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Expression of a human liver cDNA encoding a UDP-glucuronosyltransferase catalysing the glucuronidation of hyodeoxycholic acid in cell culture

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A cDNA encoding a human liver UDPGT (HLUG 25) transcribed and translated in vitro showed that the encoded protein was synthesized as a precursor and was cleaved and glycosylated when dog pancreatic microsomes were present during translation. The UDPGT cDNA was transiently expressed in mammalian cell culture (COS-7 cells) resulting in the biosynthesis of a polypeptide of 52 kDa. This expressed UDPGT glycoprotein catalysed the glucuronidation of hyodeoxycholic acid forming an ether glucuronide. These results suggest that this UDPGT isoenzyme may be responsible for the glucuronidation of 6α-hydroxy bile acids in human liver.

Glucuronosyltransferase; Bile acid; Expression; Cloning; Substrate specificity; (COS 7-cell)

1. INTRODUCTION

Bile acid glucuronides are excreted in urine of healthy humans where they represent 12 to 36% of the total daily bile acid excretion [1]. Moreover an increase in the rate of glucuronides formed, compared to glucosides, occurs in certain hepatobiliary diseases [2,3]. A major proportion of the normal bile acids found in urine are hydroxylated at C-6 of the B ring of the steroid, and 3α,6α-dihydroxy-5β-cholanic acid (hyodeoxycholic acid) has recently been identified as a quantitatively important bile acid [1]. Among the bile acids orally administered to healthy subjects, hyodeoxycholic acid can be distinguished by its high rate of glucuronidation and urinary excretion.

The high UDP-glucuronosyltransferase (UDPGT, EC 2.4.1.17) activity in vitro towards hyodeoxycholic acid found in human liver and kidney microsomes is in good agreement with in vivo data and may explain the rapid clearance of hyodeoxycholic acid in urine [4–6]. Consequently it became apparent that glucuronidation of bile acids at the 6α-hydroxy position, rather than the 3α-hydroxyl group represented a major pathway of bile acid glucuronidation in man [1].

In this paper, we present evidence that a cloned, expressed human UDPGT isoenzyme catalyses the ether glucuronidation of bile acids.

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Abbreviations: UDPGT, UDP-glucuronosyltransferase; HLUG25, human liver UDPGT cDNA; SV40, Simian Virus early gene promoter; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Chemicals*

Hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholan-24-oic acid), chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24- oic acid), ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24- oic acid), cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) were

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kindly provided by Dr P. Ross (Ninewells Hospital, Dundee, Scotland). Testosterone, androsterone, oestrone, 1-naphthol and 2-naphthol were from Sigma (Poole, Dorset, England).

2.2. *Cloning of full-length cDNA HLUG25*

A cDNA clone (HLUG25) encoding the complete sequence of a human liver UDPGT was isolated in this laboratory from a λgt11 human liver cDNA library as previously described [7].

2.3. *Expression of HLUG25 in transfected COS-7 cells*

The partial *Eco*RI digest of HLUG25 cDNA was subcloned in a modified Bluescript vector including flanking *Hin*dIII sites. The cDNA fragment including the flanking *Hin*dIII sites of this vector was ligated into the *Hin*dIII site of the expression vector pKCRH2. The correct and opposite orientations of HLUG25 cDNA with respect to the SV40 early gene transcription promoter in pKRH2 were determined by restriction mapping of plasmid minipreps before large scale DNA preparation of the (+) and (−) recombinant plasmids.

COS-7 cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% (v/v) foetal calf serum, 2 mM glutamine and 50 U/ml penicillin/streptomycin. The recombinant plasmid (30 μg/140 mm dish) was transfected to 70% confluent cells by the $CaPO_4$/glycerol shock procedure [8]. Hepes buffer, pH 7.2 (50 mM), was added to the medium to maintain constant pH. Cells were harvested 2 days after transfection, washed in 138 mM NaCl/2.7 mM KCl/1.5 mM potassium dihydrogen phosphate/8 mM disodium hydrogen phosphate/HCl, pH 7.4 (PBS) and pelleted by centrifugation at 1000 × *g* for 15 min. Disruption of the cells was achieved by a three times freezing (−50°C)-thawing (37°C) cycle before storage at −80°C.

