Carrier-mediated transport of intact UDP-glucuronic acid into the lumen of endoplasmic-reticulum-derived vesicles from rat liver

Xavier BOSSUYT and Norbert BLANCKAERT

Laboratory of Biological Chemistry, Department of Molecular Cell Biology, Catholic University of Leuven, GHB-Herestraat 49, B-3000 Leuven, Belgium

Uptake and metabolism of UDP-glucuronic acid (UDPGlcA) by rough-endoplasmic-reticulum (RER)-derived vesicles was studied. Analysis of the molecular species, double-labelling experiments and *trans*-stimulation experiments revealed that initial uptake represented entry into microsomes of predominantly intact UDPGlcA, concomitant with rapid hydrolysis of the internalized nucleotide sugar. The uptake constituted effective translocation from the medium into the lumen of the vesicles. Thus the amount of vesicle-associated label at equilibrium uptake was directly proportional to the volume of the intravesicular space. Permeabilized microsomes were unable to retain UDPGlcA. The microsomal uptake of UDPGlcA met the criteria

of bidirectional carrier-mediated translocation. Transport was time- and temperature-dependent, saturable, selective, capable of *trans*-stimulation, and operational against a concentration gradient. Microsomal uptake was inhibited by *N*-ethylmaleimide that was presented at the cytosolic side of the endoplasmic-reticulum (ER) membrane. Uptake studies performed in membrane preparations that were highly enriched in RER, smooth ER or Golgi revealed that UDPGlcA was taken up by the ER as well as by the Golgi apparatus. Our findings demonstrate the existence in rat liver ER of a carrier system mediating proper translocation of intact UDPGlcA across the membrane.

INTRODUCTION

Glucuronidation plays a major role in detoxication and elimination of a large number of hydrophobic endobiotics and xenobiotics (for reviews, see Burchell and Coughtrie, 1989; Tephly and Burchell, 1990; Miners and Mackenzie, 1991). This remarkable versatility of the glucuronidation system is due to the fact that UDP-glucuronosyltransferase (UGT) represents a supergene family of isoenzymes, located primarily in the hepatic endoplasmic reticulum (ER).

In contrast with the recent substantial advancement of our understanding of the molecular structures of the UGTs (Burchell et al., 1991), relatively little insight has been attained into the regulation of the transferases. All UGTs, which are integral membrane proteins deeply imbedded in the ER, display latency in native microsomal preparations (Dutton, 1975). Maximal transferase activity is found *in vitro* only after disruptive treatment of the membrane vesicles. Cogent evidence is accumulating that the catalytic centre of the transferase is oriented toward the lumen of the microsomal vesicle (Berry and Hallinan, 1974; Hallinan and de Brito, 1981; Mackenzie and Owens, 1984; Scragg et al., 1985; Vanstapel and Blanckaert, 1988; Shepherd et al., 1989; Blanckaert et al., 1991).

A model to explain the latency of UGT has been proposed by Berry and Hallinan (1976). They suggested that the latency results from the need to transport substrates across the ER-membrane permeability barrier, and postulated a compartmentation model in which the substrate and the co-substrate are both presented at the cytosolic side of the ER and the catalytic centre resides in a luminal domain. This topological arrangement necessitates translocation of UDP-glucuronic acid (UDPGlcA), a highly charged molecule synthesized in the cytosol, across the membrane to reach the lumen of the ER.

Bilirubin UDP-glucosyltransferase and UDP-xylosyltransferase have direct access to an intramicrosomal nucleotide sugar pool (Vanstapel and Blanckaert, 1987), and evidence for

carrier-mediated translocation for UDP-glucose (UDPGlc) has been presented (Vanstapel and Blanckaert, 1988). Hauser et al. (1988) reported carrier-mediated transport of UDPGlcA into the ER membrane, but were unable to demonstrate either the existence of an intramicrosomal pool of intact UDPGlcA or translocation of the nucleotide sugar across the membrane into the luminal space of the vesicles.

The following unresolved issues are directly addressed in the studies reported here. Is there direct evidence for uptake of intact UDPGlcA in the ER? Does uptake of UDPGlcA reflect translocation of intact UDPGlcA across the ER membrane into the intravesicular space? Is such membrane translocation mediated by a carrier system? What is the subcellular location of UDPGlcA transport? Our results demonstrate that a carrier protein mediates transmembrane transport of intact UDPGlcA into the lumen of ER-derived vesicles.

MATERIALS AND METHODS

Chemicals

UDP[¹⁴C]GlcA (11.1 GBq/mmol) and [¹⁴C]sucrose (24.9 GBq/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. [¹⁴C]Glucose (10.1 GBq/mmol), UDP[³H]galactose (407 GBq/mmol), [³H]UTP (2.07 TBq/mmol), UDP[³H]Glc (699 GBq/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. [¹⁴C]UMP (1.7 GBq/mmol), all unlabelled nucleotides, *p*-nitrophenyl dTMP, α -D-glucose 1-phosphate (Glc-1-P), BSA fraction V, Hepes, *N*-ethylmaleimide (NEM), alkaline phosphatase, nucleotide pyrophosphatase from *Crotalus adamanteus* and ovomucoid were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. 3-[(3-Chloroamidopropyl)dimethylammonio]-1-(2-hydroxy-1-propanesulphonate) (Chapso) was supplied by Bio-Rad, Richmond, CA, U.S.A. UDPGlc dehydrogenase and UDPGlc pyrophosphorylase were from Boehringer Mannheim Biochemicals, Mannheim, Germany. All other chemicals were of reagent grade quality.

Aqueous solutions were prepared with deionized water. *Staphylococcus aureus* α -toxin (from strain Wood 46) was purified by a pore-glass adsorption procedure (Harshman et al., 1988).

