Developmental aspects of a unique glutathione *S*-transferase subunit Yx in the liver cytosol from rats with hereditary hyperbilirubinuria

Comparison with rat fetal liver transferase subunit Yfetus

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The unique glutathione *S*-transferase (GST) subunit Yx, which is undetectable in normal adult rat liver cytosol, was shown to occur in the liver cytosol of rats with hereditary hyperbilirubinuria (EHB). The Yx subunit is a member of the Alpha-class GST subunits, and is immunologically closely related to the Yc subunit. The Yx subunit has an apparent M_r of 26400, different from those of Ya (M_r 25800), Yb₁ and Yb₂ (both M_r 27200) and Yc (M_r 28400). During postnatal development in livers of EHB rats, the Yx subunit concentration in either sex was highest during the first week *post partum* and declined rapidly with age. Although the concentration of subunit Yx at 8 weeks of age accounted for about 60 % in females and 40 % in males of that observed in 1-week-old 'neonatal' male EHB rats, concentrations in females thereafter increased gradually to almost the neonatal level and remained at this high level at least up to 37 weeks of age, whereas the concentration in males did not increase again. Thus the post-pubertal Yx subunit concentration was 2-fold higher in females than in males. In contrast, in normal Sprague–Dawley rat liver, the Yfetus subunit, with the same M_r as the Yx subunit, had the highest concentration in 10-day-old animals, declined rapidly thereafter, and was not detectable in the post-pubertal period. The Yfetus subunit was also immunoreactive with an antibody against GST YcYc. The analysis of GST subunits by reverse-phase h.p.l.c. revealed that the Yx subunit was eluted at a retention time different from other known subunits, but coincided with that of Yfetus. The *N*-terminal amino acid sequence of the Yx subunit displayed a high degree of sequence similarity to that of the Yfetus subunit. These data suggest that the Yx subunit in EHB rats may be very similar to, if not identical with, the Yfetus subunit.

INTRODUCTION

The glutathione *S*-transferases (GSTs) have been investigated most extensively in rats, and multiple forms have been shown (Mannervik & Danielson, 1988). Cytosolic GSTs are dimeric proteins composed of various combinations of individual subunits and are generally grouped into three classes named Alpha, Mu and Pi (Mannervik *et al.*, 1985). Normal adult rat liver cytosol abundantly contains Alpha-class subunits Ya and Yc and Mu-class subunits Yb₁ and Yb₂, whereas the Pi-class subunit Yf (Yp) is undetectable (Ketterer, 1986). We have recently identified a unique subunit, Yx, which is immunoreactive with an antibody against GST YcYc (Igarashi *et al.*, 1991). This subunit is quite different from subunit Yc in molecular size. The Yx subunit occurs in the liver cytosol of rats with hereditary hyperbilirubinuria (EHB), but is present in negligible amounts only in normal adult rat liver (Igarashi *et al.*, 1991). A novel Alpha-class subunit, Yfetus, which is immunologically related to subunit Yc and absent from adult livers, has been reported in fetal rat liver (Scott *et al.*, 1986; Scott & Kirsch, 1987). The Yfetus subunit has currently been recognized to be identical with Alpha-class subunit 10 (Ketterer *et al.*, 1988). The purpose of the present study is to examine the postnatal developmental change

of the Yx subunit in EHB rats and to elucidate its correspondence to the Yfetus subunit of pre-natal and post-natal rat liver cytosol.

MATERIALS AND METHODS

Animals

EHB rats are a Sprague–Dawley (SD) rat strain characterized by urinary bilirubin excretion and jaundice immediately after birth, discovered by Mikami *et al.* (1986). EHB and normal rats of both sexes were used. The animals had free access to water and a commercial chow (MF; Oriental Yeast Co., Tokyo, Japan). They were killed by exsanguination. The livers were quickly removed, weighed and perfused with 0.15 M-KCl. Then 20 % (w/v) homogenates were prepared in ice-cold 0.15 M-KCl in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 9000 *g* for 20 min. The resultant supernatants were further centrifuged at 105000 *g* for 60 min. The resulting clear supernatants were used as the cytosol.

