

## Acyl-CoA binding and acylation of UDP-glucuronosyltransferase isoforms of rat liver: their effect on enzyme activity

Atsushi YAMASHITA,\* Masanobu WATANABE, Takashi TONEGAWA, Takayuki SUGIURA and Keizo WAKU

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

When [ $^{14}\text{C}$ ]arachidonoyl-CoA was incubated with crude extracts of rat liver microsomes, [ $^{14}\text{C}$ ]arachidonic acid was incorporated into many proteins, suggesting that modification of these proteins with fatty acid, i.e. acylation, occurred. Using a [ $^{14}\text{C}$ ]arachidonoyl-CoA labelling assay, 50 and 53 kDa proteins were purified from rat liver microsomes to near homogeneity by sequential chromatography on Red-Toyopearl, hydroxyapatite, heparin-Toyopearl, Blue-Toyopearl and UDP-hexanolamine-agarose. Acylation of the 50 and 53 kDa proteins occurred in the absence of any other protein, suggesting that these molecules catalyse autoacylation. The acylation was dependent on the length of the incubation period and the concentration of [ $^{14}\text{C}$ ]arachidonoyl-CoA. The 50 and 53 kDa proteins also had

acyl-CoA-binding activity; initial rates of acyl-CoA binding and acylation were 0.25 and 0.004  $\text{min}^{-1}$  respectively. The proteins also had weak but distinct acyl-CoA-hydrolysing activity (0.006  $\text{min}^{-1}$ ). These results suggest that the proteins catalysed the sequential reactions of binding to acyl-CoA, autoacylation, and hydrolysis of fatty acid. N-terminal amino acid sequencing analysis showed these proteins to be UDP-glucuronosyltransferase (UDPGT) isoforms. UDPGT activity was inhibited by arachidonoyl-CoA. These results suggest that binding of acyl-CoA and acylation of UDPGT isoforms regulate the enzyme activities, implying a possible novel function for fatty acyl-CoA in glucuronidation, which is involved in the metabolism of drugs, steroids and bilirubin.

### INTRODUCTION

Long-chain fatty acyl-CoA is a metabolically active form of fatty acid. Various kinds of enzymic conversion of fatty acids, including desaturation, chain elongation and oxidation, proceed, mainly in the form of fatty acyl-CoA, in mammalian tissues [1]. Another important role of acyl-CoA is to serve as an acyl donor in the formation of various types of simple and complex lipid molecules [1]. In addition to its role as a metabolic intermediate, long-chain fatty acyl-CoA is known to possess several functional roles as a modulator of various enzymes and cell functions. Possible roles of acyl-CoA in the modulation of the activities of enzymes, such as  $\text{Na}^+, \text{K}^+$ -ATPase [2],  $\text{Ca}^{2+}$ -ATPase [3] and protein kinase C [4], and the function of thyroid hormone receptor [5] have been proposed. It has also been demonstrated that acyl-CoA is required for budding of transport vesicles from Golgi cisternae [6,7]. Protein acylation involving acyl-CoA was also reported to contribute significantly to the regulation of protein function [8,9]. Various proteins are known to be post-translationally modified by palmitoylation and myristoylation. Such binding of lipid molecules is expected to change the physical properties of the modified protein quite dramatically, because largely hydrophilic residues become hydrophobic. This will influence the interactions between such modified proteins and other molecules present in their vicinity, such as lipids and other proteins.

In the present study, we developed a [ $^{14}\text{C}$ ]acyl-CoA labelling assay using [ $^{14}\text{C}$ ]arachidonoyl-CoA to survey novel targets of protein-acylation and to search for novel actions of acyl-CoA. This assay detected various [ $^{14}\text{C}$ ]arachidonate-labelled proteins in rat liver microsomal fractions. Among these target proteins,

50 and 53 kDa proteins were purified from rat liver microsomes guided by this assay. Interestingly, these proteins were acylated in the absence of other proteins, suggesting that they catalysed autoacylation, a novel type of protein acylation. The purified proteins also possessed acyl-CoA-binding activity. We identified the 50 kDa/53 kDa proteins as UDP-glucuronosyltransferase (UDPGT) isoforms by amino acid sequencing analysis and UDPGT assay. We further examined the effects of acyl-CoA on UDPGT activity. Treatment of the 50 kDa/53 kDa proteins with arachidonoyl-CoA suppressed the UDPGT activity. The physiological significance of the binding of acyl-CoA and the acylation of the 50 kDa/53 kDa proteins is discussed.

### EXPERIMENTAL

#### Materials

[1- $^{14}\text{C}$ ]Arachidonic acid (20:4; 2.1 GBq/mmol), [1- $^{14}\text{C}$ ]arachidonoyl-CoA (1.7 GBq/mmol), UDP-[ $^{14}\text{C}$ ]glucuronic acid (10.55 GBq/mmol) and EN $^3$ HANCE were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Fatty acids (14:0, 16:0, 18:0, 18:1, 18:2 and 20:4), UDP-hexanolamine-agarose and azolectin (crude soybean lecithin) were obtained from Sigma (St. Louis, MO, U.S.A.). CoA was obtained from Kyowa Hakko, Ltd. (Tokyo, Japan). CHAPS was purchased from Dojindo Laboratories (Kumamoto, Japan). TSKgel AF Red-, Blue- and heparin-Toyopearl 650 ML were purchased from Tohso (Tokyo, Japan). Bio-gel HTP was from Bio-Rad (Richmond, CA, U.S.A.). Sephacryl S300 HR and CoA-agarose were from Pharmacia LKB (Uppsala, Sweden).

Abbreviations used: DTT, dithiothreitol; UDPGT, UDP-glucuronosyltransferase. Fatty acids are designated in terms of the number of carbon atoms: the number of double bonds, e.g. 20:4 denotes arachidonic acid.

\* To whom all correspondence should be addressed.

TLC plates (silica gel 60, 5721) were from Merck (Darmstadt, Germany). Acyl-CoA esters with various fatty acid moieties were synthesized as described previously [10]. All other reagents were of reagent or analytical grade.

#### [<sup>14</sup>C]Arachidonoyl-CoA labelling assay

Fractions to be tested (2.5 µg of protein was used unless otherwise indicated) were incubated with [<sup>14</sup>C]arachidonoyl-CoA (20 µM was used unless otherwise indicated) for various periods at 30 °C. In some cases, fractions were treated with various reagents such as *N*-ethylmaleimide prior to incubation with [<sup>14</sup>C]arachidonoyl-CoA. In other cases, fractions were incubated with [<sup>14</sup>C]arachidonoyl-CoA in the presence of various reagents, such as unlabelled acyl-CoA. The reaction was terminated by the addition of SDS (3% final concentration) and Bromophenol Blue, and the products were analysed by SDS/PAGE. The gel was stained with Coomassie Brilliant Blue, destained and subjected to fluorography using EN<sup>3</sup>HANCE at -80 °C with an intensifying screen. In some cases, bands of interest on the gels were also quantified using a liquid scintillation spectrometer.

