Photoaffinity labelling of steroid-hormone-binding glutathione *S*-transferases with [³H]methyltrienolone

Inhibition of steroid-binding activity by the anticarcinogen indole-3-carbinol

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The identification and characterization of steroid-hormone-binding glutathione *S*-transferases (GST) were undertaken using photoaffinity-labelling techniques. Irradiation of mouse liver cytosol, in the presence of 50 nM-[³H]methyltrienolone, resulted in the specific affinity labelling of five proteins. One of these proteins, designated MBP27, had an approximate molecular mass of 27 kDa under denaturing conditions and was induced by treatment of mice with either 2(3)-*t*-butyl-4-hydroxyanisole (BHA) or phenobarbital (PB). An additional affinity-labelled protein, MBP25, which was not detected in untreated mouse cytosol, was induced in the liver cytosols from BHA- and PB-treated mice. The molecular masses of these proteins and their induction by BHA and PB suggested that they may be steroid-hormone-binding GST subunits. Irradiation of mouse liver cytosol in the presence of [³H]methyltrienolone, followed by immunoprecipitation using GST-specific antibodies established that both GST μ and GST α bind [³H]methyltrienolone and both contribute to the affinity-labelled protein designated MBP27. GST Ya₁Ya₁, an Alpha class GST that is not expressed in untreated mouse liver but is induced by BHA and PB, was also found to bind [³H]methyltrienolone and is identical with the affinity-labelled protein designated MBP25. Experiments were undertaken next to assess the effects of the anticarcinogenic plant compound indole-3-carbinol (I3C) on GST-mediated steroid hormone-binding using the photoaffinity labelling techniques. Treatment of mice with I3C resulted in the induction of immunoreactive GST μ and GST Ya₁Ya₁. However, the steroid-binding activity of these proteins *in vitro* was severely inhibited by the acid-condensation products of I3C that are generated in the stomach after ingestion. These results suggest that I3C may inhibit GST-mediated steroid-binding activity which could contribute to the anticarcinogenic activity of this compound.

INTRODUCTION

The intracellular transport of lipophilic materials can be mediated by soluble transfer proteins that mobilize these compounds in the aqueous environment of the cytoplasm. Hepatic transfer proteins can prevent the reflux of compounds delivered to the liver back into the plasma through the sinusoidal membrane [1–3]. Transfer proteins also can mediate the transfer of lipophilic materials from the site of entry (i.e. sinusoidal membrane) to the endoplasmic reticulum, where they undergo metabolic conversions [4–6]. Lastly, transfer proteins can facilitate the transport of compounds to the canalicular membrane, where these materials can be transported to the bile duct by canalicular efflux proteins [7]. The glutathione *S*-transferase (GST) family of proteins has been implicated in several intracellular transport processes.

The GSTs comprise a family of dimeric proteins that catalyse the nucleophilic attack of glutathione on electrophilic centres in a wide variety of organic molecules [8–16]. The GSTs can also bind a variety of lipophilic compounds at non-catalytic binding sites, and an intracellular transfer function has been ascribed to this binding activity [17,18]. For example, the ligandins, members of the GST Alpha class, efficiently bind bilirubin [7] and bile acids [19,20] at the non-catalytic binding site. In perfused liver, these GST enzymes have been shown to prevent the back-diffusion (reflux) of bilirubin from the cytoplasm to the plasma and were shown to increase net extraction of bilirubin by the liver [2]. A member of the GST Alpha class also has been shown to bind leukotriene C₄ [21]. The GST α responsible for this binding

has been found both in liver cytoplasm and associated with the canalicular membrane, where a membrane-bound efflux protein eliminates leukotriene C₄ from the cell [21]. Conceivably, GST α may facilitate the transfer of leukotriene C₄ from the cytoplasm to the canalicular-efflux protein. Similarly, GST Pi class members are often overexpressed along with canalicular-efflux proteins ('P-glycoproteins') in cells that have developed resistance to certain drugs [18,22–24]. Although GST π has not been directly implicated in the intracellular transport of drugs, it is interesting to speculate that this GST may function in concert with P-glycoproteins in the elimination of some compounds.

