Characterization of *S*-hexylglutathione-binding proteins of human hepatocellular carcinoma: separation of enoyl-CoA isomerase from an Alpha class glutathione transferase form

Hiroko KAJIHARA-KANO*, Makoto HAYAKARI*, Kimihiko SATOH*, Yoshihisa TOMIOKA*†, Michinao MIZUGAKI*† and Shigeki TSUCHIDA*¹

*Second Department of Biochemistry, Hirosaki University School of Medicine, Hirosaki 036, Japan, and †Department of Pharmaceutical Sciences, Tohoku University Hospital, Sendai 980, Japan

Recent studies have revealed binding of mitochondrial enoyl-CoA isomerase (ECI) to *S*-hexylglutathione–Sepharose, an affinity matrix used for purification of glutathione transferases (GSTs), and the enzyme has been suggested to be identical with the Alpha class form of GST with a subunit molecular mass of about 30 kDa. In the present study, *S*-hexylglutathione-binding proteins of human hepatocellular carcinomas were characterized to examine their identity. Supernatant fractions of carcinoma and surrounding tissues were applied to an affinity column, and bound fractions were resolved into three proteins with subunit molecular masses/pI values of 33 kDa/7.0, 30 kDa/5.8 and 29 kDa/5.8 in addition to the well-characterized four GST subunits, A1, A2, M1 and P1, by two-dimensional gel electrophoresis. The proteins were further purified by chromatofocusing at pH 7.4–4.0. The 30 and 29 kDa proteins were eluted at pH 4.9 and by 1 M NaCl respectively, and could be clearly separated from each other. The 29 kDa protein exhibited a low but

significant activity towards 1-chloro-2,4-dinitrobenzene (4.25 μ mol/min per mg of protein) and reacted with anti-(GST A1-2) antibody, suggesting that it is a member of the GST Alpha class. The 30 kDa protein did not react with anti-GST antibodies and was identified as ECI by immunoblotting and N-terminal-amino-acid-sequencing analyses. The results thus indicated that the Alpha class GST form composed of the 29 kDa subunits and ECI are two different proteins. The 33 kDa protein was eluted from the chromatofocusing column at pH 7.0 and did not react with either anti-GST antibodies or antibodies against mitochondrial enzymes involved in the β -oxidation of fatty acids. However, it exhibited a carbonyl reductase activity with menadione and ubiquinone, and amino acid sequences of its peptides cleaved by *Staphylococcus aureus* V8 proteinase were consistent with those reported for the enzyme. Thus this protein binding to *S*-hexylglutathione–Sepharose was identified as carbonyl reductase.

INTRODUCTION

Glutathione transferases (GSTs) (EC 2.5.1.18) are a family of enzymes which catalyse the conjugation of many electrophiles with glutathione (GSH) [1]. They have been grouped into five classes, Alpha, Mu, Pi, Theta and Sigma, in a species-independent classification [2,3]. As human Alpha class GSTs, two subunits A1 and A2 are well characterized [4], but the presence of additional subunits has also been suggested. These include the GST-9.9 subunit of the skin [5] and a 30 kDa subunit described in several cancer cell lines [6]. Most GST forms except those of the Theta class bind to *S*-hexylglutathione– or glutathione–Sepharose and this affinity has been widely used to effect their purification [7,8]. In addition to GSTs, such matrices also bind glutathione-related enzymes such as glyoxalase I [9] and thioltransferase [10]. Furthermore, recent studies have revealed that the mitochondrial enoyl-CoA isomerase (ECI) and carbonyl reductase (CR), both unrelated to glutathione metabolism, are also retained by *S*-hexylglutathione– and glutathione–Sepharose respectively [11,12]. The binding of the 30 kDa protein ECI casts doubt on the existence of a separate putative Alpha class GST form [6], since the two proteins share similar properties [11].

