Volume 308, number 2, 161–164 FEBS 11419 August 1992

Characterisation of a human bilirubin UDP-glucuronosyltransferase stably expressed in hamster lung fibroblast cell cultures

Lesley Sutherland*, Sahidan bin Senafi, Thomas Ebner, Douglas J. Clarke and Brian Burchell

Department of Biochemical Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee, DD1 9SY, Scotland, UK

Received 29 June 1992

A cDNA encoding a human bilirubin UDP-glucuronosyltransferase has been isolated and stably expressed in Chinese hamster V79 lung fibroblast cell line. Western blotting of cell homogenates with anti-UGT antibody revealed a highly expressed protein of approx. 55.5 kDa in size. The expressed enzyme specifically catalysed the formation of bilirubin mono- and diglucuronides, and also catalysed the glucuronidation of two phenolic compounds, which are good substrates for other human UGT isoenzymes, at low rates.

Complementary DNA; Eukaryotic expression vector; Enzyme activity

1. INTRODUCTION

UDP-glucuronosyltransferases (UGT) are a large family of enzymes involved in the transfer of a glucuronide group, from the cofactor UDP-glucuronic acid (UDPGA), to a variety of endogenous and exogenous substrates. The increased polarity of the glucuronide facilitates its excretion into the bile or urine [1]. The glucuronidation of bilirubin, the end product of haem catabolism, is an extremely important biotransformation carried out by the liver. Aberrations in this function are manifested in Crigler–Najjar syndrome, a rare genetic human disease, in which the ability to glucuronidate bilirubin is severely reduced which results in neurotoxic consequences which may eventually lead to death [1]. Studies in this laboratory have sought to characterise the bilirubin UGT(s) involved in this important physiological function.

Only three UGTs have been purified to date from human liver that catalyse the glucuronidation of estriol, 4-aminobiphenyl [2] and 6α-hydroxy bile acids [3]. The purification of the elusive human bilirubin UGT has yet to be reported.

The study of human gene products has been greatly facilitated by the technology of cDNA cloning. The subsequent expression of the polypeptides encoded by these clones in heterologous mammalian tissue culture systems provides a means whereby the biochemistry of these proteins can be studied, something which is often not possible due to the scarcity of human tissue and the instability of proteins during purification. In this respect, the construction of systems which stably express the protein of interest have been particularly useful [4].

Harding et al. [5] reported the cloning and expression of the first UGT cDNA (termed HP1) from human liver. Subsequent work revealed a second, closely related isoform (HP2), also to be present in this tissue [6]. These two isoforms were extremely interesting because they shared identical 3′ halves, while having only approximately 48% identity in their 5′ half. This strongly suggested that the mRNAs encoding these isoforms were produced by alternative splicing of a primary transcript. Further work revealed there to be two more members of this gene family (HP3 and HP4), all sharing identical 3′ sequences [4,7].

Here we report the stable expression of one of these cDNA clones (HP3), which encodes a bilirubin transferase, in a tissue culture expression system. We have used this system to examine some of the biochemical properties of this important isoenzyme.

2. MATERIALS AND METHODS

2.1. *Screening of cDNA libraries*

The human liver cDNA library used for this study was a λUNIZAP library, purchased from Stratagene (La Jolla, CA, USA).

The protocols used to screen the library, and to obtain full-length clones, have been described in detail elsewhere [4,7].

2.2. *Restriction digestions*

All restriction endonucleases used in this study were purchased from Promega Corporation (Madison, USA). Reactions were carried out under the conditions recommended by the manufacturer.

* *Present address:* Institute of Genetics, University of Glasgow, Church street, Glasgow, G11 5JS, UK.

Correspondence address: B. Burchell, Department of Biochemical Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee, DD1 9SY, Scotland, UK. Fax: (44) (382) 644620.

2.3. *Construction of plasmids and transfection of UGT HP3 into tissue culture cells*

Clone pBluescript HP3 were prepared from the original λUNIZAP clone by in vivo excision of the pBluescript sequences according to the manufacturers instructions. The full-length HP3 cDNA (including non-coding DNA) was released from pBluescript HP3 by digestion with the restriction endonucleases *Bam*H1 and *Xho*I and ligated [6] into the eukaryotic expression vector pCDNA1neo (Invitrogen) to give construct pCDNA1neoHP3(+), in which the HP3 fragment was in the correct orientation to give expression from the vector CMV promoter/enhancer. Cloning of the *Bam*HI/*Hin*dIII pBluescriptHP3 fragment into the pCDNA1neo polylinker produced a control construct with the HP3 insert in the opposite (−) orientation [pCDNA1neoHP3(−)].

Plasmid DNA was propagated in the *E. coli* host strain MC1061 [6] under selection for both kanamycin and ampicillin (both 50 μg/ml, Sigma, UK) resistance. Plasmid was isolated using the alkaline lysis method with purification on CsCl gradients [8].

