Electrophoretic mobility of γ -glutamyltransferase in rat liver subcellular fractions

Evidence for structure difference from the kidney enzyme

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Adult rat liver γ -glutamyltransferase (GGT) has been poorly characterized because of its very low concentration in the tissue. In contrast with the kidney, the liver enzyme is inducible by some xenobiotics, and its relationship to hepatic ontogeny and carcinogenesis seems to be important. Liver GGT polypeptides were identified by immunoblot analysis in subcellular fractions (rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi membranes and plasma membranes). Rat liver GGT appeared as a series of polypeptides corresponding to different maturation steps. Polypeptides related to the heavy subunit of GGT were detected in rough endoplasmic reticulum at 49, 53 and 55 kDa, and in Golgi membranes at 55, 60 and 66 kDa. Two polypeptides related to the light subunit of GGT were also observed in Golgi membranes. In plasma membranes GGT was composed of 100 kDa, 66 kDa and 31 kDa polypeptides. The 66 kDa component could correspond to the heavy subunit of the rat liver enzyme, and if so has a molecular mass higher than that of the purified rat kidney form of GGT (papain-treated). These data suggest different peptide backbones for the heavy subunits of liver GGT and kidney GGT.

INTRODUCTION

γ -Glutamyltransferase (GGT, EC 2.3.2.2) catalyses the transfer of the glutamyl moiety of GSH and other γ -glutamyl compounds to a variety of amino acid and peptide acceptors. Its major location among mammalian tissues is the kidney, from which source the enzyme has been extensively studied (Matsuda, 1983). Although present at very low concentrations in the liver, the hepatic enzyme is of considerable interest. Ethanol, barbiturates or hepatocarcinogens induce its expression in the liver, and this is often associated with the appearance of the enzyme in the blood (Tazi *et al.*, 1980; Siest *et al.*, 1982; Sulakhe & Lutt, 1987). But the functional significance of GGT in normal liver remains to be elucidated, and first its structure and biosynthesis have to be defined. The few reported attempts to purify GGT from mammalian liver were mainly focused on the characterization of its kinetic properties (Shaw *et al.*, 1980; Bagrel *et al.*, 1981).

In contrast, the structure (amino acid and cDNA sequences) of the kidney enzyme as well as its post-translational maturation are well known (Matsuda, 1983; Nash & Tate, 1984; Laperche *et al.*, 1986; Coloma & Pitot, 1986a). It is a heterodimeric glycoprotein, synthesized in the rough endoplasmic reticulum (RER) as a unique precursor, which is cleaved into two subunits [heavy (H) and light (L)] and matured all along its migration pathways towards its functional location, the peripheral membranes of the cell.

In our previous studies on liver GGT, we have suggested a similar biosynthetic pathway for the liver

and the kidney enzymes, but some intraspecies dissimilarity in their molecular architecture, on the basis of immunoblotting analysis (Rahimi-Pour *et al.*, 1986; Antoine *et al.*, 1987). Our purpose was to investigate the different steps of post-translational maturation of control rat liver GGT during its intracellular transport from RER to plasma membranes (PM). The sensitivity of immunodetection of the GGT polypeptides in isolated liver subcellular fractions after Western blotting was greatly improved by the use of ¹²⁵I-Protein A. The data obtained clearly show differences between the liver GGT and the kidney GGT, without excluding some dissimilarity in the peptide backbones of their H subunits.

EXPERIMENTAL

Liver subcellular fractionation

Adult male Sprague–Dawley rats weighing 180–200 g were used for liver subfractionation into rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), Golgi membranes and plasma membranes (PM) from the same liver, as previously described (Antoine *et al.*, 1987). Briefly, liver was perfused with 0.15 M-NaCl, cut into small pieces and carefully homogenized (in a Dounce homogenizer) in the following buffer systems: (a) 1 mM-sodium bicarbonate buffer, pH 7.4, for preparation of PM by the procedure of Neville (1975); (b) 0.5 M-sucrose/5 mM-MgCl₂/50 mM-Tris/HCl buffer, pH 7.0, for preparation of SER, RER and Golgi membranes (Morr *et al.*, 1972). SER and RER were separated by centrifugation on a discontinuous sucrose

Abbreviations used: GGT, γ -glutamyltransferase (EC 2.3.2.2); H-GGT, heavy subunit of GGT; L-GGT, light subunit of GGT; PAGE, polyacrylamide-gel electrophoresis; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; PM, plasma membranes.

