Application of 5-Azido-UDP-glucose and 5-Azido-UDP-glucuronic Acid Photoaffinity Probes for the Determination of the Active Site Orientation of Microsomal UDP-glucosyltransferases and UDP-glucuronosyltransferases*

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A new approach to determining the active site orientation of microsomal glycosyltransferases is presented which utilizes the photoaffinity analogs [32P]5-Azido-UDP-glucose ([32P]5N,UDP-Glc) and [32P]5-Azido-UDP-glucuronic acid ([32P]5N,UDP-GlcA). It was previously shown that both photoprobes could be used to photolabel UDP-glucose:dolichol phosphate glucosyltransferase (Glc-P-Dol synthase), as well as the family of UDP-glucuronosyltransferases in rat liver microsomes. The effects of detergents, proteases, and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) on the labeling of these enzymes were examined in intact rat liver microsomes. Photolabeling of Glc-P-Dol synthase by either photoprobe was the same in intact or disrupted vesicles, was susceptible to trypsin digestion, and was inhibited by the nonpenetrating inhibitor DIDS. Photolabeling of the UDP-glucuronosyltransferases by [32P]5N,UDP-GlcA was stimulated 1.3-fold in disrupted vesicles as compared to intact vesicles, whereas photolabeling of these enzymes by [32P]5N,UDP-Glc showed a 14-fold increase when vesicles were disrupted. Photolabeled UDP-glucosyltransferases were only susceptible to trypsin digestion in disrupted vesicles, and this was further verified by Western blot analyses. The results indicate a cytoplasmic orientation for access of UDP-sugars to Glc-P-Dol synthase and a lumenal orientation of most UDP-glucuronosyltransferases.

The glycosyltransferase reactions involved in the biosynthesis of N-linked glycoproteins are known to occur in the endoplasmic reticulum (ER) and Golgi membranes. Additionally, specific UDP-glucuronosyltransferases which require exclusively UDP-GlcA as the glucuronosyl donor (3) have been demonstrated in the livers of all mammalian species that have been studied (4), and these enzymes are engaged in the glucuronidation of a variety of endogenous and exogenous substrates. Since these UDP-glucuronosyltransferases are membrane-associated, determination of their active site orientation, whether cytoplasmic or lumenal, and their overall topology within the membrane remain critical problems in understanding the above biosynthetic pathways. In the present studies, the orientations of UDP-Glc:Glc-P-Dol glucosyltransferase (or Glc-P-Dol synthase, GPDS), involved in glycoprotein biosynthesis, and UDP-glucuronosyltransferases were studied.

Initial studies on the orientation of the glucosyltransferases involved in the biosynthesis of the lipid-linked oligosaccharides of the N-linked glycoprotein pathway were done by observing the effect of trypsin treatment on glycosyltransferase activities in intact and detergent disrupted rat liver microsomes (5). In this system, and also in hen-oviduct microsomes (6), GPDS activity was found to decrease in the presence and absence of detergents following trypsin treatment, suggesting a cytoplasmic orientation for this enzyme. In another experiment using the nonpenetrating anion transport inhibitor, DIDS, and calf thyroid microsomes, GPDS activity was inhibited (7), again suggesting a cytoplasmic orientation. However, none of the above experiments could preclude the possibility that a transporter of UDP-Glc was affected independently of GDPs activity. Indeed, translocation of UDP-Glc into sealed rat liver ER vesicles has been reported (8, 9), as well as a lumenal ER glucosyltransferase which utilizes UDP-Glc to directly glucosylate glycoproteins (10).

The transmembrane orientation of the UDP-glucuronosyltransferase, which has been extensively studied during recent years, and evidence has accumulated for a lumenal orientation of the active site of this family of enzymes in rat liver microsomes (4). Most of this evidence has centered on transport of the substrate UDP-GlcA across the ER membranes; a lumenal orientation would require availability of UDP-GlcA inside the microsomal vesicles. Evidence that UDP-GlcA might be transported across the membrane by the same transport system as UDP-Glc was suggested by cis inhibition and trans stimulation of UDP-Glc transport by UDP-GlcA (9, 11). Recently, the transmembrane transport of UDP-GlcA and presence of intact UDP-GlcA in the microsomal vesicles of rat liver has been demonstrated (12). Additionally, studies in sealed rat liver microsomes in conjunction with protease treatment (13), immunoblotting analysis (14), and computer predictions of UDP-glucuronosyltransferase sequences (15, 16) indicated that most of the UDP-glucuronosyltransferases...
are located on the luminal side of the ER.

