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Characterization of UDP-glucuronic acid transport in rat liver microsomal vesicles with photoaffinity analogs

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Abstract

The endoplasmic reticulum (ER) of rat liver contains several well characterized UDP-glucuronosyltransferases (UGTs), membrane-bound proteins of 50–54 kDa, and also less well identified UDP-glucosyltransferases, with nucleotide binding sites located on the lumenal surface. There is evidence that the substrates for these enzymes, UDP-glucuronic acid (UDP-GlcUA) and UDP-glucose (UDP-Glc), biosynthesized in the cytosol, are transported into the lumen of the ER via unknown mechanisms, the characteristics of which are poorly defined. A new approach for the study of the transport process has been devised using two active-site directed photoaffinity analogs, [β-^{32}P]5-azido-UDP-GlcUA and [β-^{32}P]5-azido-UDP-Glc. Photoincorporation of these probes into the lumenally oriented UGTs of intact rat liver microsomal vesicles was used as an indicator of transport. In intact vesicles, [^{32}P]5N_3UDP-GlcUA was efficiently incorporated into UGTs in a time, temperature and concentration dependent manner. In contrast, [^{32}P]5N_3UDP-Glc apparently was not transported effectively; maximal photolabeling of the 50–54 kDa proteins by this probe was dependent on detergent disruption of the vesicles. Vesicular uptake of and subsequent photolabeling of the 50–54 kDa proteins by [^{32}P]5N_3UDP-GlcUA were inhibited by UDP-GlcUA and 5N_3UDP-GlcUA while UDP-Glc, 5N_3UDP-Glc, UDP-xylose and UDP-*N*-acetylglucosamine were less inhibitory, suggesting a high degree of specificity for the uptake/photolabeling process. The anionic transport inhibitors DIDS and SITS inhibited [^{32}P]5N_3UDP-GlcUA photoincorporation into UGTs in intact vesicles, but also inhibited photolabeling of these and other enzymes in detergent disrupted vesicles. These data suggest the presence in rat liver microsomal vesicles of a specific, carrier-mediated transport process for UDP-GlcUA which is distinct from the mechanism of UDP-Glc transport.

Keywords: Photoaffinity labeling; Nucleotide sugar transport; UDP-glucuronosyltransferase

1. Introduction

The UDP-glucuronosyltransferases (UGTs) (EC 2.4.1.17) are a multigenic family of membrane-bound enzymes involved in the detoxification of drugs and endogenous compounds such as bilirubin, steroids, thyroxin and bile acids. They catalyze the transfer of glucuronic acid from UDP-GlcUA, the essential cosubstrate for all UGTs, to a large variety of structurally unrelated compounds with hydroxyl-, carboxyl-, amino- and sulfhydryl groups, leading to the formation of water-soluble β-D-glucuronides [1]. Thus, one of the physiological functions of the liver, the detoxification of these compounds and facilitation of their secretion into bile or urine, is achieved, among other mechanisms, by the UDP-GlcUA-dependent glucuronidation reactions catalyzed by microsomal UGTs.

Abbreviations: 5N_3UDP-GlcUA, 5-azido-UDP-glucuronic acid; 5N_3UDP-Glc, 5-azido-UDP-glucose; UDP-GlcUA, UDP-glucuronic acid; UDP-Glc, UDP-glucose; UDP-Gal, UDP-galactose; UDP-Xyl, UDP-xylose; UDP-GlcNAc, UDP-*N*-acetylglucosamine; PNP, *p*-nitrophenol; UGT, UDP-glucuronosyltransferase; ER, endoplasmic reticulum; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

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Accumulating evidence from studies using photoaffinity labeling [2,3], proteinases [4] and proteinases in conjunction with antibodies [5,6], as well as computer-based DNA sequence analysis [7–9], overwhelmingly favors the view that the catalytic binding site(s) of the UGTs is located on the lumenal aspect of the ER. Since the obligatory substrate for the glucuronidation reaction, UDP-GlcUA, is biosynthesized in the cytosol [10], the need for UDP-GlcUA translocation into the lumen of the ER is evident. Based on these data and theoretical considerations, transport of the negatively charged UDP-GlcUA into the lumen of the ER by a protein-mediated mechanism coupled to the antiport of UMP has been proposed [1,11]. Attempts have been made to examine UDP-GlcUA transport from the cytosol into the ER of rat hepatocytes employing a rapid filtration technique, which demonstrated the presence of a membrane transporter involved in UDP-GlcUA translocation [12]. However, a study using similar techniques, while demonstrating UDP-GlcUA transport in Golgi, showed less evidence of UDP-GlcUA transport in ER [13]. Transport of UDP-Glc between the cytosol and the lumen of ER has also been documented but, in these studies, transport of UDP-GlcUA could not be demonstrated [4,14]. Therefore, it is evident that the issue of ER UDP-GlcUA and UDP-Glc transport remains controversial. Other characteristics of the putative UDP-GlcUA transporter, such as the identity and structure of the protein(s), the substrate specificity and the mechanism of transport are unknown.

