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PURIFICATION AND CHARACTERIZATION OF A CATALYTICALLY ACTIVE HUMAN LIVER UDP-GLUCURONOSYLTRANSFERASE EXPRESSED AS A FUSION PROTEIN IN *E. coli*

Thierry PILLOT [a], Mohamed OUZZINE [a], Sylvie FOURNEL-GIGLEUX [a], Chantal LAFAURIE [a], Anna RADOMINSKA [b], Roger LESTER [b], Richard DRAKE [b], Susan TREAT [b], Gérard SIEST [a], and Jacques MAGDALOU [a]

[a] Centre du Médicament, URA CNRS 597, 30 rue Lionnois, 54000 Nancy, FRANCE

[b] University of Arkansas for Medical Science, Gastroenterology Division, Little Rock, 72205, AR

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SUMMARY The purification and the characterization of functional human liver UDP-glucuronosyltransferase 2B4 produced as a *Staphylococcus aureus* protein A fusion protein in *E. coli* are described. The purified fusion protein was able to catalyze the glucuronidation of hyodeoxycholic acid, the major substrate described for this isoform to date. The effects of the amount and the nature of the phospholipids upon reconstitution into phospholipid micelles were investigated. Apparent determined K_m values for hyodeoxycholic acid and UDP-glucuronic acid were 0.55 and 0.43 mM, respectively. Moreover, photoaffinity labelling of the fusion protein with a photoactivatable analog of UDP-glucuronic acid strongly suggested that this recombinant protein exhibited similar binding properties as the microsomal protein, which emphasizes its use for further structural analyses.

INTRODUCTION The superfamily of UDP-glucuronosyltransferases (EC 2.4.1.17, UGT) (1) catalyzes the conjugation of glucuronic acid from UDP-glucuronic acid (UDP-GlcU) to a large variety of drugs, and endogenous compounds, such as bilirubin or bile acids (2). The instability of these phospholipido-dependent membrane-bound enzymes has delayed the purification and characterization of human UGT isoforms. To date, only three human isoenzymes were purified to homogeneity (3, 4). The development of cDNA cloning and heterologous expression led to the identification and to the partial characterization of the substrate specificity of at least nine human UGT isoforms (1). We previously described the production of a transfected V79 cell line stably expressing a human liver UGT isoform (UGT2B4) exhibiting an apparent molecular mass of 52 kDa and specifically glucuronidating hyodeoxycholic acid (HDCA) (5). In the present study, we describe the production in *E. coli* of the human liver UGT2B4 as a protein fused with the staphylococcal protein A (SpA). The fusion protein was purified by one immuno-affinity chromatography step on IgG-Sepharose matrix. The enzymatic activity of the purified fusion protein was reconstituted into phospholipid micelles (PLM) and the catalytic properties of the fusion protein were determined. Moreover, photolabelling was used to investigate the binding of the co-substrate to the purified enzyme.

ABBREVIATIONS: HDCA, hyodeoxycholic acid; OLPC, oleoyl-lysophosphatidylcholine; PLM, phospholipid micelles; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SpA, Staphylococcal protein A; UGT, UDP-glucuronosyltransferase; UDP-Glc, UDP-glucose; UDP-GlcU, UDP-glucuronic acid.

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MATERIALS AND METHODS

Materials. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and were used according to the supplier's recommendations. The *E. coli* strain N4830-1 (F', *su*, *his*, *ilv*, *gal* K8, (*cblD-pgl*), [1, Bam, N^+, cI857]), the pRIT2T expression vector, and the IgG-Fast Flow Sepharose gel were from Pharmacia. DNase I, lysozyme, HDCA, phenylmethylsulfonylfluoride (PMSF), oleoyl-lysophosphatidylcholine (OLPC), dimyristoyl-lysophosphatidylcholine (DMPC), and egg lysophosphatidylcholine (LPC) were purchased from Sigma. UDP-GlcU was from Boehringer Mannheim. Tritium-labeled [^{3}H]-HDCA was synthesized as previously described (6).

Protein analyses. Protein concentration was measured according to Bradford method using bovin serum albumin as standard (7). The proteins were analyzed by SDS-PAGE according to Laemmli (8) and transferred onto Immobilon-P^R membrane (Millipore, France). The fusion protein was detected by using a monoclonal anti-SpA alkaline-phosphatase conjugated antibody (TEBU, France).