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gradient of the previous post-mitochondrial supernatant (mainly devoid of Golgi membranes, nuclear membranes and PM) at 100 000 *g* in an SW 28 rotor for 3 h. SER was restricted to vesicles with a relative density of 1.13 or less, and RER was restricted to vesicles with a relative density of 1.25 or more. Golgi membranes and PM are respectively enriched in trans-Golgi membranes and bile-canalicular membranes. Their purity was assessed by two-dimensional electrophoresis and marker-enzyme determinations (Antoine *et al.*, 1987).

Purification of rat kidney GGT and preparation of antibodies

Rat kidney GGT was purified from papain-treated cortical slices, by a procedure similar to that described by Tate & Meister (1975). The purity of the preparation and the specificity of the antiserum raised in rabbit were described previously (Rahimi-Pour *et al.*, 1986). Namely, these anti-(kidney GGT) antibodies were able to precipitate GGT activity from liver PM, but with less affinity than for the kidney enzyme. For purification of specific antibodies against each GGT polypeptide, purified kidney GGT was analysed by SDS/PAGE and transferred to a nitrocellulose sheet. The 95 kDa, 50 kDa and 30 kDa bands were then cut out and antibodies were affinity-purified from total antiserum on their respective nitrocellulose strips as described by Olmsted (1981). Antibody specificity for GGT was checked as described by Rahimi-Pour *et al.* (1986). The 'impoverished' total antiserum was obtained as follows. Total antiserum was incubated with a saturating concentration of purified kidney GGT in 0.15 M-NaCl for 16 h at 4 °C. After centrifugation to remove the immunoprecipitate (10 000 *g* for 10 min), GGT activity was measured in the supernatant by the method of Szasz (1969). The loss of activity of the pure GGT added was 90 %. The saturating concentration of purified GGT was calculated from the immunoprecipitation curve previously published (Rahimi-Pour *et al.*, 1986). The batch of pure kidney GGT used for immunoprecipitation was not the same as that used to obtain the antiserum. Antiserum against deglycosylated GGT was kindly donated by Dr. J. Coloma, and had been obtained in a rabbit by immunization with an anhydrous-HF-treated pure kidney GGT preparation (Coloma & Pitot, 1986b).

Gel electrophoresis and immunoblots

Proteins from different subcellular fractions were solubilized in 0.2 M-Tris containing 0.5 M-sucrose, 5 mM-EDTA, 1 % (w/v) methionine, 0.01 % Bromophenol Blue, 2 % (w/v) SDS and 5 mM-dithiothreitol. Iodoacetamide (50 mM) was added before PAGE in the presence of 0.1 % SDS according to the procedure of Laemmli (1970). Western blotting was performed as described by Burnette (1981). The time needed for effectively complete transfer of the protein from polyacrylamide to nitrocellulose was about 16 h. Procedures used for pre-hybridization and hybridization depended on the method used for the detection of immune complexes. When 125 I-Protein A was used, nitrocellulose sheets were pre-hybridized with 1 % (w/v) haemoglobin in Tween/Tris-buffered saline (0.05 % Tween 20 in 0.5 M-NaCl/20 mM-Tris/HCl buffer, pH 7.5), then incubated with specific antiserum (1:500) in 0.5 % haemoglobin in Tween/Tris-buffered saline for 2 h and with 125 I-Protein A (5×10^6 c.p.m.) in 0.5 % haemoglobin in Tween/Tris-

buffered saline for 1 h. Protein A was iodinated to the extent of 2×10^6 c.p.m./ μ g with Na 125 I and chloramine-T (Greenwood *et al.*, 1963). Radioactive bands were detected by autoradiography on Kodak X-Omat film after 18 h exposure at -80 °C.

In some studies (e.g. Fig. 3a) the use of peroxidase-conjugated Protein A to reveal goat anti-(human albumin) antibody required that gelatin (3 %, w/v) be employed as a saturator because of the peroxidase activity of haemoglobin. For the blots in Fig. 1 the nitrocellulose sheet was saturated by newborn-calf serum and incubated with peroxidase-conjugated goat anti-(rabbit IgG) antibodies. The substrate used for detecting peroxidase activity was *o*-diaminobenzidine.