Photoaffinity analogs of UDP-GlcUA and UDP-Glc, 5-azido-UDP-GlcUA ($5N_3$UDP-GlcUA) and 5-azido-UDP-Glc ($5N_3$UDP-Glc), have proven to be useful tools for studying membrane-associated UDP-glycosyltransferases in both microsomal and purified protein preparations [3,15,16]. In previous studies with rat liver microsomes, it was observed that UGTs (or UGT-like proteins with 50–54 kDa molecular weight) could be specifically photolabeled with both [^{32}P]$5N_3$UDP-GlcUA and [^{32}P]$5N_3$UDP-Glc [3,15]. It also was observed that [^{32}P]$5N_3$UDP-GlcUA was photoincorporated into the lumenally oriented UGTs of intact rat liver microsomal vesicles and that detergent treatment of the vesicles prior to photolabeling did not significantly increase the degree of photoincorporation. In contrast, effective photolabeling of the lumenal UGTs with [^{32}P]$5N_3$UDP-Glc was highly dependent on detergent disruption of the vesicles [3]. This suggested the presence of different translocation systems for the two nucleotide-sugars.

In this report, the fact that both photoprobes could be used to photolabel UGTs in rat liver microsomes was utilized to characterize some of the properties of UDP-GlcUA transport. The effects of time, temperature and concentration of [^{32}P]$5N_3$UDP-GlcUA on transport and photolabeling in intact rat liver microsomes are presented. Other structurally related compounds and previously reported effectors of UDP-GlcUA transport, such as UDP-GlcNAc, SITS and DIDS were also tested for their effects on UGT photolabeling. For comparison, the photolabeling of the UGTs with [^{32}P]$5N_3$UDP-Glc and its relation to UDP-GlcUA transport are also presented.

2. Materials and methods

[β-^{32}P]$5N_3$-UDP-GlcUA and [β-^{32}P]$5N_3$-UDP-Glc (2–5 mCi/μmol) were synthesized and purified as described previously [15,17]. [^{32}P]P_i and [γ-^{32}P]ATP were obtained from ICN. Reagents such as Triton X-100, Brij 58, sugar nucleotides, saccharolactone, trypsin (Type IX, from porcine pancreas), DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) and SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) were from Sigma. Intact liver microsomes from male rats (Sprague-Dawley, 220–250 g) were prepared as previously described [4], except that they were not further subfractionated into rough and smooth ER fractions. The mannose-6-phosphatase latency determinations were performed as previously described [16]. Autoradiographs were analyzed by densitometry with a Zeineh Video Densitometer (Biomed Instruments). The Western blot analysis was performed by the method of Towbin et al. [18]. Blotted proteins were then examined by using a rabbit anti-3-hydroxysteroid (3-OH steroid) UGT antibody which was a generous gift from M. Green and Dr. T. Tephly, University of Iowa, Iowa City, Iowa. Plastic-backed cellulose thin-layer plates were from Kodak.

2.1. Photoaffinity labeling with [^{32}P]$5N_3$UDP-GlcUA and [^{32}P]$5N_3$UDP-Glc

The standard protocol for photolabeling of rat liver microsomes with either photoprobe was as follows: rat liver microsomes (50 μg protein) were incubated for 10 min in the presence or absence of 0.05% Triton X-100 on ice in 150 mM Hepes, pH 7.0, and 10 mM $MgCl_2$ in a total volume of 25 μl. Either [^{32}P]$5N_3$UDP-GlcUA or [^{32}P]$5N_3$UDP-Glc (2–5 mCi/μmol) was added (final concentration: 40 μM) and allowed to equilibrate for 1 min, followed by ultraviolet-irradiation with a hand-held lamp (UVP-11, 254 nm, Ultraviolet Products) for 90 s at room temperature. Variations of this standard protocol are described in the appropriate figure legends. Reactions were terminated and processed for SDS-PAGE as described previously [17]. Proteins were separated on 10% SDS-polyacrylamide gels [19], followed by autoradiography for 1–2 days.

Table 1
Quantitation of the effect of detergent, cold nucleotides and trypsin on photoaffinity labeling

Addition	Photoincorporation % of control			
	$5N_3$UDP-GlcUA		$5N_3$UDP-Glc	
	−Triton	+Triton	−Triton	+Triton
None	100	100	11	100
250 μM UDP-GlcUA	23	32	9	9
250 μM UDP-Glc	43	57	6	5
25 μg trypsin	51	99	8	61

The autoradiographs shown in Fig. 1A and B were analyzed by densitometry. Photoaffinity labeling was carried out in the absence or presence of 0.05% Triton- X-100 as described under Materials and methods with 40 μM [β-^{32}P]$5N_3$UDP-GlcUA (A) and [β-^{32}P]-$5N_2$UDP-Glc (B). The description of the experiments is presented in the legend to Fig. 1. The results are from a representative experiment.