Construction of the expression vector. Cloning and sequencing of HLUG25 cDNA encoding the UGT2B4 isoform have been previously described (9). The total coding sequence has been isolated from a pKCRH2 expression vector and subcloned into the prokaryotic expression vector pRIT2T in two steps, according to classical molecular biology technics (10). Briefly, a 686-bp *SacI-DdeI* restriction fragment from HLUG25 cDNA, encoding the last 298-526 amino acids was inserted into the *SacI-HincII* sites of pUC19, to produce the pUC19-SH25 plasmid. A *PvuII-HindIII* restriction fragment from HLUG25 cDNA encoding the 14-526 amino acids was subcloned into the *HincII-HindIII* sites of pUC19. This plasmid was digested with both *NdeI* and *SacI*, and the resulted fragment was ligated into the same sites of the pUC19-SH25 plasmid. The resultant vector was digested with *BamHI* and *PstI* and the fragment encoding the full length sequence of UGT2B4 was ligated into the same restriction sites of pRIT2T, in frame with the 3' sequence of SpA, under the control of the λP_R promoter.

Production and purification of the fusion protein. *E. coli* N4830-1 harboring the recombinant vector were grown at 30°C in 100 ml of Luria and Bertani medium supplemented with ampicillin (100 µg/ml) and 0.2 % (w/v) glucose. When the absorbance at 600 nm reached 0.6-0.7, the production of the fusion protein was achieved by a temperature shift to 42°C. At various times, aliquots of the culture medium and the production of the recombinant protein was followed by SDS-PAGE and immunoblot analysis. The bacterial pellet resulting from a 1,000 x *g* centrifugation, was resuspended in 10 ml of lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM glucose, 1 mM EDTA, 1 mM PMSF and 1 mg/ml lysozyme) and incubated for 30 min on ice. DNase I (1 µg/ml) and $MgCl_2$ (10 mM) were added and the suspension was incubated at 37°C for 10 min, followed by centrifugation at 12,000 x *g* for 10 min at 4°C. The pellet was treated with 8 M urea in order to solubilize inclusion bodies and renature the proteins according to Marston (11). The solubilized and renatured proteins from inclusion bodies were loaded onto 1 ml of IgG-Sepharose affinity matrix and the fusion protein was purified according to Nilsson *et al.* (12). The eluted fractions were concentrated and analyzed by SDS-PAGE.

Enzyme assays and photolabelling. PLM were prepared as previously described (13), and stored under nitrogen before utilization. 12 µg of purified fusion protein were incubated with increasing amounts of PLM for 30 min on ice, then for 5 min at 37°C. Glucuronidation of HDCA was assayed according to Radominska *et al.* (14), in the presence of mixed micelles of cold and [^{3}H]-HDCA (1 mM, 1.0 µCi). Photoaffinity labelling of the purified fusion protein was achieved according to a previous work (15). 10 µg of purified fusion protein were photolabelled for 90 s in the presence of 40 µM of [β-^{32}-P]5N_3UDP-GlcU or [β-^{32}-P]5N_3UDP-Glc.

Kinetic constant determination. Apparent kinetic constants (K_m and V_m) were determined by linear least-squares regression analysis of values from double-reciprocal plots. The concentration of HDCA varied from 0.05 to 2.0 mM for a constant concentration of UDP-GlcU of 1.0 mM. The glucuronic acid concentration was varied from 0.1 to 4.0 mM for a constant concentration of HDCA of 0.5 mM.

RESULTS

Production and purification of the fusion protein. In the aim to produce a cytoplasmic SpA fusion protein, a cDNA fragment encoding the 14-526 amino acids of UGT2B4 was subcloned into the expression vector pRIT2T. The final map of the recombinant vector, named pRIT2T-UGT2B4, is presented the Fig. 1. The correct orientation of the subcloned fragment and the respect of the reading frame of SpA were verified by restriction mapping (data not shown), and confirmed by SDS-PAGE analysis. The recombinant clones, harboring the pRIT2T-UGT2B4 vector, produced a recombinant protein of an apparent molecular mass of

