Glutathione S-Transferases, Structure, Regulation, and Therapeutic Implications*

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The glutathione S-transferases (GSTs) are a family of enzymes that catalyze the nucleophilic addition of the thiol of reduced glutathione to a variety of electrophiles (1-9). In addition, the GSTs bind with varying affinities a variety of hydrophobic compounds such as heme, bilirubin, polycyclic aromatic hydrocarbons, and dexamethasone (1-9). It is now generally accepted that the GSTs are encoded by at least five different gene families (9-12). Four of the gene families encode the cytosolic GSTs whereas the fifth encodes a microsomal form of the enzyme (13, 14). In this review, we have focused on three GST research areas: 1) structure-function analysis of GSTs; 2) regulation of GST expression; and 3) GSTs as therapeutic targets in disease. Since our review will not be comprehensive, we would like to direct the reader to several excellent recent reviews focusing on various aspects of GSTs (4, 8, 9).

Structure-Function Analysis of GSTs

The active site of the GSTs has been shown to contain two binding sites, one binding site for glutathione, G-site, and a second site for substrate binding, H-site (for reviews see Refs. 2 and 7). Experiments based on kinetic and chemical modification techniques indicated that the active site might contain either histidine, cysteine, tryptophan, arginine, or aspartic acid (15-21). Mutagenesis of several of the putative active-site residues have ruled out the involvement of histidine (22-29), cysteine (22-26), tryptophan (26, 30), aspartic acid (30), and arginine (30) in the catalytic mechanism of the GSTs.

Recently, the three-dimensional structures of two \( \pi \) class GSTs with the inhibitors glutathione sulfonate and \( \beta \)-hexylglutathione (31, 32) and the rat 3-3 isozyme complexed with glutathione (33, 34) have been solved (see Fig. 1 for GST 3-3 structure).

The three-dimensional structure of the porcine \( \pi \) homodimeric GST (31) revealed a globular protein with unit cell dimensions of 55 \( \times \) 52 \( \times \) 45 \( \AA \). Each of the subunits in the homodimer was folded into two domains of different structures. The first domain, residues 1-74, consisted of a central four-stranded \( \beta \)-sheet flanked on one side by two \( \alpha \)-helices and on the other side by a bent irregular helix structure. The second domain, residues 81-207, contained five \( \alpha \)-helices. In the crystal structure, the dimeric GST binds two molecules of glutathione sulfonate at a site on domain I, the G-site. The side chains lining the G-site include Tyr-7, Gly-12, Arg-13, Trp-38, Lys-42, Gln-49, Pro-51, Gln-62, Ser-63, and Glu-95. Interestingly, no cysteine residue was shown to be part of the G-site. The sulfonate group of the inhibitor was shown interacting with Tyr-7 (Tyr-6 in rat GST 3-3) and the hydroxyl group of tyrosine 6 is within hydrogen bonding distance of the thiolate anion of glutathione. Even though the overall topology of the two GSTs is quite similar, significant differences also exist (see Ref. 34).

Based on the alignment of cDNA sequence and the crystallographic data described above, several groups have investigated changes in the amino acid sequences involved in the formation of both the G- and H-sites of several GSTs. The tyrosine residue, which is conserved in at least 12 mammalian GSTs, was studied using site-directed mutagenesis by several groups. The tyrosine residue was replaced by several amino acids in the rat 1-1 and 3-3 forms and the human A1-1 and P1-1 forms of GST (29, 30, 35, 36). In all cases, mutation of the conserved tyrosine resulted in a decrease of at least 90% in specific activity of the GST. These results are consistent with the crystallographic data, which suggest that tyrosine 6 plays an essential role in stabilizing the thiolate anion of glutathione through hydrogen bonding.

Regulation of GST Gene Expression

Okuda et al. (37) have reported the isolation and characterization of the rat \( \pi \) (Yp) gene. Sequence analysis indicates that the gene contains 7 exons and 6 introns and spans approximately 3 kilobase pairs. Sakai et al. (38) fused various regions of the 5'-flanking regulatory region of the rat GST \( \pi \) gene to the CAT struc-

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tural gene and transfected them into a rat hepatoma cell line. Two enhancing elements were found at 2.5 and 2.0 kilobase pairs upstream from the transcription start site and designated GPEI and GPEII. A consensus sequence for the phorbol 12-O-tetradecanoate 13-acetate-responsive element, AP-1 binding (TRE) site, was identified in GPEI. GPEII contained two SV40 enhancer corelike sequences and one polyoma corelike sequence. A silencing element was found 400 base pairs upstream from the cap site. The functional silencer unit consists of several cis-acting elements that function cooperatively. At least three proteins bind to the silencer, one of which (designated SF-A), has been identified as the trans-activator LAP-IL-DBP (39, 40).