3. Results

3.1. Effect of nucleotide sugars and trypsin treatment on photoaffinity labeling of intact and detergent treated microsomes

Since a previous photoaffinity study [16] suggested that [^{32}P]$5N_3$UDP-GlcUA was transported into intact rat liver vesicles, this phenomenon was studied systematically using the photoincorporation of [^{32}P]$5N_3$UDP-GlcUA into 50–54 kDa UGTs as an indicator of transport. In Fig. 1A, the effects of detergent disruption, unlabeled UDP-GlcUA and UDP-Glc and trypsin digestion on photoincorporation of either [^{32}P]$5N_3$UDP-GlcUA or -Glc into intact rat liver microsomes are shown. Detergent disruption of the microsomal vesicles did not alter [^{32}P]$5N_3$UDP-GlcUA photoincorporation into 50–54 kDa UGTs (Fig. 1A and Table 1). Both UDP-GlcUA and UDP-Glc at 250 μM concentrations effectively inhibited photoincorporation of [^{32}P]$5N_3$ UDP-GlcUA into the UGTs. Also shown in Fig. 1, and in most other figures, is the photolabeling of a 37 kDa protein previously identified as the cytoplasmically oriented UDP-glucose:dolichylphosphate glucosyltransferase (Glc-P-Dol synthase or GPDS) [3,15,16]. The 62 kDa protein photolabeled with [^{32}P]$5N_3$UDP-Glc, which labels in the absence of ultraviolet irradiation, has previously been shown to be phosphoglucomutase [20].

Treatment of intact vesicles with trypsin, followed by photolabeling, resulted in decreased [^{32}P]$5N_3$UDP-GlcUA photoincorporation into UGTs, although the electrophoretic mobility of these proteins was not affected (Fig. 1A, compare lanes 1 and 4). Trypsin digestion of detergent-disrupted vesicles did not affect photoincorporation of [^{32}P]$5N_3$UDP-GlcUA into UGTs but did result in an increase in electrophoretic mobilities (Table 1, Fig. 1A) The observed inhibition of photoincorporation into UGTs in the intact, trypsin digested vesicles raises the possibility that protein-mediated transport of the photoprobe was altered. Additionally, the results demonstrating decreased photolabeling of UGTs in intact vesicles exposed to trypsin are compatible with the localization of the putative transporter on the cytoplasmic surface of the ER. However, other interpretations of the data, such as interaction of trypsin with the vesicle membrane, are possible.

Similar studies were performed using [^{32}P]$5N_3$UDP-Glc (Fig. 1B). The major difference from the above results with [^{32}P]$5N_3$UDP-GlcUA was that there was no significant photolabeling of proteins in the 50–54 kDa range in intact vesicles. However, photolabeling of detergent disrupted vesicles resulted in the 50–54 kDa proteins being labeled with an intensity comparable to that observed with [^{32}P]$5N_3$UDP-GlcUA. In these same disrupted vesicles, there was inhibition of photolabeling by both UDP-GlcUA and UDP-Glc and trypsin exposure resulted in a shift in the electrophoretic mobility of the UGTs.

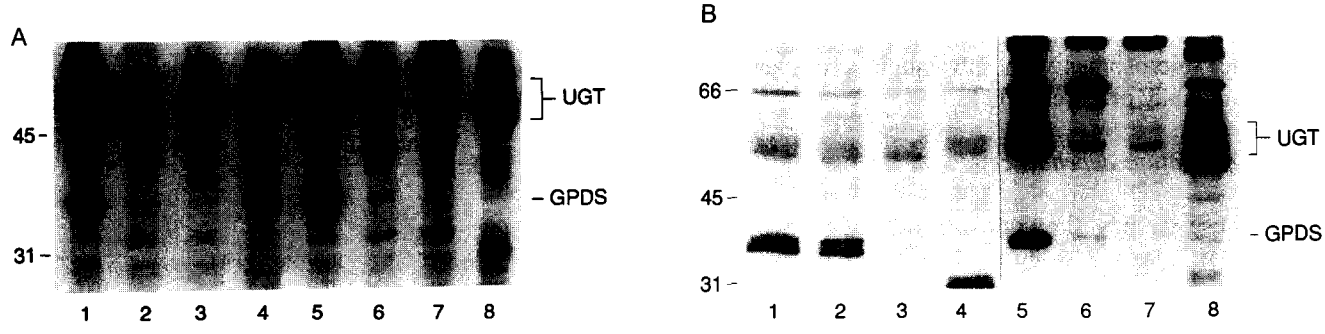

Fig. 1. [^{32}P]$5N_3$UDP-GlcUA and [^{32}P]$5N_3$UDP-Glc labeling of intact and detergent-treated membrane proteins. The effect of UDP-GlcUA and UDP-Glc and trypsin digestion. Autoradiograph of rat liver microsomes (50 μg) photolabeled with either (A) 40 μM [^{32}P]$5N_3$UDP-GlcUA or (B) 40 μM [^{32}P]$5N_3$UDP-Glc. Photolabeling was performed as described under Materials and methods; the ER vesicles were preincubated with detergent, cold nucleotides and trypsin for 10 min on ice, photolabeled and then processed for SDS-PAGE. For both A and B, lanes 1–4 are intact vesicles and lanes 5–8 are detergent-disrupted vesicles. Additions to incubations: lanes 1 and 5: none; lanes 2 and 6: 250 μM UDP-GlcUA; lanes 3 and 7: 250 μM UDP-Glc; lanes 4 and 8: 25 μg trypsin. Mannose-6-phosphatase latency of intact vesicles was 92%.