Synthesis of [^3H]UDPGlcA, [^{14}C]GlcA and [^{14}C]GlcA-1-P

UDPGlcA, radiolabelled in the uridine moiety, was synthesized enzymically. The reaction mixture comprised 200 μM [^3H]UTP (300 $\mu\text{Ci}/\text{mmol}$), 500 μM Glc-1-P, 200 μM MgCl_2 , 20 mM Tris/HCl (pH 7.8) and 40 units of pyrophosphorylase/ml. This mixture was incubated for 30 min at 37 °C. Thereafter 1 mM NAD^+ , 140 μM EDTA and 0.23 unit of UDPGlc dehydrogenase/ml were added. The reaction mixture was kept at 37 °C for 30 min. After this 1 h incubation period, the enzymes were inactivated by boiling. [^3H]UDPGlcA was isolated by h.p.l.c. (see below). The [^3H]UDPGlcA-containing fractions collected from several chromatographic runs were pooled, freeze-dried and desalted on a DEAE-cellulose column (17 cm $\times$ 25 cm). The column was equilibrated in 2 M KHCO_3 and washed with 500 ml of 200 mM triethylamine bicarbonate (pH 7.0). The sample, dissolved in 5 ml of 100 mM triethylamine bicarbonate, was loaded and the column was washed with 200 ml of 100 mM triethylamine bicarbonate (pH 7.0). The flow rate was 0.5 ml/min.

[^{14}C]GlcA-1-P was prepared by enzymic cleavage of UDP[^{14}C]GlcA by *C. adamanteus* nucleotide pyrophosphatase. A reaction mixture containing 200 μM UDP[^{14}C]GlcA, 20 units of pyrophosphatase/ml, 10 mM Hepes (pH 7), 250 mM sucrose, 150 mM KCl and 6 mM MgCl_2 was incubated at 37 °C for 30 min. Such incubation resulted in complete conversion of UDP[^{14}C]GlcA into [^{14}C]GlcA-1-P. For the preparation of [^{14}C]GlcA, alkaline phosphatase was added at that time, and the incubation was prolonged for another 30 min. Enzymes were inactivated by boiling. The sugar and sugar phosphate were isolated on a DEAE-cellulose column (17 cm $\times$ 25 cm). The column was equilibrated in 2 M KHCO_3 and washed with 500 ml of 200 mM triethylamine bicarbonate (pH 7.0). The sample was added and the column washed with 200 ml of triethylamine bicarbonate (pH 7.0) (200 mM or 300 mM, respectively, for the elution of GlcA or GlcA-1-P).

Preparation and characterization of microsomes

Microsomes enriched with rough ER (RER) or smooth ER (SER) were obtained as described previously (Vanstapel and Blanckaert, 1988) from liver of adult male Wistar rats weighing 200–300 g. The pellet was washed and resuspended in 250 mM sucrose containing 1 mM disodium EDTA buffered to pH 7.4 with 5 mM Hepes. The microsomes were stored under an argon atmosphere at -70°C . The RER fraction was used for UDPGlcA-transport studies.

Structural intactness of the microsomes was routinely verified by determination of mannose-6-phosphatase latency (Vanstapel et al., 1986), which was $> 95\%$ in all preparations used. Specific activity of mannose-6-phosphatase in Chapso-disrupted RER microsomes was 222 ± 31 nmol/min per mg of protein ($n = 8$). This amounted to, on average, 5.16 times the specific activity found in homogenate (43 ± 6 nmol/min per mg of protein; $n = 8$). In comparison with the RNA content of SER microsomes (114 ± 44 μg of RNA/mg of protein; $n = 3$) and homogenate (77 ± 18 μg of RNA/mg of protein; $n = 3$), RER microsomes (271 μg of RNA/mg of protein; $n = 3$) were enriched, respectively, 2.4-fold (range 1.8–4.4; $n = 3$) and 3.5-fold (range 2.7–5.1; $n = 3$). Washing RER-enriched microsomes with EDTA-containing buffer stripped about 60% (range 35–65%; $n = 3$) of the RNA content. RNA content was determined by u.v. absorption

(Fleck and Munro, 1962). The Golgi marker UDPGal galactosyl-transferase, with ovomucoid (17.5 mg/ml) as acceptor substrate (Bretz and Stäubli, 1977), was assayed in RER membrane preparations after pretreating the microsomes (2 mg of protein/ml) with 4 mM Chapso for 1 h at 0 °C. Specific activity of this marker was 44 ± 12 pmol/min per mg of protein ($n = 7$), 103 ± 29 pmol/min per mg of protein ($n = 7$) and 19 ± 10 pmol/min per mg of protein ($n = 6$) in, respectively, RER, SER and homogenate. Activities of alkaline phosphodiesterase, a plasma-membrane marker (Beaufay et al., 1974) were 165 ± 10 pmol/min per mg of protein ($n = 3$), 174 ± 17 pmol/min per mg of protein ($n = 3$) and 124 ± 4 pmol/min per mg of protein ($n = 3$) in, respectively, RER, SER and homogenate.

Microsomes which had been preincubated for 150 min at 37 °C before uptake studies were started are denoted as 'preincubated microsomes'. Such preincubation was performed to remove a luminal pool of endogenous nucleotides (Vanstapel and Blanckaert, 1987).

Preparation of Golgi membrane vesicles

The Golgi fraction was isolated as described by Howell et al. (1978). The discontinuous sucrose gradient produced a pooled light fraction and an intermediate fraction at the 0.25 M/0.86 M sucrose interface (designated G_1), and a heavy fraction at the 0.86 M/1.15 M sucrose interface (designated G_2) (Howell et al., 1978; Higgins, 1984). The pellet and the fraction that remained in the 1.15 M sucrose layer were designated, respectively, R and S (Higgins, 1984). These four fractions were collected, washed, and resuspended in 250 mM sucrose containing 1 mM disodium EDTA buffered to pH 7.4 with 5 mM Hepes. Fractions used for electron microscopy were washed and resuspended in 250 mM sucrose.

Morphological procedure and electron-microscopic examination

Pellets were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and processed for electron microscopy.

Electron-microscopic examination of fractions G_1 , G_2 , S and R (see 'Preparation of Golgi membrane vesicles') showed that G_1 contained numerous Golgi cisternae and small dense vesicles. G_2 contained numerous small dense vesicles, often with heterogeneous contents, some free ribosomes, some smooth vesicles, and a small number of Golgi cisternae. S showed mainly small smooth vesicles, some free ribosomes and some ribosome-studded vesicles. R displayed only vesicles of the RER.