#### Purification of acyl acceptor proteins from rat liver microsomes

Male rats of the Wistar strain, about 300 g in body weight (Sankyo Lab Service Co., Tokyo, Japan), were used. All animals were maintained under an alternating light-dark cycle (light from 8:00 to 18:00 h), and fed a standard diet *ad libitum*. All procedures were carried out at 4 °C. Livers (2 livers, approx. 28 g) were rapidly isolated and microsomes were prepared by differential centrifugation as described previously [10,11].

Microsomes were treated with 1% CHAPS, 20% ethylene glycol, 50 mM Tris/HCl (pH 7.4) (detergent:protein ratio was approx. 2; total volume, 160 ml), and then carefully sonicated in a Branson Sonifier (type 250; Danbury, CT, U.S.A.) equipped with a microtip (working at one-tenth of its maximum output for 1 min exposures with a 1 min interval, 2 cycles) in an ice bath. The clear solution was centrifuged at 105 000 g for 60 min and the resultant supernatant, designated 'CHAPS extract', was pooled. This CHAPS extract was applied to a TSKgel AF Red-Toyopearl 650 ML column (2.5 cm diam. × 8 cm), pre-equilibrated with 25 mM Tris/HCl (pH 7.4), 1% CHAPS, 20% ethylene glycol, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 0.2 mg/ml azolectin (buffer A). The column was washed with buffer A until the absorbance at 280 nm fell to the baseline (approx. 100 ml). The retained proteins were eluted with a linear gradient of NaCl in buffer A (0–2 M, total volume 200 ml) and each fraction was collected. When the [<sup>14</sup>C]arachidonoyl-CoA labelling assay was performed with each fraction, various acceptor proteins were eluted in a broad range of fractions. The 50 and 53 kDa proteins were eluted at around 0.2–0.5 M NaCl. These fractions were pooled and applied to a hydroxyapatite, Bio-Gel HTP column (1.5 cm diam. × 15 cm), pre-equilibrated with buffer A. The column was washed and eluted with a linear gradient of potassium phosphate in buffer A (0–0.5 M; total volume 120 ml). The 50 and 53 kDa proteins were eluted at around 0.2 M potassium phosphate. These fractions were pooled and diluted with 3 vol. of buffer A, and then further applied to a TSKgel AF-heparin-Toyopearl column (1 cm diam. × 6 cm) pre-equilibrated with buffer A. The proteins were eluted with a linear gradient of NaCl in buffer A (0–1 M; total volume 80 ml). The 50 and 53 kDa proteins were eluted at around 0.4 M NaCl. Several proteins labelled with [<sup>14</sup>C]acyl-CoA were enriched through these procedures, but the 50 and 53 kDa proteins were the major components. After desalting, the active fraction was further applied to a TSKgel AF-Blue-Toyopearl column ( $V_i$  =

5 ml) pre-equilibrated with buffer A. When the proteins were eluted with a linear gradient of NaCl in buffer A (0–1 M; total volume 60 ml), the 50 and 53 kDa proteins were eluted around 0.2 M NaCl. This fraction was pooled and most of the experiments were performed using this fraction (termed the 'Blue-Toyopearl fraction').

For complete purification, the pooled Blue-Toyopearl fraction was applied to UDP-hexanolamine-agarose ( $V_i$  = 2 ml) after desalting, and eluted with 5 mM UDP-glucuronic acid (10 ml). Elution of the proteins was confirmed by SDS/PAGE followed by staining with silver and the [<sup>14</sup>C]arachidonoyl-CoA labelling assay.

#### Hydrolysis of labelled proteins *in situ*

To test the stability of the interaction between labelled arachidonoyl-CoA and proteins, [<sup>14</sup>C]arachidonoyl-labelled proteins (2.5 µg) were subjected to SDS/PAGE and the gel was fixed and stained. The gel was further incubated with 1 M hydroxylamine (pH 7.0), 0.1 M KOH in 20% methanol, or 0.1 M HCl for 90 min at room temperature [8,9,12] and then subjected to fluorography.

#### Binding of 50 kDa/53 kDa proteins to CoA-agarose

The Blue-Toyopearl fraction was desalted using Ampure SA (Amersham) gel-filtration mini-columns. The fraction (50 µg of protein) was applied to four columns of CoA-agarose (column volume, 0.5 ml) pre-equilibrated with buffer A. After each column was washed, the proteins were eluted with 2 ml of buffer containing 10 mM ATP, 10 mM CoA, or 0.5 or 1 mM arachidonoyl-CoA. Aliquots of each eluate were analysed by SDS/PAGE and silver staining.

#### [<sup>14</sup>C]Arachidonoyl-CoA binding assay

The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5 µg) were incubated with 20 µM [<sup>14</sup>C]arachidonoyl-CoA for various periods at 30 °C. Protein-bound and free [<sup>14</sup>C]arachidonoyl-CoA fractions were separated by gel filtration. The mixture was loaded on to Sephacryl S300 HR ( $V_i$  = 30 ml) pre-equilibrated with 25 mM Tris/HCl (pH 7.4) containing 0.1% CHAPS and 1 mM DTT, and eluted with the same buffer, with collection of 1 ml fractions. The radioactivity of each fraction was determined using a liquid scintillation spectrometer.

#### Acyl-CoA hydrolase assay

The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5 µg of protein) were incubated with 20 µM [<sup>14</sup>C]arachidonoyl-CoA for various periods at 30 °C and with various concentrations of [<sup>14</sup>C]arachidonoyl-CoA for 3 h. The reaction was stopped by adding an equal volume of methanol, and unlabelled fatty acid was further added as a carrier. The mixture was directly applied to TLC plates, which were developed with petroleum ether/diethyl ether/acetic acid (70:30:1, by vol.). The spots of fatty acids were each scraped off into counting vials and the radioactivity was measured.

#### N-Terminal amino acid sequence analysis and protein homology search

The 50 and 53 kDa proteins were separated by SDS/PAGE and blotted on to polyvinylidene difluoride membranes with 50 mM 3-cyclohexylaminopropanesulphonic acid (pH 11.5) in 10% methanol [13]. The membranes were stained with Coomassie

Brilliant Blue, and the bands of the 50 and 53 kDa proteins were each excised. The N-terminal amino acid sequences were analysed by stepwise Edman degradation using a gas-phase automated protein sequenator (Applied Biosystems, Model 477A). Protein homology was searched in a database using DNASIS.