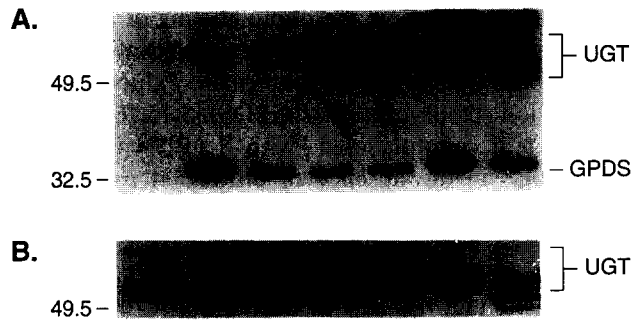

Fig. 2. Western blot analysis of rat liver microsomal UGT treated with trypsin and photolabeled with [^{32}P]5N$_3$UDP-Glc. Intact and 0.05% Triton X-100 disrupted vesicles (50 μg protein) incubated with different concentrations of trypsin prior to photolabeling with 40 μM [^{32}P]5N$_3$UDP-Glc were separated by SDS-PAGE and blotted onto nitrocellulose as described in Materials and methods. (A) Autoradiograph of the photolabeled UGTs. (B) Western blot analysis was performed by using a anti 3-OH steroid-UGT-specific antibody. For both A and B: lane 1: intact vesicles, no ultraviolet; lane 2: intact vesicles; lane 3: intact vesicles, 15 μg of trypsin; lane 4: intact vesicles, 25 μg trypsin; lane 5: intact vesicles, 31 μg trypsin; lane 6: disrupted vesicles; lane 7: disrupted vesicles, 15 μg trypsin. Mannose-6-phosphatase latency of intact vesicles was 92%.

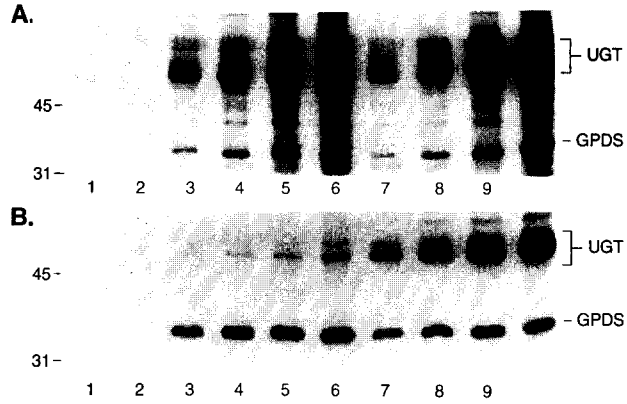

Fig. 3. The concentration dependence of [^{32}P]5N$_3$UDP-GlcUA and [^{32}P]5N$_3$UDP-Glc photolabeling. Intact and detergent-treated vesicles (10 min on ice) were pre-equilibrated for 1 min at room temperature with various concentrations of (A) [^{32}P]5N$_3$UDP-GlcUA or (B) [^{32}P]5N$_3$UDP-Glc, irradiated for 90′ and analyzed by autoradiography after SDS-PAGE as described in Materials and methods. For both A and B, lanes 1 and 3–6 are intact vesicles and lanes 2 and 7–10 are detergent-disrupted vesicles. Photoprobe concentrations are: lanes 1 and 2: 40 μM, no ultraviolet irradiation; lanes 3 and 7: 10 μM; lanes 4 and 8: 20 μM; lanes 5 and 9: 40 μM; lanes 6 and 10: 80 μM. Mannose-6-phosphatase latency was 92% for intact vesicles.

The effect of varied concentrations of trypsin on vesicles photolabeled with [^{32}P]5N$_3$UDP-Glc (Fig. 2A) was examined in more detail in conjunction with Western blot analysis using an anti-3-OH steroid UGT antibody (Fig. 2B). The radiolabeled proteins in intact microsomes were not hydrolyzed in the presence of 15, 25 and 31 μg of trypsin (Fig. 2A, lanes 3, 4 and 5), but detergent treatment of microsomes incubated with even the lowest concentration of trypsin (15 μg) resulted in complete hydrolysis of these proteins. Fig. 2B shows the Western blot of proteins from the same trypsin-digested intact (Fig. 2B, lanes 1–4) and detergent disrupted (Fig. 2B, lanes 5–7) microsomes. The results indicated that at least some of the photolabeled proteins of 50–54 kDa range were UGTs. This is consistent with previous Western blot analyses of [^{32}P]5N$_3$UDP-GlcUA photolabeled UGTs [16] which, together with the present studies, form the basis for the hypothesis of [^{32}P]5N$_3$UDP-GlcUA translocation into intact microsomes with subsequent photolabeling of the lumenally oriented UGTs.

3.2. Concentration dependence

For these studies, the concentration of both photoprobes was varied (10–80 μM) in both intact and detergent disrupted vesicles. As shown in Fig. 3A, photolabeling of UGTs by [^{32}P]5N$_3$UDP-GlcUA was saturable in both intact and disrupted vesicles, with half maximal photoincorporation at approx. 25 μM (Table 2). With [^{32}P]5N3UDP-Glc, photoincorporation into UGTs of intact vesicles was non-saturable, although in disrupted vesicles half-maximal saturation was reached at approx. 30 μM. Lanes 1 and 2 (Fig. 3A and B) demonstrate that photolabeling of the 50–54 kDa proteins was dependent on exposure to ultraviolet irradiation, regardless of whether the microsomes were intact or disrupted. As would be expected if this were a specific transport process, the saturation and linearity of UGT photolabeling was achieved.

