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PROPERTIES OF A 3-METHYLCHOLANTHRENE-INDUCIBLE PHENOL UDP-GLUCURONOSYLTRANSFERASE FROM RAT LIVER

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Abstract—Functional and molecular probes are described which are useful to identify a 3-methylcholanthrene-inducible phenol UDP-glucuronosyltransferase (GT_{MC}) from rat liver. Two different procedures for isolation of GT_{MC} were compared, method 1 utilizing DEAE-Sepharose chromatography or method 2, chromatofocusing. Method 2 appeared to be superior in separating different isoenzymes. Subsequently the enzyme was purified by affinity chromatography on UDP-hexanolamine Sepharose. With both methods a protein was purified with a subunit M_r of 55,000, catalyzing glucuronidation of a variety of planar phenols and, in particular, of benzo(*a*)pyrene-3,6-quinol to its mono- and diglucuronide. Antibodies to GT_{MC} recognized a polypeptide with a subunit M_r of 55,000 as the major 3-methylcholanthrene-inducible isoenzyme in rat liver microsomes. The described functional and molecular probes may help to differentiate GT_{MC} from similar isoenzymes conjugating planar phenols and to elucidate its regulation and biological function.

Glucuronidation is an important pathway for the metabolism and excretion of both endogenous and exogenous compounds [1]. The reaction is catalyzed by a family of isoenzymes of UDPGT,§ responsible for the transfer of the glucuronic acid moiety from UDP-glucuronic acid to the aglycone acceptor substrate. Some isoenzymes appear to be more specific for endogenous substrates such as bilirubin [2], 17β-hydroxysteroids or 3-hydroxysteroids [3, 4]. A 3-methylcholanthrene(MC)-inducible phenol UDPGT (GT_{MC}) has been described for which no endogenous substrate has yet been identified [3–5]. GT_{MC} has also been named *p*-nitrophenol UDPGT [3, 4] or GT_1 [5, 6] by various laboratories. This isoenzyme catalyzes the glucuronidation of a variety of planar phenols including toxic phenolic and polyphenolic metabolites of aromatic hydrocarbons and appears to be co-induced with other drug metabolizing enzymes involved in the metabolism of carcinogenic aromatic hydrocarbons [6–8]. The possibility emerges that more than one MC-inducible UPDGT with a similar or different subunit molecular weight may exist in rat liver microsomes from MC treated rats. The present study was performed where functional and molecular probes were used to identify GT_{MC}. Immunoblot analysis was used as a molecular probe to compare GT_{MC}. Antibodies to GT_{MC}, which were prepared different ways in different laboratories have been compared as to their specificity and their ability to serve as selective probes.

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§ Abbreviations used: UDPGT, UDP-glucuronosyltransferase (EC 2.4.1.17); MC, 3-methylcholanthrene; BP, benzo(*a*)pyrene; SDS-PAGE, sodium dodecyl sulfate polyacrylamide-gel electrophoresis.

MATERIALS AND METHODS

Chemicals

BP-3,6-quinone was obtained from the Chemical Carcinogen Reference Standard Repository, National Institute of Health, Bethesda, MD. It was reduced to the corresponding quinol by addition of 0.1 M ascorbic acid, immediately before use. Nitrocellulose sheets 15 F (0.45 μm) were obtained from Sartorius, Göttingen, F.R.G.; and horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin from DAKOPATTS, Hamburg, F.R.G.

Purification of GT_{MC}

Liver microsomes from MC-treated male Wistar rats were used as the enzyme source. MC (40 mg/kg, dissolved in olive oil) was given once i.p. and animals were killed four days after treatment. Enzyme was purified by two methods. In method 1 the enzyme was purified essentially as described by Bock *et al.* [5] with minor modifications: ammonium sulfate precipitation was replaced by precipitation with polyethylene glycol 6000 (3%–12%), and gel filtration was carried out as the last step. Other procedures included successive chromatography on DEAE-Sepharose CL-6B, UDP-hexanolamine Sepharose 4B and Bio-Gel A-1.5 m. In method 2 the enzyme was separated from other isoenzymes by chromatofocusing of Emulgen-911-solubilized liver microsomes followed by UDP-hexanolamine Sepharose 4B affinity chromatography [3].

