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An investigation of the transverse topology of bilirubin UDP-glucuronosyltransferase in rat hepatic endoplasmic reticulum

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Bilirubin UDP-glucuronosyltransferase (UDPGT) activity in sealed hepatic microsomes from clofibrate-treated rats was highly latent and was fully expressed by disruption of vesicles with detergents. Antibodies raised against purified bilirubin UDPGT were used to study the transmembrane orientation of the protein to provide a molecular understanding of the UDPGT latency. Immunoblot analysis of sealed microsomes, and microsomes after treatment with proteinases, showed that only a small portion of the protein resides on the cytoplasmic side of the microsomal vesicles. Treatment of microsomes with sodium deoxycholate allowed subtilisin and proteinase K to cleave the transferase, causing loss of activity and the release of smaller immunodetectable peptides. Treatment of the purified bilirubin UDPGT with peptide *N*-glycosidase F indicated that the enzyme was a glycoprotein. A working model of the transmembrane topology of bilirubin UDPGT is described.

INTRODUCTION

Hepatic microsomal UDP-glucuronosyltransferases (UDPGTs, EC 2.4.1.17) are a family of detoxicating isoenzymes [1]. These enzymes facilitate the elimination of drugs and other potentially toxic compounds by conjugating them with glucuronic acid, thereby increasing the polarity of the drug and allowing excretion of the product in the bile or urine [2].

Bilirubin UDPGT is the rate-limiting enzyme in the excretion of bilirubin from the body. The absence of this enzyme activity can lead to jaundice as a developmental insufficiency manifested in neonatal animals [3] and in Crigler–Najjar syndrome [4].

The transmembrane orientation of various members of this enzyme family has been a subject of controversy for many years (see [2]). The activity of bilirubin UDPGT in intact hepatic microsomes is not fully expressed and can be increased more than 10-fold by disruption of microsomal vesicles [5]. A model has been proposed which suggests that UDP-glucuronic acid (UDPGA) transport is rate-limiting for glucuronidation by intact microsomes (see [6]); disruption of the membrane barrier by detergents would allow free access of the donor substrate and reveal the full catalytic potential of the enzyme. This model would also suggest that the active site of the enzyme binding such a hydrophilic and charged substrate (UDPGA) would reside in the lumen of the endoplasmic reticulum (ER). Therefore we have investigated the transmembrane orientation of a membrane-bound UDPGT at the molecular level.

The evidence presented here indicates that most of the bilirubin UDPGT, a glycoprotein, was located on the luminal side of the ER.

MATERIALS AND METHODS

Materials

Bilirubin, UDPGA (sodium salt), sodium deoxycholate (DOC), phenylmethanesulphonyl fluoride (PMSF), 1,10-*o*-phenanthroline, subtilisin BPN, trypsin inhibitor, 4-chloro-1-naphthol and Triton X-100 were all obtained from Sigma (Poole, Dorset, U.K.). Peptide *N*-glycosidase F (PNGase F) and trypsin were from Boehringer Mannheim (Lewes, East Sussex, U.K.). Proteinase K was from Merck (BDH, Glasgow, Scotland). Lubrol 12A9 was from ICI (Macclesfield, U.K.). Fetuin was from Gibco (Paisley, Renfrewshire, Scotland, U.K.) Clofibrate was from Fluka AG (Neu-Ulm, Germany). Schleicher and Schuell nitrocellulose was obtained from Anderman (Kingston-upon-Thames, Surrey, U.K.). All other chemicals where available were of Analytical Reagent grade. Anti-sheep IgG (from donkey) and peroxidase–anti-peroxidase (PAP) complex were supplied by the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland, U.K.

Purification of bilirubin UDPGT

Male Wistar rats (200–250 g) from the Dundee Animal Unit were given an injection of 200 mg of clofibrate/kg body wt. in 0.5 ml of corn oil intraperitoneally twice daily for 4 days. The animals were killed and livers removed 24 h after the final injection. Hepatic microsomes were prepared and bilirubin UDPGT was further purified by detergent solubilization, $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography as previously described [7]. Selected fractions from the DEAE-cellulose column were concentrated approx. 25-

Abbreviations used: UDPGT, UDP-glucuronosyltransferase; PNGase F, peptide *N*-glycosidase F; UDPGA, UDP-glucuronic acid; DOC, deoxycholate; PMSF, phenylmethanesulphonyl fluoride; ER, endoplasmic reticulum; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-propanesulphonic acid.