3.3. Temperature and time dependence

It would be anticipated that protein-mediated transport of [^{32}P]5N$_3$UDP-GlcUA would be time- and temperature-dependent. To test this, intact rat liver vesi-

Table 2
Quantitation of the effect of probe concentration and detergent on photoaffinity labeling

Concentration of photoprobe (μM)	Photoincorporation % of control			
	5N$_3$UDP-GlcUA		5N$_3$UDP-Glc	
	−Triton	+Triton	−Triton	+Triton
0	0	0	0	0
10	43	51	7	33
20	82	67	8	60
40	97	100	11	91
80	100	100	26	100

The autoradiographs shown in Fig. 3A and B were analyzed by densitometry. Photoaffinity labeling was carried out in the absence or presence of 0.05% Triton X-100 as described under Materials and methods with 40 μM [β-^{32}P]5N$_3$UDP-GlcUA (A) and [β-^{32}P]-5N$_3$UDP-Glc (B). The description of the experiments is presented in the legend to Fig. 3. The results are from a representative experiment.

Table 3
Quantitation of the effect of time and temperature on photoaffinity labeling

Temp (°C)	Time min	Photoincorporation % of control	
		−Triton	+Triton
4°C	0	4	28
	1	13	30
	3	19	26
	5	25	26
24°C	0	32	96
	1	61	
	3	99	99
	5	100	99
37°C	0	51	100
	1	99	
	3	100	100
	5	100	100

The autoradiographs shown in Fig. 4A and B were analyzed by densitometry. Photoaffinity labeling was carried out in the absence or presence of 0.05% Triton X-100 as described under Materials and methods with 40 μM [β-^{32}P]5N$_3$UDP-GlcUA. The description of the experiments is presented in the legend to Fig. 4. The results are from a representative experiment.

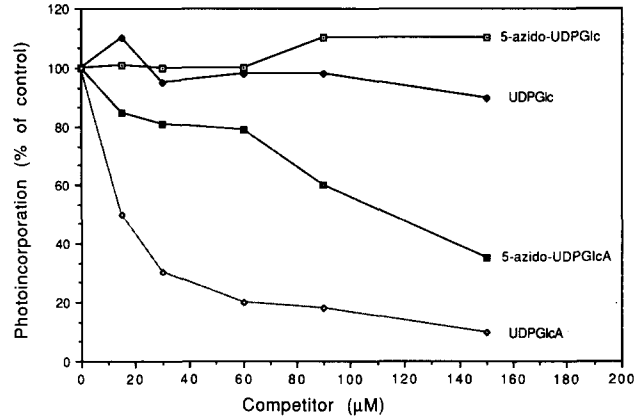

Fig. 5. Effect of unlabeled nucleotide sugars on the transport of [β-^{32}P]5N$_3$UDP-GlcUA. Intact rat liver vesicles (50 μg) were incubated with 40 μM [β-^{32}P]5N$_3$UDP-GlcUA and UDP-Glc, UDP-GlcUA, 5N$_3$UDP-Glc or 5N$_3$UDP-GlcUA: at 0, 15, 30, 60, 90 and 150 μM. Vesicles were added last to measure competition for transport. Samples were incubated for 10 min and ultraviolet-irradiated for 90'. Shown in the graph is the percent of photoincorporation into UGT measured by densitometry of the autoradiographs with each control (0 μM) represented as 100%. Mannose-6-phosphatase latency was 92% for intact vesicles.

cles were incubated with 40 μM [^{32}P]5N$_3$UDP-GlcUA for varying lengths of time (0–5 min) at different temperatures (4°, 24° and 37°C) prior to ultraviolet irradiation. As shown in the autoradiograph in Fig. 4A, enhanced photoincorporation into the lumenal UGTs was observed with increasing incubation time and temperature. At 4°C, UGT photoincorporation increased with time from 4% to 25% of the maximal values (Table 3). However, saturation of photoincorporation was observed after 3 min pre-incubation at 24°C, and after only 1 min at 37°C. As a control, vesicles were disrupted with detergent prior to addition of the photoprobe and incubated as described for the intact vesicles. As shown in the autoradiograph in Fig. 4B, detergent disruption abolished the time dependence of the UGT photolabeling. These results are consistent with a protein-mediated transport of 5N$_3$-UDP-GlcUA.

3.4. Inhibition of UGT photolabeling by unlabeled 5-azido nucleotide sugars

Our previous studies [3,15] and Fig. 1 indicated that [^{32}P]5N$_3$UDP-GlcUA photolabeling of the UGTs could be inhibited by UDP-GlcUA and UDP-Glc in concentrations 5-fold or greater than the concentrations of the probes. However, the effects of unlabeled 5N$_3$ UDP-GlcUA and 5N$_3$UDP-Glc on uptake and photolabeling have not been previously examined. Therefore, experiments were performed which directly examined competition for transport and subsequent photolabeling of UGTs in intact vesicles. As shown in Fig. 5, UDP-GlcUA and 5N$_3$UDP-GlcUA were effective competitive inhibitors of [^{32}P]5N$_3$UDP-GlcUA uptake and subsequent photoincorporation, with half-maximal in-