Transport assay

Microsomal uptake of radiolabelled nucleotide or sucrose was assessed by a rapid filtration technique using mixed cellulose filters (type HAWP 02500; 0.45 μm pore size; from Millipore Corp., Bedford, MA, U.S.A.) and an ultrafiltration manifold (FH 225V; Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Microsomes (10 mg of protein/ml) were resuspended and incubated at 37 °C in a mixture of 250 mM sucrose, 150 mM KCl, 6 mM MgCl_2 and 10 mM Hepes at pH 7 (medium A). NAD^+ (2 mM) was added to inhibit microsomal nucleotide pyrophosphatase activity. Deviations from the standard composition of the medium are indicated in the legends of Figures or Tables. The transport assay was started by addition of radio-labelled substrate (25 μM , unless specified otherwise). The reaction was stopped at selected time intervals by removing a portion (usually 20 μl) of the reaction mixture and diluting it into 1 ml of ice-cold medium A. Then 900 μl of this diluted mixture was poured over the cellulose filter under vacuum and washed

with 5 ml of ice-cold medium A. Filtration and rinsing were completed within 15–20 s. The filter was transferred from the ultrafiltration manifold into a scintillation vial, and 10 ml of scintillant (Ultima Gold MV; Packard) was added. Radioactivity on the filter was determined by liquid-scintillation spectrometry (Packard model 1900 CA).

Non-specific binding of radiolabelled substances to microsomes and filters was routinely assessed by mixing the microsomal suspension and the radiolabel-containing start solution at 0 °C. This mixture was immediately subjected to the ultrafiltration procedure. Such blank values were < 0.05 % of the total radioactivity applied to the filter and were subtracted from the test results. The ultrafiltrate contained < 3 % of the applied total protein. Throughout this study, uptake is reported as radiolabelled-nucleotide equivalents, because considerable metabolism of the tested nucleotide sugars may occur. In initial-rate experiments, the reaction was stopped at 20 s intervals. Uptake curves were non-linear. Initial rates were estimated by calculation of the second-degree polynomial equation that fitted the uptake values as a function of incubation time.

Analysis of microsomal breakdown of the substrates used in transport assay

Microsomal transformation of radiolabelled UDP-sugar to protein-linked and/or lipid-linked derivatives during incubation was assessed by measurement of radioactivity in trichloroacetic acid (TCA)-precipitable and lipid-extractable compounds by methods described by Goldelaine et al. (1977).

The radioactive species in the total incubation mixture were analysed by a TCA/ether extraction procedure. Microsomes which had been incubated with radiolabelled UDPGlcA were subjected to TCA precipitation (see above). The supernatant of the precipitate was washed seven times with ether and subjected to h.p.l.c. (see below).

The composition of the radiolabelled components present inside the RER vesicles was determined as follows. The uptake reaction was terminated by removing 50 µl from the incubation mixture and mixing that sample with 1 ml of ice-cold medium A. Then 900 µl of this mixture was poured over the cellulose filter under vacuum and washed with 5 ml of ice-cold medium A. The filter was placed in a 20 ml scintillation vial containing unlabelled carrier substances and 0.1 % Triton X-100 in 0.9 ml of water. The carrier compounds were UDPGlcA (1 mM), GlcA-1-*P* (0.2 mM) and GlcA (1 mM). The vial was placed in a boiling-water bath, to boil for 3 min. Radiolabel recovered in the boiled extract was > 70 % of the radiolabel trapped on the filter.

The radioactive species in the extravesicular medium were determined by analysing the ultrafiltrate prepared as described above in the section 'Transport assay', except that the reaction was terminated by using medium A supplemented with the above-mentioned carrier substances. The filters were not washed with 5 ml of medium A.

H.p.l.c. analysis

The soluble radioactive species in either filtrate, in filter extract, or in the total medium were separated by ion-exchange chromatography on a prepacked 150 mm × 4.6 mm RoSiL AN column (5 µm particle size; Bio-Rad). A Waters (Millipore Co., Waters Chromatography Division, Milford, MA, U.S.A.) high-pressure liquid chromatograph was used, equipped with an automated gradient controller (model 680), a set of pumps (model 510 and 501), an Intelligent Sample Processor (model 712), a u.v. detector (Lambda-Max model 481), and an electronic integrator (model 3390A). The flow rate was set at 2 ml/min. Before injection, the

solutions were degassed and filtered by using a Millipore type HV filter.

Separation of UDPGlcA, GlcA-1-*P* and GlcA

After injection, the column was washed for 5 min with 10 mM NH₄H₂PO₄ (pH 3.7). This was followed by a linear gradient over a period of 40 min to 480 mM NH₄H₂PO₄ (pH 3.7). Effluent fractions (1 ml) were collected and radioactivity was measured, with FloScint IV (Packard) as scintillant. Peaks were identified on the basis of retention times of reference GlcA, GlcA-1-*P* and UDPGlcA, which were eluted at, respectively, 4.5 min, 13 min and 17 min.

Separation of UDPGlcA, UDP, UMP, and uridine

After injection, the column was washed for 3 min with 3 mM NH₄H₂PO₄ (pH 3.7). This was followed by a linear gradient over 16 min with 96 mM-NH₄H₂PO₄ (pH 3.7) to reach the ratio 3 mM-NH₄H₂PO₄/96 mM-NH₄H₂PO₄ of 1:4. Thereafter, the column was washed with 3 mM NH₄H₂PO₄ for 7 min. Fractions (1 ml) were collected and radioactivity was measured, with FloScint IV (Packard) as scintillant. Peaks were identified by spectrophotometry (260 nm) on the basis of retention times of reference uridine, UMP, UDP and UDPGlcA, which were eluted at, respectively, 1.2 min, 6.4 min, 11.9 min and 15.4 min.

Other assays

Protein was determined by the protein-Coomassie Blue dye-binding method with BSA as calibration standard (Bradford, 1976). Phosphodiesterase was assayed as described by Beaufay et al. (1974).

Statistics

Results are given as means ± 1 S.D., with the number of observations in parentheses. Statistical analysis was carried out by Student's *t* test.