Members of the GST Mu class bind haem and have been shown to function in the transport of haem from the mitochondria, where final haem synthesis occurs [25], to the endoplasmic reticulum, where the haem is incorporated into cytochrome b₅ apoprotein [26]. In rat, GST Mu class members also have been demonstrated to non-catalytically bind bile acids and steroid hormones [17,19,27,28]. Moreover, a GST Mu class member has been identified as a DNA-binding non-histone protein in rat liver nuclei [29]. The localization of this protein has prompted suggestions of a possible role for GST μ in the metabolism and inactivation of electrophilic compounds within the nucleus [29]. However, considering the steroid hormone-binding properties of GST μ , one must also consider that this protein may function in the localization of steroid hormones in the nucleus, where these hormones may interact with their receptors [30]. In addition, the binding of genotoxic compounds to GST μ may facilitate their localization in the cell nucleus, where they can elicit toxicity.

Abbreviations used: GST, glutathione *S*-transferase; BHA, 2(3)-*t*-butyl-4-hydroxyanisole; PB, phenobarbital; I3C, indole-3-carbinol; DCC, dextran-coated charcoal; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; MBP, methyltrienolone-binding protein; CU, cumene hydroperoxide; EA, ethacrynic acid.

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Indole-3-carbinol (I3C) is a glucobrassicin derivative found in cruciferous vegetables [31] that exhibits anticarcinogenic activity [32,33]. This activity has been attributed in part to the induction of GST activity by I3C and the associated increase in the conjugation of electrophilic chemical carcinogens [34–36]. Considering the putative role of GSTs in nuclear localization of steroid hormones and carcinogens, I3C conceivably could affect this localization and associated processes of carcinogenesis. In the present study we have used photoaffinity-labelling techniques with the photoreactive steroid hormone [^3H]methyltrienolone to identify and characterize steroid-binding GSTs in mouse liver cytosol. Further, this approach was used to assess the effects of I3C and its acid-condensation products on the steroid-binding activity of the GSTs.

MATERIALS AND METHODS

Animals

Male CD-1 mice were purchased from Charles River Laboratories, Raleigh, NC, U.S.A. Animals were 8 weeks old when received, and treatment began within the next week. Mice treated with BHA (Sigma) received the drug mixed with ground rodent feed (Agway; Prolab) at a concentration of 7.5 mg of BHA/g of food for 10 days. Mice treated with PB (Merck) received that drug in their drinking water at a concentration of 1 g/litre for 9 days. Mice were treated with I3C, incorporated into their feed, at concentrations of 1.75, 3.5 and 5.25 mg/g for 7 days. On the basis of feed-consumption rates and size of the mice (35 g), these concentrations provided calculated average daily oral I3C doses of 250, 500, and 750 mg/kg. Control animals were age-matched and were provided the same diet without the addition of the drugs. Animals were maintained under a photoperiod of 12 h.

Preparation of hepatic cytosols

Immediately after the mice had been killed by cervical dislocation, livers were removed and minced in ice-cold 1.15% KCl, frozen in liquid N_2 , and stored at -80°C . Frozen liver samples of approx. 2 g each were subsequently homogenized with a Dounce tissue homogenizer in 8.0 ml of cytosol buffer [10 mM-Hepes (pH 7.4)/1.0 mM-EDTA/10% glycerol]. Homogenates were centrifuged at 100000 g for 1 h. The supernatants were diluted with cytosol buffer to a protein concentration of 10 mg/ml, separated into 0.5 ml aliquots, and stored at -80°C . Protein concentrations were determined as described by Bradford [37] using commercially available reagent (Bio-Rad) with BSA as a standard.

Photoaffinity labelling of GST proteins

Cytosolic proteins were subjected to photoaffinity labelling with the photoreactive steroid hormone [^3H]methyltrienolone (New England Nuclear). Mouse liver cytosol (10 μl of a 10 $\mu\text{g}/\mu\text{l}$ solution) was incubated on ice for 1.5 h with 50 nM- ^3H]methyltrienolone (81.8–87.0 Ci/mmol) in cytosol buffer containing no glycerol (PL buffer). The total volume of the mixture was 60 μl . Samples then were irradiated for 10 min at 4–7 $^\circ\text{C}$ with a 450 W mercury-vapour lamp in a photoaffinity labelling apparatus as described by LeBlanc & Waxman [38]. Unbound [^3H]methyltrienolone was removed by incubation of the mixture for 10 min with 20 μl of dextran-coated charcoal (DCC; 1.25 mg of acid-washed charcoal and 0.125 mg of Dextran T70/ml of PL buffer) and subsequent precipitation of the DCC by centrifugation. The individual cytosolic proteins were separated by SDS/PAGE with 12.5% (w/v) gels. Proteins were transferred to nitrocellulose (Bio-Rad), and the nitrocellulose was then dried for 10 min in an oven at 80 $^\circ\text{C}$. The dried nitrocellulose was

sprayed with En 3 Hance (New England Nuclear), and the [^3H]methyltrienolone-labelled proteins were detected by fluorography. Band intensities on the autoradiographs were measured by laser densitometry [39].

