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Isolation and characterisation of a new hepatic bilirubin UDP-glucuronosyltransferase

Absence from Gunn rat liver

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A novel UDP-glucuronosyltransferase that conjugates bilirubin IXα, bilirubin monoglucuronide and an arylalkanoic acid was purified to homogeneity from clofibrate treated Wistar rats. The enzyme displayed a subunit molecular mass of 54 kDa, a pI of 7.6 and was demonstrated to be *N*-glycosylated. Sequence analysis of peptides derived by endoproteinase Glu-C cleavage of the purified enzyme indicated that it was a new member of the recently identified *UGT1* subfamily. Immunoblot analysis demonstrated that this enzyme was absent from Gunn rat liver. The molecular derivation of this enzyme and the lack of it in Gunn rats is discussed.

UGT1 subfamily; Arylalkanoic acid; Glycosylation; Congenital jaundice; Acylglucuronide

1. INTRODUCTION

UDP-glucuronosyltransferases (UDPGTs, EC 2.4.1.17) are a family of isoenzymes which are responsible for the glucuronic acid conjugation of potentially toxic xenobiotic and endogenous compounds allowing their excretion in bile and urine [1].

Bilirubin UDPGT activity performs the critical function of catalysing the glucuronidation of bilirubin IXα, the toxic end product of haem catabolism thereby facilitating its safe elimination from the body [1]. Jaundice (hyperbilirubinaemia) caused by the genetic deficiency of this enzyme activity has been demonstrated to result in serious physiological consequences that can lead to death [1]. Indirect evidence such as the additive inheritance of bilirubin UDPGT inducibility in mice [2] and the two peaks of bilirubin UDPGT activity observed by chromatofocusing solubilised microsomes [3] have indicated the existence of more than one bilirubin UDPGT in rodents. Recently a short communication describing a rat bilirubin UDPGT cDNA has appeared [4]. In our laboratory we have previously reported the existence of a UDPGT that mediates the glucuronidation of bilirubin and arylalkanoic acids, which was absent from congenitally jaundiced Gunn rats [5]. Analysis of this transferase is important due to the possible toxicological consequences of arylcarboxylic acids causing aberrations in bilirubin excretion and due to it catalysing the formation of acylglucuronides which have been implicated in toxic drug reactions [6]. Here we report the isolation and biochemical analysis of this enzyme which represents a second bilirubin UDPGT in rat liver and discuss the molecular basis for its absence in the mutant Gunn rat.

A preliminary communication of this study was reported previously [7].

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2. MATERIALS AND METHODS

2.1. *Materials*

Bilirubin, UDP-glucuronic acid (ammonium salt), Nitro blue tetrazolium, 5-bromo-4-chloro-3-indoyl phosphate and alkaline phosphatase-conjugated rabbit anti-goat IgG were all obtained from Sigma (Poole, UK). Ethylanthranilate was from Kodak (Rochester, NY). Nitrocellulose was obtained from Anderman (Kingston-upon-Thames, Surrey, UK). Bovine serum albumin fraction V and glycopeptide *N*-glycosidase F were from Boehringer Mannheim (Sussex, UK).

Egg yolk phosphatidylcholine was from Lipid Products (Surrey, UK). Lubrol 12A9 was from ICI Organics Division (Manchester, UK). Ultrafiltration apparatus was from Amicon (Gloucestershire, UK). Mono P HR5/20 columns, Polybuffer 96, Q-Sepharose fast flow, Sephacryl S-300, Sephadex G25M PD10 columns and FPLC system were from Pharmacia (Milton Keynes, UK). All other chemicals were of analytical grade from Merck (Poole, UK).

2.2. *Animals*

Wistar and Gunn rats were from the colonies maintained at the Animal Unit, Ninewells Hospital and Medical School, Dundee.

2.3. *Enzyme assays and protein determination*

Bilirubin and 7,7,7-triphenylheptanoic acid glucuronidation were

measured as previously described [5]. HPLC analysis of bilirubin conjugates was by the method of Odell et al. [8]. Phosphatidylcholine reconstitution of purified UDPGT fractions was as described in [9].