In this report, we present a new approach to studying the active site orientation and topology of membrane-associated glycosyltransferases utilizing two recently developed phot affinity analogs, 5-azido-UDP-Glc ([\(^{32}\)P]5N3UDP-Glc) (17) and 5-azido-UDP-GlcA ([\(^{32}\)P]5N3UDP-GlcA) (18), and intact rat liver microsomes. ([\(^{32}\)P]5N3UDP-Glc has been used extensively in the study of membrane-associated glycosyltransferases (19, 20), especially GPDS (21-23). ([\(^{32}\)P]5N3UDP-GlcA has been used to photolabel UDP-glucuronosyltransferases in control and phenobarbital-induced rats, and it was shown to also photoincorporate into GPDS in rat and human livers (18). In addition, data are presented in this report which show that ([\(^{32}\)P]5N3UDP-Glc can photoincorporate into UDP-glucuronosyltransferases. Taking advantage of the fact that both photoprobes can be used to photolabel both GPDS and UDP-glucuronosyltransferases and that intact rat liver microsomal vesicles could be prepared, the effect of detergents, trypsin, and DIDS, on the photolabeling of these two families of enzymes was examined in relation to their active site orientations. Evidence is presented that indicates that GPDS has a cytoplasmic orientation while UDP-glucuronosyltransferases have a luminal orientation.

MATERIALS AND METHODS

[\(^{32}\)P]5-azido-UDP-glucose and [\(^{32}\)P]5-azido-UDP-glucuronic acid were synthesized and purified as previously described (17, 18). [\(^{32}\)P]Pi and [\(\gamma^{32}\)P]ATP were from ICN. Dolichyl phosphate, mannose 6-phosphate, Brij 58, nucleotides, saccharolectine, trypsin, and hexokinase were from Sigma. Plastic-backed cellulose thin layer chromatography sheets were from Kodak. Intact rat liver microsomes were prepared as previously described (9), except that they were not further fractionated into rough and smooth ER fractions.

Enzyme Assays—GPDS was assayed as described previously (22) with the following differences. Reaction mixtures containing 250-300 \(\mu\)g of microsomes, 60 mM HEPES, pH 6.5, 3 mM MgCl\(_2\), and 5 mM saccharolactone were incubated at 37 °C for 10 min, and then 5 \(\mu\)M [\(^{14}\)C]UDP-Glc was added for 10 min at 37 °C. All assays were done in a final volume of 0.1 ml, and any variation to the above protocol is described in the figure legend. When used, exogenous dolichyl phosphate (4 \(\mu\)g) was added in a final concentration of 0.1% Triton X-100.

Bile acid UDP-glucuronosyltransferase activity was measured with radioactive bile acids as substrates as described previously (24, 25). Bile acids were dissolved and added in methanol (1.6% final concentration) or in the form of micelles with Brij 58 as previously described (24). For assays with intact microsomal proteins, the bile acid substrates (0.1 mg of microsomal proteins) were incubated in 60 \(\mu\)l of 100 mM HEPES-NaOH buffer, pH 6.5, 5 mM MgCl\(_2\), 5 mM saccharolactone, 4.2 mM UDP-GlcA, and 50 \(\mu\)g of freshly prepared microsomal proteins. After 10 min at 37 °C, the reaction was stopped by the addition of 20 \(\mu\)l of ethyl acetate, and 80 \(\mu\)l of the mixture was directly applied to the preadsorbent layer of 19-channel preparative silica gel TLC plates. The glucuronidated bile acids and the unreacted substrate were separated by two TLC developments in chloroform/methanol/glacial acetic acid/water (65:25:2:4, v/v/v/v). Hydroxyl- and carbonyl-linked glucuronides are clearly separated under the latter conditions. Radioactive compounds were localized on cellulose TLC plates and developed in methanol, 88% formic acid, H\(_2\)O (80:15:5, v/v/v) (28). Radiochemical yields of [\(^{32}\)P]Man-6-P averaged above 95% as determined by autoradiography and scintillation counting of the TLC plates. To the reaction mix was added an equal volume of 30 mM Man-6-P and it was stored at -20 °C for subsequent use in the latency assays.