Chemicals

1-Chloro-2,4-dinitrobenzene and GSH were purchased from Wako Pure Chemicals, Tokyo, Japan. Other reagents used were of analytical grade.

Abbreviations used: GST, glutathione *S*-transferase [in the present paper we have employed the 'Y' nomenclature for GST subunits; correspondence to the numerical nomenclature is as follows: Ya, 1; Yb₁, 3; Yb₂, 4; Yc, 2; Yf(Yp), 7; Yk, 8; Yfetus, 10]; EHB rat, Eisai hyperbilirubinuria rat; SD rat, Sprague–Dawley rat.

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Determination of bilirubin in the plasma

Total and conjugated bilirubin were determined according to the method of Witmans *et al.* (1961). The value for unconjugated bilirubin was calculated by subtracting the amount of conjugated bilirubin from total bilirubin.

Determination of GST activity

GST activities were assayed according to the method of Habig *et al.* (1974).

SDS/PAGE

Electrophoresis was performed as described by Laemmli (1979) in 12.5% (w/v) polyacrylamide gels.

Analysis of GST subunits by reverse-phase h.p.l.c.

H.p.l.c. analysis of GSTs was carried out according to the method of Ostlund Farrants *et al.* (1987) with modifications as follows. The separation of GST subunits was performed with a TSK-gel Octadecyl-4PW reverse-phase column (150 mm $\times$ 4.6 mm internal diameter) (Tosoh Co., Tokyo, Japan). The solvents were 35% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (solvent A) and 85% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (solvent B). The sample was injected at 100% (v/v) solvent A, and a linear gradient was run from 0% to 40% (v/v) solvent B over 60 min at a flow rate of 0.7 ml/min. Polypeptides were detected at 220 nm.

Immunoblot analysis

Antibodies against rat GST YaYa, YcYc, Yb₁Yb₁ and Yb₂Yb₂ were prepared as described previously (Igarashi *et al.*, 1985). Western-Blot analysis was carried out as described previously (Igarashi *et al.*, 1988). In the experiment investigating developmental changes in GST subunits, each cytosol (2 μ g of protein) at different ages was subjected to SDS/PAGE on the same gel. After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane and the resolved proteins were probed for cross-reactivity with antisera. The intensity of immunoreactive bands was measured by densitometric scanning with a Shimadzu CS-910 dual-wavelength t.l.c. scanner (Shimadzu, Kyoto, Japan). The value was expressed as a percentage of that obtained for 1-week-old (e.g. Fig. 2a and Fig. 4c) or 10-week-old (e.g. Fig. 4b) male animals, which was defined as 100%.

N-Terminal amino acid sequence analysis

N-Terminal amino acid sequence analysis was performed with an Applied Biosystems 470A protein sequencer (Foster City, CA, U.S.A.). Phenylthiohydantoin derivatives of amino acids were identified by h.p.l.c. according to the method of Glajch *et al.* (1985).

Protein determination

Protein contents were determined according to the method of Lowry *et al.* (1951) or the Coomassie Brilliant Blue G-250 dye-binding assay method of Bradford (1976), with BSA as the standard.

RESULTS

Fig. 1 shows the Western-blot analysis of GST subunits in normal adult SD rat and EHB rat liver cytosol. Anti-(GST YcYc) serum cross-reacted weakly but clearly with an additional subunit (referred to as Yx) (M_r 26400) as well as Yc subunit (M_r 28400) in EHB rats. The Yx subunit exhibited an intermediate mobility between Ya (M_r 25800) and Yb (M_r 27200) on SDS/PAGE, as reported previously (Igarashi *et al.*, 1991). The Yx subunit was

neither detected in normal adult animals nor did it react with antibodies against GST YaYa, Yb₁Yb₁ or Yb₂Yb₂. Except for the Yx subunit, no marked differences between SD and EHB rats were observed in the Western-blot analysis.

