The development of glutathione *S*-transferase subunits in rat liver

Sensitive detection of the major subunit forms of rat glutathione *S*-transferase by using an e.l.i.s.a. method

Fiona M. G. McCUSKER, Sinead J. BOYCE and Timothy J. MANTLE*

Department of Biochemistry, Trinity College, Dublin 2, Ireland

The development of the subunits of glutathione *S*-transferase in rat liver shows that there is a co-ordinated development of the Ya, Yb₁, Yb₂ and Yc subunits but that the Yf and Yk subunits show unique patterns of development. The Yk subunit is the only form that is expressed at relatively high levels during the foetal period as well as during the adult period. In contrast with all other forms, the Yf subunit in the rat declines rapidly during the last few days before parturition and is virtually undetectable in hepatocytes of adult animals. The expression of the Yf subunit in foetal liver presents a 'patchy' appearance that is similar to that induced by the administration of lead acetate and may reflect cell-cycle-associated regulation of expression.

INTRODUCTION

The glutathione *S*-transferases (GSTs) exist in multiple forms in all mammalian species studied to date (for a recent review see Mannervik, 1985). All of the subunits that have been studied in sufficient detail can be grouped in three species-independent families, which have been termed Alpha, Mu and Pi (Mannervik *et al.*, 1985). In the rat, which is the most studied species, these correspond (Hayes & Mantle, 1986a) to groups I (subunits Ya, Yc and Yk) II (Yb₁, Yb₂ and Yn) and III (subunit Yf). [The relationship between the Bass-Hayes nomenclature (see, e.g., Hayes & Mantle, 1986a) and that of Jakoby *et al.* (1984) is given in Mantle (1987).] Although sequence data, substrate-specificities, inhibitor-sensitivities and immunological relationships between these subunits have been described, there is little information on the development of these forms in rat liver. Earlier studies on the development of the multiple forms of GST have measured activity with substrates that are not particularly discriminating with regard to the different subunits (Igarashi *et al.*, 1987) or have utilized a limited range of antisera (Hales & Neims, 1976) with specificities that are not well defined in the light of recent classifications (Mannervik *et al.*, 1985; Hayes & Mantle, 1986a). Recent advances in understanding the transcriptional regulation of the various GST subunits have localized 5'-upstream genomic sequences that may function as enhancer and silencer elements, and it may be expected that factors affecting both tissue and temporal development will soon be identified (Rothkopf *et al.*, 1986; Okuda *et al.*, 1987; Lai *et al.*, 1988). It is important therefore to examine the development of the various subunits at the protein level to establish a 'baseline' and also to attempt to obtain an insight into factors that may be important in the complex regulation of this supergene family. Recently Strange and co-workers (Fryer *et al.*, 1986; Hirrell *et al.*, 1987; Faulder *et al.*, 1987) have presented data on the ontogeny of the human GSTs in

foetal and neonatal tissue, and in the present paper we describe the developmental patterns of the GST subunits in rat liver.

MATERIALS AND METHODS

Enzyme purification and antibody production

Purification of the various forms has been described previously, together with details of raising polyclonal antisera (Hayes & Mantle, 1986a; Hayes, 1986). We have described the difficulties in obtaining antisera that are monospecific for Yb₁ and Yb₂ subunits previously (Hayes & Mantle, 1986a). In the present study antisera specific for Yb₁ and Yb₂ subunits were obtained by immunosorbing out the 'contaminating' antibodies on Yb₂ subunit-Sepharose and Yb₁ subunit-Sepharose respectively.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970) in 15%-polyacrylamide gels containing 0.09% (w/v) bisacrylamide (Hayes & Mantle, 1986b).

Immunoblotting

Proteins were transferred electrophoretically on to nitrocellulose and then probed with antisera as described by Towbin *et al.* (1979).

Development of an e.l.i.s.a. for the GST subunits

Polyclonal antisera that were shown to be monospecific by immunoblotting were used to coat 96-well micro-titre plates (Nunc Immunoplate I), and non-specific sites were blocked by using 5% (w/v) bovine serum albumin in Tween/phosphate-buffered saline (0.05% Tween 20 in 0.14 M-NaCl/0.037 M-KH₂PO₄/0.011 M-Na₂HPO₄ titrated to pH 7.2 with 1 M-NaOH). Rat liver cytosol (0.25–5 µg diluted in Tween/phosphate-buffered saline)

Abbreviation used: GST, glutathione *S*-transferase.