RESULTS

Optimization of transport assay conditions

Study of UDPGlcA transport across the ER membrane was hindered by breakdown of the UDPGlcA substrate, catalysed

Table 1 Metabolism of UDPGlcA in total incubation medium

RER microsomes were incubated at 37 °C with 25 µM UDP[¹⁴C]GlcA in a medium containing no (control) or 2 mM NAD⁺. Radiolabel present in intact UDPGlcA recovered after various periods of incubation was determined by h.p.l.c. analysis of the total medium. Values represent means ± 1 S.D. for the numbers of determinations shown in parentheses in at least three separate microsomal preparations. When indicated by * (*P* < 0.005) mean is significantly different from mean of corresponding control condition.

Tested inhibitor	Time (min)	Composition of the radiolabelled species (% of total radiolabel)		
		UDPGlcA	GlcA-1- <i>P</i>	GlcA
Control	3	32 ± 8 (4)	47 ± 7 (4)	21 ± 3 (4)
	6	8 ± 10 (4)	53 ± 8 (4)	39 ± 6 (4)
	9	2 ± 2 (5)	56 ± 6 (5)	42 ± 6 (5)
NAD ⁺	3	76 ± 5 (4)*	5 ± 2 (4)*	19 ± 4 (4)
	6	51 ± 5 (4)*	4 ± 2 (4)*	45 ± 5 (4)
	9	36 ± 5 (5)*	5 ± 3 (5)*	59 ± 8 (5)

Figure 1 Microsomal uptake of UDP[^{14}C]GlcA

Vesicle-associated radiolabel was monitored as a function of incubation time. In panel (a), the initial UDP[^{14}C]GlcA substrate concentration was 25 μM . Data shown are mean values $\pm$ 1 S.D. for eight separate experiments, using different microsomal preparations. Panel (b) shows the time courses for uptake of 200 μM (∇), 400 μM ($\blacktriangledown$), 600 μM ($\square$), 800 μM ($\blacksquare$) and 1000 μM ($\triangle$) UDP[^{14}C]GlcA.

by microsomal nucleotide pyrophosphatase and alkaline phosphatase. Incubation of microsomes at 37 $^{\circ}\text{C}$ with 25 μM UDP[^{14}C]GlcA resulted in rapid breakdown of the sugar nucleotide. After 9 min of incubation, > 90 % of the ^{14}C label was found in the breakdown products GlcA and GlcA-1-*P* (Table 1). As NAD^{+} provided considerable substrate protection (Table 1) and did not significantly interfere with UDPGlcA uptake (results not shown; $n = 5$), we included 2 mM NAD^{+} in all subsequent experiments. Nucleotide pyrophosphatase activity (0.19 ± 0.09 nmol/min per mg; $n = 3$), assayed with 1.5 mM *p*-nitrophenyl dTMP as substrate in the presence of 2 mM NAD^{+} , averaged 13 % of the activities (1.4 ± 0.6 nmol/min per mg; $n = 3$) found in control microsomes, incubated in the absence of NAD^{+} .

Time course of microsomal UDPGlcA uptake

Figures 1(a) and 1(b) show the time curves for uptake of radiolabel by RER vesicles incubated with various UDP[^{14}C]GlcA concentrations. At the low substrate concentrations, uptake of ^{14}C label consistently displayed an initial overshoot phase, during which radioactivity in the microsomes transiently exceeded the amount of label present in the vesicles at equilibrium. Microsomes incubated with 25 μM UDP[^{14}C]GlcA (Figure 1a) contained, after 3 min, 330 ± 100 pmol of

UDP[^{14}C]GlcA equivalents/mg of protein ($n = 23$). From the measured intravesicular volume (2 μl /mg of protein; see below) we calculate that, during overshoot, the putative transporter produced an UDPGlcA concentration gradient of up to 6.6-fold across the membrane. This biphasic uptake was no longer observed at substrate concentrations exceeding 0.6 mM (Figure 1b). At equilibrium uptake, the amount of vesicle-associated label was linearly related to the initial substrate concentration. Evidence that the overshoot phenomenon most likely reflected *trans*-stimulation of UDPGlcA transport by endogenous solutes contained in the microsomes is presented below.

Metabolism of vesicle-associated and extravesicular UDPGlcA

Incubation of microsomes with 25 μM UDP[^{14}C]GlcA resulted in formation of a major TCA-precipitable fraction and of a minor lipid-extractable portion. After 3 min and 20 min we found, respectively, 17 ± 4 % ($n = 3$) and 17 ± 4 % ($n = 3$) of the vesicle-associated label in the TCA precipitate, and 2 ± 1 % ($n = 3$) and 4 ± 1 % ($n = 3$) was recovered in the Folch extract. By contrast, when the UDPGlcA substrate was labelled in the uridine portion, only a small amount of label was recovered in the TCA precipitate and Folch extract. Using 25 μM [^3H]UDPGlcA and incubation for 3 min, we found 0.3 % of the total vesicle-associated label in the TCA-precipitable fraction and 0.1 % of the total vesicle-associated label in the Folch-extractable fraction. Collectively, our findings indicated that glucuronic acid or a part of UDPGlcA containing the sugar moiety, but not the uridine moiety, became incorporated into macromolecular acceptors.

Upon incubation of microsomes with radiolabelled UDPGlcA, the vesicle-associated label comprised, in addition to authentic UDPGlcA, considerable amounts of hydrolysis products (Table 2). After incubation for 3 min with 25 μM UDP[^{14}C]GlcA, UDPGlcA accounted for 33 ± 4 % ($n = 5$) of the total radiolabel. GlcA, uridine and UMP were the main hydrolysis products (Table 2). Prolonged incubation resulted in gradual decrease in the relative amount of vesicle-associated intact UDPGlcA.

H.p.l.c. analysis of the extravesicular medium revealed considerable breakdown of UDPGlcA. After 3 min, authentic UDPGlcA amounted to 78 ± 7 % ($n = 5$) of the total radiolabel when microsomal vesicles were incubated with 25 μM UDP[^{14}C]GlcA (Table 2). Extravesicular UDPGlcA was completely hydrolysed after 20 min.