The post-natal developmental alterations in the GST Yx subunit concentrations in liver cytosol obtained from EHB rats of both sexes were examined by immunoblotting (Fig. 2a). When the Yx subunit concentration in 1-week-old male rats is defined as 100%, the female Yx subunit concentration was slightly higher at this age and declined transiently to about 60% at 8 weeks of age. Thereafter female Yx subunit concentrations increased to reach 90–100% of the 1-week-old male concentration at 20 weeks of age and older. In contrast, male Yx subunit concentrations in 8-week-old animals were decreased to about 40% of the concentration at 1 week, and thereafter remained nearly constant, although a slight transient increase to about 55% of the concentration at 1 week was observed in 12-week-old animals. Thus the GST Yx subunit concentration in livers of adult EHB rats was 2-fold higher in females than in males. The developmental patterns of the Yx subunit seemed to correlate reasonably well with the developmental profiles of conjugated plasma bilirubin concentrations in EHB rats of both sexes (Fig. 2b). Plasma concentrations of conjugated rather than unconjugated bilirubin in post-pubertal EHB rats were significantly different between males and females and changed with age.

We compared the concentrations of Yx subunit in female adult SD and EHB rats with the GST subunit present in the neonatal female SD rat (7 days *post partum*) liver cytosol by immunoblotting with anti-(GST YcYc) antibody (Fig. 3). Neonatal liver cytosol of the SD rat strongly immunoreacted with a GST subunit different from Yc that showed the same mobility as the Yx subunit. The former subunit in neonatal rat liver, having a lower M_r than the Yc subunit, seemed to be identical with Yfetus reported by Scott *et al.* (1986).

Fig. 4 indicates the developmental changes in liver cytosolic GST activities towards 1-chloro-2,4-dinitrobenzene and the relative concentrations of subunits Yc and Yfetus in normal SD rat liver. GST activity in both sexes increased gradually with age and reached a plateau about 10 weeks *post partum*. The Yc subunit concentration rose progressively during the development in parallel with the GST activities towards 1-chloro-2,4-dinitrobenzene. In contrast, the Yfetus subunit concentration increased progressively after parturition and reached a maximum during the first week of age in both sexes. Thereafter the concentration

Fig. 1. Western-blot analysis of liver cytosolic GST subunits from EHB and normal SD rat

Lane 1, normal SD rat (female, 10-week-old); lane 2, EHB rat (female, 10-week-old). Normal SD rat liver cytosol and EHB rat liver cytosol (both 2 μ g of protein) were subjected to SDS/PAGE and then blotted on to a nitrocellulose membrane. The membranes were incubated with (a) anti-(GST YaYa), (b) anti-(GST Yb₁Yb₁), (c) anti-(GST Yb₂Yb₂) and (d) anti-(GST YcYc) sera. Subunits Ya, Yb (Yb₁/Yb₂), Yc and Yx are indicated.

Fig. 2. Post-natal developmental changes in the Yx subunit concentrations (a) and plasma bilirubin concentrations (b) in male and female EHB rats

(a) Each cytosol (2 μ g of protein) from male (○) and female (●) EHB rats at different ages was applied to SDS/polyacrylamide gels on the same gel. After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane and the resolved proteins were probed for cross-reactivity with antisera raised against GST YcYc. The intensity of the immunoreactive bands was measured by densitometric scanning. The value was expressed as a percentage of that obtained for 1-week-old male EHB rats, which was defined as 100%. Each point represents the mean $\pm$ S.E.M. from three experiments from different animals. (b) The values represent the means $\pm$ S.E.M. from four to five animals. ▨, Male animals; ▤, female animals. * $P < 0.05$, ** $P < 0.01$ compared with the corresponding male rat. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ compared with the corresponding 1-week-old (a) or 5-week-old (b) animal.