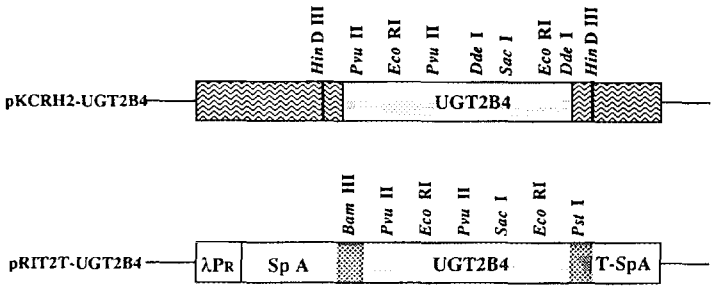

Fig. 1. Restriction map of the recombinant expression vector.

87 kDa (Fig. 2a, *lane 2*), which is in good agreement with the predicted molecular mass of the fusion protein. This protein was absent in control cells harvested before induction (Fig. 2a, *lane 1*). Maximal level of expression occurred after an induction of 2.5 h (data not shown) and the protein precipitated mainly into inclusion bodies. As shown on Fig. 2b, the recombinant protein was found to be associated with the insoluble material, following several washing procedures with detergent (Fig. 2b, *lanes 2* and *3*). The inclusion bodies were isolated and were solubilized by 8 M urea. The renatured proteins from inclusion bodies were directly loaded on the IgG-Sepharose gel and the fusion protein was purified by one immuno-affinity chromatography step. SDS-PAGE analysis of the eluted fractions showed that the fusion protein could be purified to homogeneity (Fig 3, *lanes 1* and *2*).

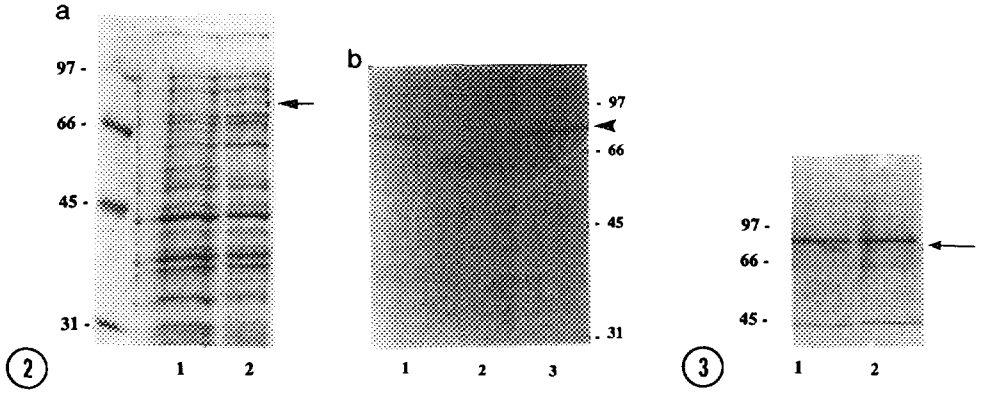

Fig. 2. Production of the SpA-UGT2B4 fusion protein.
a. 10 % SDS-PAGE analysis of the produced fusion protein. *Lanes 1*, 50 µg of bacterial homogenate before induction ; *2*, 50 µg of bacterial homogenate after 2.5 hr of induction. Standard molecular mass (kDa) are indicated on the left.
b. Immunoblot analysis of the solubility of the produced fusion protein. *Lanes 1*, total cell homogenate (50 µg proteins) ; *2*, supernatant of lysis (50 µg of proteins) ; *3*, insoluble material (50 µg of proteins). Standard molecular mass (kDa) are indicated on the right.

Fig. 3. Purification of the fusion protein.
Eluted fractions from immuno-affinity chromatography were subjected to SDS-PAGE followed by silver staining (*lanes 1 and 2*, 2.0 and 5.0 µg proteins, respectively).

Reconstitution of the enzymatic activity. The effect of various types of PLM on the HDCA glucuronidation rate were investigated. As shown in Table 1, the delipidated enzyme presented very low level of HDCA glucuronidation. Reconstitution procedures using increasing amounts of various PLM showed that OLPC enhanced the enzymatic activity in a concentration-dependent manner, to a maximum of 58 pmol/min x mg protein for a PLM/protein weight ratio of 10.0. This ratio was used for further experiments. On the contrary, DMPC or LPC showed a slight activating effect on the glucuronidation of HDCA (Table 1).