Protein content of microsomal samples was determined by the method of Lowry et al. [10] and in purified fractions by the Bradford method [11] using bovine serum albumin as standard.

2.4. *Electrophoresis and immunoblot analysis*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis using an alkaline phosphatase linked detection system were carried out as described previously [12].

The production and characterization of anti-rat liver UDPGT (RAL1), anti-rat kidney bilirubin/phenol UDPGT (RAK1) and anti-rat testosterone UDPGT antibodies have been described elsewhere [13–15].

2.5. *Large scale purification of bilirubin UDPGT (pI 7.6)*

Solubilised microsomal fractions isolated from the livers of 25–30 male Wistar rats (200–300 g) pretreated with clofibrate were used for the preparation of a 20–60% ammonium sulphate precipitated microsomal fraction as previously described [5]. The final ammonium sulphate precipitate was dissolved in and extensively dialysed against 20 mM Tris-HCl, pH 8.0, 0.05% w/v Lubrol PX, 5 mM mercaptoethanol, 5% v/v glycerol (buffer A). The resultant fraction was then subjected to FPLC anion-exchange chromatography using a high-capacity Q-Sepharose fast-flow and a Pharmacia XK column (5×50 cm) at 200 ml/h. Bilirubin UDPGT activity was eluted from the column using a 4 litre linear gradient of 0–0.4 M KCl in buffer A. This allowed the partial resolution of bilirubin UDPGT activity from other UDPGT activities producing a similar elution profile to that previously obtained with DEAE cellulose [5] but greatly increasing the yield. Selected fractions were concentrated and applied to a Sephacryl S-300 column (1.5×66 cm) in buffer A with 0.1 M NaCl and bilirubin UDPGT activity eluted at a flow rate of 10 ml/h. Bilirubin UDPGT enriched fractions from the gel filtration step were applied to a Mono P chromatofocusing column fitted to an FPLC system and a pH gradient of 9.0–6.0 over 35 ml was generated according to the manufacturer's instructions (Pharmacia) with the inclusion of 0.05% w/v Lubrol PX, 5 mM 2-mercaptoethanol, 5% v/v glycerol in the buffers. Buffer A was 74 mM Tris-Cl pH 9.3, Buffer B was 10% polybuffer 96 pH 6.0.

2.6. *Deglycosylation with peptide N-glycosidase F*

The purified protein (2 μg) was incubated with 2 U of peptide *N*-glycosidase F (EC 3.2.2.18) as previously described [16] in the presence of 10 mM 1,10-*o*-phenanthroline for 18 h at 37°C.

2.7. *Preparation of peptides and sequence analysis*

The purified enzyme was fragmented with *Staphylococcus aureus* endoproteinase Glu-C using conditions that generated a relatively small number (<15) of large fragments (10–30 kDa) (0.05% SDS, 53 mM sodium phosphate pH 7.0, at a protease/protein substrate ratio of 0.2 at 18°C for 4 h). Cleaved peptides were purified by electroelution from Tricine gels [17,18]. The purified protein and derived peptide fragments were prepared and coupled via their N-terminus and lysyl side chains to *p*-phenylene diisothiocyanate activated glass and sequenced by automated solid-phase Edman degradation essentially as described by Findlay et al. [18].

3. RESULTS AND DISCUSSION

3.1. *Enzyme isolation and characterization*

FPLC chromatography of bilirubin UDPGT activity from ammonium sulphate fractionated microsomes by anion exchange and gel filtration led to a 20-fold purification of the activity (Table I). Chromatofocusing of the high activity fractions from the gel filtration step led to a major peak of bilirubin UDPGT activity eluting at pH 7.6. The specific activity of the purified preparation towards bilirubin and 7,7,7-triphenylheptanoic acid was 110–142 nmol/min/mg and 63–75 nmol/min/mg, (three preparations), respectively, representing a 129-fold purification over ammonium sulphate fractionated microsomes (Table I). HPLC analysis of bilirubin glucuronides formed by this enzyme indicated it was capable of both mono- and diglucuronidation of bilirubin (not shown).