RESULTS

Purified kidney GGT is composed of two subunits, termed H-GGT and L-GGT (heavy and light), that migrate in SDS/PAGE as two broad bands (50–55 kDa and 25–30 kDa respectively) because of the presence of differentially glycosylated isoforms (Fig. 4, lane 1) (Coloma & Pitot, 1986b). The first attempts made to characterize rat liver GGT in PM generally reported a major or unique band at about 100 kDa, when recognized by an anti-(kidney GGT) serum [Fig. 1 (lane a) and Castle *et al.* (1985)]. In our experiments this 100 kDa

Fig. 1. Immunoblotting analysis of rat liver PM (80 μ g of protein) after SDS/PAGE (13% acrylamide)

The detection of GGT-related polypeptides was performed on nitrocellulose sheets by using peroxidase-conjugated anti-(rabbit IgG) antibodies (Rahimi-Pour *et al.*, 1986). Specific antibodies were affinity-purified from total antiserum on the respective nitrocellulose slips obtained after electroblotting of pure kidney GGT as shown in Rahimi-Pour *et al.* (1986). Lane a, total anti-(kidney GGT) serum; lane b, anti-(kidney 95 kDa polypeptide) serum; lane c, anti-(kidney H-GGT) serum; lane d, anti-(kidney L-GGT) serum; lane e, Coomassie Blue staining.

Fig. 2. Identification of the detected polypeptides in liver RER and PM as GGT-related

Electrophoresis and Western-blotting conditions were as indicated in Fig. 1 legend. The detection of GGT-related bands was done with three different antisera: (a) anti-(rat kidney GGT) serum after immunoprecipitation with a preparation of pure kidney enzyme (loss of 90 % of the specific activity in the supernatant); (b) total anti-(rat kidney GGT) serum; (c) anti-(rat kidney deglycosylated GGT) serum prepared by acidic treatment of pure kidney GGT before rabbit immunization (Coloma & Pitot, 1986b). Lanes 1, liver PM (100 μ g of protein); lanes 2, liver RER (100 μ g of protein); lanes 3, purified rat kidney GGT (1 μ g).

polypeptide was shown to be GGT-related because of the sharing of antigenic epitopes with both kidney H-GGT and L-GGT subunits, as shown by the use of immunoblotting analysis with immunoaffinity-purified anti-(kidney H-GGT) and anti-(kidney L-GGT) antibodies that were previously characterized by Rahimi-Pour *et al.* (1986) (Fig. 1, lanes c and d).

The detection of GGT-related polypeptides in subcellular fractions of control rat liver was further improved by the use of 125 I-Protein A as immune-complex detector. After SDS/PAGE and Western blotting, the whole anti-(rat kidney GGT) serum recognized several bands in rat liver PM and RER. In PM 100 kDa, 66 kDa and 31 kDa GGT-related polypeptides were observed. In RER three bands of 49 kDa, 53 kDa and 55 kDa were revealed (Fig. 2b, lanes 1 and 2).

To assess the GGT identity of each liver polypeptide that was detected by the anti-(kidney GGT) serum, three other experiments were performed.

(i) The total antiserum was 'impoverished' of its content of anti-GGT antibodies by immunoprecipitation with a purified kidney GGT preparation. The new antiserum obtained had largely lost its capacity to recognize kidney H-GGT (Fig. 2a, lane 3), and subsequently in the liver the 55 kDa band in RER (compare Fig. 2a, lane 2, and Fig. 2b, lane 2) and the 66 kDa band in PM (compare Fig. 2a, lane 1, and Fig. 2b, lane 1). These two liver polypeptides are thus confirmed to be H-GGT-related. In PM the 100 kDa band is still recognized by the 'impoverished' antiserum, confirming the existence of common epitopes with the kidney L-GGT (Fig. 2a, lane 1). The two bands still observed in RER (Fig. 2a, lane 2) could be immature polypeptides of liver GGT.

(ii) An antibody directed only against kidney 'deglycosylated' GGT (Coloma & Pitot, 1986b) was able to recognize the 53 kDa band and, weakly, the 49 kDa band in RER (Fig. 2c, lane 2), but not the purified kidney GGT (Fig. 2c, lane 3), nor any polypeptide in PM (Fig.