For the latency assays, assay buffer or photolabeling buffer was used. Membranes were incubated in the presence or absence of detergent for 10 min at 37 °C, and then [\(^{32}\)P]Man-6-P (1.5 mM final) was added for 15 min at 37 °C. Aliquots were removed from each reaction at various time points and spotted on cellulose TLC plates. The TLC plates were developed in the solvent described above, autoradiographed for 2 h, and scanned for scintillation counting. The percentage of [\(^{32}\)P]Man-6-P, present in the stock solution was subtracted from the [\(^{32}\)P]Pi, value determined in the latency experiments. Using this technique, values of 85-90% Man-6-P latency for up to 15 min were routinely observed for intact vesicles, similar to the values previously reported employing phosphate assays (29, 30).

RESULTS

Effects of Detergent, Protease Treatment, and DIDS on Enzymatic Activity of Intact Microsomes—In determining the orientation of the active sites of GPDS and UDP-glucuronosyltransferases, it was first necessary to characterize their respective activities in rat liver microsomes. The effects of trypsin and DIDS on the formation of two types of bile acid glucurononides in sealed and detergent treated microsomes was studied (Table I). The treatment of hepatic microsomes with the optimal concentrations of Brij 58 and Triton X-100 (0.6 mg of detergent/mg of protein) resulted in a 2-4-fold increase in the formation of the 3-O-glucuronidase of lithocholic acid. Using lithocholic acid prepared in form of Brij 58 micelles as a substrate, an 8-fold stimulation of 3-OH steroid UDP-glucuronosyltransferase activity was obtained. After treatment of sealed microsomes with trypsin (1.2 mg/mg of protein), a slight increase of lithocholic acid UDP-glucuronosyltransferase activity was observed. When the detergent-solubilized microsomes were further treated with trypsin, no apparent loss or stimulation of lithocholic acid UDP-glucuronosyltransferase was observed. The addition of the anion transport inhibitor DIDS resulted in a decrease of 35 and 53% in the lithocholic acid UDP-glucuronosyltransferases activities in intact vesicles and over 60% in disrupted microsomes.

In contrast, as shown in Table II, the activity of GPDS in intact vesicles was reduced in the presence of detergent and was susceptible to trypsin digestion. The reduction in the formation of radiolabeled Dol-P-Glc in the presence of DIDS was decreased by 27% in intact microsomes and by 64% in disrupted. If exogenous dolichol phosphate was added, which also requires addition of detergent, the activity was increased 5-fold and was even more sensitive to trypsin and DIDS. These observations are consistent with what has been previously reported for GPDS (5-7), leading to the conclusion by these investigators that GPDS was cytoplasmic in orientation. However, transport of UDP-Glc into the lumen cannot be
The results shown were with microsomes obtained from Sprague-Dawley rat livers. Lithocholic acid glucuronidation reactions were carried out as described under "Materials and Methods." The substate concentration was 100 μM. For disruption of vesicles, microsomal proteins were incubated for 10 min prior to assay with 0.05% Brij 58 or Triton X-100 in the same incubation medium. For experiments involving trypsin, 60 μg of trypsin was incubated with 50 μg of microsomal protein for 15 min at 37 °C before addition of the assay mixture. When included, DIDS was present at 0.2 mM final concentration. LA = lithocholic acid. Specific activities of enzymes are expressed as pmol/min × mg protein. Mannose-6-phosphatase latency in intact microsomes was 89% and was abolished in the presence of 0.05% detergents.

TABLE I

| Type of glucuronidation | Hydroxyl-linked | Carboxyl-linked |
|-------------------------|----------------|----------------|
| Intact microsomes       | 0.81           | 0.23           |
| + 0.05% Brij 58         | 3.5            | 0.75           |
| + 0.05% Triton X-100    | 1.75           | 0.76           |
| + Trypsin               | 1.2            | 0.27           |
| + 0.05% Brij 58 + trypsin | 3.3        | 0.91           |
| + 0.05% Triton X-100 + trypsin | 2.6    | 0.48           |
| LA in form of Brij 58 micelles | 6.8      | 1.51           |
| Intact + DIDS           | 0.53           | 0.11           |
| 0.05% Brij 58 + DIDS    | 1.38           | 0.27           |