Protein was determined according to Lowry *et al.* [9]. SDS-PAGE was carried out according to Laemmli [10]. The following protein standards were used: bovine serum albumin (M_r 68,000), bovine liver catalase (M_r 58,000), glutamate dehydrogenase

Table 1. GT activities towards various substrates in rat liver microsomes and in purified GT_{MC}

Substrate	GT activities (nmol/min/mg protein)			
	Microsomes (untreated)	Microsomes (MC-treatment)	Purified enzyme (method 1)	Purified enzyme (method 2)
1-Naphthol	40 ± 6	149 ± 26	2900 (1900–3800)	1633 (1500–1700)
4-Methyl-umbelliferone	55 ± 10	154 ± 18	1570	1334
BP-3,6-quinol	5.6 ± 0.9	64 ± 10	250 (150–310)	552 (200–700)
BP-3,6-quinol MG*	0.25 ± 0.03	10 ± 4	121 (70–130)	67 (60–80)
Testosterone	3.3 ± 0.5	4.3 ± 2	21 (10–27)	1.9

Means ± SD of four determinations are listed. When more than two determinations were available ranges are given in parenthesis for the purified enzyme at the affinity chromatography step.
* MG, monoglucuronide.

from beef liver (M_r 53,000), ovalbumin (M_r 43,000). Proteins were stained with Coomassie Blue R 250.

Assays of GT

Published methods were used to assay GT activity towards 1-naphthol [11], 4-methylumbelliferone [6], testosterone [12] and BP-3,6-quinol [8]. Rates of BP-3,6-quinol mono- and diglucuronide formation were calculated from initial rates. For calculation of total monoglucuronide formation (Table 1) the sum of the two sequential reactions is given, since diglucuronide formation includes monoglucuronide formation.

Immunoblot analysis

SDS-PAGE was carried out with 0.1 × 8 × 8 cm slab gels. Proteins were transferred from the polyacrylamide gels to nitrocellulose sheets [13]. For electrophoretic transfer a current setting of 300 mA was used for 8 hr. Nitrocellulose sheets, containing the transferred proteins, were treated for 15 min with two changes of blocking buffer consisting of 0.05% Tween 20 in PBS (phosphate buffered saline). Then the nitrocellulose sheets were incubated overnight at room temperature with rabbit anti-GT_{MC} IgG (1:2000) in blocking buffer. After two washes with PBS and an additional wash with blocking buffer the sheets were incubated for 2 hr with peroxidase-conjugated swine anti-rabbit IgG (1:100) and then washed twice with PBS. To each tray, containing nitrocellulose sheets, a fresh solution of 0.6 mM 4-chloro-1-naphthol (dissolved in methanol) was added and 2 mM H_2O_2 in 20 mM ammonium acetate/citrate buffer, pH 5 was employed to develop the stain. After about 5 min of shaking at room temperature each sheet was washed with water.

Preparation of antibodies to rat liver microsomal GT_{MC}

(a) *Antibodies of broader specificity*. The antigen employed was enzyme purified from male Wistar rats by method 1 (see above). Enzyme fractions eluting at the leading edge of Bio-Gel A-1.5 m chromatography were used for immunization. Antibodies to this phenol UDPGT preparation were raised in rabbits and IgG (about 100–150 mg protein/ml) was prepared by chromatography on Protein-A Sepharose [14].

(b) *Selective antibodies to GT_{MC}*. The antigen used was enzyme isolated by method 2 from livers of female Sprague–Dawley rats and further purified by electroelution from SDS-PAGE [4]. Antibodies to the electroeluted enzyme were raised in rabbits.