On SDS-PAGE analysis of fractions corresponding to the pI 7.6 peak a single polypeptide of molecular mass 54 kDa was apparent (Fig. 1a). Incubation of this preparation with an enzyme that catalyses the cleavage of oligosaccharide moieties from *N*-linked glycoproteins caused a decrease in the apparent molecular mass of the protein of 2 kDa, (Fig. 1a) indicating it was a glycoprotein. The protein was immunoreactive to an antibody (RAK1) raised to phenol and bilirubin UDPGTs but not to one raised to testosterone UDPGT (not shown).

3.2. *Lack of enzyme expression in Gunn rat liver*

Previously we have demonstrated that the reactions catalysed by this purified enzyme were absent from congenitally jaundiced Gunn rat liver microsomes [5]. Immunoblot analysis with a broad specificity anti-UDPGT antibody (RAL1) indicated that there was no

Table I

Purification of hepatic bilirubin UDPGT (pI 7.6) from Wistar rats

Fraction	Total protein (mg)	Specific activity (U*/mg)	Total activity (U*)	Yield %	Purification factor
$(NH_4)_2SO_4$ fractionated microsomes	5148	1.1	5662	100	1
Q-Sepharose FF chromatography	175	13.9	2433	43	12.6
Sephacryl S-300 chromatography	63.2	22.4	1416	25	20.4
Chromatofocusing	1.94	142	275.5	4.9	129

*1 U represents 1 nmol of bilirubin glucuronide formed per min of incubation. Data were from a single representative experiment.