Evidence that intact UDPGlcA enters the microsomes

Considering the rapid hydrolysis of UDPGlcA, it was of paramount importance to verify that uptake of radiolabel reflected uptake of intact UDPGlcA and not of radiolabelled breakdown product of UDPGlcA.

We present three lines of experimental evidence indicating that authentic UDPGlcA, and not a product of hydrolysis, entered the microsomes. First, neither GlcA nor GlcA-1-*P*, both tested at a 80-fold molar excess over the 25 μM UDP[^{14}C]GlcA substrate, displayed any *cis*-inhibitory effect on microsomal uptake of ^{14}C label. This observation demonstrated that the microsomal uptake of radioactivity did not reflect transport of hydrolytic products from UDPGlcA.

In a second approach, we found that neither GlcA nor GlcA-1-*P* was able to *trans*-stimulate efflux of radiolabel present in microsomes that had been incubated with UDP[^{14}C]GlcA (Table 3). By contrast, unlabelled UDPGlcA effectively *trans*-stimulated the transport of the radiolabel taken up by microsomes that had been preincubated with UDP[^{14}C]GlcA (Figure 2). These findings

Table 2 Determination of the radiolabelled species in the extravesicular and intravesicular media upon incubation of microsomes with radiolabelled UDPGlcA

Microsomes were incubated with 25 μ M UDP[14 C]GlcA (upper part of Table) or with 25 μ M [3 H]UDPGlcA (lower part of Table). At the indicated incubation time, the composition of the radiolabelled substances in the intravesicular and extravesicular media was determined by h.p.l.c. (see the Materials and methods section). Data from two to five different microsomal preparations are presented as means $\pm$ 1 S.D., for the number of determinations shown in parentheses; n.d., not detectable.

Medium	Time (min)	Distribution of radioactivity (% of total radiolabel)		
		UDP[14 C]GlcA	[14 C]GlcA-1-P	[14 C]GlcA
Intravesicular	3	33 $\pm$ 4 (5)	3 $\pm$ 3 (5)	64 $\pm$ 5 (5)
	9	27 $\pm$ 5 (3)	2 $\pm$ 1 (3)	71 $\pm$ 5 (3)
	20	21 $\pm$ 4 (3)	4 $\pm$ 1 (3)	75 $\pm$ 4 (3)
Extravesicular	3	78 $\pm$ 7 (5)	2 $\pm$ 2 (5)	20 $\pm$ 8 (5)
	9	26 $\pm$ 12 (3)	n.d.	74 $\pm$ 13 (3)
	20	5 $\pm$ 4 (3)	n.d.	95 $\pm$ 5 (3)

Medium	Time (min)	Distribution of radioactivity (% of total radiolabel)			
		[3 H]UDPGlcA	[3 H]UDP	[3 H]UMP	[3 H]Uridine
Intravesicular	3	27 $\pm$ 10 (4)	6 $\pm$ 1 (4)	30 $\pm$ 4 (4)	37 $\pm$ 8 (4)
	9	23 $\pm$ 16 (4)	11 $\pm$ 2 (4)	32 $\pm$ 9 (4)	34 $\pm$ 8 (4)
Extravesicular	3	62 $\pm$ 14 (4)	2 $\pm$ 1 (4)	21 $\pm$ 5 (4)	15 $\pm$ 9 (4)
	9	18 $\pm$ 14 (4)	2 $\pm$ 1 (4)	33 $\pm$ 4 (4)	47 $\pm$ 11 (4)

Figure 2 *Trans*-stimulation of microsomal UDP[14 C]GlcA efflux by unlabelled UDPGlcA

Microsomal vesicles (11.4 mg/ml) were incubated at 37 °C with 28.5 μ M UDP[14 C]GlcA in a total volume of 140 μ l. Microsomal uptake of label was monitored as a function of time. Portions (30 μ l) were removed and subjected to ultrafiltration in order to determine 14 C label, at 55 s and 90 s. After 60 s, 20 μ l of either medium A (see the Materials and methods section) (O) or a 16 mM UDPGlcA stock solution (●) was added. Values represent means $\pm$ 1 S.D. for four determinations on two separate microsomal preparations.

provide strong evidence that translocation of radiolabel reflected transport of intact UDPGlcA rather than of its hydrolysis products. Another important conclusion could be drawn from the results of these stimulation experiments. Because transport of both GlcA and GlcA-1-P was excluded and the 14 C label resided in the sugar moiety of the nucleotide sugar, efflux of radiolabel from the microsomal vesicles unquestionably corresponded with efflux of intact radiolabelled UDPGlcA, rather than of UMP or UDP.

A third approach to verify transport of intact UDPGlcA comprised experiments in which uptake of radiolabel was studied

in microsomes incubated simultaneously with UDP[14 C]GlcA, labelled in the sugar moiety, and [3 H]UDPGlcA, labelled in the uridine portion. Time curves for microsomal uptake of the 3 H label and 14 C label, both expressed as percentage of the corresponding total radiolabel, coincided (Figure 3a). This provided evidence that the radiolabelled species being translocated was authentic UDPGlcA. By contrast, the time curves for microsomal uptake of 3 H label and 14 C label were widely divergent when microsomes were incubated with both [3 H]UDPGlcA and [14 C]UMP (Figure 3d). Similarly, time curves for microsomal uptake of 3 H label diverged from time curves of 14 C-label uptake when microsomes were incubated with [3 H]UDPGlcA and [14 C]GlcA, or with [3 H]UDPGlcA and [14 C]GlcA-1-P (Figures 3c and 3b).

Collectively, these observations demonstrate that microsomes incubated with radiolabelled UDPGlcA take up the intact nucleotide sugar. After uptake, rapid hydrolysis as well as incorporation into lipid- and protein-linked derivatives of the internalized UDPGlcA occurs.