### Measurement of UDPGT activity

UDPGT activity was measured according to the method of Bansal and Gessner [14] with minor modifications. The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5  $\mu$ g) in Buffer A were incubated with 0.8 mM UDP-[ $^{14}$ C]glucuronic acid (3.7 Bq) and 5 mM MgCl<sub>2</sub> in the presence of 200  $\mu$ M testosterone or androsterone. In some cases, the fractions were incubated with arachidonoyl-CoA. Incubation was carried out at 30 °C for 30 min, and the reaction was terminated by addition of 2 vol. of ethanol. The ethanolic mixtures were evaporated and applied to silica-gel TLC plates; the chromatograms were then developed with n-butanol/acetone/acetic acid/ammonia/water (70:50:18:1.5:60, by vol.). After development, the plates were subjected to fluorography. The areas corresponding to [ $^{14}$ C]glucuronides were scraped off into counting vials and the radioactivity was measured.

### Protein assay

Protein contents were determined by the method of Lowry et al. [15] using BSA as a standard.

## RESULTS

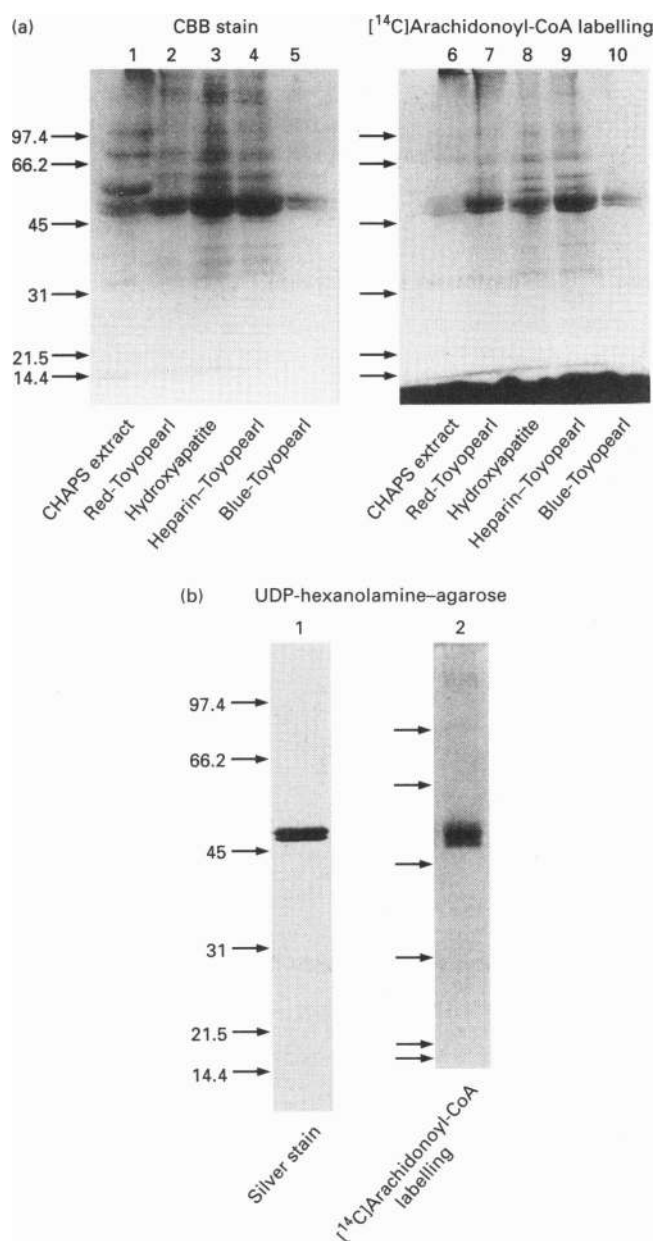
### Detection of [ $^{14}$ C]acyl-CoA-labelled proteins in rat liver microsomes

We developed [ $^{14}$ C]acyl-CoA labelling as a simple method to detect target proteins of acylation. The assay consisted of incubating fractions of proteins from various purification steps with a [ $^{14}$ C]fatty acyl-CoA and analysing the labelled proteins by SDS/PAGE. The autoradiograph of the crude extracts revealed several bands (Figure 1a). The  $^{14}$ C labelling occurred only upon mixing and incubation of fractions with [ $^{14}$ C]acyl-CoA. Since  $^{14}$ C labelling was detected after SDS/PAGE, i.e. after denaturation of proteins by SDS and staining/destaining in acetic acid/methanol, the modification of proteins with [ $^{14}$ C]arachidonate was considered to be covalent (i.e. acylation).

To study the labelling of the proteins by acyl-CoA further, we attempted to purify acceptor proteins of labelled fatty acyl moieties from rat liver microsomal proteins.

### Purification of 50 and 53 kDa proteins

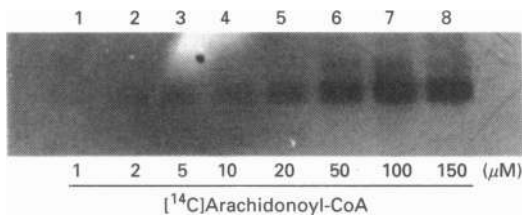
We purified two major acceptor proteins of 50 and 53 kDa from the microsomal fraction of rat liver. The purification consisted of the solubilization of microsomes with CHAPS and sequential chromatography on Red-Toyopearl, hydroxyapatite, heparin-Toyopearl, Blue-Toyopearl and UDP-hexanolamine-agarose as described in the Experimental section. The elution pattern of each column was checked by SDS/PAGE stained with Coomassie Brilliant Blue and [ $^{14}$ C]acyl-CoA labelling. The purification procedure is summarized in Figure 1. Aliquots from each step of the purification were analysed by SDS/PAGE stained with Coomassie Brilliant Blue and labelled with [ $^{14}$ C]acyl-CoA (Figure 1a). The 50 kDa/53 kDa proteins were enriched in the Blue-Toyopearl fraction. Although several minor components were observed by protein staining and [ $^{14}$ C]acyl-CoA labelling, the content of the 50 and 53 kDa proteins reached over 95% (Figure