Purification of GST proteins and catalytic-activity measurements

Mouse liver GST proteins were purified by affinity chromatography using a GSH-agarose matrix (Sigma) [40]. The purified GST proteins were revealed, and their apparent molecular masses were determined, by SDS/PAGE [41] on 12.5% gels stained with Coomassie Brilliant Blue R-250. The GST activity assays, using 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide (CH), 1,2-dichloro-4-nitrobenzene (DCNB) and ethacrynic acid (EA) as substrates, were conducted as described previously [42].

Immunodetection of GST proteins

Immunoblots were performed as described previously [43]. Polyclonal antibodies to mouse GST α , GST μ , and GST π were provided by Dr. Ann Benson (Department of Biochemistry and Molecular Biology, University of Arkansas, Little Rock, AR, U.S.A.) whereas polyclonal antibody to human GST π and rat GST YaYa were purchased from Bioproducts for Science, Indianapolis, IN, U.S.A. Comparative experiments performed with polyclonal antibodies to mouse GST π and human GST π indicated that these two antibodies exhibited the same protein recognition specificity. After SDS/PAGE [41] and transfer to nitrocellulose membrane, protein-bound antibodies were detected either colorimetrically [43] or by chemiluminescence (Bio-Rad) [44]. GST abundance was quantified by laser densitometry [39] by scanning the nitrocellulose blots when using the colorimetric method or X-ray films when using the chemiluminescent method.

Immunoprecipitation of GST proteins

Cytosolic proteins were subjected to photoaffinity labelling with [^3H]methyltrienolone as previously described. After irradiation, samples were incubated with primary antibody on ice for 1 h. Secondary antibody (anti-IgG) then was added to the samples, and the mixtures were incubated at 4 $^\circ\text{C}$ for an additional 1 h. Protein A-Sephadex beads (50 μg) were then added to each sample, and the tubes were incubated at 4 $^\circ\text{C}$ for 3 h. The Protein A-Sephadex beads were pelleted by centrifugation and were washed three times with cytosol buffer. Bound proteins were released from the Protein A-Sephadex beads by heating for 10 min at 100 $^\circ\text{C}$ in electrophoresis sample buffer [41]. The Protein A-Sephadex beads again were pelleted, and the supernatants were subjected to SDS/PAGE [41]. Immunoprecipitated GST proteins were identified by immunoblot analyses as described previously. Bound [^3H]methyltrienolone associated with the immunoprecipitated proteins was detected by fluorography as described previously.

Acidification of I3C

I3C (Sigma) was incubated in 0.05 M-HCl, pH 1.3, for 100 min with constant stirring to generate acid-condensation products [45]. After this acidification, the products were extracted into methanol and stored at -20°C . Acid-condensation products were analysed by h.p.l.c. using a C_{18} reverse-phase column and a mobile phase consisting of an acetonitrile gradient ranging from 10 to 100% (v/v). 3,3'-di-indolylmethane used as a standard during h.p.l.c. analyses was kindly provided by Dr. L. F. Bjeldanes, Department of Nutritional Studies, University of California, Berkeley, CA, U.S.A. Inhibition of GST-mediated steroid binding by I3C and its acid condensation products was determined by adding the desired amount of compound, dissolved in methanol, to the reaction tube, then evaporating the methanol

under a stream of nitrogen before adding the other constituents of the mixture.

RESULTS

Photoaffinity labelling of cytosolic steroid-binding proteins

Irradiation of mouse liver cytosol in the presence of [3 H]methyltrienolone resulted in the photoaffinity labelling of five major methyltrienolone-binding proteins (MBPs) (Fig. 1a). These MBPs were assigned numerical designations based upon their apparent molecular masses. The band designated MBP27 appeared to be composed of more than one protein having similar molecular masses. Treatment of mice with BHA or PB, two inducers of GST proteins [46,47], increased the labelling of MBP27 and induced a labelled protein, designated MBP25, that was not detectable in untreated mouse liver cytosol (Fig. 1b). PB treatment also increased the labelled protein designated MBP58.