2.4. *Labelling and immunoprecipitation*

At 72 h post-transfection COS-7 cells were washed, incubated 1 h in methionine free DMEM medium and labelled for 4 h with 50 μCi of L-[^{35}S]methionine (1420 Ci/mmol). Cells were rinsed twice in ice-cold PBS, harvested and centrifuged at 1500 × *g* for 15 min. The pellets were resuspended, washed and pelleted again. The pellet was solubilised with 0.5% (w/v) deoxycholate, 1% Triton X-100, 0.1% (w/v) SDS in 10 mM Tris-HCl, pH 7.4, by gentle hand homogenisation. The mixture was centrifuged at 16000 × *g* and the supernatant was incubated with preimmune serum and with sheep anti-rat UDPGT antiserum as previously described, except that incubations with antiserum were overnight in ice [9]. The final products were analysed by SDS gel electrophoresis on 7.5% gels [10] followed by fluorography [11].

2.5. *In vitro transcription/translation of HLUG25 cDNA*

The partial *Eco*RI digest of HLUG25 cDNA was ligated into the *Eco*RI site of the plasmid Gemini 1 (Promega Biotec, Madison, WI, USA). After transformation into *E. coli* JM 101, plasmids containing inserts in the correct orientation for transcription of a functional RNA and those in the reverse orientation were isolated and linearised with *Eco*RV. RNA was synthesized in vitro by T7 RNA polymerase, capped using G5′ppp5′ and added to a messenger-dependent reticulocyte lysate system with 15 μCi of L-[^{35}S]methionine in the presence or absence of dog pancreas microsomes [12,13]. Immunoprecipitation and SDS-PAGE of the translation products were carried out as described above.

2.6. *UDPGT activity assay in the transfected COS-7 cells and human microsomes*

The cell homogenate (protein content measured according to the procedure of Lowry et al. [14]) was assayed for UDPGT activities by a thin-layer chromatographic method as previously described [15]. Incubation conditions were the same as in [16], except that the reaction was performed using 18 μM (8 μM ^{14}C-labelled, 0.15 μCi) UDP-glucuronic acid, 0.5 mM aglycone and 0.1 mg cell homogenate protein in 100 mM Tris/maleate, 10 mM $MgCl_2$, 2 mg/ml bovine serum albumin buffer, pH 7.0, in the presence of egg lecithin liposomes (0.2 mg/mg protein) to facilitate access of the bile acids to the enzyme. The enzyme reaction was stopped after 60 min at 37°C. This low concentration of UDP-glucuronic acid was used to improve the sensitivity of the assay by allowing a greater proportion of the radioactive label to be incorporated into the glucuronides synthesized (limit of detection: 0.1 pmol/min per mg protein). The assay occurred at linear rates under these conditions. Human liver microsomes were prepared as previously described [17] and protein content measured according to Lowry et al. [14]. Glucuronidation of the various substrates in human liver microsomes was evaluated as described above except protein content was 0.05 mg per assay and incubation time was reduced to 15 min.

3. RESULTS AND DISCUSSION

3.1. *Expression of UDPGT protein in vitro and in COS-7 cells in culture*

The human liver HLUG25 cDNA (2104 base-pairs long) [7] was expressed in both in vivo and in vitro systems. HLUG25-RNA was transcribed from a Gemini vector and, when translated in vitro, produced a 53 kDa polypeptide (fig.1A, lanes 1 and 2). When dog pancreatic microsomes were added to the translation mixture primed with HLUG25, a higher rate of translation occurred as can be seen by comparison of the radiolabelled translation products in lanes 1 and 3 (fig.1A), which correspond to the same exposure times. The comparison of lanes 1 and 2 with lane 3 revealed two additional faster migrating polypeptides of 50 and 52 kDa, when the translation was performed in the presence of dog pancreatic microsomes. They correspond to the cleaved protein and cleaved plus glycosylated protein, respectively, in agreement with the presence of a signal sequence and one potential *N*-glycosylation site identified from the nucleotide sequence of the cDNA [7]. Radiolabelled proteins immunoprecipitable with specific anti-UDPGT antisera could only be seen with HLUG25-RNA in the correct orientation (not shown). The other minor products which were