**Figure 1** Purification of 50 and 53 kDa acyl-acceptor proteins in rat liver microsomes

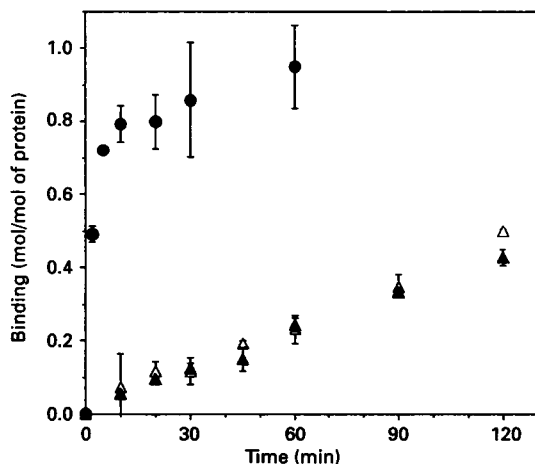
(a) The [ $^{14}$ C]arachidonoyl-CoA labelling assay was performed at each of the steps of purification. Aliquots were incubated with 25  $\mu$ M [ $^{14}$ C]arachidonoyl-CoA and separated by SDS/PAGE. The gel was stained with Coomassie Brilliant Blue (left-hand panel) and analysed by fluorography (right-hand panel). Lanes 1 and 6, CHAPS extract (20  $\mu$ g); lanes 2 and 7, Red-Toyopearl (10  $\mu$ g); lanes 3 and 8, hydroxyapatite (25  $\mu$ g); lanes 4 and 9, heparin-Toyopearl (20  $\mu$ g); lanes 5 and 10, Blue-Toyopearl (2.5  $\mu$ g). Marker proteins: phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). (b) Purification of the 50 and 53 kDa proteins on UDP-hexanolamine-agarose. Blue-Toyopearl fraction was applied to a UDP-hexanolamine-agarose column, which was washed and eluted with 5 mM UDP-glucuronic acid. The eluates were analysed by SDS/PAGE and bands were detected by silver staining (lane 1) and [ $^{14}$ C]arachidonoyl-CoA labelling (lane 2).

1a). Usually, the 50 kDa/53 kDa proteins in this fraction possessed acylation activity of approx. 0.1 mol of fatty acid incorporated/mol of protein during 30 min incubation. Most of



**Figure 2** Dose-dependency of the acylation

The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5  $\mu\text{g}$ ) were incubated with various concentrations of [ $^{14}\text{C}$ ]arachidonoyl-CoA (lanes corresponding to 1, 2, 5, 10, 20, 50, 100 and 150  $\mu\text{M}$ ) for 30 min and each was analysed by means of SDS/PAGE and fluorography.



**Figure 3** Time courses of acylation and [ $^{14}\text{C}$ ]arachidonoyl-CoA binding

The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5  $\mu\text{g}$ ) were incubated with 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]arachidonoyl-CoA for various periods of time. Each was analysed by means of SDS/PAGE and fluorography. Radioactivity incorporated into the 50 kDa ( $\Delta$ ) and 53 kDa proteins ( $\blacktriangle$ ) was measured. The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5  $\mu\text{g}$ ) were incubated with 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]arachidonoyl-CoA for various periods and then applied to Sephacryl S300 HR for separation of bound/free. The radioactivity of the bound form was measured ( $\bullet$ ).

the experiments were performed in this fraction (defined as the 'Blue-Toyopearl fraction').

Complete purification of the 50 and 53 kDa proteins was achieved on UDP-hexanolamine-agarose (Figure 1b); the minor contaminants were removed. Radioactivity was still incorporated into the purified proteins upon incubation with [ $^{14}\text{C}$ ]arachidonoyl-CoA, suggesting that the acylation is catalysed by the proteins themselves, i.e. autoacylation, rather than by contaminating enzymes.

#### Properties of the acylation of 50 kDa/53 kDa proteins

Several properties of the acylation of the 50 and 53 kDa proteins by [ $^{14}\text{C}$ ]arachidonoyl-CoA were examined. When the proteins were incubated with various concentrations of [ $^{14}\text{C}$ ]arachidonoyl-CoA for 30 min, the labelling increased in a concentration-dependent manner (Figure 2). Apparent half-maximal labelling was seen at approx. 20–50  $\mu\text{M}$  [ $^{14}\text{C}$ ]arachidonoyl-CoA. When the purified 50 and 53 kDa proteins were incubated with 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]arachidonoyl-CoA for various periods of time, the label was incorporated into each protein in a time-dependent manner (Figure 3). Acylation proceeded slowly and the incorporation of

fatty acid into the 50 and 53 kDa proteins was 0.11 and 0.12 mol/mol of protein, respectively, over a 30 min incubation. The initial rates of activity were calculated as approx. 0.0036 (50 kDa) and 0.0040 (53 kDa)  $\text{min}^{-1}$ .

The acyl-CoA specificity of the acylation of the 50 and 53 kDa proteins was examined by a competitive labelling assay with [ $^{14}\text{C}$ ]arachidonoyl-CoA and various unlabelled acyl-CoAs (Table 1). The labelling was inhibited by unlabelled arachidonoyl-CoA, linoleoyl-CoA, oleoyl-CoA, stearoyl-CoA, palmitoyl-CoA and myristoyl-CoA. The results indicated that the acylation has a broad specificity for acyl-CoA. This broad specificity for fatty acyl-CoA esters indicated that the acylation reaction is distinct from myristoylation and palmitoylation, both of which exhibit tight fatty acid specificities [8,9]. Compounds such as CoA and ATP were next tested as potential competitors. Even 1 mM ATP and CoA failed to inhibit the acylation potently. This result suggests that the 50 kDa/53 kDa proteins have higher affinities for acyl-CoA than CoA and ATP. UTP and UDP-glucuronic acid were also ineffective.

We then searched for activators and inhibitors of the acylation reaction (Table 2). Pretreatment of purified 50 and 53 kDa proteins with DTT (1 mM or 5 mM) enhanced the acylation (the buffer for the purification of the 50 and 53 kDa proteins already contained 1 mM DTT), while treatment with 2.5% 2-mercaptoethanol partially inhibited the reaction. In addition, SH reagents such as 5,5'-dithiobis-2-(nitrobenzoic acid) and *N*-ethylmaleimide inhibited the acylation in a dose-dependent manner. Acylation was completely inhibited by these reagents at 1 mM. These results suggest that SH groups are involved in the acylation of the 50 kDa/53 kDa proteins. Acylation was completely inhibited by pretreatment with 3% SDS, and by heat denaturation (100  $^{\circ}\text{C}$ , 5 min). These results suggest that the acylation of these proteins is an enzyme-catalysed reaction.

#### Analysis of the mode of labelling

We examined the mode of labelling of the 50 and 53 kDa proteins with [ $^{14}\text{C}$ ]arachidonoyl-CoA (Table 2). Labelled proteins in the gel were treated with hydroxylamine (pH 7.0), KOH in methanol, and HCl. The radioactivity was reduced by hydroxylamine and KOH/methanol, but not by HCl. Since hydroxylamine (pH 7.0) cleaves thioester linkages and KOH in methanol cleaves ester linkages [8,9,12], it appears that [ $^{14}\text{C}$ ]arachidonic acid was bound to the 50 and 53 kDa proteins via a thioester linkage.