2c, lane 1). These 49 kDa and 53 kDa bands in RER could correspond to not-entirely core-*N*-glycosylated GGT-related polypeptides. The same antibody was, in some experiments, able to recognize weakly in RER a 24 kDa band that is probably (from its molecular mass) an immature form of liver L-GGT.

(iii) The 66 kDa polypeptide seen in PM was shown not to be albumin (Fig. 3a). It is not immunodetected by an antibody directed against human albumin, which, as expected, recognized rat albumin, either pure (Sigma Chemical Co.) or in serum and in liver RER. Fig. 3(b) (lane 3) confirms that our anti-GGT serum does not cross-react with rat albumin. Finally, a GGT-related polypeptide was immunodetected at about 70 kDa in the serum of a phenobarbital-treated rat (Fig. 3b, lane 4).

The possible maturation pathway of liver GGT along its migration from its site of synthesis, the RER, towards PM is illustrated by Fig. 4. In both RER and SER, liver H-GGT appears as a series of three polypeptides (49 kDa, 53 kDa and 55 kDa) that could correspond to the precursors of differentially core-*N*-glycosylated mature proteins (as suggested by Yamashita *et al.*, 1983) (Fig. 4, lanes 4 and 5). In Golgi membranes is mainly recovered the largest RER polypeptide (i.e. 55 kDa), which is further matured by successive glycosylation steps to first a 60 kDa and then a 66 kDa polypeptide (Fig. 4, lane 3). This 66 kDa band would correspond to the mature form of liver H-GGT in its final location, the PM (Fig. 4, lane 2).

In a previous investigation, where subcellular fractions were solubilized with 8 M-urea for two-dimensional electrophoresis, L-GGT was not immunodetected in the liver PM by the anti-(kidney GGT) serum, but was immunodetected in another tissue, the brain (Rahimi-Pour *et al.*, 1986; Antoine *et al.*, 1986). In the present study liver L-GGT still remains undetectable in RER and SER (Fig. 4, lanes 4 and 5), but would be recognizable in its mature form in Golgi membranes and in PM as a

Fig. 3. Verification of the non-cross-reactivity between the anti-GGT serum and albumin by immunoblotting analysis

Panel (a) shows the immunodetection of rat liver or rat serum albumin by an anti-(human albumin) antibody. The cross-reactivity between human and rat albumin was expected, by reason of the high degree of homology found among the amino acid sequences in various mammals (see Putnam, 1984). The bands were detected by means of peroxidase-conjugated Protein A. Panel (b) shows in parallel the immunodetection of rat liver GGT or rat serum GGT for comparison. The procedure used was as indicated in Fig. 1 legend. Lanes 1, liver RER (80 μ g of protein); lanes 2, liver PM (80 μ g of protein); lanes 3, pure rat albumin (Sigma Chemical Co.) (1 μ g); lanes 4, serum of phenobarbital-treated rat (0.1 μ l equivalent).

31 kDa polypeptide (Fig. 4, lanes 2 and 3). In some experiments additional bands at 24 kDa in RER and at 27 kDa in Golgi membranes were seen, suggesting a total maturation of liver L-GGT from at least a 24 kDa polypeptide to a 31 kDa polypeptide.

DISCUSSION

Knowledge of adult liver GGT (structure and maturation), which is of great interest in pharmacology, has hitherto been rather restricted to data obtained in a rat hepatoma cell line (Barouki *et al.*, 1984). However, GGT is considered to be an oncoprotein, and its gene expression in hepatoma is perhaps no longer strictly adult-liver-specific (Tsuchida *et al.*, 1979). Thus, before understanding the ontogenic reversion of GGT to fetal expression in hepatoma (Yamashita *et al.*, 1983), it is fundamental to characterize the adult-liver-specific GGT in control animals. During electrophoresis, even in denaturing conditions, liver GGT subunits, as in any other tissue with low enzyme concentration, tend to aggregate as a major 100 kDa band (Castle *et al.*, 1985). Our previous studies using two-dimensional electrophoresis have first permitted the immunodetection of liver H-GGT in PM, as two polypeptides with different isoelectric points (Rahimi-Pour *et al.*, 1986).