* To whom correspondence should be addressed.

was then incubated in the pre-coated well for 1 h at room temperature. After a washing with Tween/phosphate-buffered saline the adsorbed antigen was detected by incubating with the appropriate biotinylated monospecific antiserum in Tween/phosphate-buffered saline (Watt & Watt, 1983) for 1 h at room temperature followed by incubation with streptavidin-biotinylated horseradish peroxidase conjugate and subsequent reaction with *o*-phenylenediamine and H_2O_2 .

Immunocytochemical staining

Paraffin-embedded sections of liver (5 μ m) that had been fixed in Bouin's fluid were stained by using GST antiserum by the method of Hsu *et al.* (1981).

RESULTS AND DISCUSSION

Fig. 1 shows immunoblots of liver cytosols obtained from rats at different stages of development. It should be noted that the gestation period of the rat is 21 days. It is clear that the antisera are monospecific and in addition that there appear to be three classes of ontogenetic profile. The first is seen only with the Yf subunit, which is expressed at detectable levels before birth, but declines during the last 2 days of foetal development so that the amounts are almost undetectable after parturition. When sections of liver from adult rats (male or female) are examined immunocytochemically there is intense staining, with anti-(Yf subunit) serum, of the bile-duct epithelium (arrow c in Fig. 2a), and very low concentrations are just detectable in the adult hepatocyte (Fig. 2a). When similar sections from foetal rats are examined there is a very 'patchy' expression of Yf subunit in hepatocytes (compare arrows d and e) but little staining of the bile ducts (Fig. 2b). A similar result has been reported by Hiley *et al.* (1988) in the foetal human liver. It may be noted that a similar 'patchy' expression can be seen in adult rat liver after intracardiac administration of lead acetate or lead nitrate (Fig. 2c). It has been suggested that one effect of administering lead salts in this way is to stimulate a mitotic wave that originates from a population arrested in G₂ phase (Choi & Richter, 1978). It is currently unclear as to whether the 'patchy' expression of the Yf subunit in these two situations is associated with cell-cycle events.

A second ontogenetic profile is seen for the Yk subunit (Figs 1b and 3), which is expressed at relatively high levels in the foetus and then doubles during the first 2 weeks of life. This conclusion is not immediately apparent in Fig. 1(b), where the increase in Yk subunits with age results in an increase in the width of the band, which has not stained with a corresponding intensity in the immunoblot. The results are presented in Fig. 3 of a more quantifiable study that measured the amount of the various subunits in cytosols by using an e.l.i.s.a. that we have developed for the Ya, Yb₁, Yc and Yk subunits. It is clear from this experiment that the concentrations of the Yk subunit are relatively high in the foetal liver, rising only 2-fold between days 5 and 20 to plateau at the adult concentration of 0.12 ng/ μ g. The Yk subunit (subunit 8 in the nomenclature of Jakoby *et al.*, 1984) is particularly active with 4-hydroxyalken-2-als (Jensson *et al.*, 1986); however, as lipid peroxidation is reported to be low in foetal tissues (Barber & Wilbur, 1959), it is unlikely that the relatively high concentration of the Yk

Fig. 1. Development of GST subunits monitored by immunoblotting

Rat liver cytosol (200 μ g) from animals of different ages was subjected to SDS/polyacrylamide-gel electrophoresis, electrophoretically transferred to nitrocellulose and immunoblotted with (a) anti-(Yf subunit) serum, (b) anti-(Yk subunit) serum, (c) anti-(Yb₁/Yb₂ subunit) serum, (d) anti-(Ya subunit) serum and (e) anti-(Yc subunit) serum. The dilution of primary antibody was 1:200 and of the second antibody 1:1000. The lane marked 'Std' contained the relevant purified subunit as standard, and the other lanes contain cytosols from animals aged -2, -1, 0, 5, 10, 20, 30 and 40 days respectively. The negative notation denotes days before birth.

subunit in foetal liver is involved in detoxifying these toxic products.