Evidence that UDPGlcA enters the lumen of the microsomal vesicles

Figure 4 shows the relationship between the membrane-enclosed intravesicular volume, as assessed by sucrose-accessible space, and the equilibrium uptake of either the total vesicle-associated UDP[14 C]GlcA-derived radiolabel or the TCA-precipitable UDP[14 C]GlcA-derived radioactivity. As the intravesicular volume decreased, equilibrium uptake of total vesicle-associated UDP[14 C]GlcA-derived radiolabel diminished. This linear relationship indicates that UDPGlcA was transported into a closed, osmotically sensitive, intravesicular space. Extrapolating the line describing the relationship between the sucrose space and the total vesicle-associated UDP[14 C]GlcA-derived radiolabel to zero sucrose-accessible space shows a binding component of about 1.17 nmol of UDPGlcA equivalents/mg of protein. This calculated value is in close agreement with our experimental results for

Figure 3 Translocation of a mixture of [^3H]UDPGlcA and UDP[^{14}C]GlcA, a mixture of [^3H]UDPGlcA and [^{14}C]UMP, a mixture of [^3H]UDPGlcA and [^{14}C]GlcA, or a mixture of [^3H]UDPGlcA and [^{14}C]GlcA-1-P, into microsomes

Microsomes were incubated with 25 μM UDPGlcA containing [^3H]UDPGlcA ($\circ$) and UDP[^{14}C]GlcA ($\bullet$) (a), with 25 μM [^3H]UDPGlcA ($\circ$) and 25 μM [^{14}C]GlcA-1-P ($\blacksquare$) (b), with 25 μM [^3H]UDPGlcA ($\circ$) and 25 μM [^{14}C]GlcA ($\blacktriangle$) (c), or with 25 μM [^3H]UDPGlcA ($\circ$) and 25 μM [^{14}C]UMP ($\square$) (d). Vesicle-associated label was monitored as a function of incubation time and expressed as a percentage of the respective total radiolabel. Data points represent means $\pm$ S.D. for three (a and b) or four (c and d) microsomal preparations.

Figure 4 Effect of osmolarity of the medium on UDPGlcA and sucrose-accessible space in RER microsomes

Microsomes were incubated for 15 min at 37 $^{\circ}\text{C}$ in incubation buffer containing various concentrations of raffinose (0 mM, 50 mM, 100 mM, 150 mM and 250 mM). Thereafter, 2 mM UDP[^{14}C]GlcA or 10 mM [^{14}C]sucrose was added. Vesicle-associated radioactivity was determined at equilibrium uptake, after 40 min of incubation. The sucrose-accessible volume is represented on the abscissa. The corresponding total vesicle-associated UDP[^{14}C]GlcA equivalents ($\circ$) and UDP[^{14}C]GlcA equivalents recovered in TCA-precipitated compounds ($\bullet$) are represented in the ordinate. Each data point represents the mean $\pm$ S.D. for five different microsomal preparations.

Figure 5 Effect of *Staph. aureus* α -toxin on microsomal UDPGlcA uptake

Microsomes were preincubated for 10 min at 37 $^{\circ}\text{C}$ in the absence ($\circ$) or presence ($\bullet$) of 125 μg of *Staph. aureus* α -toxin/mg of protein. Thereafter 25 μM [^3H]UDPGlcA was added and vesicle-associated label was monitored as a function of time. Data points are means $\pm$ S.D. for three different microsomal preparations.

equilibrium uptake of TCA-precipitable UDP[^{14}C]GlcA-derived radioactivity. We found that approx. 0.72 nmol of UDPGlcA equivalents/mg of protein, corresponding to 17 % of equilibrium uptake, could be accounted for by incorporation of radiolabel into macromolecules and remained unaffected by microsomal volume.

Additional corroborating evidence that uptake corresponded to entry of UDPGlcA in the microsomal lumen was obtained in uptake studies performed with microsomal vesicles permeabilized by treatment with the pore-forming agent *Staph. aureus* α -toxin. When incubated with [^3H]UDPGlcA, such permeabilized microsomal preparations were unable to retain the radiolabel (Figure 5), as is expected when uptake is based on retention of radio-labelled substrate in the lumen of the microsomal vesicles.

Dependence of microsomal UDPGlcA uptake on temperature and on protein concentration

The initial rate of uptake of UDPGlcA by microsomes was clearly temperature-dependent. Transport rates fell by 85 % when the assay temperature was decreased from 37 $^{\circ}\text{C}$ to 0 $^{\circ}\text{C}$. Based on the Arrhenius plot shown in Figure 6, the energy of activation for uptake reaction was estimated at 36.4 ± 1.2 kJ/mol ($n = 3$). Tested with 25 μM UDP[^{14}C]GlcA, initial uptake rates were proportional to microsomal protein concentration over the range 2.5–20 mg of protein/ml.

Uptake overshooting: evidence for coupled transport

Several lines of evidence support the hypothesis that the overshoot phenomenon observed in native microsomes (Figure 1a) reflected *trans*-stimulation. Such stimulation may be caused by countertransport of endogenous solutes sharing the putative UDPGlcA carrier for transport across the membrane. This hypothesis is consistent with the previous observation that sealed microsomal vesicles contain a luminal pool of endogenous UDPGlc, which can be depleted by preincubation of the microsomes (Vanstapel and Blanckaert, 1987). As previously observed for microsomal uptake of UDPGlc (Vanstapel and Blanckaert, 1988), we found that preincubation of native microsomes at

Figure 6 Temperature-dependence of microsomal uptake of UDPGlcA

Preincubated microsomes were thermally equilibrated at 0 °C, 10 °C, 20 °C, 30 °C and 37 °C. Uptake was then started by addition of 5 μ M UDP[14 C]GlcA and initial rates were estimated. The Arrhenius plot is presented. Data points represent means $\pm$ S.D. for three microsomal preparations.

Figure 7 *Trans*-stimulation of microsomal uptake of UDPGlcA

Panel (a) shows the time curves for uptake of 14 C label by vesicles that had been preincubated for 0 min ($\circ$), 30 min ($\square$), 60 min ($\blacksquare$), 90 min ($\blacktriangle$) or 150 min ($\bullet$) at 37 °C before radiolabelled substrate was added. The data shown are from one representative experiment out of three. Panel (b) illustrates the effect of preloading the microsomes with unlabelled UDPGlc. Preloading consisted of incubation at 37 °C for 15 min of preincubated microsomes in incubation buffer without ($\circ$) or with 5 mM UDPGlc ($\bullet$). This was followed by re-isolation and washing (twice) of the vesicles by ultracentrifugation (104 000 g for 30 min at 4 °C). Thereafter uptake was started by addition of 25 μ M UDP[14 C]GlcA. Each data point represents the mean $\pm$ S.D. for three separate microsomal preparations.