#### Binding activity of the 50 and 53 kDa proteins to acyl-CoA

Since purified 50 and 53 kDa proteins were acylated by themselves, the proteins should have a binding activity towards acyl-CoA. Thus several experiments were conducted to examine the binding activity.

Acyl-CoA binding of the 50 kDa/53 kDa proteins was examined by bound/free separation using gel filtration. The 50 kDa/53 kDa proteins were incubated with [ $^{14}\text{C}$ ]arachidonoyl-CoA and then loaded on to the Sephacryl S300 HR. Two peaks of  $^{14}\text{C}$  label were observed in the elution profile. Separation of the protein-bound form and free form of [ $^{14}\text{C}$ ]acyl-CoA was achieved by the column. The binding (first peak of radioactivity) included both covalent and non-covalent binding (binding of [ $^{14}\text{C}$ ]arachidonoyl-CoA plus [ $^{14}\text{C}$ ]acylation).

Using this assay, acyl-CoA binding to the 50 kDa/53 kDa proteins was examined. The 50 kDa/53 kDa proteins were incubated with 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]arachidonoyl-CoA for various periods (Figure 3). The binding was dependent on the incubation periods, and reached a maximal level (approx. 1 mol of acyl-CoA

**Table 1** Effects of various reagents on acylation of the 50 kDa/53 kDa proteins

The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5  $\mu\text{g}$  of protein) were incubated with 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]arachidonoyl-CoA for 30 min in the presence of indicated competitors. Values represent means  $\pm$  S.D. (% of control). The 100% values were  $0.11 \pm 0.01$  and  $0.10 \pm 0.01$  (mol/mol of protein) for the 50 and 53 kDa proteins respectively.

Competitors	Concentration ( $\mu\text{M}$ )	Acylation (% of control)	
		50 kDa protein	53 kDa protein
Arachidonoyl-CoA	200	9.6 $\pm$ 3.4	14.1 $\pm$ 6.8
Linoleoyl-CoA	200	18.1 $\pm$ 1.1	8.8 $\pm$ 2.0
Oleoyl-CoA	200	21.3 $\pm$ 2.2	12.5 $\pm$ 1.0
Stearoyl-CoA	200	19.1 $\pm$ 2.4	10.0 $\pm$ 4.1
Palmitoyl-CoA	200	20.2 $\pm$ 5.6	16.3 $\pm$ 5.1
Myristoyl-CoA	200	26.6 $\pm$ 4.5	26.3 $\pm$ 6.1
CoA	1000	52.1 $\pm$ 5.6	70.0 $\pm$ 8.2
ATP	1000	78.7 $\pm$ 3.4	86.2 $\pm$ 1.0
UTP	1000	91.5 $\pm$ 10.1	117.5 $\pm$ 2.6
UDP-glucuronic acid	1000	71.3 $\pm$ 1.1	81.2 $\pm$ 14.3

**Table 2** Effects of various treatments on acylation of the 50 kDa/53 kDa proteins

(a) The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5  $\mu\text{g}$  of protein) were pretreated with various reagents and then incubated with 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]arachidonoyl-CoA for 30 min. Values represent means  $\pm$  S.D. (% of control). The 100% values were  $0.11 \pm 0.01$  and  $0.10 \pm 0.01$  (mol/mol of protein) for the 50 and 53 kDa proteins respectively. (b) The 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5  $\mu\text{g}$  of protein) were incubated with 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]arachidonoyl-CoA and then separated by SDS/PAGE. Labelled proteins in the gel were treated with the indicated reagents. The 100% values were  $0.11 \pm 0.00$  and  $0.10 \pm 0.02$  (mol/mol of protein) for the 50 and 53 kDa proteins respectively. Abbreviations: DTNB, 5,5'-dithiobis-2-(nitrobenzoic acid); NEM, *N*-ethylmaleimide.

Treatment	Concentration	Acylation (% of control)	
		50 kDa protein	53 kDa protein
(a) Pretreatment			
DTT	1 mM	117.7 $\pm$ 12.2	119.3 $\pm$ 4.3
	5 mM	122.3 $\pm$ 10.2	118.2 $\pm$ 3.1
2-Mercaptoethanol	2.5%	43.7 $\pm$ 2.0	44.7 $\pm$ 4.7
DTNB	0.1 mM	67.0 $\pm$ 2.1	89.8 $\pm$ 2.8
	1 mM	0	0
NEM	0.1 mM	76.1 $\pm$ 22.3	97.7 $\pm$ 20.5
	1 mM	0	0
SDS	3%	0	0
Boiling	0	0	0
(b) Treatment after acylation			
Hydroxylamine (pH 7.0)	1 M	44.8 $\pm$ 6.3	31.8 $\pm$ 9.4
KOH in methanol	0.1 M	30.2 $\pm$ 5.2	25.9 $\pm$ 5.9
HCl	0.1 M	104.2 $\pm$ 11.5	97.6 $\pm$ 28.2

binding/mol of protein) during 30 min incubation. At this point, the acylation was about 10% of total binding, and binding was mostly accounted for by non-covalent binding. This assay showed that the proteins have acyl-CoA-binding activity (non-covalent binding). Non-covalent binding of acyl-CoA to the proteins proceeded more rapidly than acylation, with an initial rate calculated as  $0.25 \text{ min}^{-1}$  (0.5 mol of acyl-CoA binding/mol of protein during 2 min incubation).

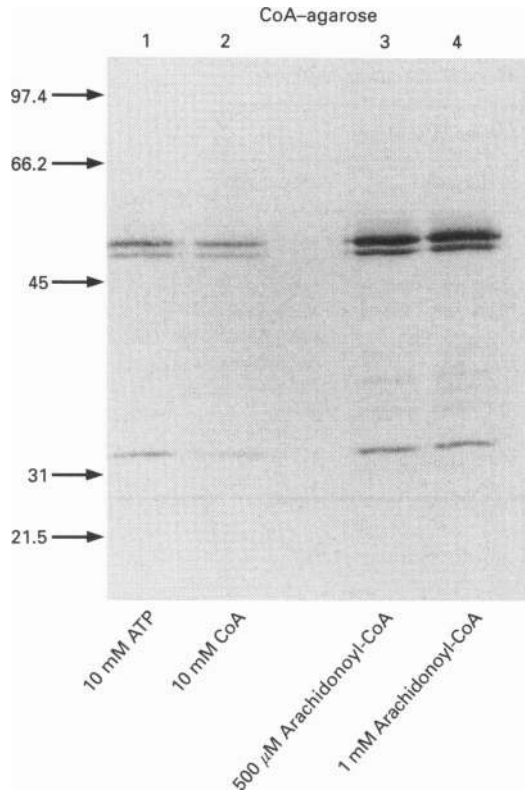
Acyl-CoA-binding activity of these proteins was also confirmed by elution through a CoA-agarose column (Figure 4). This affinity resin resembles acyl-CoA since CoA is bound to agarose by a thioester linkage with a six-carbon spacer in the resin. When the Blue-Toyopearl fraction was applied to mini-columns of CoA-agarose, the 50 and 53 kDa proteins were retained in the column, suggesting that the proteins interacted with (acyl-)CoA. The proteins were partially, and gradually, eluted by ATP and CoA at higher concentrations (10 mM). The proteins seemed to recognize a part of the adenine nucleotide moiety of acyl-CoA. These proteins were, however, eluted more efficiently with lower