RESULTS

Chromatofocusing of solubilized microsomal proteins (method 2) allows for the resolution of several UDPGTs that catalyze the glucuronidation of phenolic substrates (Fig. 1) and confirms previous results [3, 4, 15]. The activity towards 1-naphthol and 4-methylumbelliferone, which elutes at an isoelectric point of 8.5 (GT_{MC}) was markedly increased in liver microsomes obtained from MC-treated rats. In contrast, UDPGT activity towards testosterone was unchanged. UDPGT activities were also separated by DEAE-Sepharose chromatography (method 1; [5, 14]). Subsequent affinity chromatography of GT_{MC} fractions, isolated by either method 1 or 2, led to the purification of enzymes with similar substrate specificity towards various phenols and polyphenols (Table 1). BP-3,6-quinol appears to be a selective substrate for GT_{MC} since high induction factors of 10-fold for monoglucuronide formation and 40-fold for diglucuronide formation have been found using this substrate [8]. Methylumbelliferone has been shown to react with GT_{MC} and not with testosterone UDPGT [3].

As shown in Fig. 2, SDS-PAGE of the proteins isolated by methods 1 (A) or 2 (B) shows similar protein bands at M_r 55000. The proteins or protein monomer isolated as described in Materials and Methods were used to raise antibodies in rabbits. These antibodies, which precipitated and inhibited GT activities [14, 16] were used for immunoblot analysis of GT polypeptides. As shown in Fig 3B selective antibodies were obtained when the antigen was purified by method 2 and where the 55,000 M_r monomer was electroeluted from SDS-PAGE [4]. The polypeptide at M_r 55000 was clearly MC-inducible. Using method 1 for enzyme purification