Fig. 3. Comparison of the concentrations of Yx subunit from adult female SD and EHB rats with the Yfetus subunit concentration from a neonatal female SD rat by immunoblotting using anti-(GST YcYc) serum

Protein (2 μ g) from 10-week-old SD rat (lane 1), 10-week-old EHB rat (lane 2) and 1-week-old rat (lane 3) liver cytosol was subjected to SDS/PAGE and then blotted on to a nitrocellulose membrane. The membrane was incubated with anti-(GST YcYc) serum. Subunits Yc and Yx/Yfetus are indicated.

of the Yfetus subunit rapidly declined in both males and females and it was virtually undetectable 8 weeks later.

To examine the relationship between Yx subunit in adult EHB rats and Yfetus subunit in neonatal rats, an h.p.l.c. analysis of the GST subunits was performed using the corresponding GSH-affinity-purified GST fractions (Fig. 5). After h.p.l.c. each GST

subunit was identified by SDS/PAGE and/or immunoblotting. The Yx subunit in EHB rats was eluted after subunit Yc, whereas this Yx subunit peak was hardly detectable in adult normal rat liver. As shown in Fig. 5(c), however, h.p.l.c. analysis of affinity-purified GSTs from neonatal rat liver cytosol resulted in a novel peak that was eluted at the same position as the Yx subunit. This peak has been identified to be Yfetus by SDS/PAGE and immunoblotting. It should be noted that the Yk subunit is expressed at relatively high levels during the neonatal period. It should also be noted that the as yet unidentified subunit Yb₁', which is immunologically related to the Yb₁ subunit (results not shown), is eluted at an intermediate position between Yb₁ and Yb₂. The N-terminal amino acid sequence of the Yx subunit, which was obtained by collecting the corresponding fraction on reverse-phase h.p.l.c., is compared with known N-terminal sequences of other Alpha-class GST subunits (Table 1). The Yx subunit was not blocked at the N-terminus. The N-terminal amino acid sequence of the first 28 residues of the Yx subunit was found to be completely identical with that of Yfetus and to differ at only one position with respect to the Yc subunit.

DISCUSSION

EHB is a rat strain showing jaundice and hyperbilirubinuria from 1 day of age throughout life (Mikami *et al.*, 1986). Neonatal (7-day-old) EHB rats had a 50-fold higher plasma bilirubin concentration than unaffected rats (11.4 ± 0.30 versus 0.21 ± 0.08 mg/dl). Bilirubin is generally regarded as being potentially cytotoxic. However, it has recently been suggested that it might function as a possible physiological antioxidant (Stocker *et al.*, 1987a,b). The Yx subunit of GST from EHB rats expressed very high GSH peroxidase activity (Igarashi *et al.*, 1991). In the

Fig. 4. Developmental changes in (a) GST activity and (b) Yc as well as (c) Yfetus subunit concentrations in liver cytosol in normal rats

The concentrations of GST subunits Yc and Yfetus were determined as described in Fig. 2 legend. The values for the Yc and Yfetus subunits were expressed as percentages of those obtained for 10-week-old male and 1-week-old male SD rats respectively, which were defined as 100%. The given values indicate the means. The number of animals used is as follows: before birth, $n = 4-5$ for both sexes; after birth, $n = 2-6$ for males, $n = 3-6$ for females. ○, Males; ●, females.

Fig. 5. GST subunit analysis by reverse-phase h.p.l.c.

Each GST subunit obtained by affinity chromatography was applied to an Octadecyl-4PW column as described in the Materials and methods section. (a) 37-week-old female EHB rat; (b) 37-week-old female SD rat; (c) 1-3-week-old male SD rat.

Table 1. N-Terminal amino acid sequences of the Yx subunit and other subunits of Alpha-class GST

The underlined amino acids indicate residues different from the Yx subunit.