Table 3 *Trans*-stimulation of UDPGlcA efflux from microsomal vesicles by structural analogues of UDPGlcA

The experimental set-up was as outlined for the experiment described in Figure 2 legend. Instead of UDPGlcA, various nucleoside derivatives were tested for their ability to *trans*-stimulate UDP[14 C]GlcA efflux. Values, which are means $\pm$ S.D. of five determinations in three different microsomal preparations, represent the vesicle-associated 14 C label found after 90 s of incubation, expressed as percentage of vesicle-associated label found after 55 s of incubation. When indicated by * ($P < 0.01$) or ** ($P < 0.001$), mean is significantly different from mean of control condition.

Tested compound	Vesicle-associated label after 1.5 min (expressed as % of vesicle-associated label after 55 s)
None (control)	110 $\pm$ 12
UDPGlcA	35 $\pm$ 10**
GlcA	104 $\pm$ 12
GlcA-1- <i>P</i>	107 $\pm$ 8
UMP	80 $\pm$ 4*
5-Bromo-UMP	93 $\pm$ 9
UDP	81 $\pm$ 24
UTP	88 $\pm$ 6*
UDPGlcNAc	45 $\pm$ 10**
UDPGlc	72 $\pm$ 10*
UDPGal	74 $\pm$ 8*
UDP-xylose	44 $\pm$ 9**
GDP-mannose	107 $\pm$ 12
GDP-Glc	101 $\pm$ 16
GTP	110 $\pm$ 8
CMP	96 $\pm$ 5
CDP-choline	102 $\pm$ 4
AMP	100 $\pm$ 26
ADP	104 $\pm$ 9
ATP	99 $\pm$ 19

37 °C resulted in a gradual decrease in the overshoot peak for UDPGlcA uptake (Figure 7a). Overshooting was completely abolished when preincubation at 37 °C was extended to 150 min. The long preincubation did not significantly alter the intactness of the microsomal vesicles, since the mannose-6-phosphatase latency of microsomes that had been preincubated for 150 min at 37 °C was still 96.5%, compared with 97.1% before preincubation.

Secondly, we were able to reconstitute an overshoot phenomenon in preincubated RER-derived microsomal vesicles, which did not display the overshoot phenomenon. To reconstitute overshooting, such vesicles were preloaded with driver solute, by incubating for 15 min at 37 °C with a high concentration (5 mM) of UDPGlc. After washing and resuspension of the microsomes, UDP[14 C]GlcA uptake (25 μ M) unmistakably exhibited overshooting (Figure 7b), which was not seen in control vesicles preincubated in the absence of UDPGlc. These observations are consistent with massive net efflux of unlabelled endogenous UDPGlc causing *trans*-stimulation of UDP[14 C]GlcA influx as the mechanism underlying the uptake overshooting observed in native microsomes (Figure 1a). Demonstration of such coupling in solute translocation provides strong evidence for bidirectional carrier-mediated transport of UDPGlcA.

Specificity of UDPGlcA translocation: *trans*-stimulation of microsomal UDPGlcA efflux by structurally related compounds

The specificity of the postulated carrier system was investigated by testing the effectiveness of different unlabelled structural

Figure 8 Dependence of rate of microsomal UDPGlcA uptake on substrate concentration

Initial rate of microsomal uptake of radiolabel was assessed for UDP[^{14}C]GlcA concentrations ranging between 25 μM and 2 mM by using preincubated microsomes. The findings are shown in a Scatchard plot. Data points are means $\pm$ S.D. for three different microsomal preparations.

Figure 9 Effect of NEM on microsomal UDPGlcA uptake

Microsomes were preincubated in the presence (●) or absence (○) of 2.5 mM NEM at 4 °C during 15 min. The uptake reaction was started by addition of 25 μM UDP[^{14}C]GlcA, and vesicle-associated radiolabel was determined as a function of incubation time. Each data point represents the mean $\pm$ S.D. for five microsomal preparations.

analogues, added in excess, to act as *trans*-stimulator for efflux of radiolabelled UDPGlcA. Results are shown in Table 3. Only 5'-uridine nucleotides (UDPGlcA, UDPGlc, UDPGlcNAc, UDP-xylose, UMP, UTP) effectively *trans*-stimulated efflux of UDPGlcA. Cytidine-, adenosine- and guanosine-containing nucleotides were markedly less effective or exerted no effect.

Substrate concentration-dependence

Further insight in the mechanism of action of the postulated carrier came from experiments on the effect of substrate concentration on the rate of transmembrane transport. The Eadie-Scatchard presentation of the data revealed complex kinetic behaviour (Figure 8). We were thus unable to calculate reliable kinetic parameters.

Table 4 Subcellular distribution of UDPGlcA translocation

Subfractionation into Golgi and ER subfractions (G_1 , G_2 , S, and R) was performed as described in the Materials and methods section. Specific catalytic activities of the UDPGal:ovomucoid galactosyltransferase and mannose-6-phosphatase marker enzymes were determined in each subcellular fraction. Rates of translocation of UDPGlcA (4.4 μM) were determined in preincubated vesicles of each subcellular fraction. Values represent means $\pm$ 1 S.D., for the number of determinations shown in parentheses, in at least three different preparations.