Characterization of catalytic properties of the fusion protein. The apparent kinetic constants toward HDCA and UDP-GlcU were determined after reconstitution into OLPC micelles at an OLPC/protein weight ratio of 10.0 (Table 2), in the presence of 10 µg of purified fusion protein. We previously verified the linearity of the HDCA glucuronidation reaction under these conditions for a protein amount up to 40 µg and for a reaction time up to 1 h (data not shown). The fusion protein presented apparent K_m values of 0.55 ± 0.08 and 0.43 ± 0.05 mM for HDCA and UDP-GlcU, respectively. The apparent V_m for the glucuronidation of HDCA was 0.120 ± 0.06 pmol/min x mg protein, resulting to an apparent velocity, represented by the V_m/K_m ratio, of 0.28. These values were compared to the apparent constants described for human microsomes or for the recombinant UGT2B4 stably expressed in a transfected V79 cell line (5). As shown in Table 2, the apparent affinity constants of the purified fusion protein for either substrate were found very close to those observed for UGTs from other sources glucuronidating HDCA.

Photoaffinity labelling of the purified fusion protein. In an attempt to characterize the binding properties of the fusion protein toward the co-substrate of the glucuronidation reaction, the protein was labelled with photoactivatable analogs of UDP-GlcU. The effects of the phospholipids on the photoincorporation of [β-^{32}-P]5N_3UDP-GlcU were first investigated. As shown on Fig. 4a, increasing the concentration of OLPC in the irradiation medium led to an increase of the photoincorporation of the probe, with a maximal effect for an OLPC/protein weight ratio of 10.0 (Fig. 4a, *lane 3*), in good agreement with the effect of OLPC determined from enzymatic data. We previously verified the non-incorporation of this probe in the absence of UV irradiation, and on the purified SpA (data not shown). Moreover, an analog of UDP-Glc, [β-^{32}-P]5N_3UDP-

Table 1. Phospholipid effects on the HDCA glucuronidation rate by SpA-UGT2B4

	PLM / Protein (w / w)				
PLM	0.0	2.0	6.0	10.0	25.0
DMPC	ND(1)	ND	ND	1.6 (2)	ND
LPC	ND	ND	2.7	4.5	ND
OLPC	ND	13.2	28.4	58.6	20.5

(1) Non detectable, values less than 0.1 pmol/min x mg protein.
(2) Specific HDCA glucuronidation activity in pmol/ min x mg protein.
Values are the average of three independent determinations. Standard deviations were less than 0.5 pmol/min x mg protein and were omitted to the table.

Table 2. Apparent kinetic constants of various sources glucuronidating HDCA

	K_m (mM)	V_m (nmol/min x mg protein)	V_m / K_m
Human microsomes			
HDCA	0.25	0.51	2.04
UDP-GlcUA	0.21	0.86	4.09
V79-UGT2B4(1)			
HDCA	0.27	0.036	0.13
UDP-GlcUA	0.12	0.040	0.34
SpA-UGT2B4(2)			
HDCA	0.55 ± 0.08	0.092 ± 0.05	0.17
UDP-GlcUA	0.43 ± 0.05	0.12 ± 0.06	0.28

(1) According to Fournel-Gigleux *et al.* (5).
(2) Values are the average of two independent determinations.

Glc, did not react with the fusion protein (data not shown). The effect of various cold ligands on the level of photoincorporation of [β-^{32}-P]5N_3UDP-GlcU was determined. As shown on Fig. 4b, a total protection of the photolabelling occured by the presence in the irradiation medium of both UDP-GlcU, UDP or glucuronic acid (0.25 mM final concentration) (Fig. 4b, *lanes 2, 4* and *5*), whereas UDP-Glc showed slight effect (Fig. 4b, *lane 3*). These results strongly suggested specific interactions between the probe and a functionnal co-substrate binding site.