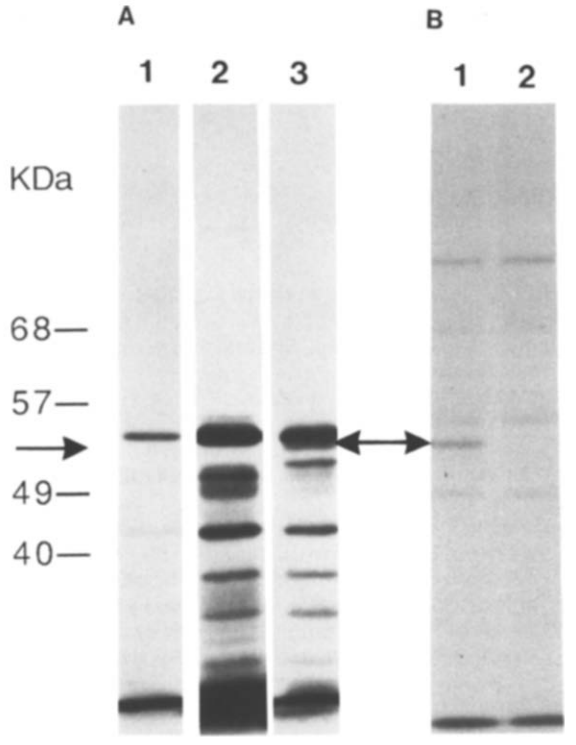

Fig.1. Expression of HLUG25 cDNA in vitro and in vivo. (A) HLUG25 mRNA was translated in the absence (lanes 1 and 2) or presence (lane 3) of dog pancreatic microsomes. The translation mixture was analysed by SDS-PAGE and visualised by autoradiography. Lanes 1 and 2 are the same except exposure time was respectively overnight and three days, lane 3 corresponds to an overnight exposure time. (B) COS-7 cells were translated with HLUG25 cDNA, proteins were labelled with L-[^{35}S]methionine 72 h later and UDPGT was specifically immunoprecipitated. The immunoprecipitates were analysed by SDS-PAGE and visualised by autoradiography. Lanes 1 and 2 correspond respectively to cells being transfected with the expression vector including the HLUG25 cDNA in the correct and reverse orientation. The arrow indicates the expressed 52 kDa protein.

precipitated with this antibody appear to result from incorrect initiation of translation or from proteolytic degradation. They are likely to be artefacts of the in vitro system as they are not observed by in vivo expression in mammalian cell cultures (fig.1B).

The HLUG25 cDNA expressed in the mammalian COS-7 cells allowed synthesis of a labelled immunoprecipitable glycopeptide of 52 kDa (fig.1B, lane 1), corresponding to the matured and enzymatically active UDPGT isoform.

3.2. *Substrate specificity of the UDPGT expressed in COS-7 cells*

The transfection and expression of the UDPGT cDNA in cell culture provided a stable source of a catalytically active single enzyme for the study of its individual substrate specificity.

Table 1 shows that the transferase synthesized from HLUG25 cDNA COS-7 cells effectively catalysed the glucuronidation of hyodeoxycholic acid, whereas no UDPGT activity was detected in the control mock-transfected cells using this substrate. This radioactive conjugate was hydrolysed by treatment with rat preputial β-glucuronidase. The hydrolysis was specifically inhibited by saccharo-1,4-lactone (not shown) indicating that this conjugate was a glucuronide. This bile acid has several potential sites of conjugation. We tested other bile acids and steroids with different combinations of hydroxyl groups on the steroid nucleus in the 3, 6, 7, 12 and 17 positions. Human liver microsomes were able to glucuronidate the bile acids and steroid hormones tested under the conditions used (table 1). COS-7 cells transfected with HLUG25 only expressed UDPGT activity towards hyodeoxycholic acid. However, some of the compounds used were glucuronidated at low rates by liver microsomes

Table 1

Catalytic activity of the expressed UDPGT in COS-7 cells

	UDPGT activity		
	Human liver microsomes (pmol/min per mg protein)	Mock transfected COS-7 cells (pmol/min per mg cell protein)	HLUG25 COS-7 cells (pmol/min per mg cell protein)
Hyodeoxycholic acid	449.0	ND	1.8
Lithocholic acid	8.6	ND	ND
Ursodeoxycholic acid	2.5	ND	ND
Chenodeoxycholic acid	5.0	ND	ND
Cholic acid	ND	ND	ND
Androsterone	98.3	ND	ND
Testosterone	39.1	ND	ND
Oestrone	12.7	ND	ND

ND, not detectable. Results show the mean values obtained using four batches of transfected cells and a single preparation of human liver microsomes

and it is likely that only androsterone glucuronidation would have been detected due to the limits of sensitivity of the assay system. Therefore, we could not assess most of the bile acids as substrates for the expressed UDPGT. We found detectable UDPGT activities towards 1-naphthol and 2-naphthol in the HLUG25 COS-7 cells at the same level as the activity in the control transfected cells (not shown). It thus appears that the UDPGT activity towards 1- and 2-naphthol was not due to the newly expressed UDPGT isoform.