Fig. 1. Photoaffinity-labelled [3 H]methyltrienolone-binding proteins in mouse liver cytosol

(a) Liver cytosol prepared from adult male CD-1 mice was irradiated in the presence of [3 H]methyltrienolone and the affinity-labelled proteins were revealed after electrophoresis as described in Materials and methods section. (b) Induction of [3 H]methyltrienolone-labelled MBP27 and MBP25 in liver cytosols prepared from mice treated with BHA (lane 2) or PB (lane 3). Lane 1 is the control (untreated).

Fig. 2. GST protein levels in liver cytosols from untreated (□) mice and mice treated with 2(3)-t-butyl-4-hydroxyanisole (▨) or phenobarbital (▤)

Protein levels were quantified by immunoblotting with GST-protein-specific antibodies as described in the Materials and methods section. Results (mean $\pm$ S.D., $n = 3$) were significantly different from the controls at $P < 0.05$ (*) or $P < 0.01$ (**) (Student's *t* test).

Photoaffinity labelling of serum or liver microsomes demonstrated that the affinity-labelled proteins in the cytosol did not originate from these potential contaminating sources. In contrast, a ~ 66 kDa protein that weakly labelled in liver cytosol was present at high concentrations in serum and was presumed to be albumin (results not shown). The addition of excess unlabelled methyltrienolone to the assay mixture eliminated the labelling of the five MBPs, indicating that the [3 H]methyltrienolone was specifically bound to these proteins (results not shown).

Induction of GST catalytic activities and proteins by BHA and PB

The apparent molecular masses of MBP27 and MBP25, and their co-induction by BHA and PB, suggested that these proteins may be GST subunits. Indeed, the BHA and PB treatments that elevated the affinity-labelled MBP27 and MBP25 also induced GST activity with DCNB and CDNB but not with EA or CH (results not shown). GST activity towards the substrate DCNB was affected most prominently by these treatments. GST μ is the largest single contributor to this catalytic activity [48,49].

Immunoblot experiments then were performed with GST-isoenzyme-specific antibodies in order to determine whether BHA and PB treatment induced any individual GST proteins in a manner that paralleled the induction of MBP27 and MBP25. GST μ protein levels were elevated significantly by both treatments, whereas GST α protein levels were elevated only by PB treatment (Fig. 2). GST π protein levels were suppressed by these treatments. These results confirmed the conclusion, suggested by catalytic activities, that GST μ was the GST most prominently induced by both BHA and PB. Furthermore, the induction of GST μ by BHA and PB paralleled the increased photoaffinity labelling of MBP27 by these treatments (Fig. 1b).

Immunoblots were also performed using antibody prepared against the rat class Alpha GST $Y_{a1}Y_{a1}$. Mice express an inducible GST of the Alpha class, designated $Y_{a1}Y_{a1}$ [49] that is not expressed in untreated mice, but is induced by BHA [49,50]. Mouse GST $Y_{a1}Y_{a1}$ has a subunit molecular mass of approx. 25 kDa, is immunochemically more closely related to rat GST $Y_{a1}Y_{a1}$ than to mouse GST α and is immunoreactive with antibodies to rat GST $Y_{a1}Y_{a1}$ [49]. GST $Y_{a1}Y_{a1}$ was not detectable in hepatic cytosols from untreated mice, but was significantly induced by treatment with either BHA or PB (Fig. 2). The molecular mass of GST $Y_{a1}Y_{a1}$, its virtual absence in untreated mice and its induction by both BHA and PB suggested that this protein may be identical with MBP25.

Purification of GST proteins

GST proteins were purified from mouse liver cytosol to establish further whether some GST isoenzymes were susceptible to photoaffinity labelling with [3 H]methyltrienolone. A GSH-agarose column retained three proteins from untreated mouse liver cytosol having apparent molecular masses after SDS/PAGE of 27, 26 and 23 kDa. The molecular masses of these proteins were comparable with those of the three major GST subunits in mouse liver cytosol [51]. Column fractions containing these three proteins exhibited high GST activity (results not shown). These observations suggested that at least one, and probably all three, proteins were GST subunits.