Chinese hamster V79 lung fibroblasts were routinely grown as described previously [9]. Recombinant plasmid DNA (5 μg) was linearised with *Sac*II and mixed with the transfection reagent DOTAP (Boehringer Mannheim) to facilitate introduction into the cell line and recombination into the genome. Stably transfected colonies (G418-resistant) were isolated and cultured as described by Fournel-Gigleux et al. [9]. Cells were maintained in selective medium until harvesting.

2.4. *Analysis of tissue culture cell homogenates and liver microsomes*

V79 cells stably transfected with HP3 cDNA were grown to 70% confluency and then harvested. Cells were washed twice with PBS solution prior to further analysis. Liver microsomes were prepared from tissue samples as previously described [1]. SDS-polyacrylamide gel electrophoresis and Western blotting of the tissue samples was as described previously [7,9].

Assay of UGT activities in cell homogenates and human liver microsomes was determined by TLC assay [10] using 0.5 mM substrate and 2 mM UDP glucuronic acid (containing 0.2 μCi/0.66 nmol ^{14}C label from NEN, UK). Bilirubin UGT activity was determined by incubation of cell homogenates or human liver microsomes with 122 μM bilirubin and 10 mM UDP glucuronic acid; the glucuronides formed were analysed by HPLC as previously described by Odell [11]. Protein levels were assayed by the Lowry method [12] using bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

3.1. *Isolation of a full length cDNA encoding human bilirubin UDPGT*

A DNA probe prepared from the human UGT cDNA HP2 [6] was used to screen a human liver λ-Unizap cDNA library under conditions of relaxed stringency. A number of related UGT cDNA's were isolated using this procedure. One clone, SH48, was judged, by comparison with other cloned UGT [5,6], to be long enough to encode a full-length UGT protein, with 18 nucleotides upstream of the AUG initiation codon. Clone SH48 was identical with sequences which had previously been characterised in this laboratory as partial UGT cDNAs and classified as HP3 [4] and to the HBr1 cDNA isolated by [13]. We therefore decided to use clone SH48 in a study of the activity of isoform HP3 in a tissue culture expression system.

3.2. *Expression of UGT-HP3 in tissue culture cells*

To check that the pCDNA1neoHP3 constructs were expressing UGT-HP3 correctly, Cos-7 cells were transiently transfected with plasmids pcDNA1neoHP3(+) and pcDNA1neoHP3(−). Transfected cells were harvested and assayed for UDPGT activity with bilirubin provided as the substrate. Cells which had been transfected with pcDNAneoHP3(+) showed high UGT activity towards bilirubin, whereas, as expected, the pcDNA1neoHP3(−) transfected cells were inactive towards bilirubin (9 data not shown). The same HP3 expression constructs were therefore used to stably transfect V79 cells using G418 resistance as the dominant selectable marker. V79 cells had been assayed previously and found to have no detectable UDPGT activity towards bilirubin. Six G418^r colonies were isolated and the cells from each were grown and assayed for bilirubin UGT activity. Five of the six cell lines were found to have high UDPGT activity towards this substrate. The cell line V79-HP3.3 was chosen for further study because it showed the highest activity, comparable to those found in human liver microsomes (Table I). The activity of this cell line appeared to be stable with increasing passage number, providing the G418 selection was maintained (data not shown).

Western blotting of cell homogenate from this line, using an anti UGT antibody, showed high levels of a major immunoreactive protein, 55.5 kDa, to be present (Fig. 1). Some smaller molecular weight proteins were also immuno-detected which are presumably degradation products of the major protein. The expressed bilirubin UGT protein exhibited a similar molecular weight to the expressed phenol UGTs, HP1 was 55 kDa and HP4 was 56 kDa (Fig. 1).

3.3. *UGT-HP3 catalyses the glucuronidation of bilirubin*

The ability of UGT HP3 to glucuronidate the endogenous substrate bilirubin was determined by HPLC assay of V79-HP3 cells. A typical HPLC elution profile is shown in Fig. 2. The results show that UGT-HP3 catalysed the conjugation of glucuronic acid with bilirubin at a rate comparable to that observed with human liver microsomes (Table I). This high activity system avoids the potential problems associated with using extremely low UDP-glucuronic acid concentrations, large amounts of protein or excessively long incubation times, required by other expression systems [13]. No activity towards bilirubin was detected in transfected V79 cells stably expressing other UGT-HP proteins (Table I).