TABLE II

Enzymatic assays of rat liver GPDS

Rat liver Glc-P-Dol synthase was assayed as described under "Materials and Methods." Activity is expressed in pmol of [14C]Glc-P-Dol produced/min/mg of total protein. Where indicated, 4 μg of dolichyl phosphate (Dol-P) in 0.1% Triton X-100, 0.2 mM DIDS, or 100 μg of trypsin were present. Mannose-6-phosphatase latency in intact microsomes was 89% and greater than 95% in the presence of DIDS. Latency was abolished in the presence of detergent and Dol-P.

| Sample            | Activity pmol/min/mg protein |
|-------------------|-----------------------------|
| Intact            | 0.30                         |
| + 0.05% TX        | 0.17                         |
| + 0.05% Brij 58   | 0.17                         |
| + Trypsin         | 0.21                         |
| + Dol-P           | 1.50                         |
| + Dol-P, Trypsin  | 0.56                         |
| Intact + DIDS     | 0.22                         |
| 0.05% TX + DIDS   | 0.11                         |
| + Dol-P + DIDS    | 0.38                         |

excluded in interpreting these activities. Also, it is possible that the trypsinization of a cytoplasmic domain of GPDS independent of the active site domain(s) results in an unstable conformation of the enzyme and thus loss of activity.

Photoaffinity labeling of intact and disrupted rat liver vesicles. Autoradiograph of rat liver microsomes (140 μg) photolabeled with either 30 μM [32P]5N3UDP-Glc (A) or [32P]5N3UDP-GlcA (B) as described under "Materials and Methods." Lanes 1 and 2, intact vesicles; lanes 3 and 4, 0.05% Brij 58; lanes 5 and 6, 0.5% Triton X-100. Lanes 1, 3, and 5 were samples which were incubated with photoprobe but not UV irradiated. Lane 7, intact vesicles plus 0.2 mM UDP-Glc; lane 8, 0.05% Triton X-100 plus 0.2 mM UDP-Glc; lane 9, intact vesicles plus 0.2 mM UDP-GlcA; lane 10, 0.05% Triton X-100 plus 0.2 mM UDP-GlcA; lane 11 trypsin digestion of intact, photolabeled vesicles; lane 12, trypsin digestion of 0.05% Triton X-100-disrupted, photolabeled vesicles; lane 12 trypsin digestion of 0.05% Triton X-100-disrupted, photolabeled vesicles. Mannose-6-phosphatase latency was 89% for intact vesicles.

UDP-Glc decreased the photolabeling of GPDS by both photoprobes by over 90% (lanes 7 and 8). The presence of 0.2 mM UDP-GlcA resulted in a 54 and 65% decrease in [32P]5N3UDP-GlcA photolabeling (Fig. 1A, lanes 1 and 2) of [32P]5N3UDP-Glc photolabeling of GPDS (Fig. 1A, lanes 3 and 4), and over a 90% decrease in [32P]5N3UDP-GlcA photolabeling (Fig. 1B, lanes 9 and 10). Intact and disrupted vesicles were photolabeled with either photoprobe and then digested with trypsin for 10 min. As shown in Fig. 1, lanes 11 and 12, the photolabeled GPDS band was decreased by greater than 90% after trypsin digestion whether in intact or disrupted vesicles.

Photoaffinity labeling of the UDP-glucuronosyltransferases with [32P]5N3UDP-GlcA was not changed in Brij 58-disrupted vesicles (Fig. 1B, lane 4) and was enhanced 1.3-fold in Triton X-100 (lane 6) relative to the intact vesicles (lane 2). In intact vesicles photolabeled with [32P]5N3UDP-Glc (Fig. 1A, lane 2), only one weakly photoincorporated band in the 51-56 kDa region was observed. In the presence of Brij 58 (Fig. 1A, lane 4) or Triton X-100 (lane 6), [32P]5N3UDP-Glc photolabeling of proteins in the 51-56 kDa region increased by 2- and 14-fold, respectively. In intact vesicles, the presence of 0.2 mM UDP-Glc resulted in no photolabeling of UDP-glucuronosyltransferases with [32P]5N3UDP-Glc (Fig. 1A, lane 7) and only a 33% decrease (compared to lane 2) in [32P]5N3UDP-GlcA photolabeling of UDP-glucuronosyltransferases (Fig. 1B, lane 7). In Triton X-100-disrupted vesicles, 0.2 mM UDP-Glc...
decreased photolabeling of the UDP-glucuronosyltransferases by 88% (Fig. 1A, lane 8) and 52% (Fig. 1B, lane 8). In intact and disrupted vesicles, the addition of 0.2 mM UDP-GlcA resulted in greater than 95% inhibition of [32P]5N5UDP-GlcA photoinsertion into UDP-glucuronosyltransferases (Fig. 1B, lanes 9 and 10). Trypsin digestion of intact vesicles after photolabeling with [32P]5N5UDP-GlcA had no effect on the photoinsertion into the UDP-glucuronosyltransferases (Fig. 1B, lane 11), whereas the presence of detergent plus trypsin caused a 75% reduction in the labeling of the 51–56-kDa UDP-glucuronosyltransferases (lane 12). A similar effect was observed in [32P]5N5UDP-Glc photoinsertions of UDP-glucuronosyltransferases in disrupted vesicles after trypsin treatment (Fig. 1A, lane 12).