ECI catalyses the isomerization of 3-enoyl-CoA to 2-*trans*-enoyl-CoA esters in the process of β -oxidation of unsaturated fatty acids, these products then being converted into β -

hydroxyacyl-CoA by mitochondrial enoyl-CoA hydratase (ECH) and the peroxisomal bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) [13,14]. Mitochondrial ECI is significantly homologous (about 30% identity) to ECH in amino acid sequence [15,16]. The mitochondrial fatty acid β -oxidation cycle in mammals consists of acyl-CoA dehydrogenase, ECH, 3-hydroxyacyl-CoA dehydrogenase (HAD) and 3-oxoacyl-CoA thiolase [17]. The subunit molecular masses of ECH, HAD and 2,4-dienoyl-CoA reductase (DCR) are reported to be 27–33 kDa [15,18,19]. During purification of GSTs from human hepatocellular carcinoma tissues, we found three proteins with subunits of molecular masses 33, 30 and 29 kDa, in addition to well-characterized GST forms, which bound to *S*-hexylglutathione–Sepharose. In the present study we demonstrated that the 29 kDa subunit protein is a member of the Alpha class GSTs and clearly separable from the 30 kDa ECI. We also provide evidence to indicate that the 33 kDa protein is CR and not ECH or another mitochondrial enzyme involved in fatty acid β -oxidation.

MATERIALS AND METHODS

Materials

Epoxy-activated Sepharose 6B, Polybuffer exchanger (PBE 118 and PBE 94), Polybuffer 74 and Pharmalyte (pH 8–10.5) were

Abbreviations used: GST, glutathione transferase; ECH, enoyl-CoA hydratase; ECI, enoyl-CoA isomerase; HAD, 3-hydroxyacyl-CoA dehydrogenase; DCR, 2,4-dienoyl-CoA reductase; CR, carbonyl reductase; CDNB, 1-chloro-2,4-dinitrobenzene.

¹ To whom correspondence should be addressed.

obtained from Pharmacia Biotech (Uppsala, Sweden). Crotonyl-CoA, acetoacetyl-CoA and *Staphylococcus aureus* V8 proteinase (Type XVII-B) were from Sigma (St. Louis, MO, U.S.A.). Nitrocellulose membrane and anti-rabbit IgG antibody conjugated to horseradish peroxidase were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Daunorubicin was from Wako Pure Chemicals (Osaka, Japan). Ubiquinone was kindly donated by Eisai Co. (Tokyo, Japan). All other chemicals were of highest purity grade commercially available.

Protein purification

Human hepatocellular carcinoma tissues and surrounding non-hepatoma samples were obtained at surgery or at autopsy from four patients (55–65-year-old men) in Hirosaki University Hospital and stored at -80°C . On thawing the tissue specimens (5–10 g) were homogenized in 9 vol. of 10 mM Tris/HCl, pH 7.4, containing 0.2 M NaCl. The homogenates were centrifuged at 105000 *g* for 45 min and the supernatant fractions applied to an *S*-hexylglutathione–Sepharose column (2 cm $\times$ 10 cm) equilibrated with the homogenizing buffer. After washing, the bound fractions were eluted with 5 mM *S*-hexylglutathione in the buffer and the eluates were subjected to chromatofocusing at pH 7.4–4.0, essentially according to the previously reported method [20]. Unbound fractions were applied to a chromatofocusing gel PBE 118 column [20] to purify GST A1-1 and A1-2.

Enzyme assays

GST activity was assayed with CDNB or other substrates by the method of Habig et al. [21]. Glutathione peroxidase activity towards cumene hydroperoxide was determined by the method of Lawrence and Burk [22]. ECH activity was assayed with crotonyl-CoA as a substrate, as described by Steinmann and Hill [23], and CR activity with menadione and other substrates by the method of Wermuth [24]. HAD activity was assayed in the reverse direction with acetoacetyl-CoA as a substrate by the method of Osumi and Hashimoto [18]. Protein content was measured by the method of Bradford [25] using BSA as a standard.