concentrations of arachidonoyl-CoA, at least 26 times lower than the ligand concentration in agarose (because 13  $\mu\text{mol}$  of CoA was bound to 1 ml of agarose the ligand concentration was calculated to be approx. 13 mM). These results also confirmed that the 50 and 53 kDa proteins had higher affinities for acyl-CoA than ATP and CoA. Free fatty acids such as arachidonic acid failed to elute the 50 and 53 kDa proteins from CoA-agarose (results not shown). These results suggest that the proteins interact with acyl-CoA but not with free fatty acids.

These experiments suggested that the 50 and 53 kDa proteins are acyl-CoA-binding proteins, and that the acylation occurs by self-catalysis (i.e. autoacylation), as described above.

#### The 50 and 53 kDa proteins have acyl-CoA-hydrolysing activity

Acyl-CoA hydrolase activity was assayed with the purified proteins. As shown in Figure 5, very low acyl-CoA-hydrolysing activity was detected (approx. 111 pmol/min per mg of purified protein, or  $0.006 \text{ min}^{-1}$ ). Hydrolase activity depended on the



**Figure 4** Binding of 50 and 53 kDa proteins to CoA-agarose

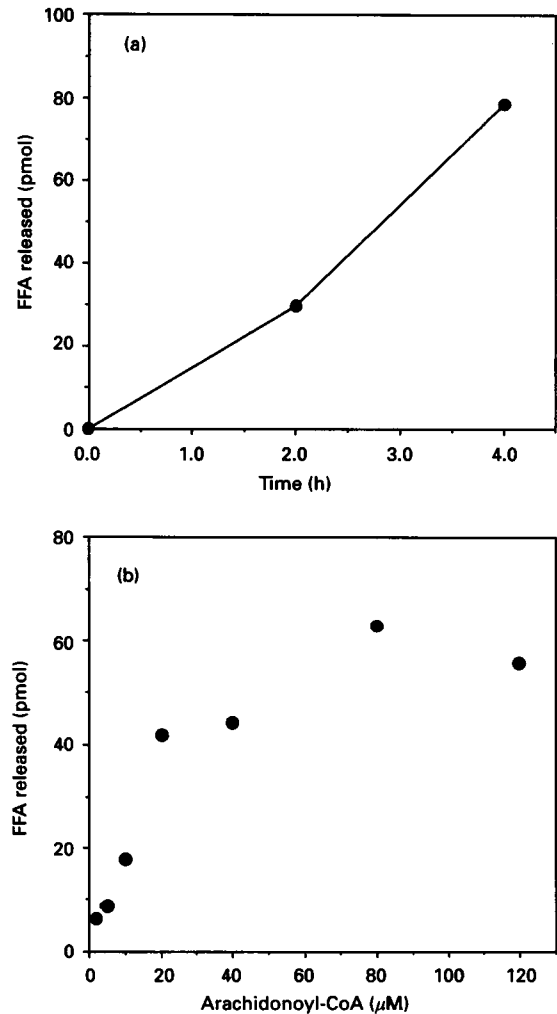
(a) The 50 kDa/53 kDa proteins (Blue-Toyoppearl fraction, 50  $\mu$ g) were applied to four columns of CoA-agarose (column volume, 0.5 ml), pre-equilibrated with buffer A. Each column was washed and eluted with 2 ml of buffer containing 10 mM ATP (lane 1), 10 mM CoA (lane 2), or 0.5 (lane 3) or 1 mM arachidonoyl-CoA (lane 4). Aliquots of each fraction were analysed by SDS/PAGE and silver staining.

concentration of [ $^{14}$ C]arachidonoyl-CoA, and the apparent  $K_m$  value for this substrate was 15  $\mu$ M (Figure 5b). Acyl-CoA specificity of the hydrolase activity of the 50 kDa/53 kDa proteins was also examined by a competitive assay with [ $^{14}$ C]arachidonoyl-CoA and various unlabelled acyl-CoAs. [ $^{14}$ C]Arachidonoyl-CoA hydrolase activity was inhibited by various unlabelled acyl-CoAs, suggesting that the hydrolase reaction has a broad acyl-CoA specificity (results not shown). This specificity was the same as that of the acylation reaction. The results suggested that covalently bound fatty acid is turned over by the hydrolase activity.

The fraction had no detectable non-specific carboxylesterase activity towards *p*-nitrophenyl acetate (results not shown).

#### The 50 and 53 kDa proteins are isoforms of UDPGT

The N-terminal amino acid sequences of the 50 and 53 kDa proteins were analysed up to 21 residues; GKVLVWPMEFS-HWMNIKTILD and GKVLVWPMDFSHWMNIKIILD respectively. These results strongly suggest that the two proteins are related. A protein homology search in a database showed that the N-terminal sequence of the 50 kDa protein was identical to previously reported partial sequences of UDPGT isoforms, UDPGTr-3 [16] and UDPGTr-5 [17], and that the corresponding sequence of the 53 kDa protein was identical to that of UDPGTr-4 [18] (Table 3). We have already confirmed the binding activity of the 50 and 53 kDa proteins to UDP-hexanolamine-agarose



**Figure 5** Acyl-CoA hydrolase activity of the 50 kDa/53 kDa proteins

(a) The 50 kDa/53 kDa proteins (Blue-Toyoppearl fraction, 2.5  $\mu$ g) were incubated with 20  $\mu$ M [ $^{14}$ C]arachidonoyl-CoA for 2 and 4 h. (b) The proteins (2.5  $\mu$ g) were incubated with various concentrations of [ $^{14}$ C]arachidonoyl-CoA for 3 h. After the reaction was terminated, the mixture was separated by TLC, and the radioactivity of fatty acid was measured.

(UDP-sugar analogue, Figure 1b). Furthermore, the purified proteins possessed the enzyme activities of UDPGT, 197.1 and 193.8 nmol/min per mg of protein against testosterone and androsterone respectively (Table 4). These are putative substrates of UDPGTr-3/UDPGTr-5 and UDPGTr-4 (Table 3). These UDPGT activities against testosterone and androsterone were enriched about 109.5- and 65.7-fold respectively, compared with the microsomal activities after the purification procedures. The observation that the proteins possess this enzyme activity strongly supports the conclusion that they are UDPGT isoforms.