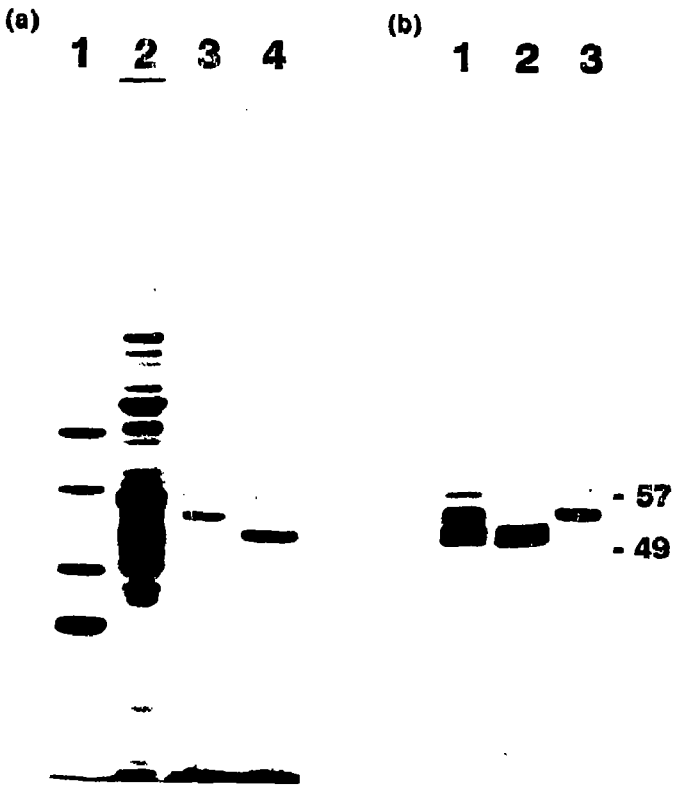

Fig. 1. Molecular analysis of the purified UDPGT and its absence in Gunn rats. (a) SDS-PAGE of the purified UDPGT and evidence of glycosylation. Lane 1, protein molecular weight standards: albumin (68 kDa), pyruvate kinase (57 kDa), fumarase (49 kDa) and aldolase (40 kDa); lane 2, 20 μg of Wistar rat liver microsomal protein; lane 3, 2 μg of purified bilirubin UDPGT; lane 4, 2 μg of purified bilirubin UDPGT treated with glycopeptidase-*N*-glycosidase F as described in section 2. Proteins were visualized using Coomassie blue. (b) Immunoblot analysis of liver microsomes prepared from Wistar and Gunn rats and the purified UDPGT. Lane 1, 20 μg of Wistar rat liver microsomal proteins; lane 2, 20 μg of Gunn rat liver microsomal proteins; lane 3, 0.5 μg of purified bilirubin UDPGT. The relative mobilities of molecular weight marker proteins in kDa, as determined on an identical Coomassie blue-stained gel run at the same time, are shown on the right.

immunoreactive protein in Gunn rat liver isolate that co-migrated with the purified protein in contrast to Wistar rat extracts (Fig. 1b). Thus the lack of enzyme activity towards 7,7,7-triphenylheptanoic acid and bilirubin appeared to be due to the absence of expression of this UDPGT isoenzyme.

3.3. *Evidence for the existence of a second bilirubin UDPGT in rats*

To determine whether or not this was the same bilirubin UDPGT that was cloned by Sato et al. [4] we prepared peptides for microsequence analysis. Sequencing of the native 54 kDa protein indicated that the sample was homogeneous as there was cleavage of only one amino acid per Edman degradation cycle. Comparison of this N-terminal sequence and amino acid sequences derived from endoproteinase Glu-C cleavage

A.

54kDa peptide	1	XKLLVIPIDGSHWL	14
26kDa peptide	1	XKILVIP	7
pSK1	26	G.v..f.me.....	39
4NPGT	26	D....v.Q.....	39
HUG-Br1	28	G.i.l..v.....	41
HUG-Br2	29	G.v..v.T...P..	42
RLUG 38	24	G.v..W.meF...m	37
A-18	30	G.....	43

B.

26.5kDa peptide	1	XXFYTMRKYPVXFQNEXVXAXF (L)	22
pSK1	68	GED.f.lqT.aFPyTK.EYQREI	90
4NPGT	68	ESKy.RRkSf..PynL.ElRtRy	90
HUG-Br1	70	DGA...lkT...P..R.D.KES.	92
HUG-Br2	71	EEK.f.lTA.a.PwTqKEFDRVT	93
RLUG 38	68	KSPD.KPETf.TSVSKdElENY.	90
A-18	72	EGS.....P....N.T.A.	94

C.

12kDa peptide	1	XTQXPAPLSYVPFXLXXNXXXMNF (K)	24
pSK1	180	EA..C.L.S..i.NL..TMLSDH.T.	204
4NPGT	178	HIG.S.S.v....rFYTKFSDH.T.	202
HUG-Br1	182	EA..C.N.f....rP.SSHSDH.T.	206
HUG-Br2	183	KG..C.N.S..i..L.TT.SDH.T.	207
RLUG38	182	SSGrFIL.P....VI.SGMGGP.T.	206
A-18	185	EA..C.....S.SS.TDR...	209

D.

28kDa peptide (a)	1	FEAYVNASGEHGIXXFXL	18
28kDa peptide (b)	1	AYVNASGEHXIVXFXLXXM	19
pSK1	287	E.....V.S.GS.	308
4NPGT	285	E.....V.S.GS.	306
HUG-Br1	289	E....i.....V.S.GS.	310
HUG-Br2	290	E....i.....V.S.GS.	311
RLUG38	292	dM.Df.qs.....v.V.S.GS.	313
A-18	290	E.....V.S.GS.	311

E.