SDS/PAGE

SDS/PAGE was performed with 12.5% acrylamide gels by the method of Laemmli [26]. Two-dimensional gel electrophoresis was carried out as reported previously [27] and proteins were stained with Coomassie Brilliant Blue R-250. Immunoblotting was performed by the method of Towbin et al. [28]. Anti-GST A1-2 antibody was raised in a rabbit as described previously [20]. Anti-rat ECI antibody [29] and anti-rat DCR antibody [30] were prepared as reported previously. Anti-ECH [31] and anti-(rat HAD) antibodies [18] were generously donated by Dr. T. Hashimoto, Shinshu University School of Medicine, Matsumoto, Japan.

N-terminal-amino-acid-sequence analysis

Edman degradations were performed with a gas-phase protein sequencer (model 492; Applied Biosystems, Foster, CA, U.S.A.). Amino acid phenylthiohydantoin derivatives were identified and quantified as reported previously [27].

Protease digestion and peptide separation

Aliquots (50 μg) of the purified 33 kDa protein dissolved in 100 mM Tris/HCl, pH 7.8, were digested with 1 μg of V8

proteinase at 37°C for 24 h. Peptides were separated by reverse-phase HPLC on a TSK-GEL ODS-80TS column (4.6 cm $\times$ 250 mm, Tosoh, Tokyo, Japan) with a gradient of 0–80% acetonitrile in aq. 0.1% trifluoroacetic acid.

Inhibition of GST or CR activities by antibodies

Activity inhibition testing using anti-(GST A1-2) antibody was performed as follows: anti-(GST A1-2) antibody (up to 100 μg of protein) was added to the 29 kDa protein (5 μg of protein) or GST A1-2 (0.5 μg of protein) and the final volumes were made up to 400 μl with PBS containing 0.1% (w/v) BSA. The mixture was left at 4°C overnight and then centrifuged at 16000 *g* for 10 min. The supernatant was used for assay of the remaining GST activity with CDNB. As a control, non-immune γ -globulin was used instead of the antibody. Inhibition testing of CR activity was similarly carried out using an anti-(33 kDa protein) antibody raised in a rabbit by immunizing with the 33 kDa protein as described above.

RESULTS

Purification of 29, 30 and 33 kDa proteins

Cytosolic extracts from human hepatocellular carcinoma tissues were applied to *S*-hexylglutathione–Sepharose and the column was eluted with 5 mM *S*-hexylglutathione. The eluates were resolved into several spots by two-dimensional gel electrophoresis (Figure 1a). Among them, four spots were identified as GST subunits A1 (molecular mass 26 kDa/pI 7.7), A2 (26/7.2), M1

Figure 1 Two-dimensional gel electrophoresis of GST and other proteins bound to *S*-hexylglutathione–Sepharose

Supernatant fractions from 10 g samples of human hepatocellular carcinoma (a) and surrounding non-hepatoma (b) tissues obtained from the same patient were individually applied to an *S*-hexylglutathione–Sepharose column (2 cm $\times$ 10 cm) equilibrated with 10 mM Tris/HCl, pH 7.4, containing 0.2 M NaCl. GSTs and other proteins were eluted with 5 mM *S*-hexylglutathione in the buffer. Aliquots (50 μg) of the eluted proteins were subjected to two-dimensional gel electrophoresis and then stained with Coomassie Brilliant Blue. A1, A2, M1 and P1 indicate the GST subunits. Numbers on the electrophoretogram 1, 2 and 3 indicate the spots for the 33 kDa/pI 7.0, 30 kDa/pI 5.8 and 29 kDa/pI 5.8 proteins respectively.