The 50 and 53 kDa proteins are multifunctional enzymes with both acyl-CoA hydrolase and UDPGT activity, although the magnitudes of the activities differed by about 1000 times. The 50 and 53 kDa proteins seem to have different catalytic sites for different enzyme activities, because acylation was not inhibited by UDP-glucuronic acid (Table 1).

#### Inhibition of UDPGT activity by acyl-CoA

The effects of acyl-CoA treatment on the UDPGT activity of the

**Table 3 Amino acid sequence comparison of the 50 and 53 kDa proteins with UDPGT isoforms**

The N-terminal amino acid sequences of the 50 and 53 kDa proteins were analysed using a protein sequenator. Corresponding deduced amino acid sequences of UDPGT isoforms are shown. The portions from positions 1 to 23 are signal peptides cleaved in mature enzymes [16–18].

Protein	Amino acid sequence	Putative substrates	References
50 kDa	1 GKVLVWPMFESHWMNIKTILD 21		
53 kDa	1 GKVLVWPMDFSHWMNIKIILD 21		
UDPGT-3	21 GNCGKVLVWPMFESHWMNIKTILDELVQR 49	Testosterone, dihydrotestosterone, $\beta$ -oestradiol	[16]
UDPGT-5	21 AKCGKVLVWPMFESHWMNIKTILDELVQR 49	Testosterone, dihydrotestosterone, $\beta$ -oestradiol	[17]
UDPGT-4	21 GHCCKVLVWPMDFSHWMNIKIILDELVQR 49	Etiocolanolone, androsterone, lithocholic acid	[18]

**Table 4 Relationship of UDPGT activity, binding of acyl-CoA and autoacylation**

UDPGT activities in purified 50 kDa/53 kDa proteins (Blue-Toyopearl fraction, 2.5  $\mu$ g of protein) or microsomal fraction (200  $\mu$ g) were measured against testosterone and androsterone in the presence of the indicated amounts of arachidonoyl-CoA. [ $^{14}$ C]Arachidonoyl-CoA binding and acylation of the 50 kDa/53 kDa proteins over 30 min were measured at the indicated concentrations of [ $^{14}$ C]arachidonoyl-CoA. Values given in parentheses are in terms of % of controls.

Protein	Activity	Treatment (substrate)	Arachidonoyl-CoA ( $\mu$ M)		
			0	20	50
50 kDa/53 kDa protein	UDPGT activity (nmol/min per mg of protein)	Testosterone	197.1 $\pm$ 25.5 (100)	27.9 $\pm$ 7.6 (14.2)	20.3 $\pm$ 2.3 (10.3)
		Androsterone	193.8 $\pm$ 2.9 (100)	92.6 $\pm$ 4.3 (47.8)	52.3 $\pm$ 7.0 (27.0)
	Acyl-CoA binding (mol/mol of protein) (over 30 min)	—	0.95 $\pm$ 0.05	0.98 $\pm$ 0.02	
	Acylation (mol/mol of protein) (over 30 min)	50 kDa protein	—	0.12 $\pm$ 0.01	0.22 $\pm$ 0.02
		53 kDa protein	—	0.13 $\pm$ 0.02	0.23 $\pm$ 0.02
Microsomes	UDPGT activity (nmol/min per mg of protein)	Testosterone	1.80 $\pm$ 0.06 (100)	0.79 $\pm$ 0.05 (43.9)	0.62 $\pm$ 0.02 (34.4)
		Androsterone	2.95 $\pm$ 0.02 (100)	1.63 $\pm$ 0.06 (55.3)	0.82 $\pm$ 0.02 (27.8)

purified proteins and microsomal fraction were examined. As shown in Table 4, when the purified proteins were assayed in the presence of 20  $\mu$ M arachidonoyl-CoA, UDPGT activities towards testosterone and androsterone were reduced to 14.2% and 47.8% respectively, compared with the mock treatment. The inhibition of the enzyme activity by arachidonoyl-CoA was dose-dependent. A similar inhibition was also observed in the case of microsomal enzymes. These results suggest that glucuronidation of these hormones is regulated by the acyl-CoA level.

We next examined the correlation of the binding of acyl-CoA and/or autoacylation of the proteins with inhibition of UDPGT activity. As shown in Table 4, severe inhibition was observed with 20  $\mu$ M arachidonoyl-CoA. However, only 10% of the 50 and 53 kDa proteins was acylated under these experimental conditions. The inhibition of UDPGT activity seemed to be correlated with acyl-CoA binding to the proteins. This indicates that the inhibition may be mainly caused by the binding of the acyl-CoA to the proteins. UDPGT activity was more inhibited by 50  $\mu$ M arachidonoyl-CoA than 20  $\mu$ M (dose-dependent),

although the binding was already saturated even at 20  $\mu$ M. Therefore, UDPGT activity is inhibited not only by the binding of acyl-CoA but also by autoacylation.

## DISCUSSION

In this study, we have purified the 50 and 53 kDa proteins, two major acyl-acceptor proteins, to near homogeneity from a rat liver microsomal fraction. The detection of  $^{14}$ C-labelling in these proteins after SDS/PAGE suggested that covalent modification, i.e. acylation, had occurred. Acylation occurred in the absence of any other proteins, suggesting autoacylation.

Several experiments suggested that the 50 and 53 kDa proteins were acyl-CoA-binding proteins. Acyl-CoA binding to the proteins occurred rapidly, whereas the autoacylation occurred slowly (Figure 3). These proteins also possess weak acyl-CoA hydrolase activity (Figure 5). The rates of the three phenomena were 0.25 (binding), 0.004 (acylation), and 0.006  $\text{min}^{-1}$  (hydrolysis), respectively. These results demonstrated that these proteins

catalysed the sequential reactions of binding of acyl-CoA, autoacylation and hydrolysis of the fatty acid.

The physiological significance of the sequential reactions of the 50 kDa/53 kDa proteins with acyl-CoA remains to be established. Several lines of experimental evidence indicated that these proteins are UDPGT isoforms (Figure 1b and Tables 3 and 4). UDPGT catalyses the glucuronidation of endogenous (bilirubin and steroids) as well as xenobiotic compounds, and is mainly located in the endoplasmic reticulum of hepatocytes [19–21]. Many isoforms, which exhibit different substrate specificities, are known to exist. Recent cDNA cloning studies of UDPGT have confirmed the existence of isoenzymes [16–18,20,21].