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fold with an ultrafiltration unit (Amicon) fitted with a PM-10 Diaflo membrane. This protein concentrate was equilibrated with 0.05% (w/v) Lubrol 12A9/75 mM-Tris/acetate/5% (v/v) glycerol/5 mM-2-mercaptoethanol (buffer A), pH 9.3. The eluted protein was then applied to a Mono P chromatofocusing column fitted to fast-protein-liquid-chromatographic equipment (Pharmacia). Bilirubin UDPGT activity was eluted with 10 ml of buffer A, followed by 30 ml of 0.05% (w/v) Lubrol 12A9/5% (v/v) glycerol/5 mM-2-mercaptoethanol/10% (v/v) Polybuffer adjusted to pH 6.0 with acetic acid. The chromatofocused enzyme was eluted at pH 7.65. Bilirubin UDPGT activity was measured as previously described [8], using 4 mM-UDPGA and 122 μM-bilirubin.

Deglycosylation of purified bilirubin UDPGT with PNGase F

Aliquots of purified bilirubin UDPGT were incubated with 2 units of PNGase F (EC 3.2.2.18) as previously described [9] in the presence of 10 mM-1,10-*o*-phenanthroline for 18 h at 37 °C. Some samples of the purified enzyme were also incubated with 5 units of PNGase F in a non-denaturing modified buffer system (20 mM-K_2HPO_4/20 mM-EDTA/143 mM-2-mercaptoethanol/120 μM-DOC, pH 7.5) for 3 h at room temperature.

Proteolysis of bilirubin UDPGT in hepatic microsomal fractions

Freshly prepared hepatic microsome suspensions (2 mg of protein in 0.25 M-sucrose/5 mM-Hepes, pH 7.4) from clofibrate-treated or untreated 4-week-old rats were incubated with 0.2–0.4 mg of detergent/mg of protein in 150 mM-Tris/maleate buffer, pH 7.4, containing 5 mM-$MgCl_2$ for 30 min at 20 °C (solubilized microsomes). Controls were incubated with buffer alone (sealed microsomes). Proteinases were added in the same buffer and the incubation mixtures were made up to 1800 μl. Proteolysis was performed for 30 min at 20 °C and then 200 μl of 2 mM-PMSF/4 mM-1,10-*o*-phenanthroline in 40% (v/v) ethanol was added to inhibit subtilisin BPN and proteinase K. A 20 mg portion of trypsin inhibitor in 200 μl of water was added to inhibit trypsin. The incubation mixtures were stored at 0 °C for assay of bilirubin UDPGT activity. Aliquots (200 μl) were removed, and an additional 20 μl of proteinase inhibitors (20 mM-PMSF and 100 mM-1,10-*o*-phenanthroline) was added to prevent proteolysis during denaturation in SDS before gel electrophoresis.

Immunoblot analysis of untreated, detergent-solubilized and proteinase-treated hepatic microsomes

Samples of the various treated or untreated microsomes (50 μg of protein) were mixed with 25 μl of 0.23 M-sucrose/2.8 M-2-mercaptoethanol/0.28 M-SDS/0.5 M-Tris buffer, pH 6.8, incubated at 100 °C for 3 min, and the microsomal proteins were separated by 10%-polyacrylamide-gel electrophoresis in the presence of SDS [10]. The separated proteins were transferred from the gel to nitrocellulose as described by Towbin *et al.* [11]. Blotted proteins were then examined by using a goat anti-(bilirubin UDPGT) antibody as previously described [12].