As to the identity of the other rat liver vesicle proteins which have incorporated radioactivity, the 62-kDa protein in Fig. 1A which labels in the absence of UV irradiation (lanes 1, 3, and 5) has previously been shown to be phosphoglucomutase, and the labeling has been attributed to trace amounts of contaminating [32P]Glc-l-P in the photoprobe preparations (31). The identities of the polypeptides at 54 and 31 kDa which also label in the presence of [32P]5N5UDP-Glc and in the absence of UV irradiation have not been determined, nor is the identity of the labeling species known. The identity of the 71-kDa protein which was photolabeled by both photoprobes preferentially in disrupted vesicles is not known, although it has the potential of being an additional marker of vesicle latency. Photolabeling of this 71-kDa protein was increased 86% in disrupted vesicles, a value similar to the 89% latency determined for these microsomes by mannose-6-phosphate assays.

Immunorecognition of Photoincorporated UDP-glucuronosyltransferases—To verify that the 51–56-kDa proteins that become photolabeled with both photoprobes were indeed UDP-glucuronosyltransferases, an antibody known to recognize UDP-glucuronosyltransferases was used in Western blot analyses of photolabeled vesicles. As shown in Fig. 2, the proteins recognized by the antibody also photoincorporate [32P]5N5UDP-Glc and [32P]5N5UDP-GlcA in the 51–56 kDa region. As also shown in Fig. 1, photolabeled UDP-glucuronosyltransferases were susceptible to trypsin degradation only in disrupted vesicles (Fig. 2, A and B, lanes 5 and 11). Addition of chymotrypsin had no effect on the photolabeling of UDP-glucuronosyltransferases in intact microsomes, although there was slight degradation in detergent-disrupted vesicles (Fig. 2, A and B, lanes 6 and 12). The similarity of the [32P]5N5UDP-Glc and [32P]5N5UDP-GlcA photolabeling patterns of the 51–56-kDa proteins to the polypeptides recognized in the Western blot suggests that they are UDP-glucuronosyltransferases or closely related enzymes.

Effects of DIDS on GPDS Photolabeling—A previous study indicated that the anion transport inhibitor DIDS was an inhibitor of GPDS in porcine thyroid vesicles (7). In rat liver vesicles, GPDS activity was also inhibited by DIDS independent of added exogenous dolichol-P (see Table II). The effect of DIDS on GPDS photolabeling with [32P]5N5UDP-Glc is shown in Fig. 3. Increasing concentrations of DIDS resulted in an almost complete inhibition of GPDS photolabeling at 0.3 mM concentrations in both disrupted and intact vesicles. Photolabeling of all other [32P]5N5UDP-Glc-photoinserted polypeptides, including the UDP-glucuronosyltransferases, were also decreased with increasing DIDS concentrations, suggesting that DIDS could be a nonspecific inhibitor of many enzymes. However, the mannose-6-phosphatase latency increased by 5% in intact vesicles treated with DIDS, thus not changing the interpretation that DIDS is nonpenetrating and that the observed inhibition suggests a cytoplasmic orientation of the GPDS active site.