Figure 2 Separation of proteins bound to S-hexylglutathione-Sepharose by chromatofocusing at pH 7.4–4.0

Proteins bound to the affinity column were dialysed against 25 mM imidazole/HCl, pH 7.4, and then applied to a column (1 cm × 16 cm) of chromatofocusing gel PBE 94 equilibrated with the same buffer. Elution was carried out with 230 ml of Polybuffer-74/HCl, pH 4.0, diluted 12-fold with de-aerated water and then with 1 M NaCl as described previously [17]. The flow rate was 15 ml/h and fractions (2.6 ml each) were collected. ●, GST activity towards 1-chloro-2,4-dinitrobenzene; — — —, pH. The individual GST peaks were identified as A1-1/A1-2 (fractions 10–16), M1-1 (fractions 50–52), A2-2 (fractions 70–72) and P1-1 (fractions 86–88), as indicated in the Figure. Spots 1, 2 and 3 of Figure 1a were eluted at pH 7.0 (fractions 32–36), pH 4.9 (fractions 81–83) and by 1 M NaCl (fraction 100) respectively.

(27/6.0) and P1 (24.5/5.4) by their molecular masses and pI values. Three additional spots, with subunit molecular masses 33 kDa/pI 7.0 (spot 1), 30/5.8 (spot 2) and 29/5.8 (spot 3), were also detected. As shown in Figure 1a, another spot (32 kDa/pI 7.3) adjacent to spot 1, was observed in carcinoma samples and it, as well as the spot-1 protein, was markedly increased as compared with the surrounding non-carcinoma cases (Figure 1b). To separate these components, the eluates were subjected to chromatofocusing at pH 7.4–4.0 (Figure 2). Assay for GST activity with CDNB revealed that GST forms were resolved into four peaks and SDS/PAGE demonstrated that the individual peaks contained A1-1/A1-2 in unbound fractions, M1-1 (pH 6.0), A2-2 (5.2) and P1-1 (4.7) (Figure 3a), consistent with our previous results [20]. The 33 and 30 kDa proteins were eluted from the column at pH 7.0 (fractions 32–36, Figure 2) and 4.9 (fractions 81–83) respectively. Traces of GST activity were detected in these fractions, presumably reflecting slight contamination with GSTs. The 29 kDa protein was retained in the column when the pH value was decreased to 4.3, but was eluted by 1 M NaCl (fraction 100). This fraction exhibited low activity towards CDNB. The protein was homogeneously purified and contamination with other GST forms shown to be negligible by SDS/PAGE (Figure 3a). The 32 kDa protein corresponding to the spot adjacent to the spot 1 (Figure 1a) was eluted from the column at pH 7.2 (fractions 22–26), but was found to be contaminated with significant amounts of GST A1-1 and A1-2.

Figure 3 SDS/PAGE

GSTs and other proteins separated by chromatofocusing at pH 7.4–4.0 were subjected to SDS/PAGE. (a) Protein staining. (b) and (c) Immunoblots with anti-ECI and anti-(GST A1-2) antibodies respectively. Lane 1, molecular-mass standard proteins from top to bottom: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa). Lane 2, S-hexylglutathione-Sepharose-bound fractions (20 µg); lane 3, the breakthrough fractions of chromatofocusing at pH 7.4–4.0 (fraction 14 in Figure 2, 4 µg); lane 4, GST A1-2 (fraction 20, 2 µg); lane 5, 33 kDa protein (fraction 34, 1 µg); lane 6, GST M1-1 (fraction 50, 1 µg); lane 7, GST A2-2 (fraction 70, 0.5 µg); lane 8, 30 kDa protein (fraction 82, 1 µg); lane 9, GST P1-1 (fraction 86, 0.5 µg); lane 10, 29 kDa protein (fraction 100, 1 µg).

Identification of the 29 kDa protein as an Alpha class GST form

The 30 and 29 kDa proteins thus purified were assayed for ECH, HAD and CR activities as described in the Materials and methods section. GST activities were also determined with CDNB and other substrates. As summarized in Table 1, the 29 kDa protein showed significant GST activity towards CDNB, but not other substrates, including cumene hydroperoxide. The value of the specific activity towards CDNB was about 10 % of those for GST A1-1 and A2-2. It was devoid of ECH, HAD and CR activities. The 30 kDa protein lacked these as well as any significant GST activity.