Immunoblot experiments were performed with the purified proteins to establish whether the three proteins were GSTs and to determine to which specific GST family each belonged (Fig. 3). The 27 kDa purified protein reacted with antibodies to mouse GST μ , but not with antibodies to GST α or π . The 26 kDa protein reacted exclusively with antibodies to mouse GST α ,

Fig. 3. Immunoidentification of affinity-purified GSTs

Three proteins from mouse liver cytosol eluted from a GSH-agarose affinity column were analysed by immunoblotting using GST-protein-specific antibodies. The purified proteins were probed for reactivity with antibodies raised against (1) GST α , μ and π consecutively, (2) GST μ , (3) GST α and (4) GST π .

whereas the 23 kDa protein reacted only with antibodies to both human and mouse GST π . An immunoblot of the purified proteins, consecutively developed with antibodies to the three GSTs, confirmed that the purified 27, 26 and 23 kD proteins were GST μ , α and π respectively (Fig. 3, lane 1).

Photoaffinity labelling of purified GST proteins

The affinity-purified proteins were subjected to photoaffinity labelling with [3 H]methyltrienolone to determine whether any of these proteins might be MBP27, a protein that had been identified in crude cytosolic preparations. A protein of approx. 27 kDa was labelled strongly by the radioligand, and this labelling was largely eliminated in the presence of excess unlabelled methyltrienolone (results not shown). In contrast, the 23 kDa GST π was labelled weakly by methyltrienolone, and this binding was non-specific, as demonstrated in the presence of excess unlabelled methyltrienolone (results not shown). Experiments were next performed during which cytosol from BHA-treated mouse liver was irradiated in the presence of [3 H]methyltrienolone, then used in the affinity purification of GSTs. SDS/PAGE followed by autoradiography revealed that MBP27 and MBP25 were affinity-purified with the GSH-agarose column (results not shown). Taken together, these results provided further evidence that MBP27 may be identical with the 27 kDa GST μ and MBP25 may be identical with the 25 kDa GST $Y_aY_a_1$. However, the close proximity of the 26 kDa GST α to GST π after electrophoresis precluded us from eliminating the possibility that this protein may contribute to the affinity labelled MBP27.

Immunoprecipitation of MBP proteins

Mouse liver cytosol was subjected to photoaffinity labelling with [3 H]methyltrienolone followed by immunoprecipitation with GST isoenzyme-specific antibodies in order to establish definitively whether GST proteins were photoaffinity-labelled by [3 H]methyltrienolone in the crude cytosolic preparations. Immunoblot analyses of the immunoprecipitates derived with each of the GST-specific antibodies confirmed that the antibodies had precipitated the targeted proteins. Protein precipitated by antibodies to a specific GST immunoreacted only with that antibody during immunoblotting, thus confirming the specificity of the immunoprecipitation.

Autoradiographs of immunoprecipitated proteins after electrophoresis demonstrated conclusively that both GST μ and GST α in the mouse liver cytosol were photoaffinity labelled by [3 H]methyltrienolone and both likely contribute to the affinity-labelled protein designated MBP27 (Fig. 4, lanes 2 and 3).

Fig. 4. Photoaffinity labelling of proteins immunoprecipitated from mouse liver with GST-specific antibodies

Liver cytosol from mice treated with BHA was irradiated in the presence of [3 H]methyltrienolone and samples were then immunoprecipitated with antibodies raised against specific GST proteins as described in the Materials and methods section. Affinity labelled precipitated proteins were then revealed on X-ray film. Lane 1 contains photoaffinity-labelled cytosol. Lanes 2-5 contain immunoprecipitates obtained with antibodies to GST μ (2), GST α (3), GST $Y_aY_a_1$ (4) and GST π (5).

Fig. 5. GST protein levels in liver cytosols from mice treated with I3C

Protein levels were quantified by immunoblotting with GST-isoenzyme-specific antibodies as described in the Materials and methods section. Results (means $\pm$ s.d., $n = 3$) were significantly different from the controls at $P < 0.05$ (*) or $P < 0.01$ (**) (Student's t test).