The third ontogenetic profile for GST forms in the rat is seen with the Ya, Yb, and Yc subunits (Figs. 1 and 3). The concentrations of these subunits are low in the foetal liver and rise slowly between days 5 and 40. Close inspection of the profiles in the case of these forms suggests that there may be neonatal and pubertal 'spurts'. We have been unable to generate sufficient monospecific anti-(Yb₂ serum) to develop an e.l.i.s.a., and initially used *trans*-4-phenylbut-3-en-2-one conjugation as a relatively specific measure of the Yb₂ subunit (Mannervik *et al.*, 1985). However, it is clear from Fig. 3 that the development of the *trans*-4-phenylbut-3-en-2-one conjugating activity lags significantly behind that of the

Fig. 2. Immunocytochemical staining of rat liver with anti-(Yf subunit) serum

Sections of liver ($5\ \mu\text{m}$) were prepared as described in the text from (a) adult rat, (b) foetal rat (3 days *pre-partum*) and (c) adult rat 31 h after receiving an intracardiac injection of lead acetate ($100\ \mu\text{mol/kg}$). It should be noted that for (a) the animal was not fasted, to emphasize the very slight nuclear staining (arrowed a), and that this should be compared with the negatively stained nuclei (arrowed b) in the hepatocytes of animals receiving lead acetate (c). This has been a very reproducible result (S. J. Boyce & T. J. Mantle, unpublished work) in several serial sections from male and female rats.

Fig. 3. Development of GST subunits monitored by e.l.i.s.a.

Results are shown for the Ya (○), Yb₁ (■), Yc (◇) and Yk (●) subunits. The adult values (day 40) were 1.2, 3.3, 8.5 and 0.13 ng/μg respectively. The standard curves for the Ya, Yb₁, Yc and Yk subunits covered the ranges 0.1–10, 0.1–10, 0.1–10 and 0.01–1 ng respectively. The amount of antigen in cytosols was determined by using a range of dilutions (0.25–5 μg of protein). Data are only presented for determinations at two different dilutions (at least in duplicate) that were shown to be on the linear part of the standard curve. Also shown (□) is the development of GST activity with *trans*-4-phenylbut-3-en-2-one as substrate. The between-batch coefficient of variation is less than 5%.

Fig. 4. Development of the Yb₁ and Yb₂ subunits

A Y fraction was prepared from rats at different stages of development by affinity purification on *S*-hexylglutathione-Sepharose, and the Yb₁ and Yb₂ subunits were separated by isoelectric focusing (in 8 M-urea) and then detected by Coomassie Blue staining. Only the relevant portion of the isoelectric-focusing gel is shown for days 5, 30 and 40 in lanes, 1, 2 and 3 respectively. A sample of GST Yb₁Yb₂ is shown in the lane marked 'Std'.

Ya, Yb₁ and Yc subunits in the 'third' developmental profile. It is unclear what the activity with *trans*-4-phenylbut-3-en-2-one is monitoring as we can show by focusing an affinity-purified 'Y fraction' (prepared by step elution from *S*-hexylglutathione-Sepharose) that the Yb₁ and Yb₂ subunits exhibit a common developmental profile (Fig. 4). Recently sequences coding for the Yb₃ (Abramovitz & Listowsky, 1987) and the Yb₄ (Lai *et al.*, 1988) subunits have been reported, so that

developmental and activity profiles for these subunits should be examined. It is unclear as to the nature of the regulatory mechanisms involved in the control of the development of expression of the various GST subunits.

The current work provides a basis for re-evaluating the effects of hormones on the development of particular subunits. In the case of the Pi class it appears that different mechanisms of regulation are operative in the three species (mouse, rat and human) that have been most extensively studied. The expression of the mouse Yf subunit is enhanced significantly by testosterone (Hatayama *et al.*, 1986), although it should be noted that there is significant expression in female animals and in males after castration. In the rat it is clear from the present work that there is no stimulation of the Yf subunit in the male rat at puberty, and indeed there is a down-regulation of the rat Yf subunit at birth. We have obtained evidence for a similar down-regulation of the Yf subunit in female rats (F. M. G. McCusker & T. J. Mantle, unpublished work). This down-regulation is reversed dramatically in preneoplastic nodules (Satoh *et al.*, 1985), and, as shown in the present study, to a lesser extent after the administration of lead acetate. In the case of the human Yf subunit there is a further variation in that the de-repression seen in preneoplastic foci with the rat analogue does not appear to be mirrored in human liver (Hayes *et al.*, 1987; Kano *et al.*, 1987). As the structural elements of the Yf genes are probably similar in the rat, human and mouse, further work is necessary to define the different regulatory elements operative in the different species (Kano *et al.*, 1987).