14kDa peptide	1	XRXXXXTLNVLEMXXDXLXXA	21
4NPGT	403	ET.GAGV.....TA.D.EN.	424
pSK1	401	ET.GAGV.....TA.D.EN.	422
HUG-Br1	405	ETkGAGV.....TSeD.EN.	426
HUG-Br2	406	ETkGAGV.....TSeD.EN.	427
RLUG 38	408	VHkGAAV...iRT.SKSD.FN.	429
A-18	406	ET.GAGV.....K★	418

Fig. 2. Sequences of bilirubin UDPGT (pI 7.6) peptides and their homology with deduced amino acid sequences from UDPGT cDNAs. Sequence data from the bilirubin UDPGT peptides were aligned with similar regions of other UDPGTs. pSK1, rat bilirubin UDPGT [4]; 4NPGT, rat 4-nitrophenol UDPGT [19]; HUG-Br1, HUG-Br2, human bilirubin UDPGTs [20]; RLUG38, rat testosterone UDPGT [21]; A-18-truncated mutant Gunn rat UDPGT [22]. Two amino acids at the same position in the peptide's sequence denote two possible assignments derived from microsequence analysis. Numbers at the N-terminal and C-terminal of sequences denote positions from the amino terminal amino acid in the respective protein sequences. Identical residues with the bilirubin UDPGT (pI 7.6) peptides are indicated by a dot (•). Conservative replacements [23] are denoted by the lower case letter representing the particular amino acid, whereas more radical changes are shown in upper case. *N*-Linked glycosylation consensus sequences are underlined. The ★ indicates the relative position of the stop codon in the A-18 cDNA sequence.

peptides with deduced amino acid sequences of rat UDPGT cDNAs indicated that a novel bilirubin UDPGT had been isolated (Fig. 2). The 12, 26.5 and 54 kDa peptide sequences were most identical with a cloned human bilirubin UDPGT (HUG-Br1) (Fig. 2) The residues identified in the 14 and 28 kDa peptides were identical with rat 4-nitrophenol UDPGT and the other rat bilirubin UDPGT (Fig. 2) indicating that this

transferase belongs to the recently identified *UGT1* subfamily of UDPGTs that share identical C-terminal domains [24].

It was noteworthy that the amino-terminal residue of the native purified protein corresponded to the 24–30th residue of the cDNA derived protein sequences (Fig. 2). This suggested that like 4-nitrophenol UDPGT [19] and testosterone UDPGT [21] a signal peptide was removed during the biogenesis of this enzyme. Interestingly, the residues identified in the overlapping 28 kDa peptides contained an *N*-linked glycosylation consensus sequence Asn-Ala-Ser (Fig. 2) [25] with an asparagine HPLC elution profile characteristic of oligosaccharide modified asparagines in other glycoproteins (Keen, J.N. and Findlay, J.B.C., unpublished work).

3.4. *The molecular basis for the lack of the UDPGT in Gunn rats*

Whilst this manuscript was being prepared a very recent report by Iyanagi [22] described 3 new subfamily *UGT1* cDNAs from the Gunn rat with a 3'-terminal half identical to a mutant non-functional Gunn rat phenol UDPGT previously identified [26] (Fig. 2). The homologues of these new cDNAs in 'normal' rats have not been identified nor the function of the proteins they encode assigned.

Comparison of the partial amino acid sequences of the new bilirubin UDPGT presented in this paper with Iyanagi's sequences indicated that all but the 14 kDa peptide sequence were identical with the deduced protein sequence of the Gunn rat A-18 cDNA (Fig. 2). The non-identical sequence corresponded to the site of the proposed mutation that would result in a truncated Gunn rat UDPGT protein [22,26] (Fig. 2).

It therefore appears likely that this mutation was the cause of the absence of this enzyme from Gunn rats.

4. CONCLUDING REMARKS

This study has demonstrated that a second bilirubin UDPGT exists in rats which belongs to the recently identified *UGT1* subfamily, the members of which have been proposed to be produced via alternative splicing of a common transcript [24]. The lack of this and at least a further 4 *UGT1* gene subfamily members in Gunn rats [22] (Fig. 1) suggests that a single genetic mutation in these animals results in the loss of several UDPGT proteins.

The high identity of the new rat isoform reported here to a human bilirubin UDPGT (HUG-Br1) suggest that this isoform may mediate toxic acylglucuronide formation in man.

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