Immunoblot analysis indicated the 29 kDa protein to react with the anti-GST A1-2 antibody (Figure 3b), but not the anti-ECI antibody (Figure 3c). Furthermore, the GST activity of the protein was inhibited by anti-(GST A1-2) antibody, but to a lesser extent than GST A1-2 itself (Figure 4), suggesting that it is different from GST A1-1 and A2-2, but is nevertheless a member of the Alpha class. On the other hand, the 30 kDa protein was stained by immunoblotting with the anti-ECI but not the anti-(GST A1-2) antibody (Figure 3). The N-terminal amino acid sequence of the 30 kDa protein was also consistent with that reported for mitochondrial ECI [11] (results not shown).

The 33 kDa protein lacked ECH and HAD activities, but exhibited CR activity towards menadione, ubiquinone and daunorubicin (Table 1). Since the N-terminal amino acid sequence of the 33 kDa protein could not be determined, possibly because of blocking of the N-terminus, the peptides digested with V8 proteinase were resolved by HPLC and subjected to the analysis (Figure 5). The sequences of four peptides examined were all in agreement with those reported for human CR [32]. To confirm the difference of the 33 kDa protein from ECH and other enzymes involved in β -oxidation of fatty acids, the protein was examined by immunoblotting. It did not react with antibodies against ECH, HAD and DCR (results not shown). The anti-(33 kDa protein) antibody dose-dependently inhibited the CR activity (results not shown), providing supporting evidence that the protein is identical with CR. Furthermore, this antibody also reacted with the 32 kDa protein eluted from the chromatofocusing column at pH 7.2.

Table 1 GST, ECH, HAD and CR activities of the 33, 30 and 29 kDa proteins resolved by chromatofocusing

The 33, 30 and 29 kDa proteins were resolved by chromatofocusing, as shown in Figure 2 and the assays were performed as described in the text. Data for GST A1-1 and GST A2-2 are also included for comparison. Abbreviation: n.d., not detectable.

Enzyme and substrate	Enzyme activity ($\mu\text{mol}/\text{min}$ per mg of protein)				
	33 kDa protein	30 kDa protein	29 kDa protein	GST A1-1	GST A2-2
Glutathione transferase					
1-Chloro-2,4-dinitrobenzene	0.05	0.08	4.25	52.4	25.8
1,2-Dichloro-4-nitrobenzene	n.d.	n.d.	n.d.	0.03	0.02
Cumene hydroperoxide	n.d.	n.d.	n.d.	2.51	21.4
Ethacrynic acid	n.d.	n.d.	n.d.	0.04	0.51
Enoyl-CoA hydratase					
Crotonyl-CoA	n.d.	n.d.	n.d.	n.d.	n.d.
3-Hydroxyacyl-CoA dehydrogenase					
Acetoacetyl-CoA	n.d.	n.d.	n.d.	n.d.	n.d.
Carbonyl reductase					
Menadione	5.20	n.d.	n.d.	n.d.	n.d.
Ubiquinone	4.52	n.d.	n.d.	n.d.	n.d.
Daunorubicin	0.85	n.d.	n.d.	n.d.	n.d.

Figure 4 Inhibition of the 29 kDa protein GST activity by anti-(GST A1-2) antibody

Activity-inhibition testing was carried out as described in the text. Mixtures of the 29 kDa protein or GST A1-2 and anti-(GST A1-2) antibody were left at 4 °C overnight and then centrifuged. The supernatants were used for assay of the remaining GST activity. ○, 29 kDa protein; ●, GST A1-2; △, 29 kDa protein and non-immune γ -globulin instead of anti-(GST A1-2) antibody.