Time Course of GPDS Photolabeling—Since the degree of GPDS photolabeling with [32P]5N5UDP-Glc did not increase in disrupted vesicles, was inhibited by the nonpenetrating DIDS, and was sensitive to trypsin degradation in intact vesicles, access of UDP-Glc to the GPDS active site appears to be cytoplasmic. To further confirm this, the time length of preincubation of the vesicles and [32P]5N5UDP-Glc prior to UV irradiation was varied. As shown in Fig. 4 in the absence (lanes 1–7) or presence of 0.05% Triton X-100 (lanes 8–14), [32P]5N5UDP-Glc photoincorporation into GPDS did not increase over a 10-min preincubation time. As an internal control, the photolabeling of UDP-glucuronosyltransferases was not observed until after 20 s of preincubation in the disrupted vesicles (see lane 9). If [32P]5N5UDP-GlcA was used, the same type of GPDS photolabeling was observed (data not shown).

**DISCUSSION**

The active site orientation of membrane-associated glycosyltransferases and the way in which their substrates and/or...
products move across membranes remains uncharacterized in many biosynthetic pathways. In the biosynthesis of Asn-linked glycoproteins, it is known that the donor lipid-linked oligosaccharide is transferred to protein in the lumen of the ER (32, 33), yet sites of synthesis of the precursor dolichol-PP-oligosaccharides have been localized to both luminal and cytosolic locations (2). Unfortunately, the techniques employed to date to study some of these problems have proved less than conclusive largely due to the inherent instability of sealed microsomal vesicles to exogenous perturbations. Interpretation of trypsin inactivation in sealed vesicles has always been hindered by the possibility that the active site was actually on the opposite side of the membrane, and the observed loss of activity was due to proteolytic-induced conformational changes in the membrane-spanning domain of the enzyme. In this report, we have taken advantage of two photoaffinity analogs of UDP-Glc and UDP-GlcA to examine substrate accessibility to the active sites of two ER-associated glycosyltransferases. When combined with the previous methods used, such as the effects of adding detergents, trypsin, and transport inhibitors (2), this technique allows direct visualization of the results. Moreover, use of $^{32}$P$^5$NaUDP-Glc or $^{32}$P$^5$NaUDP-GlcA allows the photolabeling of all UDP-glucuronosyltransferases present, rather than individual enzymes.

As shown in Fig. 1, photolabeling of GPDS with both photoprobe was independent of vesicle integrity and was protected by UDP-Glc. Also, lengthening the preincubation time prior to photolysis did not enhance $^{32}$P$^5$NaUDP-Glc photoaffinity labeling of GPDS (see Fig. 4). Treatment of microsomes with trypsin after photolabeling resulted in loss of the photolabeled 37-kDa GPDS band. Consistently, a 33-kDa photoaffinity band was observed after trypsin digestion which is probably derived from native GPDS. This 33-kDa protein could be photoaffinity labeled whether trypsin was added before or after photolysis and could be protected by UDP-Glc (data not shown). Although final verification of this 33-kDa protein as a derivative of GPDS must await further studies, it is hoped that the purification of this band along with native GPDS will enhance our efforts to isolate the active site peptides of GPDS. Inhibition of GPDS photolabeling by DIDS is also consistent with the enzymatic assays as well as a previous study (7). In the presence of detergent, a greater inhibition of GPDS activity and photolabeling was observed with DIDS when compared to the same concentration of DIDS in intact vesicles (see Table II and Fig. 3). The photolabeling demonstrates that the interaction of DIDS with GPDS was active site directed, although the specificity appears to be broad and include all UDP-Glc- and UDP-GlcA-utilizing proteins. An explanation for some of the results observed with DIDS is not known, although these results suggest that continued use of it as a specific inhibitor of nucleotide transport or enzyme activity should be carefully evaluated. Together, these data demonstrate that the active site orientation of GPDS for UDP-Glc is cytoplasmic.