In humans, approximately 80% of bilirubin conjugates excreted in bile are diglucuronides and the remainder being monoglucuronides [14] UGT-HP3 homogenates clearly catalysed the formation of both mono- and diglucuronides in in vitro incubation mixtures. Previously the question has arisen whether the formation of the two natural bilirubin monoglucuronide isomers (C-8 and C-12) depends on discrete enzyme forms. In this study we unequivocally demonstrate that both these

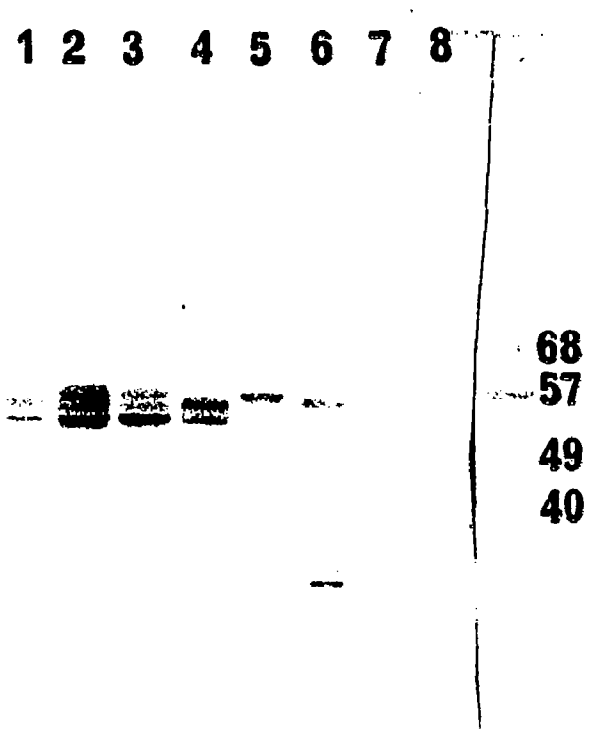

Fig. 1. Immunoblot analysis of human liver microsomes and V79 cells stably expressing UGTs using an anti-UGT antibody. Lanes 1–3, human liver microsomes from three different individuals (20 μg); lane 4, human kidney microsomes (20 μg); lane 5, V79 cells expressing UGT HP4; lane 6, homogenate V79 cells expressing HP3; lanes 7 and 8, V79 cells expressing HP1. Lanes 4–7, 20 μg, lane 8, 40 μg of cell homogenate protein was loaded. Molecular weight markers, from the same gel used for the immunoblot, stained with Ponceau red are shown on the right.

isomers are formed by a single human UGT. The percentage of bilirubin diglucuronides formed (53%) was greater than formed by incubation with human liver microsomes (36%) under equivalent conditions. The proportion of diglucuronides formed during in vitro incubations with liver extracts is dependent on the concentration of substrates used in the assay [15], and this variation of product dependent on substrate concentration was also demonstrated using V79-HP3 cell homogenates (data not shown). Incubation of whole cells in culture with bilirubin may produce the glucuronide formation pattern observed in vivo.

3.4. *Activity of UGT-HP3 towards xenobiotic substrates*

The activity of the UGT-HP3 protein towards a further three substrates was tested by TLC assays. Other cell lines stably expressing two other UGT-HP proteins (HP1 and HP4) were also studied as comparative controls. The results are shown in Table I. UGT-HP3 showed a small level of activity towards 1-naphthol but this substrate was more specifically glucuronidated by UGT-HP1. 4-Methylumbelliferone was observed to be relatively non-specifically glucuronidated at a similar rate by all UGT isoenzymes confirming the value of this compound for general detection of glucuronidation, but also that it is useless as a specific probe for an individual UGT isoform. The conjugation of 1-naphthol and 4-methylumbelliferone by UGT-HP3 indicates that as well as forming ester glucuronides of bilirubin, this enzyme was also capable of the conjugation of small phenols. Such a result indicates that saturation of this en-

Table I

Substrate specificity of UGTs stably expressed in V79 cells

Substrates	UGT activities (nmol/min/mg protein)			
	HP1	HP3	HP4	HLM
Bilirubin	n.d.	0.4	n.d.	0.9 ± 0.5
Propofol	n.d.	n.d.	0.9	1.8 ± 1.1
4-Methylumbelliferone	1.1	0.8	1.5	26 ± 6.3
1-Naphthol	3.2	0.3	0.2	5.7 ± 3.2

Data reported for cell homogenates are the average of three separate preparations. HLM, data from six preparations of human liver microsomes and S.D.