Subunit	Sequence																								Reference				
	1	5	10	15	20	25																							
Yx	P	G	K	P	V	L	H	Y	F	D	G	R	G	R	M	E	P	I	R	W	L	L	A	A	G	V	E	Present study	
Yfetus	P	G	K	P	V	L	H	Y	F	D	G	R	G	R	M	E	P	I	X	X	L	L	A	A	G	V	X	Meyer <i>et al.</i> (1991)	
Tc	P	G	K	P	V	L	H	Y	F	D	G	R	G	R	M	E	P	I	R	W	L	L	A	A	G	V	<u>D</u>	Mannervik & Danielson (1988)	
Ya	<u>S</u>	G	K	P	V	L	H	Y	F	<u>I</u>	<u>A</u>	R	G	R	M	E	<u>C</u>	I	R	W	L	L	A	A	G	V	E	Pickett <i>et al.</i> (1984)	
Yk	<u>E</u>	<u>V</u>	K	P	<u>K</u>	<u>L</u>	<u>Y</u>	<u>Y</u>	<u>F</u>	<u>Q</u>	G	R	G	R	M	E	<u>V</u>	I	R	W	L	L	A	<u>T</u>	A	G	V	E	Ålin <i>et al.</i> (1989)

present study, adult male EHB rats with higher bilirubin concentrations than females possessed a lower Yx subunit concentration than females and vice versa.

Analysis of affinity-purified GSTs from EHB and neonatal SD rat liver cytosol by reverse-phase h.p.l.c. resulted in a peak for subunit Yx that was different from other subunits and eluted at the same position as the Yfetus subunit. Most of the Alpha-class GST subunits including Yfetus are N-terminally blocked (Mannervik *et al.*, 1985; Ålin *et al.*, 1985; Meyer *et al.*, 1991), whereas the Yc subunit is not (Del Boccio *et al.*, 1987). Analysis of the N-terminal amino acid sequence of subunit Yx has revealed that it is not N-terminally blocked and that it has a high degree of identity with subunit Yfetus. Based on immunological properties, electrophoretic mobility on SDS/PAGE, retention times on reverse-phase h.p.l.c. and N-terminal amino acid sequence, the Yx subunit may be very similar to, if not identical with, the Yfetus subunit.

The developmental expression of the GST subunits Yc and Yx in SD rat liver revealed that the Yc subunit is expressed in a co-ordinated developmental fashion. An almost similar post-natal developmental pattern for the Ya subunit has been reported in rat liver (Hales & Neims, 1976). However, the Yfetus subunit, which can form a heterodimer with the Yc subunit, showed a unique pattern of development different from those of other GST subunits. The present study has demonstrated that expression of the Yfetus subunit is much higher in the neonatal stage than in fetal liver, although the Yfetus subunit has previously been purified and characterized from the livers of 17- or 18-day fetuses (Scott *et al.*, 1986; Scott & Kirsch, 1987). The elevated expression of the Yfetus subunit during the neonatal period may thus be regulated by some components in maternal milk.

Spearman & Leibman (1984) have reported that the individual GST isoenzymes are selectively regulated and express distinct developmental patterns. The present data support these findings. McCusker *et al.* (1989) have reported that the Pi-class Yf (Yp) subunit in the rat declines rapidly during the last few days before parturition and is virtually undetectable in hepatocytes of adult animals. As shown in Fig. 5(c), the contribution of the Yfetus subunit to the total rat liver GST activity during the first 30 days *post partum* seems to be very high. The distribution of GST isoenzymes may vary considerably from one tissue to another (Ketterer, 1986). In view of the distinct functional properties of the different forms of GST, the differential tissue distribution is also recognized to be of importance with respect to the responsibility for tissue-selective toxicity. The Yfetus subunit has been reported to possess high activity towards peroxides such as cumene hydroperoxide (Scott & Kirsch, 1987). However, it is currently unclear whether the relatively high concentrations of Yfetus subunit during the first postnatal week are involved in detoxifying toxic products as lipid peroxides.

The present study indicates that the Yfetus subunit may be down-regulated in adult (8 weeks of age) rats. This down-regulation of the Yfetus subunit can be reversed dramatically in hyperbilirubinuria, although the exact mechanism of regulation of Yfetus (Yx) expression in EHB rats remains unclear. The

present study suggests that de-repression of the Yfetus subunit occurs in EHB rats. The presence of high concentrations of bilirubin during the neonatal period may be required for the establishment of the postpubertal pattern of Yx subunit development in EHB rats.

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