RESULTS AND DISCUSSION

Effect of proteolytic treatment on bilirubin UDPGT activities of untreated sealed and detergent-disrupted microsomes

Hepatic microsomes from clofibrate-treated rats were incubated with various amounts of proteinases in the presence and absence of deoxycholate. The microsomal preparations were assayed for bilirubin UDPGT activity to assess the integrity of the sealed, intact, microsomes before and after proteinase treatment. This crucial assessment indicated the sidedness of the proteolytic attack. In intact sealed microsomes, proteinases should be able to attack proteins exposed on the cytoplasmic surface of ER vesicles; in detergent-solubilized microsomes, proteinases should operate at both luminal and cytoplasmic surfaces [13]. Assay of the latent UDPGT activities is an excellent indicator of the integrity of the ER membrane. The latency of 1-naphthol UDPGT and bilirubin UDPGT have been shown to correlate exactly (r = 0.998) with that of the classical mannose-6-phosphatase assessment of ER integrity [14,15].

The results of optimal activition of bilirubin UDPGT activity in hepatic microsomes using Lubrol (0.2 mg of detergent/mg of protein) indicated that greater than 90% of the microsomes were sealed even after incubation for 60 min at 20 °C (results not shown). The results of the assessment of intactness using deoxycholate also indicated that 80% of microsomal vesicles were sealed (Table 1). After treatment of sealed microsomes with proteinases, an increase of bilirubin UDPGT activity was observed, and the integrity of the vesicles was, not surprisingly, decreased to approx. 60%. However, when the proteinase-treated 'sealed' microsomes, where pro-

Table 1. Effect of proteinase treatment on bilirubin UDPGT activity in intact and DOC-treated microsomes

Values in parentheses indicate the percentage of activity remaining after treatment with proteinases. The results shown are means ± S.D. for data from at least six separate experiments using microsomal preparations from different pools of rat livers.

		Bilirubin UDPGT activity (nmol/min per mg of protein)					
			DOC after proteolysis	DOC before proteolysis			
Proteinase	Concn. (μg/mg of protein)	Intact microsomes	(0.2 mg/mg of protein)	(0.2 mg/mg of protein)		(0.4 mg/mg of protein)	
None	–	0.29 ± 0.09	1.42 ± 0.18	1.42 ± 0.19	(100)	0.94	(100)
Trypsin	10	0.60 ± 0.13	1.45 ± 0.06	1.50 ± 0.63		0.99 ± 0.16	
Subtilisin BPN	50	0.64 ± 0.25	1.30 ± 0.16	1.32 ± 0.11	(93)	0.68 ± 0.26	(72)
Proteinase K	50	0.72 ± 0.27	1.37 ± 0.20	1.12 ± 0.10	(79)	0.38 ± 0.25	(40)

teinase action had been terminated, were further treated with optimal amounts of detergent, no apparent loss of bilirubin UDPGT protein was observed, as indicated by the retention of transferase activity (Table 1).

These data agree with the previous suggestion [16] that, when 'sealed' microsomes were used, the proteinases may damage the cytoplasmic face of the microsomes and allow entry of low-M_r compounds such as UDPGA, but that the active site of UDPGT was apparently unaffected.

Treatment of microsomes with optimal amounts of detergent (0.2 mg of deoxycholate/mg of protein) does not solubilize the microsomes [17] and did not allow proteolytic destruction of the bilirubin UDPGT activity (Table 1). Higher levels of detergent, up to 0.4 mg of deoxycholate/mg of protein, which solubilized and partially denatured the transferase, as indicated by the decrease of enzyme activity from 1.42 units/mg of protein to 0.94 units/mg of protein, facilitated proteolytic degradation of the bilirubin UDPGT (Table 1). Vanstapel & Blanckaert [15] have recently demonstrated that Nagarse only significantly decreased bilirubin UDPGT activity after disruption of the microsomal fraction with the detergent, CHAPS, in agreement with the results reported here.

Immunoblot analysis of bilirubin UDPGT after proteolytic treatment of 'intact' and detergent-treated microsomes

Intact and detergent-treated microsomes were analysed by immunoblotting. Fig. 1 shows the results of these experiments. Treatment of 'intact' microsomes with all proteinases caused a slight increase in the mobility of bilirubin UDPGT, indicating the removal of approx. 2 kDa of protein (Fig. 1*a*). Mixing the untreated and trypsin-treated microsomes before immunoblotting confirmed that only the 2 kDa fragment was removed (Fig. 1*b*). Subtilisin BPN and proteinase K behave similarly in that they both cause extensive destruction of the bilirubin UDPGT protein in the presence of high levels of detergent (Fig. 1*a*). Here we were able to relate this proteolytic degradation to the loss of bilirubin UDPGT activity (Table 1) and have observed the appearance of immunodetectable peptides of approx. 20 kDa (Fig. 1*a*).