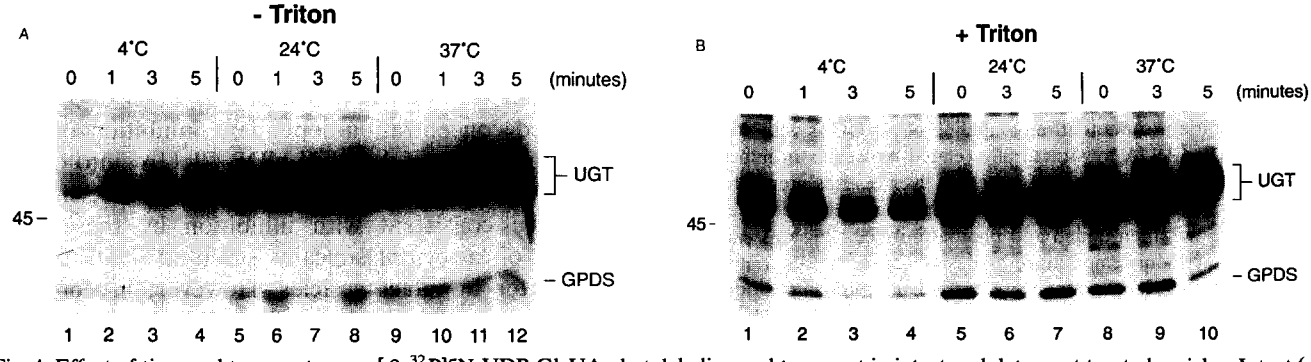

Fig. 4. Effect of time and temperature on [β-^{32}P]5N$_3$UDP-GlcUA photolabeling and transport in intact and detergent treated vesicles. Intact (A) and detergent treated vesicles (B)were prepared and incubated with 40 μM [β-^{32}P]5N$_3$UDP-GlcUA for 0–5 minutes prior to ultraviolet-irradiation at 4°C (A and B, lanes 1–4), 24°C (A, lanes 5–8; B, lanes 5–7) and 37°C (A, lanes 9–12; B, lanes 8–10). Proteins were equilibrated for 5 min at each temperature before addition of photoprobe. Mannose-6-phosphatase latency was 92% for intact vesicles.

hibition at 15 μM and 100 μM, respectively. UDP-Glc showed only slight inhibition (10% of control) at 150 μM, while $5N_3$UDP-Glc had no apparent competitive effect in intact vesicles. In detergent disrupted vesicles, 150 μM UDP-Glc or $5N_3$UDP-Glc inhibited UGT photolabeling by 47% and 48%, respectively (data not shown). These results are consistent with the notion that a specific transporter protein(s) may be involved in transport of [^{32}P]$5N_3$UDP-GlcUA, and that the mechanism operative for UDP-Glc transport and subsequent interaction with UGTs is distinct from that of the UDP-GlcUA system.

3.5. Effects of other uridine sugar nucleotides and nucleotides on photoaffinity labeling

The specificity of the postulated transport of $5N_3$UDP-GlcUA was examined by the incubation of several structural analogs, in concentrations 5- or 10-fold higher than that of the probe, with intact microsomal vesicles prior to the addition of photoprobe and ultraviolet-irradiation. Of the substrates or analogs shown in Fig. 6, UDP-GlcUA (200 μM) was the most effective inhibitor (reducing photoincorporation to 15% of control), followed by UDP-Glc (65%), UDP-Xyl (64%) and UDP-Gal (61%). Other compounds, such as 5-bromo-dUMP, ATP and GTP were weak inhibitors of photoaffinity labeling (Fig. 6, lanes 8–10).

UDP-GlcNAc has been implicated as an activator of UGTs in vivo, acting either as an allosteric effector or by enhancing access of UDP-GlcUA to the enzyme [21–23]. As shown in Fig. 6 (lanes 6, 7), 200 and 400 μM concentrations of UDP-GlcNAc did not affect the photolabeling of UGTs with [^{32}P]$5N_3$UDP-GlcUA. Also, no significant effect on [^{32}P]$5N_3$UDP-GlcUA photoincorporation in detergent-treated vesicles was observed (data not shown). These results may indicate that UDP-GlcNAc does not modulate UDP-GlcUA transport in ER, or since UDP-GlcNAc apparently affects the rate of transport, these effects might not be demonstrable with the experimental conditions employed here.

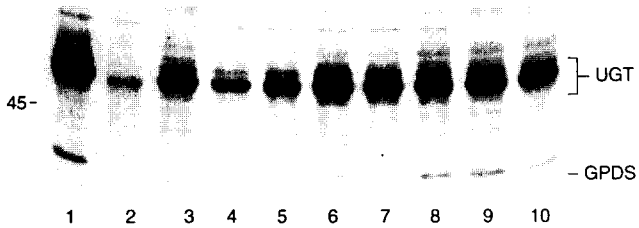

Fig. 6. Inhibition of photoaffinity labeling of rat UGTs with 40 μM [^{32}P]$5N_3$UDP-GlcUA by structurally related molecules in intact rat liver vesicles. Autoradiogram of intact rat liver vesicles preincubated for 10 min at room temperature with competing nucleotide. Incubation mixture was then added and photoaffinity labeling was carried out for 90′ at room temperature as described under Materials and methods. Control incubations were carried out with nucleotide replaced with water. Lane 1: intact vesicles; lane 2: 200 μM UDP-GlcUA; lane 3: 200 μM UDP-Glc; lane 4: 200 μM UDP-Gal; lane 5: 200 μM UDP-Xyl; lane 6: 200 μM UDP-GlcNAc; lane 7: 400 μM UDP-GlcNAc; lane 8: 200 μM 5-bromo-dUMP; lane 9: 200 μM ATP; lane 10: 200 μM GTP. Mannose-6-phosphatase latency was 92% for intact vesicles.