A possible explanation for the role of the interaction of the 50 kDa/53 kDa proteins with acyl-CoA (binding of acyl-CoA, autoacylation, and ester bond hydrolysis) is that the processes affect the UDPGT activity. In fact, acyl-CoA treatment inhibited the UDPGT activity of the purified proteins and microsomal fractions in the *in vitro* assay (Table 4). This inhibition was mainly due to the binding of acyl-CoA. The acylation of the proteins also contributed to the inhibition of UDPGT activity. Acylation may involve more stable inhibition of the enzyme activity (Table 4).

The question of whether acyl-CoA is involved in the regulation of UDPGT activity within intact cells is critically important. Recently, Zhong et al. [22] reported that long-chain fatty acids inhibited the glucuronidation of benzopyrene *in situ* during liver perfusion. They attributed the inhibition to acyl-CoA derived from a free fatty acid, but not to free fatty acids themselves. The findings of the present study seem to account for the mechanism of inhibition of UDPGT activity in this *in situ* assay. Krcmery and Zakim also reported that physiological concentrations of oleoyl-CoA inhibited microsomal UDPGT activities towards 4-nitrophenol and 1-naphthol [23]. These reports suggest the possible regulation of UDPGT activity by acyl-CoA not only *in vitro*, but also within intact cells.

Various lipid-binding proteins are known to exist within cells. Glutathione S-transferases are fatty acid-binding proteins and their activities are also regulated by fatty acid [24]. Both glutathione S-transferases and UDPGT are involved in the conjugation of hydrophobic molecules. It is very interesting that these enzymes, which have similar functions, are lipid-binding proteins and are regulated by lipids.

We detected several acyl-acceptor proteins by [<sup>14</sup>C]acyl-CoA labelling assay even after several purification steps (Figure 1a). These proteins also seemed to catalyse the autoacylation reaction, suggesting the diversity of autoacylation. Previously, a protein capable of catalysing an autoacylation reaction was detected and purified from bovine liver extract using an <sup>125</sup>I-labelled myristoyl-CoA analogue [25,26]. This protein was identified as methylmalonate-semialdehyde dehydrogenase and the enzyme activity was also inhibited by acyl-CoA. Yeast aldehyde dehydrogenase and bovine liver glutamate dehydrogenase were also regulated by acyl-CoA via autoacylation [26]. These enzymes, however, are clearly different from the 50 and 53 kDa proteins in several characteristics such as tissue and subcellular distribution, and relative molecular mass. Although auto-

acylation is a novel and unique concept for the regulation of the function of enzymes by fatty acid modification, information regarding proteins which undergo autoacylation is extremely limited. Using these assays, acyl-acceptor proteins other than UDPGT isoforms and these dehydrogenases may also be detected. The accumulation of evidence for autoacylation of various proteins is necessary to understand the precise role of this type of protein modification.

In conclusion, we have demonstrated that UDPGT isoforms are novel acyl-CoA-binding and fatty acid-modified proteins. These modifications are proposed to be part of a novel system of regulation of UDPGT activity by acyl-CoA. Fatty acyl-CoA may modulate the glucuronidation involved in the metabolism of drugs, steroids and bilirubin.

We are grateful to Mr. Hideo Tanihata and Ms. Yuko Hosono for their technical assistance. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

## REFERENCES

- 1 Van Golde, L. M. G. and Van den Bergh, S. G. (1977) in *Lipid Metabolism in Mammals* (Snyder, F., ed.), vol. 1, pp. 35–149. Plenum Press, New York
- 2 Huang, W.-H., Wang, Y. and Askari, A. (1989) *J. Biol. Chem.* **264**, 2605–2608
- 3 Deeney, J. T., Tornheim, K., Korchak, H. M., Prentki, M. and Corkey, B. E. (1992) *J. Biol. Chem.* **267**, 19840–19845
- 4 Bronfman, M., Morales, M. N. and Orellana, A. (1988) *Biochem. Biophys. Res. Commun.* **152**, 987–992
- 5 Li, Q., Yamamoto, N., Inoue, A. and Morisawa, S. (1990) *J. Biochem. (Tokyo)* **107**, 699–702
- 6 Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V. and Rothman, J. E. (1989) *Cell* **59**, 95–102
- 7 Ostermann, J., Orci, L., Tani, K., Amherdt, M., Ravazzola, M., Elazar, Z. and Rothman, J. E. (1993) *Cell* **75**, 1015–1025
- 8 Towler, D. A., Gordon, J. I., Adams, S. P. and Glaser, L. (1988) *Annu. Rev. Biochem.* **57**, 69–99
- 9 Schults, A. M. and Henderson, L. E. (1988) *Annu. Rev. Cell Biol.* **4**, 611–647
- 10 Sugiura, T., Masuzawa, Y. and Waku, K. (1988) *J. Biol. Chem.* **263**, 17490–17498
- 11 Yamashita, A., Sato, K., Watanabe, M., Tokudome, Y., Sugiura, T. and Waku, K. (1994) *Biochim. Biophys. Acta* **1211**, 263–269
- 12 Magee, A. I. and Schlesinger, M. J. (1982) *Biochim. Biophys. Acta* **694**, 279–289
- 13 Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038
- 14 Bansal, S. K. and Gessner, T. (1980) *Anal. Biochem.* **109**, 321–329
- 15 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 16 Mackenzie, P. I. (1987) *J. Biol. Chem.* **262**, 9744–9749
- 17 Mackenzie, P. I. (1990) *J. Biol. Chem.* **265**, 8699–8703
- 18 Mackenzie, P. I. (1986) *J. Biol. Chem.* **261**, 14112–14117
- 19 Dutton, G. J. (1980) in *Glucuronidation of Drugs and Other Compounds* (Dutton, G. J., ed.), pp. 69–78. CRC Press, Inc., Boca Raton, FL
- 20 Mulder, G. J. (1992) *Annu. Rev. Pharmacol. Toxicol.* **32**, 25–49
- 21 Burchell, B., Nebert, D. W., Nerson, D. R., Bock, K. W., Iyanagi, T., Jansen, P. L. M., Lancet, D., Mulder, G. J., Roy Chowdhury, J., Siest, G., Tephly, T. R. and Mackenzie, P. I. (1991) *DNA Cell Biol.* **10**, 487–494
- 22 Zhong, Z., Kauffman, F. C. and Thurman, R. G. (1991) *Cancer Res.* **51**, 4511–4515
- 23 Krcmery, M. and Zakim, D. (1993) *Biochem. Pharmacol.* **46**, 897–904
- 24 Li, M. and Ishibashi, T. (1990) *J. Biochem. (Tokyo)* **108**, 462–465
- 25 Deichaitte, I., Berthiaume, L., Peseckis, S. M., Patton, W. F. and Resh, M. D. (1993) *J. Biol. Chem.* **268**, 13738–13747
- 26 Berthiaume, L., Deichaitte, I., Peseckis, S. M. and Resh, M. D. (1993) *J. Biol. Chem.* **268**, 13738–13747