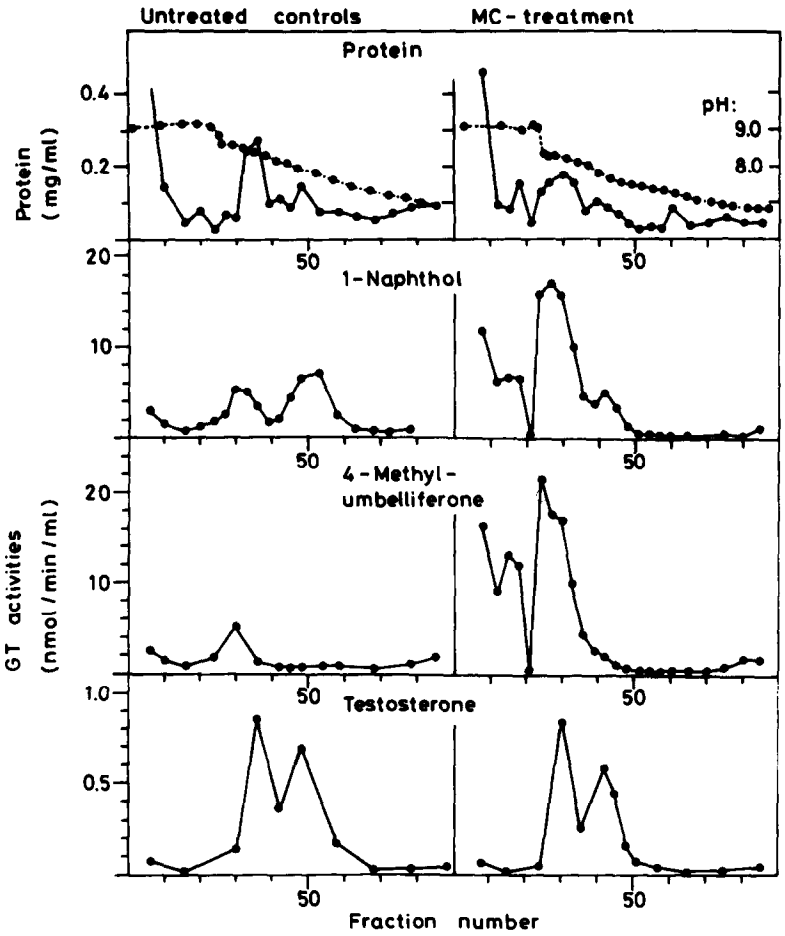

Fig. 1. Separation by chromatofocusing of liver microsomal UDPGT activities from MC-treated rats and untreated controls. Emulgen 911-solubilized microsomes (100 mg protein) were applied to a chromatofocusing column PBE 94 and eluted with Polybuffer 96-HAc, pH 7.0, to generate a pH 9–7 gradient. UDPGT activities were determined using the substrates listed. The pH gradient is indicated by the dotted line.

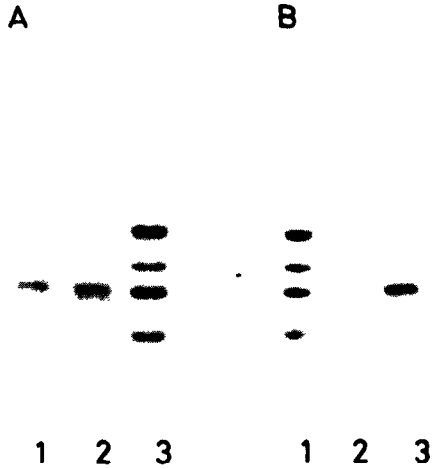

Fig. 2. Electrophoresis of purified phenol GT_{MC} isolated by DEAE-Sepharose chromatography (A, method 1) or chromatofocusing (B, method 2). (A) 1 and 2, enzyme purified according to method 1 (1.0 and 1.5 μg protein, respectively): 3, M_r protein standards. (B) 1, M_r protein standards: 2 and 3, enzyme purified according to method 2 (0.5 and 1.2 μg protein, respectively).

and antigen production with the holoenzyme fraction yielded anti-GT_{MC} antibodies with broader specificity (Fig. 3, A). Polypeptides at M_r 50000 and 52000 could be identified as 17β-hydroxysteroid GT and 3α-hydroxysteroid GT, respectively, using antibodies to the purified proteins ([3, 4] not shown). Again the polypeptide at M_r 55000 was the major MC-inducible polypeptide (Fig. 3 A). The antibody raised against the 55,000 M_r monomer of liver GT_{MC} from female Sprague–Dawley rats (prepared in Iowa) clearly recognized liver GT_{MC} purified from male Wistar rats (Fig. 3A, lane 5). This observation suggests similar GT_{MC} isoenzymes in the two rat strains. Vice versa the antibody of broader specificity, prepared in Göttingen, recognized GT_{MC} purified in Iowa (not shown). Hence GT_{MC} isoenzymes isolated in the two laboratories appear to be similar proteins. The reported differences in subunit M_r 56,000 [3, 4] and 54,000 [5, 6] appear to be related to different electrophoretic systems. Recalculation with the use of protein standards nearer the molecular weight of the GT_{MC} has been carried out and the best estimate of the molecular weight is 55,000. It is possible that another protein band arises after