Subcellular fraction	Enzymic activity (nmol/min per mg of protein)		Transport rate (pmol/min per mg of protein UDPGlcA)
	Galactosyltransferase	Mannose-6-phosphatase	
Homogenate	0.028 $\pm$ 0.009 (6)	48 $\pm$ 8 (6)	4 $\pm$ 2 (5)
G_1	1.272 $\pm$ 0.331 (6)	42 $\pm$ 20 (6)	14 $\pm$ 7 (5)
G_2	0.370 $\pm$ 0.164 (6)	138 $\pm$ 35 (6)	14 $\pm$ 8 (5)
S	0.016 $\pm$ 0.006 (6)	226 $\pm$ 64 (6)	16 $\pm$ 6 (5)
R	0.005 $\pm$ 0.003 (6)	255 $\pm$ 52 (6)	24 $\pm$ 8 (5)

Inhibition of UDPGlcA uptake by NEM

Evidence that a protein in the membrane was involved in the transport process came from the following study. No overshoot could be discerned when uptake was measured after preincubation with 2.5 mM of the thiol reagent NEM (Figure 9). There would be no reason to suspect that any molecular entering a membrane by simple diffusion would be blocked by this compound.

Subcellular localization of carrier-mediated UDPGlcA translocation

Evidence was sought to exclude the possibility that the carrier-mediated UDPGlcA translocation found in our RER preparations was dependent on contamination with Golgi membranes, for which UDPGlcA uptake was described previously (Nuwayhid et al., 1986). Using a discontinuous sucrose-density gradient, we separated the microsomes into four fractions, designated G_1 , G_2 , S and R (in order of increasing density). The results of our electron-microscopy studies (see the Materials and methods section) and of the characterization of our subcellular fractions using marker enzymes (Table 4) confirm the characteristics of preparations highly enriched in Golgi membranes for G_1 , in SER for S, and in RER for R.

Our results (Table 4) indicate that UDPGlcA transport does occur in ER membranes, and are inconsistent with UDPGlcA transport solely residing in Golgi membranes. UDPGlcA transport rates were highest in those fractions (R and S fractions) which displayed the highest ER membrane marker-enzyme activities (mannose-6-phosphatase) and lowest Golgi marker-enzyme activities (galactosyltransferase). On the other hand, our findings also show that Golgi membranes do take up UDPGlcA, since the G_1 fraction exhibited considerable transport activities despite the near absence of ER membranes from this fraction (as evidence by the very low activity of mannose-6-phosphatase).

DISCUSSION

Two previous studies have reported on UDPGlcA uptake in the ER (Hauser et al., 1988; Nuwayhid et al., 1986). Both of these studies failed to provide conclusive evidence for transport of intact UDPGlcA into the lumen of the ER. Metabolism, hydrolysis and incorporation of GlcA into macromolecular acceptors was not investigated in either of the two aforementioned studies. The present investigation reveals that UDPGlcA

is extensively and rapidly metabolized. Microsomal breakdown of UDPGlcA was not examined or even considered in the previous reports on UDPGlcA transport. Taking this hydrolysis into account, we contrived several experimental approaches to exclude the possibility that uptake of radiolabel did reflect uptake of a radiolabelled hydrolysis product and to demonstrate that uptake of radiolabel reflected transport of authentic UDPGlcA.

In agreement with Hauser et al. (1988), we found that radionucleotide binding by microsomes incubated with radio-labelled UDPGlcA exhibited features of carrier-mediated uptake, including temperature-dependency, saturation and substrate specificity. However, neither Hauser et al. (1988) nor Nuwayhid et al. (1986) demonstrated the presence of *trans*-stimulation, which contributes a persuasive argument for carrier-mediated transport. In the present study we have shown that uridine-containing nucleotides can effectively *trans*-stimulate influx and efflux of UDPGlcA. Neither Hauser et al. (1988) nor Nuwayhid et al. (1986) recognized the transient *trans*-stimulation of radio-labelled UDPGlcA uptake which we observed in native well-sealed microsomes. However, the presence of a pool of endogenous nucleotides must be taken into consideration in kinetic studies of nucleotide sugar transport across the ER membrane. At variance with the results of Hauser et al. (1988), we found that uptake of UDPGlcA corresponded to complex kinetics and did not conform with simple Michaelis-Menten behaviour. The presence of a pool of endogenous nucleotides which *trans*-stimulates uptake of low concentrations of UDPGlcA should also be considered when comparing specific uptake activities in Golgi, SER- and RER-derived membranes. We found that UDPGlcA was taken up by the ER as well as by Golgi, and could not confirm the findings of Nuwayhid et al. (1986), who report higher uptake rates in Golgi membranes compared with ER-derived membranes.

Another main and important difference between our findings and the previous work by Hauser et al. (1988) relates to the question whether UDPGlcA is transported into the lumen of the ER. Hauser et al. (1988) contend, on the basis of experiments claimed to modulate the intravesicular space, that UDPGlcA is translocated into the ER membrane rather than across the membrane and into the intravesicular space. If this hypothesis were correct, then *trans*-stimulation by intravesicular nucleotide sugars would not be possible, since, according to the assumption of the aforementioned authors, this highly charged nucleotide would remain trapped within the ER membrane. The observations of overshooting uptake and *trans*-stimulation therefore disprove the proposition by Hauser et al. (1988) and suggest a transport system which has access to the cytoplasmic as well as to the luminal side of the ER membrane. In the present paper we have provided two independent experimental approaches which unambiguously demonstrate that UDPGlcA is transported into the lumen of ER-derived vesicles. First, we found that the apparent space occupied by vesicle-associated UDPGlcA equivalents under equilibrium conditions corresponded to an osmotically sensitive compartment. Second, we showed that microsomes permeabilized with *Staph. aureus* α -toxin were not able to retain radiolabel.

The findings reported in this paper have major implications

for glucuronidation reactions. The demonstration of a carrier protein mediating transport of UDPGlcA is consistent with a compartmentation model for regulation of UGT activities. However, one cannot pass from the premiss that there is incontrovertible evidence for the existence of carrier-mediated transport of UDPGlcA, the most important donor-substrate for UGT, to the conclusion that this carrier is indispensable for UGT. To establish that a UDPGlcA carrier is a prerequisite for glucuronidation reactions, it will be necessary to demonstrate that selective inhibition of the carrier affects glucuronidation reactions in native sealed microsomes. In preliminary experiments, we found that UGT activity, assayed under the conditions of our transport assay, was inhibited by NEM. NEM, which inhibits UDPGlcA transport, did not affect total UGT activity in permeabilized microsomes. These results implicate that the transport system is important for regulation of UGT.

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