The 3α-hydroxy position represents the primary site of conjugation of the steroids lacking 6α-hydroxyl group. Androsterone, which has been shown to be glucuronidated by a specific UDPGT isoenzyme conjugating 3α-steroids in rat and human [18,19], was not apparently a substrate for the expressed HLUG25. It has been shown that monohydroxylated bile acids could also form carboxyl-linked glucuronides [20]. However, it was recently demonstrated by gas chromatography/mass spectrometry analysis that the glucuronides of hyodeoxycholic acid enzymatically synthesized with microsomes of human liver and kidney were exclusively conjugated at the 6α-hydroxy position [6]. The hyodeoxycholate conjugate was not alkali-labile after an overnight exposure to ammonia vapour [21], whereas bilirubin glucuronides were ammonia-lysed (not shown), suggesting that the bile acid glucuronide formed was not ester linked.

Hyodeoxycholic acid is a secondary bile acid formed from hyocholic acid by bacterial 7α-dehydroxylation [22] or by 6α-hydroxylation from lithocholic acid [23]. It is tempting to speculate that 6α-glucuronidation plays a major physiological role in conditions where 6α-hydroxylase activity is highly expressed, like in the foetus, or when the biliary excretion of 3α-glucuronides of bile acids is impaired, as in cholestatic disease. The cloning and expression of a specific isoform catalysing the glucuronidation of 6α-bile acids, provides the appropriate probe for regulation studies and will help in particular to investigate whether or not a possible coupling of 6α-hydroxylation and 6α-glucuronidation is an important detoxification process in man.

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REFERENCES

[1] Almé, B. and Sjovall, J. (1980) J. Steroid Biochem. 13, 907–913.
[2] Frohling, W. and Stiehl, A. (1976) Eur. J. Clin. Invest. 6, 67–74.
[3] Almé, B., Nordén, Å. and Sjovall, J. (1978) Clin. Chim. Acta 86, 251–259.
[4] Parquet, M., Pessah, M., Sacquet, E., Salvat, C., Raizman, A. and Infante, R. (1985) FEBS Lett. 189, 183–187.
[5] Parquet, M., Pessah, M., Sacquet, E., Salvat, C. and Raizman, A. (1988) Eur. J. Biochem. 171, 329–334.
[6] Marshall, H.U., Matern, H., Egestad, B., Matern, S. and Sjovall, S. (1987) Biochim. Biophys. Acta 921, 392–397.
[7] Jackson, M.R., McCarthy, L.R., Harding, D., Wilson, S., Coughtrie, M.W.H. and Burchell, B. (1987) Biochem. J. 242, 581–588.
[8] Parker, B.A. and Stark, G.R. (1979) Virology 31, 360–369.
[9] Jackson, M.R., Kennedy, S.M.E., Lown, G. and Burchell, B. (1986) Biochem. Pharmacol. 35, 1191–1198.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Skinner, M.K. and Griswold, M.D. (1983) Biochem. J. 209, 281–284.
[12] Walter, P., Ibrahimi, I. and Blobel, G. (1981) J. Cell. Biol. 91, 545–551.
[13] Clemens, M.J. (1984) in: Transcription and Translation: A Practical Approach (Hames, B.D. and Higgins, S.J. eds) pp.231–270, IRL Press, Washington.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.R. (1951) J. Biol. Chem. 193, 265–275.
[15] Bansal, S.K. and Gessner, T. (1980) Anal. Biochem. 109, 321–329.
[16] Jackson, M.R., Fournel-Gigleux, S., Harding, D. and Burchell, B. (1988) Mol. Pharmacol., in press.
[17] Coughtrie, M.W.H., Burchell, B., Leakey, J.E.A. and Hume, R. (1988) Mol. Pharmacol., in press.
[18] Falany, C.N., Green, M.D., Swain, E. and Tephly, T.R. (1986) Biochem. J. 238, 65–73.
[19] Mackenzie, P.I. (1986) J. Biol. Chem. 261, 6119–6125.
[20] Radominska-Pyrek, A., Zimniak, P., Chari, M., Golunski, E., Lester, R. and Pyrek, J.S. (1986) J. Lipid Res. 27, 96–100.
[21] Odell, G.B., Cukier, J.O. and Gourley, G.R. (1981) Hepatology 1, 307–315.
[22] Bergstrom, S., Danielsson, H. and Goranson, A. (1959) Acta Chem. Scand. 13, 776–783.
[23] Elliott, W.H. (1985) in: Sterols and Bile Acids (Danielsson, H. and Sjovall, J. eds) pp.303–329, Elsevier, Amsterdam.