Further, immunoprecipitated GST $Y_aY_a_1$ also was affinity-labelled by the [3 H]methyltrienolone, confirming that this GST corresponds to the BHA- and PB-inducible MBP25 (Fig. 4, lane 4). In contrast, immunoprecipitated GST π was not susceptible to photoaffinity labelling by the [3 H]methyltrienolone (Fig. 4, lane 5). An affinity-labelled protein corresponding to MBP34 was also immunoprecipitated with each antibody used (Fig. 4), but was not detected in immunoblots of the precipitated proteins. These observations suggest that this protein was not specifically immunoreactive with the GST antibodies, but rather may have been susceptible to complexation with the antigen-antibody matrices that were precipitated.

Fig. 6. Inhibition of [3 H]methyltrienolone binding to MBP27 (circles) and MBP25 (triangles) by I3C (filled symbols) and its acid-condensation products (open symbols)

Liver cytosols from mice treated with BHA were incubated with 50 nM [3 H]methyltrienolone and increasing concentrations of I3C or its acid condensation products. Samples were then irradiated and the effects of the indolic compounds on the photoaffinity labelling of the proteins by the [3 H]methyltrienolone were measured as described in the Materials and methods section. Results are presented as percentage labelling as compared with cytosols that were not incubated with the indolic compounds.

Effects of I3C and its acid condensation products on GST steroid-binding activity

I3C has been demonstrated to induce GST catalytic activity. Increased metabolism/elimination of some chemical carcinogens by the induced GST has been postulated to contribute to the anticarcinogenic activity associated with this compound [34–36]. Conceivably, effects of I3C on the binding and transport of steroid hormones and carcinogens by GST also could contribute to the anticarcinogenic activity of this compound. The photoaffinity-labelling approach was thus used to assess the effects of I3C on GST-mediated steroid-binding activity.

Immunoblots performed with liver cytosols from I3C-treated mice revealed that steroid-binding GST μ and GST Ya₁Ya₁ protein levels both were modestly elevated, whereas GST α protein levels were not affected by this treatment (Fig. 5). Protein levels of GST π , which did not have steroid-binding activity, also were not affected by treatment with I3C. Experiments were then undertaken to assess the direct effects of I3C on the affinity labelling of MBP27 (GST μ/α) and MBP25 (GST Ya₁Ya₁) by [3 H]methyltrienolone. The photoaffinity labelling of both MBP27 and MBP25 was modestly inhibited by I3C (Fig. 6) with approx. 50% inhibition of both proteins occurring at an I3C concentration of 170 μ M (22 μ g/ml).

Since I3C is converted into several acid-condensation products in the stomach after ingestion [52], we were interested in determining the effect of these more physiologically relevant I3C derivatives on GST-mediated steroid-binding activity. Acidification of I3C resulted in the virtually complete conversion of this compound into three major products having different mobilities during h.p.l.c. on a C₁₈ reverse-phase column (results not shown). One of these products was coeluted with 3,3'-diindolylmethane, whereas the other two products were presumed to be trimeric derivatives of I3C [57]. Photoaffinity-labelling experiments revealed that the acid-condensation products of I3C were significantly more inhibitory towards the binding of [3 H]methyltrienolone by MBP27 and MBP25 than was I3C measured on a w/v basis (Fig. 6). Considering the multimeric

composition of the acid-condensation products [57], the difference in GST inhibition between I3C and its acid-condensation products would be appreciably greater when measured on a molar basis than when measured on a w/v basis. Thus although ingestion of I3C results in a modest elevation in steroid-binding GST protein levels, the steroid-binding activity of the GSTs is severely inhibited by the acid-condensation products of I3C.

DISCUSSION

The purpose of the present study was to identify and characterize steroid-hormone-binding GST proteins using photoaffinity-labelling techniques with [3 H]methyltrienolone. Results demonstrated that cytosols from untreated mice express a protein, designated MBP27, that was specifically affinity-labelled by [3 H]methyltrienolone. Liver cytosols from mice treated with BHA or PB expressed MBP27 at elevated levels and expressed an additional protein, designated MBP25, that was affinity-labelled by [3 H]methyltrienolone. On the basis of molecular-mass comparisons, induction profiles and immunoprecipitation experiments, GST μ and GST α were identified as contributors to the affinity-labelled MBP27. In addition, MBP25 was immunochemically identified as GST Ya₁Ya₁. GST π , the major GST in untreated male mice [58] was not susceptible to affinity labelling by [3 H]methyltrienolone.