In the present study, using a more sensitive procedure, we show that mature H-GGT could be present in control rat liver PM, as a higher-molecular-mass polypeptide

Fig. 4. Immunodetection of GGT-related polypeptides in the different subcellular membranes of rat liver after SDS/PAGE (11% acrylamide)

After electrophoresis, proteins of each subfraction (75 μ g) were blotted on nitrocellulose sheets (16 h), then immunodetected with an anti-(rat kidney GGT) serum. The immune complexes were detected by using 125 I-Protein A and autoradiography. Lane 1, purified rat kidney GGT (2 μ g); lane 2, liver PM; lane 3, liver Golgi membranes; lane 4, liver SER; lane 5, liver RER.

(66 kDa) than in the rat kidney (55 kDa). The association of this form with the L-GGT subunit could be the origin of the 100 kDa band always present in rat liver PM.

But the question arising was: are these intraspecies differences supported by glycosylation dissimilarities only, as already known (Yamashita *et al.*, 1983), or as interspecies variation (Tate & Khadse, 1986), or are they, in addition, the result of different primary sequences?

Rat kidney GGT is composed of two subunits (H and L) whose peptide backbones have molecular masses of 42 kDa and 19 kDa respectively. After co-translational *N*-glycosylation, H-GGT could carry up to three high-mannose-type oligosaccharide chains [i.e. $42 + (3 \times 3 \text{ kDa}) = 51 \text{ kDa}$] and L-GGT could carry two [i.e. $19 + (2 \times 3 \text{ kDa}) = 25 \text{ kDa}$]. Then further glycosylation steps in the Golgi membranes could result in the conductance of mature 55 kDa and 30 kDa polypeptides into the brush-border membrane (Capraro & Hughey, 1983; Nash & Tate, 1984; Coloma & Pitot, 1986b).

In the liver, the different co-translationally core-*N*-glycosylated products obtained in RER are polypeptides with molecular masses of 55 kDa, 53 kDa and 49 kDa. The 53 kDa and 49 kDa polypeptides, being recognized by an anti-(kidney deglycosylated GGT) antibody, would carry fewer high-mannose-type oligosaccharide chains than the mature enzyme. Purified rat kidney GGT was shown to carry possibly from three to five oligosaccharide chains per molecule (Yamashita *et al.*, 1983), and from the analysis of its cDNA sequence there exist five putative glycosylation sites (Asn-Xaa-Ser and Asn-Xaa-Thr)

(Laperche *et al.*, 1986). Thus, if the 49 kDa polypeptide is carrying a single high-mannose-type chain (i.e. 3 kDa), and the 55 kDa is carrying three chains as in the kidney (i.e. 3×3 kDa), the peptide backbone of liver H-GGT would have a molecular mass of about 46 kDa, i.e. slightly higher than the kidney one (42 kDa) (Tate & Khadse, 1986). Such a hypothesis was in fact recently supported by Chobert *et al.* (1987), who showed that liver GGT mRNA was larger than kidney GGT mRNA by 0.4 kb, i.e. by 18 %.

Difficulties in the recognition of the L-GGT subunit with a total antiserum have often been reported, even in the kidney (Capraro & Hughey, 1983; Coloma & Pitot, 1986b). From our data, the more the liver L-GGT is mature, the more it is recognizable by our anti-(kidney GGT) antibody (in PM and Golgi membranes). In RER a 24 kDa polypeptide is barely detected with the anti-(kidney deglycosylated GGT) serum. Thus, as far as can be seen, rat liver L-GGT does not seem to differ greatly from kidney or hepatoma L-GGT in terms of size and biogenesis (Nash & Tate, 1984; Barouki *et al.*, 1984), but would seem to do so at least in terms of epitope accessibility or identity.

In conclusion, liver GGT was shown to differ from the kidney enzyme (i) very probably not only in terms of oligosaccharide chains for the H-GGT and (ii) at least by differences in conformational structure (immunological misrecognition) for the L-subunit. Moreover, the recent evidence for a longer GGT mRNA in the liver than in the kidney does not exclude the hypothesis of a longer GGT gene coding sequence in the liver, their gene expression (i.e. non-coding sequences) being already suspected to differ because of their respective regulation (inducibility, ontogenesis) in the two tissues.

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