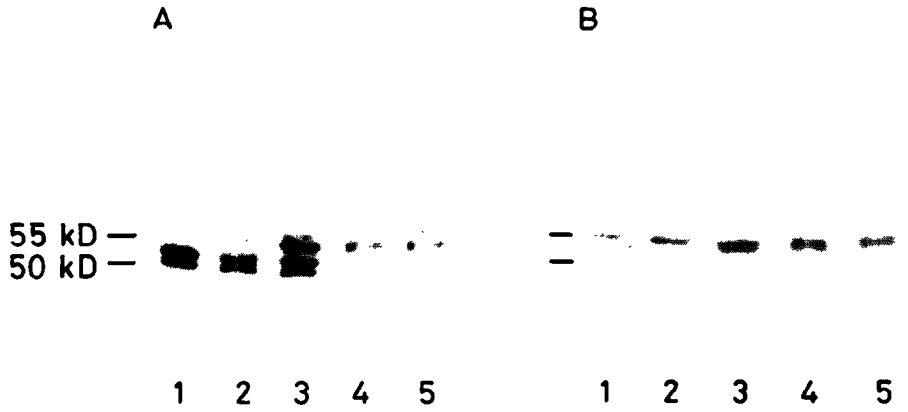

Fig. 3. Immunoblot analysis of liver microsomal UDPGT polypeptides using anti-GT_{MC} antibodies of broader specificity (A) and more selective antibodies (B). 1–3, microsomes from untreated controls, from phenobarbital- and MC-treated rats, respectively (2.5 μg protein); 4 and 5, enzymes from Wistar rats purified according to method 1 or 2, respectively (0.25 μg protein).

MC treatment somewhat below the major band (Fig. 3, lane 3). This may cause a possible discrepancy in estimating a molecular weight when large amounts of protein are used and this lower band blends into the upper band. However, it appears that the GT_1 as described by Bock *et al.* [5, 16] and the PNP-UDPGT (MC-inducible) described by Falany and Tephly [3] are the same or very similar proteins. The question of whether another MC-inducible protein appears or that unprocessed GT_{MC} is present cannot be answered at this time.

DISCUSSION

The results demonstrate that a distinct rat liver microsomal GT_{MC} isoenzyme can be isolated by anionic exchange chromatography (method 1) or chromatofocusing (method 2). Separation of different UDPGT isoenzymes appears to be more efficient using chromatofocusing. The properties of liver GT_{MC} isolated in different laboratories from different rat strains appear to be similar. GT_{MC} appears to be immunochemically distinct from steroid GTs since antibodies raised to purified 17β-hydroxysteroid GT or 3α-hydroxysteroid GT did not cross-react with GT_{MC} (unpublished results). Moreover these isoenzymes differ in their monomeric M_r values [3, 4]. However, it should be noted that both 17β-hydroxysteroid GT and GT_{MC} conjugate simple phenols such as 4-nitrophenol and 1-naphthol [3, 4]. Therefore, more selective functional probes are needed. 4-Methylumbelliferone does not react with 17β-hydroxysteroid UDPGT [3]. BP-3,6-quinol glucuronidation also appears to be a selective functional probe for GT_{MC}. GT_{MC} appears to be present at very low levels in liver microsomes from untreated controls and phenobarbital-treated rats and under these conditions both BP-3,6-quinol diglucuronide formation (Table 1) and the level of the M_r 55000 polypeptide as determined by Western blots was low (Fig. 3). Exposure to MC-type inducers led to marked increase of diglucuronide formation and antibody binding. These experiments do not rule out the possibility that another 3-MC inducible enzyme that cannot be separated from GT_{MC} is present and responsible for BP-quinol glucuronide formation.

The present study describes functional and immunochemical properties of an MC-inducible phenol UDPGT (GT_{MC}) in rat liver. The isoenzyme with a subunit M_r of 55,000 conjugates simple planar phenols such as 4-methylumbelliferone, 1-naphthol and 4-nitrophenol as well as phenolic metabolites of polycyclic aromatic hydrocarbons such as 3-hydroxy-BP and BP-3,6-quinol [3–6]. Whether glucuronidation of the new anticancer drug mitoxantrone proves to be a selective probe for rat liver GT_{MC} remains to be established [17]. Other drug metabolizing enzymes are known to be induced by MC-type inducers [6, 7, 18]. This pleiotropic response may represent an adaptive program evolved to metabolize aromatic hydrocarbons [19, 20]. It is also interesting to note that GT_{MC} appears to be permanently increased in rat liver foci and nodules after treatment with several hepatocarcinogen initiators [19, 21, 22]. Recently cDNA encoding GT_{MC} has been cloned which led to prediction of the complete amino acid sequence of this isoenzyme [23]. The availability of selective functional and molecular probes should facilitate identification, quantification and localization of GT_{MC} and to elucidate its interesting regulatory and biological properties.

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