcUA binding site-directed inhibitors (16) on the photoaffinity labeling of the UGT1′6 isoenzyme was evaluated (fig. 7). Among the eight compounds examined, PP36 (DHPGAdU) and PP56B (D-DPMSU) did not show any significant inhibitory effect. The other inhibitors produced a concentration-dependent decrease in photoincorporation. In most cases, a concentration of 150 μM (fig. 3) was sufficient to significantly decrease the incorporation of [β-32P]SN,UDP-GlcUA into UGT1′6. Interestingly, PP56B (D-DPMSU) did not affect the photoincorporation of [β-32P]SN,UDP-GlcUA, and no concentration-dependent effect was observed, even though in previous studies its inhibitory potency towards the glucuronidation of 4-methylumbellifl erone (4-MU) was the best observed and the compound behaved like a competitive inhibitor towards both UDP-GlcUA and 4-MU binding sites (16). Preincubation of PP56B (D-DPMSU) with enriched membrane fractions at 37°C for up to 30 min did not affect the binding of [β-32P]SN,UDP-GlcUA to UGT1′6, thus excluding a slow binding process for this inhibitor (not shown). The apparent discrepancy between strong competitive inhibition towards UDP-GlcUA in the enzymatic assay (16) and lack of this inhibitory effect on photoincorporation of [β-32P]SN,UDP-GlcUA into UGT1′6 could be explained by a requirement for the presence of the cosubstrate and/or the aglycone for binding to the enzyme active site. The binding of UDP-GlcUA and/or 4-MU could induce a modification of the active site in such a way as to enable subsequent binding of PP56B (D-DPMSU). Whether both cosubstrate and 4-MU are necessary for inhibition is not yet known. The effect of these inhibitors on the GPDS has been described elsewhere (8).

Since photoincorporation of the radiolabeled analog is covalent in nature, purification techniques not normally useful for membrane-associated UGTs can be used. Preparative gel electrophoresis, which allows simultaneous elution and collection of fractions containing electrophoretically separated proteins, has been used by us in the course of purification of photolabeled GPDS (10, 15). This technique has also been used successfully for isolation and purification of photolabeled UGT1′6 expressed in V79 cells (fig. 7). The identity of photolabeled and purified polypeptides as UGT1′6 was confirmed by Western blot using specific sheep anti-N-terminal UGT1′6 antibody. This method supplies us with protein purified to apparent homogeneity with highly radioactive [β-32P]SN,UDP-GlcUA covalently bound to its binding site. This complex will be used for the identification of peptides of the UDP-GlcUA binding site.

The results summarized above firmly establish [β-32P]SN,UDP-GlcUA photolabeling as an effective approach in the investigation of human recombinant UGTs and other UDP-GlcUA-utilizing proteins expressed in V79 cells. For these studies we used a well-characterized recombinant protein, UGT1′6, with known substrate specificity. Moreover, a polyclonal monospecific antibody for this enzyme is available. The data presented show several important applications of
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.

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References