DISCUSSION

Since the 29 kDa protein eluted from the chromatofocusing column with 1 M NaCl in the present study showed significant activity towards CDNB and reacted with anti-(GST A1-2) antibody, it was considered as a homodimeric form of the Alpha class. However, it differed from GST A1-1 and A2-2 in subunit molecular mass, pI and specific activity towards CDNB (Table 1). Immunoprecipitation testing with anti-(GST A1-2) antibody also demonstrated partial differences (Figure 4), while confirming that the 29 kDa protein itself possesses GST activity. Furthermore, non-identity with the GST-9.9 (molecular mass 27.5 kDa) purified from human skin, the possible equivalent of rat GST 2-2 [5], was concluded since the latter form is reported to possess a basic pI. The acidic form in this study was similar in subunit molecular mass to the Alpha class form reported to be expressed in several cancer cell lines [6]. However, the 29 kDa protein was also detected in the surrounding non-hepatoma

tissues with no differences in amount between the two cases, in contrast with the clear decrease in A1- and A2-subunit levels in most hepatoma samples. Amounts of M1 and P1 subunits in carcinoma tissues were hardly altered as compared with those in non-hepatoma tissues (results not shown).

The 30 kDa protein exhibited a pI value of 5.8 on two-dimensional gel electrophoresis (Figure 1a), giving results in line with those for ECI reported by Takahashi et al. [11]. Identity of the protein with ECI was also indicated by immunoblotting (Figure 3b) and N-terminal-amino-acid-sequence results. Although a polyclonal antibody against rat ECI was used in this study, cross-reactivity with the human enzyme has been reported [29,33]. The present study confirmed that ECI does not bind to glutathione-Sepharose [11]. Thus binding specificity was suggested to be dependent on the S-hexyl side chain of the affinity ligand. Some mitochondrial enzymes are synthesized in the cytosol as precursor forms containing N-terminal pre-sequences of about 30 amino acid residues [15,34]. This raises the possibility that the ligand may interact with the ECI presequence consisting of many hydrophobic amino acid residues. However, it is unlikely because the data for N-terminal amino acid sequencing revealed the 30 kDa protein to be mature ECI, devoid of any such presequence. Further studies are needed to clarify the nature of the binding. Our 30 kDa protein was increased in two of four hepatocellular carcinoma samples as compared with the respective non-hepatoma tissues and ECI has been noted to be increased in about a 50 % of breast cancers [35]. Since mitochondrial ECH and ECI are similar in amino acid sequence and utilize similar substrates [15,16], we examined whether the 33 kDa protein bound to S-hexylglutathione-Sepharose may be identical with ECH, HAD or DCR with subunit molecular masses of 27–33 kDa, all contributing to β -oxidation of fatty acids. Immunoblot analysis with these antibodies ruled out such a possibility. The 33 kDa protein exhibited a specific CR activity with menadione and other substrates (Table 1), the values being comparable with that for brain CR [24]. The amino acid sequences of peptides derived from the protein were consistent with those reported for human CR [32]. Activity inhibition testing with anti-(33 kDa protein) antibody revealed identity of the protein with CR. The fact that the 32 kDa protein also seemed to be identical can be explained by the

Figure 5 HPLC pattern of V8-proteinase digests of the 33 kDa protein

The 33 kDa protein (50 μ g in 0.2 ml of 100 mM Tris/HCl, pH 7.8) was digested with 1 μ g of V8 proteinase at 37 °C for 24 h. V8-proteinase digests were separated by HPLC as described in the text. The flow rate was 0.85 ml/min and peptides were detected at 220 nm. The broken line denotes the acetonitrile concentration. Peptides identified by sequence analysis are indicated P1–P4 and their amino acid sequences determined are KRVE for P1, KILLNAXPGWVRTD for P2, GWPSSAYGVTKIGVTLSRIHARKLSE for P3 and YGGLDLVNNAGIAFKVAD for P4. 'X' in these sequences denotes an unidentified residue. The sequences of P1, P2, P3 and P4 are completely identical with ^{272}Lys – ^{275}Glu , ^{220}Lys – ^{234}Asp , ^{188}Gly – ^{214}Glu and ^{81}Tyr – ^{99}Asp respectively of human CR deduced from its cDNA [32]. The numbers include the initiator methionine.