LeBlanc & Waxman [38] detected a steroid-binding protein having an approximate molecular weight of 27 kDa in liver cytosol from both male and female rats, using photoaffinity labelling techniques similar to those used in the present study. Hypophysectomy elevated the levels of this affinity-labelled protein, and administration of growth hormone to these animals did not restore this protein to normal levels. GST levels are known to be elevated in thyroidectomized rats owing to the loss of thyroxine [53]. GST levels also would likely be elevated in hypophysectomized animals, owing to the loss of pituitary-derived thyroid-stimulating hormone and the commensurate loss of thyroxine. Thus the 27 kDa [3 H]methyltrienolone-binding protein identified in rat liver cytosol likely corresponded to a GST protein. We also have identified a 27 kDa protein in human liver cytosol that is photoaffinity labelled by [3 H]methyltrienolone (G. A. LeBlanc & D. J. Waxman, unpublished work). The labelling of this protein was inhibited by other steroid hormones and bile acids that are known to bind GST. It is likely that this protein in humans also represents a steroid-binding GST.

Homma & Listowsky [27] identified the class Mu GST Yb as the major rat liver GST affinity labelled with the synthetic glucocorticoid [3 H]dexamethasone 21-methanesulphonate. This conclusion was based upon the comparative migration of the labelled protein and GST Yb during SDS/PAGE and the retention of radioactivity on a column containing antibodies to GST Yb after passing photoaffinity labelled cytosol through the column. However, as demonstrated in the present study, GST α is also susceptible to photoaffinity labelling by [3 H]methyltrienolone and also, perhaps, [3 H]dexamethasone 21-methanesulphonate. Owing to the similarity of subunit molecular masses of these proteins, both of these GSTs may contribute to the affinity-labelled band detected after electrophoresis. Results from the present study also demonstrate that the class Alpha GST Ya₁Ya₁ is responsible for approximately half of the GST-mediated [3 H]methyltrienolone-binding activity in BHA- and PB-treated mice. This protein may also bind glucocorticoids such as dexamethasone.

Inhibition of steroid-hormone- and bile-acid-binding activity by GSH has been observed with both class Alpha and Mu GSTs [27, 54, 55]. These observations raise the question of whether the non-catalytic binding of steroid hormones and other compounds

by GSTs actually would occur in the liver, where GSH levels are high [56]. The selective inhibition of steroid-binding activity by glutathione may provide a regulatory mechanism by which certain GSTs maintain a transport function, whereas others are utilized for GSH conjugation reactions.

I3C is a component of cruciferous vegetables that has been shown to possess anticarcinogenic activity [34,35]. This activity has been attributed, in part, to the induction of GST catalytic activity, resulting in increased deactivation/elimination of chemical carcinogens [34–36]. We were interested in using the photoaffinity-labelling techniques described herein to assess the effects of I3C on the non-catalytic steroid-binding activity of GSTs, since effects on this activity could alter the intracellular transport of steroid hormones that act as promoters and chemical carcinogens that are initiators of carcinogenesis. Although oral administration of I3C elevated GST μ and GST Ya₁Ya₁ protein levels, the acid-condensation products of I3C, which are generated in the stomach after ingestion of I3C [52], severely inhibited the binding of [³H]methyltrienolone by these GSTs *in vitro*. I3C condensation products have been measured in the liver after oral administration of I3C to rats [57]. These results indicate that the I3C condensation products may inhibit the intracellular transport of compounds by GST proteins. Considering the demonstrated localization of some class Mu GSTs in the nuclei of liver cells [29], it is conceivable that GST μ could facilitate the localization of GST-bound carcinogens. The inhibitory effects of the acid condensation products of I3C on this binding activity thus could impede the localization of carcinogens in the cell nucleus and reduce associated toxicity. Additional studies are required to assess the ability of nuclear GSTs to localize ligands within this cell compartment and the effects of I3C on this process.

The demonstration that class Alpha and Mu GSTs are susceptible to photoaffinity labelling with [³H]methyltrienone provides a valuable tool for the further characterization of these proteins. As exemplified in the present study with I3C, this labelling technique can be used to directly and precisely measure GST-mediated steroid-binding activity in cytosolic preparations and to assess the effects of potential inhibitors on this activity. This method also can be used to assess steroid-binding activity in various tissue preparations and may be used to assess binding of steroids by GSTs *in situ* in various cell types. Lastly, the covalent radiolabelling of amino acids in the steroid-binding site of GST proteins will provide a means for the identification and characterization of the amino acid structure constituting the steroid-hormone-binding site of the proteins.

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