Interpreting the photolabeling of the UDP-glucuronosyltransferases is more complex due to the apparent transport of the $^{32}$P$^5$NaUDP-GlcA into the vesicles. Several photolabeled bands of UDP-glucuronosyltransferases in intact and Brij 58- and Triton X-100-treated microsomes were identified with $^{32}$P$^5$NaUDP-GlcA (Fig. 1). Since extensive studies on the glucuronidation reactions of bile acids in rat livers had been previously reported (34, 35), the hydroxyl- and carboxyl-linked activity of lithocholic acid UDP-glucuronosyltransferase was studied. In intact microsomes this activity was not fully expressed, although it could be increased about 8-fold when the lithocholic acid substrate was prepared in the form of Brij 58 micelles (Table I). In the photoaffinity labeling experiments, addition of optimal amounts of detergent for the expression of the lithocholic acid-UDP-glucuronosyltransferase activity had only a 1.3-fold increase on the $^{32}$P$^5$NaUDP-GlcA photolabeling of all UDP-glucuronosyltransferases (Fig. 1B). It is possible that the marked increase in enzyme activity in Brij 58-lithocholic acid-disrupted vesicles is due to the greater access of the bile acid hydrophobic substrate to the enzyme, independent of the UDP-GlcA substrate. Support for this has come from observations that photolabeling of the UDP-glucuronosyltransferases with $^{32}$P$^5$NaUDP-GlcA are unchanged in the presence or absence of bile acid (data not shown). A similar observation has been reported on the activity and the photolabeling of GPDS with $^{32}$P$^5$NaUDP-Glc in the presence and absence of dolichyl phosphate (22). The photoaffinity study suggests that UDP-glucuronic acid transport by intact microsomes is very effective and is only slightly increased by addition of the detergent under conditions selected for this experiment. The photolabeling of the UDP-glucuronosyltransferases by $^{32}$P$^5$NaUDP-GlcA only in disrupted vesicles suggests that the photoprobe cannot be transported into the lumen of the vesicles. Inhibition of UDP-glucuronosyltransferase photolabeling with UDP-GlcA by either photoprobe was nearly complete. In intact vesicles, addition of UDP-Glc produced only a 33% decrease in the $^{32}$P$^5$NaUDP-GlcA photolabeling of UDP-glucuronosyltransferases, while in disrupted vesicles a 52% decrease was observed (Fig. 1). Western blot analyses performed with an antibody which cross-reacts with many UDP-glucuronosyltransferases suggested that the 51–56-kDa proteins photolabeled by both photoprobe were UDP-glucuronosyltransferases (see Fig. 2). It was shown that the photolabeled UDP-glucuronosyltransferases were susceptible to trypsin digestion only in the disrupted vesicles, and a small difference in the localization of the chymotrypsin-treated proteins was also observed. These results are consistent with a previous study on protease treatment of bilirubin UDP-glucuronosyltransferase (14). Our results are most compatible with a lumenal orientation of the UDP-glucuronosyltransferase active sites and the transport of $^{32}$P$^5$NaUDP-GlcA into the vesicles.

The effects of anionic transport inhibitors and length of preincubation time before photolysis on $^{32}$P$^5$NaUDP-GlcA photolabeling of UDP-glucuronosyltransferases will be re-
ported in a separate report analyzing UDP-GlcA transport and the proteins involved. Preliminary results are consistent with a luminal orientation of UDP-glucuronosyltransferases in rat liver and transport of [32P]5N3UDP-GlcA into the vesicles. On the contrary, the time course of GPDS photolabeling with [32P]5N3UDP-Glc suggests that transport of [32P]5N3UDP-Glc was not necessary for photolabeling. There were also no major [32P]5N3UDP-Glc photoincorporated proteins which might be potential candidates for a UDP-Glc transporter protein. These results indicate that no transport of UDP-Glc is required for the activity of GPDS in rat liver and transport of [32P]5N3UDP-GlcA into the vesicles. Additional experiments specifically designed to examine UDP-Glc and 5N3UDP-Glc transport are necessary and currently in progress.

In summary, we have introduced a new technique for examining the topological orientation of membrane-associated glycosyltransferases using specific photoaffinity analogs of the nucleotide sugar substrates. This technique can be applied to any glycosyltransferase which utilizes nucleotide diphosphate sugars and has the potential to be expanded to include a new method for measuring the degree of intactness of membrane vesicles. Future directions include the characterization of nucleotide transport mechanisms in these membranes and the development of additional azido-nucleotide sugar analogs to study other microsomal glycosyltransferase topologies.