reported multiplicity of CR [24], shown to be due to post-translational carboxyethylation of its lysine residue [36]. In this context it is of interest that a rat ovary 34 kDa protein bound to glutathione–Sepharose has been identified as CR [12]. The present results revealed that human CR binds to *S*-hexylglutathione, in agreement with recent findings for the enzyme in chick liver [37]. Although the N-terminal amino acid sequence of rat CR is reported to exhibit a high degree of homology with an internal sequence of human enzyme [12], present results on the 33 kDa protein suggest blocking of the N-terminus, consistent with the finding reported for human CR [32]. Since CR is a cytosolic oxidoreductase that catalyses the NADPH-dependent reduction of a large number of biologically and pharmacologically active carbonyl compounds to the corresponding alcohols [24,38], its increase in carcinoma samples (Figure 1) might be of clinical significance because the enzyme is active towards daunorubicin, an anti-cancer drug, confirming the result reported by Wermuth [24]. CR was markedly increased in three out of four hepatocellular carcinoma samples as compared with the respective non-carcinoma tissues, but was decreased in one carcinoma sample. Further studies are needed to clarify alteration of CR in hepatocellular carcinomas. Irrespective of extensive studies on human liver GSTs, CR has not been detected in affinity-purified preparations [20,39]. As shown in Figure 1(B), CR content in the surrounding non-carcinoma tissue is much smaller than GST content, suggesting that previous failure in detecting CR may be due to a low amount in the normal liver. Use of carcinoma samples with high CR and low GST contents in the present study seems to be responsible for its detection.

In conclusion, ECI and the acidic form of Alpha class GST are different proteins, separable by chromatofocusing. The GST

form differs from GST A1-1 and A2-2 in subunit molecular mass, pI and other properties.

We thank Professor Takashi Hashimoto, Department of Biochemistry, Shinshu University School of Medicine, Matsumoto, Japan for the generously providing anti-ECH and anti-HAD antibodies. This study was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan, the Karoji Memorial Fund of Hirosaki University School of Medicine and the Ichiro Kanehara Foundation.

REFERENCES

- Mannervik, B. and Danielson, U. H. (1988) *Crit. Rev. Biochem.* **23**, 283–337
- Mannervik, B., Ålin, P., Guthenberg, C., Jonsson, H., Tahir, M. K., Warholm, M. and Jörnvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7202–7206
- Hayes, J. D. and Pulford, D. J. (1995) *Crit. Rev. Biochem. Mol. Biol.* **30**, 445–600
- Stockman, P. K., Beckett, G. J. and Hayes, J. D. (1985) *Biochem. J.* **227**, 457–465
- Del Boccio, G., Di Ilio, C., Ålin, P., Jörnvall, H. and Mannervik, B. (1987) *Biochem. J.* **244**, 21–25
- Castro, V. M., Söderström, M., Carlberg, I., Widersten, M., Platz, A. and Mannervik, B. (1990) *Carcinogenesis* **11**, 1569–1576
- Guthenberg, C. and Mannervik, B. (1979) *Biochem. Biophys. Res. Commun.* **86**, 1304–1310
- Simons, P. C. and Vander Jagt, D. L. (1977) *Anal. Biochem.* **82**, 334–341
- Hayes, J. D. (1988) *Biochem. J.* **255**, 913–922
- Shen, H., Tamai, K., Satoh, K., Hatayama, I., Tsuchida, S. and Sato, K. (1991) *Arch. Biochem. Biophys.* **286**, 178–182
- Takahashi, Y., Hirata, Y., Burstein, Y. and Listowsky, I. (1994) *Biochem. J.* **304**, 849–852
- Toft, E., Söderström, M., Ahlberg, M. B. and DePierre, J. W. (1994) *Biochem. Biophys. Res. Commun.* **201**, 149–154
- Stoffel, W. and Grol, M. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1777–1782
- Vamecq, J. and Draye, J.-P. (1989) *Essays Biochem.* **24**, 115–225
- Palosaari, P. M., Vihinen, M., Mäntälä, P. I., Alexson, S. E. H., Pihlajaniemi, T. and Hiltunen, J. K. (1991) *J. Biol. Chem.* **266**, 10750–10753