We thank the Irish Cancer Society for supporting this work.

REFERENCES

- Abramovitz, M. & Listowsky, I. (1987) *J. Biol. Chem.* **262**, 7770–7773
- Barber, A. A. & Wilbur, K. M. (1959) *Radiat. Res.* **10**, 167–175
- Choie, D. D. & Richter, G. W. (1978) *Cell Tissue Kinet.* **11**, 235–239
- Faulder, C. G., Hirrell, P. A., Hume, R. & Strange, R. C. (1987) *Biochem. J.* **241**, 221–228
- Fryer, A. A., Hume, R. & Strange, R. C. (1986) *Biochim. Biophys. Acta* **883**, 448–453
- Hales, B. F. & Neims, A. H. (1976) *Biochem. J.* **160**, 231–236
- Hatayama, I., Satoh, K. & Sato, K. (1986) *Biochem. Biophys. Res. Commun.* **140**, 581–588
- Hayes, J. D. (1986) *Biochem. J.* **233**, 789–798
- Hayes, J. D. & Mantle, T. J. (1986a) *Biochem. J.* **233**, 779–788
- Hayes, J. D. & Mantle, T. J. (1986b) *Biochem. J.* **237**, 731–740
- Hayes, P. C., Portman, B., Aldis, P. M., Williams, R. & Hayes, J. D. (1987) in *Glutathione S-Transferases and Carcinogenesis* (Mantle, T. J., Pickett, C. B. & Hayes, J. D., eds.), pp. 175–187, Taylor and Francis, London, New York and Philadelphia
- Hiley, C., Fryer, A., Bell, J., Hume, R. & Strange, R. C. (1988) *Biochem. J.* **254**, 255–259
- Hirrell, P. A., Hume, R., Fryer, A. A., Collins, M. F., Drew, R., Bradwell, A. R. & Strange, R. C. (1987) *Biochim. Biophys. Acta* **915**, 371–377
- Hsu, S. M., Rane, L. & Fanger, H. (1981) *J. Histochem. Cytochem.* **26**, 577–580
- Igarashi, T., Irokawa, N., Ono, S., Ohmori, S., Ueno, K. & Kitagawa, H. (1987) *Xenobiotica* **17**, 127–137

- Jakoby, W. B., Ketterer, B. & Mannervik, B. (1984) *Biochem. Pharmacol.* **33**, 2539–2540
- Jensson, H., Guthenberg, C., Ålin, P. & Mannervik, B. (1986) *FEBS Lett.* **203**, 207–209
- Kano, T., Sakai, M. & Muramatsu, M. (1987) *Cancer Res.* **47**, 5626–5630
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lai, H.-C. J., Qian, B., Grove, G. & Tu, C.-P. D. (1988) *J. Biol. Chem.* **263**, 11389–11395
- Mannervik, B. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol.* **57**, 357–417
- Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, K. M., Warholm, M. & Jörnvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7202–7206
- Mantle, T. J. (1987) in *Glutathione S-Transferases and Carcinogenesis* (Mantle, T. J., Pickett, C. B. & Hayes, J. D., eds.), pp. xiii–xiv, Taylor and Francis, London, New York and Philadelphia
- Okuda, A., Sakai, M. & Muramatsu, M. (1987) *J. Biol. Chem.* **262**, 3858–3863
- Rothkopf, G., Telakowski-Hopkins, C. A., Stotish, R. L. & Pickett, C. B. (1986) *Biochemistry* **25**, 993–1002
- Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. & Sato, K. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3964–3968
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Watt, T. S. & Watt, R. M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 124–128

Received 20 January 1989/28 March 1989; accepted 3 April 1989