REFERENCES

1. Kornfeld, R., and Kornfeld, S. (1985) *Annu. Rev. Biochem.* 54, 631–664
2. Hirschberg, C. B., and Snider, M. D. (1987) *Annu. Rev. Biochem.* 56, 63–87
3. Dutton, G. J. (1980) *Gluconorimidation of Drugs and Other Compounds*, CRC Press Inc., Boca Raton, FL
4. Burchell, B., and Couthie, M. W. H. (1989) *Pharmacol. Ther.* 43, 261–289
5. Snider, M. D., Sultzman, L. A., and Robbins, P. W. (1980) *Cell* 21, 385–392
6. Hanover, J. A., and Lennarz, W. J. (1982) *J. Biol. Chem.* 257, 2787–2794
7. Spiro, M. J., and Spiro, R. G. (1985) *J. Biol. Chem.* 260, 5806–5815
8. Perez, M., and Hirschberg, C. B. (1986) *J. Biol. Chem.* 261, 6822–6830
9. Vanstapel, F., and Blancaert, N. (1988) *J. Clin. Invest.* 82, 1113–1122
10. Gnan, S., Cazzulo, J., and Parodi, A. J. (1991) *Biochemistry* 30, 3098–3104
11. Vanstapel, F., and Blancaert, N. (1987) *J. Biol. Chem.* 262, 4616–4623
12. Blancaert, N., Xiao Wei, L., Bossuyt, X., and Vanstapel, F. (1991) *Workshop on Glucuronidation: Its Role in Health and Disease*, Noordwijk, The Netherlands
13. Vanstapel, F., and Blancaert, N. (1988) *Arch. Biochem. Biophys.* 265, 1196–1203
14. Shepherd, S. R. P., Baird, S. J., Hallinan, T., and Butchell, B. (1989) *Biochem. J.* 259, 617–620
15. Jackson, M. R., and Burchell, B. (1986) *Nucleic Acids Res.* 14, 779–785
16. Mackenzie, P. I. (1986) *J. Biol. Chem.* 261, 6119–6125
17. Drake, R. R. J., Evans, R. K., Wolf, M. J., and Lennarz, W. J. (1989) *J. Biol. Chem.* 264, 11925–11933
18. Drake, R. R., Zimniak, P., Haley, B. E., Lennarz, W. J., and Radominska, A. (1991) *J. Biol. Chem.* 266, 23257–23260
19. Frost, D. J., Redd, S. M., Drake, R. R., Haley, B. E., and Wasserman, B. P. (1990) *J. Biol. Chem.* 265, 12967–12975
20. Lin, F. C., Brown, R. M., Drak, R. R., and Haley, B. E. (1990) *J. Biol. Chem.* 265, 4782–4784
21. Drake, R. R., Palamarczyk, G., Haley, B. E., and Lennarz, W. J. (1990) *Bioch. Rep.* 19, 61–68
22. Drake, R. R., Kaushal, G. P., Pastuszak, I., and Lennarz, W. J. (1991) *Plant. Physiol.* 97, 396–401
23. Palamarczyk, G., Drake, R. R., Haley, B. E., and Lennarz, W. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 2666–2670
24. Radominska-Pyrek, A., Zimniak, P., Chari, M., Golunski, E., and Zimniak, M. (1990) *Bioch. Rep.* 19, 61–68
25. Zimniak, P., Shattuck, K., Lester, R., and Radominska, A. (1990) *J. Clin. Invest.* 82, 234–241
26. Laemmli, U. K. (1970) *Nature* 227, 680–685
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 4350–4354
28. Bandurski, R. S., and Axelrod, B. (1951) *J. Biol. Chem.* 193, 405–410
29. Kean, E. L. (1991) *J. Biol. Chem.* 266, 942–946
30. Arion, W. J., Ballas, I. M., Lange, A. J., and Wallin, B. K. (1976) *J. Biol. Chem.* 251, 4901–4907
31. Marchesi, R. B., Richardson, K. L., Srinivasan, C., Saunders, A. M., Drake, R. R., and Haley, B. E. (1990) *Arch. Biochem. Biophys.* 280, 122–129
32. Snider, M. D., and Robbins, P. W. (1982) *J. Biol. Chem.* 257, 6795–6801
33. Weilmy, J. K., Shenbagamurthi, P., Lennarz, W. J., and Naider, F. (1983) *J. Biol. Chem.* 258, 11856–11863
34. Zimniak, P., Radominska, A., Zimniak, M., and Lester, R. (1988) *J. Lipid Res.* 29, 183–190
35. Little, J. M., Zimniak, P., Shattuck, K., Lester, R., and Radominska, A. (1990) *J. Lipid Res.* 31, 615–622