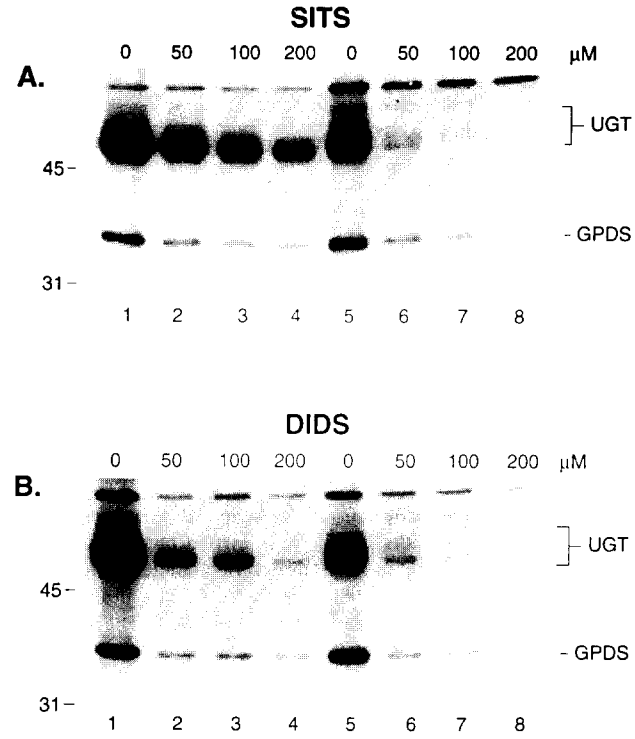

Fig. 7. Photolabeling of intact and disrupted vesicles in the presence of different concentrations of SITS and DIDS. Autoradiograph of intact and disrupted rat liver vesicles photolabeled with 40 μM [^{32}P]$5N_3$UDP-GlcUA in the presence different concentrations of SITS (A) and DIDS (B). In both A and B, lanes 1–4 are intact vesicles and lanes 5–8 are disrupted vesicles. Inhibitor concentrations: lanes 1 and 5: none; lanes 2 and 6: 50 μM; lanes 3 and 7: 100 μM; lanes 4 and 8: 200 μM. Mannose-6-phosphatase latency was 92% for intact vesicles.

3.6. Effect of SITS and DIDS on UGT photolabeling,

The effects of the non-penetrating inhibitors of anion transport, DIDS and SITS, were examined in intact and detergent disrupted vesicles (Fig. 7, Table 4). When incubated with intact vesicles, both SITS and DIDS produced concentration-dependent inhibition of photoincorporation of [^{32}P]$5N_3$UDP-GlcUA into UGTs (Fig. 7A and B, lanes 2–4 and Table 4). These findings would be consistent with inhibition by these compounds of a cytoplasmically oriented UDP-GlcUA transporter in intact vesicles. However, in detergent disrupted vesicles, both SITS and DIDS were even more effective inhibitors of UGT photolabeling (Fig. 7A and B, lanes 5–8, Table 4), suggesting a non-specific inhibition of UGTs and other enzymes, as well as the putative transporter. It is evident from these photola-

Table 4
Quantitation of the effect of SITS and DIDS on the photoaffinity labeling

Inhibitor concentration (μM)	Photoincorporation % of control	
	−Triton	+Triton
None	100	100
50 μM SITS	77	23
100 μM SITS	52	0
200 μM SITS	31	0
50 μM DIDS	37	20
100 μM DIDS	31	9
200 μM DIDS	5	5

The autographs shown in Fig. 7 were analyzed by densitometry. Photoaffinity labeling was carried out in the absence or presence of 0.05% Triton X-100 as described under Materials and methods with 40 μM [β-^{32}P]5N$_3$UDP-GlcUA. The description of the experiments is presented in the legend to Fig. 7. The results are from a representative experiment.

beling data that SITS and DIDS have a non-specific spectrum of inhibitory activity and that caution should be used in interpreting their effects as specific transport inhibitors.

4. Discussion

The existence and characterization of sugar-nucleotide transport into the lumen of the ER has been disputed [4,12–14]. At present, the identity and composition of the transporter(s) for UDP-GlcUA and other UDP-sugars utilized in UDP-glycosylation reactions are unknown, and there is only one basic method for measuring nucleotide transport [12,21,22]. The experimental approach reported here was developed to provide an alternative method for studying nucleotide sugar translocation across membranes. Based on the hypothesis that the UGTs have a lumenal orientation of the UDP-sugar binding sites, this method takes advantage of the transport of the photoaffinity analog, [^{32}P]5N$_3$UDP-GlcUA, into the lumen of intact rat liver microsomal vesicles with subsequent photolabeling of specific target enzymes (UGTs).