These results suggest that only a small portion of the bilirubin UDPGT protein is exposed on the cytoplasmic surface of the endoplasmic reticulum, and more of the enzyme was proteolytically degraded by subtilisin BPN or proteinase K in solubilized microsomes.

Deglycosylation of purified rat liver bilirubin UDPGT by PNGase F

Bilirubin UDPGT has been proposed, after the use of a staining procedure to study partially purified enzymes, to be glycoprotein [18]. Stronger evidence of glycosylation would provide some indicators of the orientation of the protein. Aliquots of purified bilirubin UDPGT were incubated with PNGase F under denaturing conditions and then examined by SDS/polyacrylamide-gel electrophoresis. Fig. 2 shows that the purified enzyme was reduced in size by approx. 2 kDa after treatment with PNGase F. Note that size changes appear larger on these 7% gels. Incubation was also conducted with fetuin, a known glycoprotein, and in the presence of 1,10-*o*-phenanthroline (to indicate that this decrease in size was not due to a proteolytic event). Incubation of bilirubin UDPGT with PNGase F under non-denaturing conditions (see [2,3]) reduced the enzyme activity by only 10%, although the decrease in size of the protein was still observed (results not shown), indicating that glycosylation is not essential for enzymic activity.

These data strongly indicate that bilirubin UDPGT is a glycoprotein, although definitive proof of glycosylation requires a chemical analysis of the purified enzyme.

A model of the transverse organization of bilirubin UDPGT in the ER

The simple picture that these results predict is of a protein slightly exposed on the cytoplasmic surface of the membrane joined by a transmembrane region to the majority of the protein on the luminal side of the ER. The luminal portion of the protein is glycosylated. Other members of this family of isoenzymes, such as androsterone UDPGT [19,20] and phenol UDPGT [21], have been

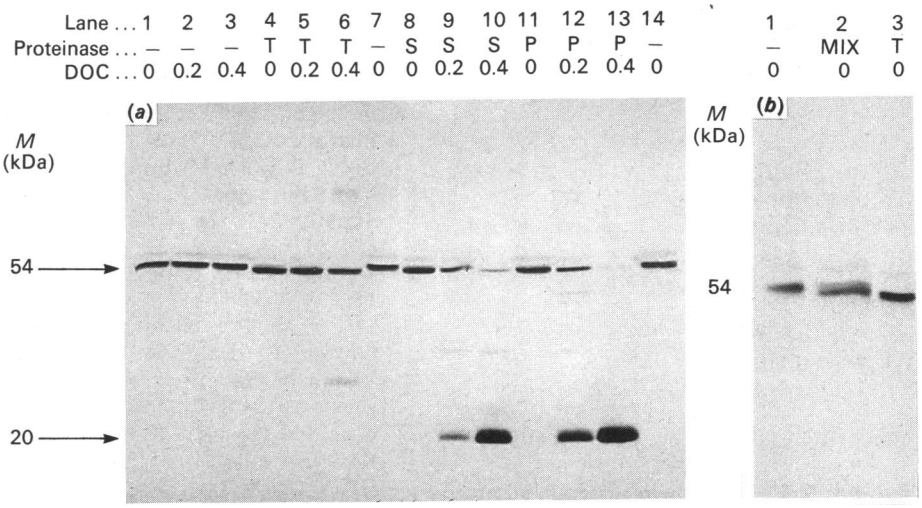

Fig. 1. Immunoblot analysis of bilirubin UDPGT after proteolysis of 'intact' and DOC-treated microsomes

DOC treatments were mg of detergent/mg of microsomal protein, and 50 μg of microsomal protein was applied to each lane. Proteins were separated on SDS/polyacrylamide gels (*a*, 10%; *b*, 7%) before blotting on to nitrocellulose. Abbreviations: T, trypsin; S, subtilisin BPN; P, proteinase K; MIX, a mixture of untreated and trypsin-treated microsomes; *M*, molecular mass.