DISCUSSION The present paper reports for the first time the expression of a human liver UGT, namely UGT2B4 (9), as a SpA fusion protein in *E. coli*. This recombinant protein was purified by one immuno-

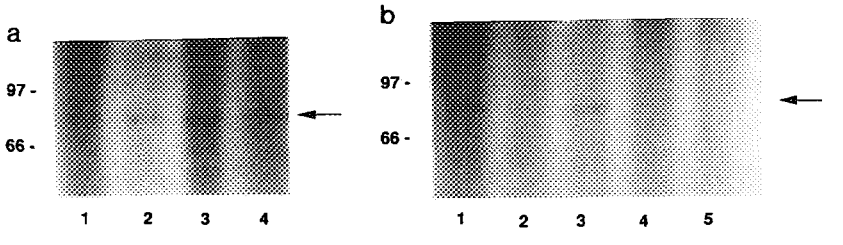

Fig. 4. Photolabelling of the purified fusion protein.
a. Influence of OLPC micelles on the photoincorporation of the [β-^{32}-P]5N_3UDP-GlcU. 10 µg of purified fusion protein was photolabelled in the presence of increasing amounts of OLPC micelles. After photolabelling reaction, the proteins were subjected to SDS-PAGE, and the radioactivity was localized by autoradiography at -80°C. The OLPC/protein weight ratios were : *lanes 1*, 0.0 ; *2*, 2.0 ; *3*, 10.0 ; *4*, 25.0.
b. Protection of the fusion protein photolabelling by various cold ligands. Before incubation with the photoprobe and irradiation, 10 µg of purified fusion protein was incubated on ice for 10 min with 0.25 mM (final concentration) of cold ligands. *Lane 1*, control ; *lanes 2, 3, 4 and 5*, UDP-GlcU, UDP-Glc, UDP and glucuronic acid, respectively.

affinity chromatography step using the capacity of SpA to bind with a high affinity to the IgG Fc fragments. Despite the fact that the fusion protein precipitated mainly into inclusion bodies, we succeeded in the restauration of the biological properties of SpA by the protocol used for the solubilization and the renaturation of proteins from inclusion bodies. After reconstitution experiments into PLM, the purified fusion protein was found to be catalytically active toward HDCA, the major substrate described for this UGT isoform. As previously described for UGT isoforms purified from mammalian liver (4, 13), the delipidated enzyme was inactive. The effects of several phospholipids on the activity of the purified fusion protein were investigated. The results showed that OLPC micelles allowed the strongest stimulation of the HDCA glucuronidation. Under these conditions, determination of the kinetic parameters toward both HDCA and UDP-GlcU and comparison with those described for other sources conjugating the same substrate, led us to suggest a correct folding of the fusion protein after reconstitution experiments. Even if the specific activity was found to be low regarding the drastic conditions used for the purification, the affinity constants for HDCA and UDP-GlcU were found to be close to those determined in human liver microsomes (4) or in the recombinant V79-UGT2B4 cell line (5). Enzymatic data were corroborated by photoaffinity labelling with a photoactivatable UDP-GlcU analog (15). Specific photoaffinity labelling with [β-^{32}-P]5N_3UDP-GlcU and protection experiments strongly supported specific interactions of UDP-GlcU with the binding site of the protein. This fusion protein seems to share identical binding properties toward the probes compared to human liver microsomes (15). It is worth to mention that UDP-Glc did not react with the fusion protein, as also described for the recombinant UGT2B4 stably expressed in transfected V79 cells (14). Moreover, effects of PLM on the incorporation of the probe were directly correlated to those observed on enzymatic data, which confirmed the involvement of phospholipids in the maintenance of the correct UGT folding.

UGT are well known as phospholipido-dependent membrane-bound proteins (2). Despite the fact that the regulation mechanisms by lipids remain poorly understood. A recent report suggested that the regulation of the UGT activity may be determined by both the charges and their repartition in the polar region of phospholipids (16), without binding of lipids into a specific(s) site(s) of the protein (17). Previous works have demonstrated the involvement of lipids in the stimulation and in the stabilization of pure forms of UGT (13, 18, 19). These authors determined that OLPC had the strongest effect on the enzyme activity, as confirmed by our findings.

This paper provides first evidence that the catalytic activity of a recombinant human UGT isoform expressed in *E. coli*, is also determined by its interactions with particular types of phospholipids. In addition, this first description of the possibility to produce a functional UGT fusion protein allowed us to propose the use of this tool for further structural and mechanistic investigations of human UGT isoforms.

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