-
- 16 Müller-Newen, G. and Stoffel, W. (1993) *Biochemistry* **32**, 11405–11412
- 17 Schulz, H. (1991) *Biochim. Biophys. Acta* **1081**, 109–120
- 18 Osumi, T. and Hashimoto, T. (1980) *Arch. Biochem. Biophys.* **203**, 372–383
- 19 Kimura, C., Kondo, A., Koeda, N., Yamanaka, H. and Mizugaki, M. (1984) *J. Biochem. (Tokyo)* **96**, 1463–1469
- 20 Soma, Y., Satoh, K. and Sato, K. (1986) *Biochim. Biophys. Acta* **869**, 247–258
- 21 Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130–7139
- 22 Lawrence, R. A. and Burk, R. F. (1976) *Biochem. Biophys. Res. Commun.* **71**, 952–958
- 23 Steinmann, H. M. and Hill, R. L. (1975) *Methods Enzymol.* **35**, 136–151
- 24 Wermuth, B. (1981) *J. Biol. Chem.* **256**, 1206–1213
- 25 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 26 Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- 27 Ishikawa, T., Tsuchida, S., Satoh, K. and Sato, K. (1988) *Eur. J. Biochem.* **176**, 551–557
- 28 Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 29 Tomioka, Y., Aihara, K., Hirose, A., Hishinuma, T. and Mizugaki, M. (1991) *J. Biochem. (Tokyo)* **109**, 394–398
- 30 Hirose, A., Kamijo, K., Osumi, T., Hashimoto, T. and Mizugaki, M. (1990) *Biochim. Biophys. Acta* **1049**, 346–349
- 31 Jiang, L. L., Kobayashi, A., Matsuura, H., Fukushima, H. and Hashimoto, T. (1996) *J. Biochem. (Tokyo)* **120**, 624–632
- 32 Wermuth, B., Bohren, K. M., Heinemann, G., von Wartburg, J.-P. and Gabbay, K. H. (1988) *J. Biol. Chem.* **263**, 16185–16188
- 33 Palosaari, P. M., Kilponen, J. M., Sormunen, R. T., Hassinen, I. E. and Hiltunen, J. K. (1990) *J. Biol. Chem.* **265**, 3347–3353
- 34 Minami-Ishii, N., Taketani, S., Osumi, T. and Hashimoto, T. (1989) *Eur. J. Biochem.* **185**, 73–78
- 35 Kelley, M. K., Engqvist-Goldstein, Å., Montali, J. A., Wheatley, J. B., Schmidt, Jr., D. E. and Kauvar, L. M. (1994) *Biochem. J.* **304**, 843–848
- 36 Krook, M., Ghosh, D., Strömberg, R., Carlquist, M. and Jörnvall, H. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 502–506
- 37 Tsai, S.-P., Wang, L.-Y., Yeh, H.-I. and Tam, M. F. (1996) *Biochim. Biophys. Acta* **1292**, 289–292
- 38 Iwata, N., Inazu, N. and Satoh, T. (1989) *J. Biochem. (Tokyo)* **105**, 556–564
- 39 Rowe, J. D., Nieves, E. and Listowsky, I. (1997) *Biochem. J.* **325**, 481–486
-