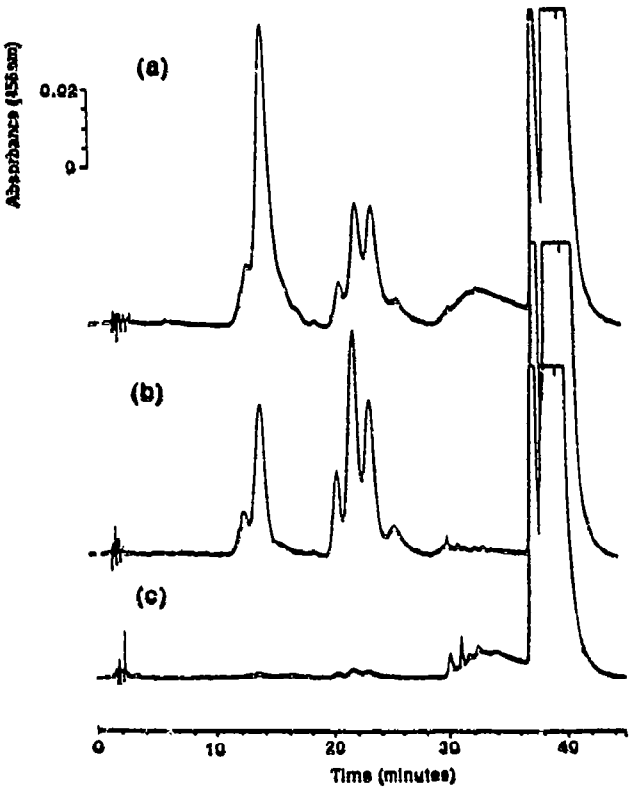

Fig. 2. HPLC elution profile of bilirubin glucuronides formed by V79-HP3 cells and human liver microsomes. (a) V79-HP3 cells; (b) human liver microsomes; (c) untransfected V79 cells. The bilirubin diglucuronides eluted between 12 and 14 min. The C8, C12 bilirubin monoglucuronides eluted between 20 and 25 min. Unconjugated bilirubin eluted at 37–41 min.

zyme by phenols (including plant alkaloids and drugs) may lead to jaundice.

Propofol (an intravenous anaesthetic), which appears to be a specific substrate for HP4, was not glucuronidated by the HP3 encoded protein.

4. CONCLUDING REMARKS

We have established a tissue culture system in which the human UGT protein HP3 is stably expressed at high levels, which provides us with a very useful model system for the study of this important human enzyme. The tissue culture system allows us to evaluate the substate specificity of this enzyme and to assess its potential role in glucuronidation of other chemicals, including drugs.

Acknowledgements: We gratefully acknowledge the financial support of the Scottish Home and Health Department, Medical Research Council and the Wellcome Trust. T.E. was sponsored by the Robert Bosch Foundation, Stuttgart, Germany, and S.S. was supported by National University of Malaysia.

REFERENCES

[1] Burchell, B. and Coughtrie, M.W.H. (1989) Pharmac. Ther. 43, 261–289.
[2] Irshaid, Y.M. and Tephly, T.R. (1987) Mol. Pharmacol. 31, 27–37.
[3] Matern, H., Lappas, N. and Matern, S. (1991) Eur. J. Biochem. 200, 393–400.
[4] Sutherland, L., Wooster, R., Jackson, M.R., Fournel-Gigleux, S. and Burchell, B., in: Hepatic Metabolism and Disposition of Endo- and Xenobiotics (K.W. Bock, W. Gerok and S. Matern, Eds.) Kluwer Academic Publishers, Lancaster, UK, vol. 57, 1991, pp. 141–150.
[5] Harding, D., Fournel-Gigleux, S., Jackson, M.R. and Burchell, B. (1988) Proc. Natl. Acad. Sci. USA 85, 8381.
[6] Harding, D., Jackson, M.R., Wooster, R., Fournel-Gigleux, S. and Burchell, B. in: Cellular and Molecular Aspects of Glucuronidation (G. Siest, J. Magdalou and B. Burchell, Eds.) John Lilley Eurotex, Montrouge, France, 1988, pp. 13–20.
[7] Wooster, R., Sutherland, L., Ebner, T., Clarke, D.J., Da Cruz e Silva, O. and Burchell, B. (1991) Biochem. J. 278, 465–469.
[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.
[9] Fournel-Gigleux, S., Sutherland, L., Sablovic, N., Burchell, B. and Siest, G. (1991) Mol. Pharmacol. 39, 177–183.
[10] Jackson, M.R., Fournel-Gigleux, S., Harding, D. and Burchell, B. (1988) Mol. Pharmacol. 34, 638–642.
[11] Odell, G.B., Mogilevsky, W.S. and Gourley, G.R. (1990) J. Chromatog. 529, 287–298.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Ritter, J.K., Crawford, J.M. and Owens, I.S. (1991) J. Biol. Chem. 268, 1043–1047.
[14] Fevery, J., Blanckaert, N., Heirwegh, K., Preaux, A.-M. and Brethelot, P. (1977) J. Clin. Invest. 60, 970–979.
[15] Blanckaert, N., Gollan, J. and Schmid, R. (1979) Proc. Natl. Acad. Sci. USA 76, 2037–2041.