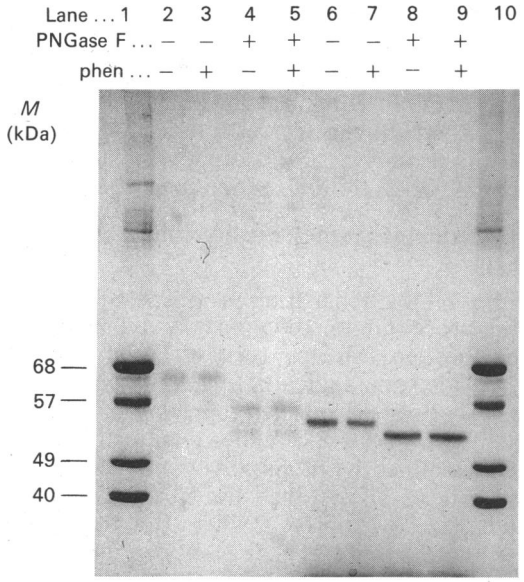

Fig. 2. Deglycosylation of purified rat liver bilirubin UDPGT by 2 units of PNGase

Lanes 1 and 10, protein molecular-mass (*M*) standards (albumin, 68 kDa; pyruvate kinase, 57 kDa; fumarase, 49 kDa; aldolase, 40 kDa). Lanes 2–5 contained 2 μg of fetuin, and lanes 6–9 contained 2 μg of bilirubin UDPGT. Proteins were subjected to SDS/polyacrylamide-gel electrophoresis on 7% gels and stained with Coomassie Blue. In some cases 1,10-*o*-phenanthroline (phen) was present.

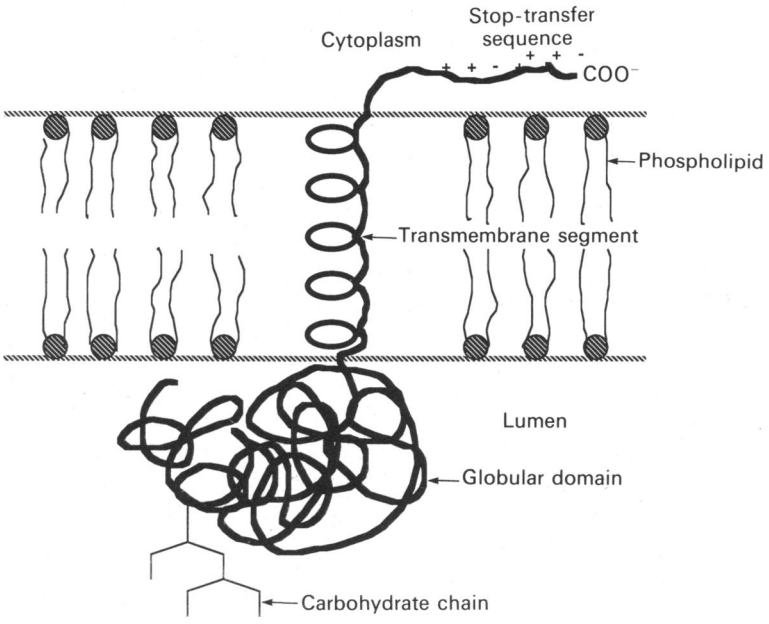

Fig. 3. Model of the transmembrane topology of bilirubin UDPGT

The model presented was devised from the evidence reported here and analysis of hydropathicity plots [22] of amino acid sequences of other isoenzymes [19] in this family.

completely sequenced, and a hydropathicity plot [22] suggests that there may be only one membrane-spanning region near the *C*-terminal end, the tail of which is highly charged and may be responsible for retention of such proteins in the ER [23; the present Fig. 3]. Although prediction is a reasonable first proposal of a topological model, improved programmes [24] might provide more structural information. Our biochemical analyses only indicated that the majority of the protein was not exposed on the cytoplasmic surface of microsomal vesicles.

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