Recent sequencing data indicate that the UGTs are anchored in the endoplasmic reticulum membrane by a carboxyl-terminal hydrophobic domain, with the bulk of the polypeptide chain located within the cisternal lumen of the ER [1,8,9,24]. There is increasing evidence that the binding of UDP-GlcUA or other UDP-sugar substrates to the carboxyl-terminal half of UGT occurs either in the cisternal lumen [4,14] or within the membrane [12]. These results strongly suggest that the UDP-GlcUA binding-sites of microsomal UGTs are located on the lumenal side of the ER [3–9,25]. A long standing problem with the interpretation of data in the UGT field is the latency of such membrane-bound enzymes and the rate limiting transport of UDP-GlcUA has been postulated as a reason for this latency [26–28]. In our system, saturable transport of [^{32}P]5N$_3$UDP-GlcUA into intact vesicles was evident and the presence of detergent and other latency decreasing factors did not significantly affect photoincorporation of the probe into the UGTs. These results suggest that UGT latency may not be solely due to rate limiting transport of UDP-GlcUA into the lumen of the ER.

As would be predicted if this model transport/photoaffinity labeling system were reflective of UDP-GlcUA transport, the photolabeling of UGTs with [^{32}P]5N$_3$UDP-GlcUA was time, temperature and concentration dependent (Figs. 3 and 4). In contrast, the lack of saturation with [^{32}P]5N$_3$UDP-Glc in intact vesicles suggested either an alternate mechanism for the observed photolabeling of UGTs with this probe or that the concentration of [^{32}P]5N$_3$UDP-Glc necessary to saturate this process is higher than those used here. These observations and, in particular, the low, but time dependent, increase in photolabeling at 4°C favor the existence of a specific transport process rather than simple, non-specific binding of [^{32}P]5N$_3$UDP-Glc to microsomal membranes.

The data presented for [^{32}P]5N$_3$UDP-Glc (Figs. 1B and 2), which has been previously shown to photoincorporate into lumenal UGTs in disrupted rat liver vesicles [3], suggests that the UDP-GlcUA transporter does not interact with 5N$_3$UDP-Glc. The presence in intact microsomes of a lumenal pool of endogenous UDP-Glc has been documented [25]. It also has been suggested that specific carrier systems exist in rat liver ER which transport nucleotide-sugars and other 5′-uridine substituted structural analogs across ER membranes [14]. These nucleotide-sugars are the substrates for glycosylation reactions catalyzed by microsomal UDP-glycosyltransferases including those involved in the biosynthesis of *N*-linked glycoproteins. Additionally, highly active nucleotide sugar transport systems have been identified in Golgi membranes [29,30]. Recently, a soluble protein isolated from the lumen of rat ER, UDP-Glc:glycoprotein glucosyltransferase has been described [31]. This enzyme, reported to be 150 kDa, dependent on calcium for activity and utilizing UDP-Glc as the glucose donor, could be photolabeled with [^{32}P]5N$_3$UDP-Glc only in disrupted vesicles and in the presence of calcium [2]. This information coupled with the fact that photolabeling of the 50–54 kDa proteins by [^{32}P]5N$_3$UDP-Glc was significant only in disrupted vesicles (Figs. 1A and 2) makes it apparent that UDP-Glc is utilized in the lumen, although the mechanism and identity of the transporter(s) remain to be established.

We conclude from these data that [^{32}P]5N$_3$UDP-GlcUA gains access to UGT binding sites and that the subsequent ultraviolet activation and photoincorpora-

tion into UGTs may be used as a specific indicator of UDP-GlcUA internalization. Though determination of the orientation of the UDP-GlcUA binding site of the UGTs in the membrane was not the purpose of these studies and remains to be definitively resolved, the photoaffinity labeling technique has allowed us to demonstrate the existence of a UDP-GlcUA transporter in rat liver ER membranes and may be the best method to use for the identification of the specific transporter protein. Since the transporter protein must, by definition, bind UDP-GlcUA, only proteins photolabeled by [^{32}P]5N$_3$UDP-GlcUA will need to be investigated and these will be isolated, purified, reconstituted and checked for transport activity. To further characterize the UDP-GlcUA transport process in hepatic microsomes by our photoaffinity method, current studies are focusing on identification of the UDP-GlcUA binding component of the UDP-GlcUA transporter by investigating the effect of a series of recently described inhibitors of UDP-glycosyltransferases [32]. In intact rat liver microsomes, some of these inhibitors produced a decrease in UGT photolabeling with [^{32}P]5N$_3$ UDP-GlcUA [33]. Moreover, the transport of [^{32}P]5N$_3$ UDP-GlcUA also has been investigated in a conventional rapid filtration UDP-GlcUA transport assay: the 5-azido derivative accumulated in the lumen of intact ER vesicles and efficiently competed with UDP-GlcUA (Berg, C., Radominska, A., Lester, R. and Gollan, J., unpublished data). The combination of these techniques with preparative electrophoresis and HPLC to isolate individual photolabeled proteins should permit identification of the UDP-GlcUA transport system and eventual cloning and expression of the protein(s).

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