5' and 3' deletion analysis of GPEI narrowed the essential sequence for the enhancing effect to a region (approximately 30 nucleotides) that contained a TRE-like octanucleotide at the 3' end. Further deletion and point mutation analysis indicated that the enhancing element was composed of two imperfect phorbol 12-O-tetradecanoate 13-acetate-responsive elements (TREs). Each of the TRE-like sequences alone had no activity, but together they acted synergistically to form a strong enhancer (41, 42). More recently, Dicianni et al. (43) have provided evidence that novel transcription factors in addition to Jun and Fos bind to this sequence and mediate transcription.

Cowell et al. (44) and Morrow et al. (45) have reported the isolation and characterization of the human GST π gene. Analysis of the human GST π promoter revealed four putative transcriptional regulatory motifs. These sequences included a TATA box, 29 base pairs upstream from the start of transcription, two Sp1 recognition sequences (GGGCGG) at nucleotides -46 to -41 and -56 to -51 and an AP-1 recognition sequence (TGACTA) at nucleotide position -69 to -63. Comparison of the human GST π gene with the homologous rat gene disclosed extensive conservation of genomic organization between the two species (44, 46, 47).

Several class μ GST genes have been isolated and characterized (48-51). Recently a class GST gene was isolated from a hamster genomic library and found to be up-regulated transcriptionally by glucocorticoids (52, 53). Using 5' deletion analysis, a major glucocorticoid-inducible regulatory region was identified. This element did not contain a classical glucocorticoid response element. Instead, several potential helix-loop-helix binding domains were contained in the glucocorticoid-inducible regulatory region. A second glucocorticoid-inducible regulatory region was also localized 3 base pairs from the helix-loop-helix domains. Transcriptional activation of the hamster class GST gene by glucocorticoids was shown to be dependent on protein synthesis, suggesting that the mechanism of induction was a secondary event.

The mechanism(s) by which xenobiotics transcriptionally activate the GST subunit 1Ya gene has been the focus of our laboratory for several years (54-57). Deletion constructs spanning the 1717 base pairs of the flanking region were transfected into HepG2 cells, and CAT activity was monitored in the presence and absence of several inducers (58-59). Using this deletion strategy, four distinct regulatory elements were identified in the rat 5'-flanking region of the Ya subunit gene (see Fig. 2).

The first regulatory element, nucleotides -860 to -850, was determined to be required for maximal basal level expression. It contained a core DNA recognition sequence that is recognized by HNF1 (hepatocyte nuclear factor 1), a liver-specific transcription factor (56, 57). A second hepatocyte-specific enhancer was localized between nucleotides -775 and -755 that contained a HNF4 (hepatocyte nuclear factor 4) recognition sequence (57).

Computer-aided sequence examination of the 5'-flanking region of the Ya gene promoter revealed the presence of a xenobiotic response element (XRE) and glucocorticoid-responsive element (GRE) that were not found during deletion analysis. An XRE core sequence (GCCGTG) was identified upstream of the HNF1 sequence between nucleotides -908 and -898 (58, 59). A consensus GRE sequence was found between nucleotides -1669 and -1590 (60). The XRE is found in multiple copies in the 5'-flanking region of the cytochrome P-450 1A1 structural gene and is recognized by the liganded Ah receptor complex. The Ah receptor has high affinity for planar aromatic compounds, and after binding of ligand the Ah receptor-ligand complex translocates to the nucleus and interacts with the XRE sequence and activates transcription (58-62).

The fifth cis-acting regulatory element in the flanking region of the Ya subunit gene was identified by its ability to mediate induction of the Ya subunit gene by β-naphthoflavone and 3-methylcholanthrene but shared no apparent sequence similarity to the XRE sequence. We subsequently named the regulatory element the antioxidant-responsive element (ARE) based on data described below (63).

Although our laboratory demonstrated that the Ah receptor was required for transcriptional activation of the Ya subunit gene by planar aromatic compounds, we demonstrated that the XRE in the Ya subunit gene was not essential for transcriptional activation of the gene by β-naphthoflavone, 3-methylcholanthrene, or benzo(a)pyrene. Several reports by Talalay and co-workers (64-67) had suggested that an Ah receptor-independent mechanism could account for the induction of several phase II drug-metabolizing enzymes by phenolic antioxidants such as tert-butylhydroquinone (t-BHQ). Using wild type and Ah receptor-deficient mouse hepatoma cell lines, we showed that the Ya subunit gene was activated through the ARE after exposure to planar aromatic compounds (e.g. β-naphthoflavone or 3-methylcholanthrene) in cells that expressed functional Ah receptors and active cytochrome P-450 1A1 (63). Cells deficient in either the Ah receptor or P-450 1A1 failed to increase transcription of the ARE when exposed to planar aromatic compounds. However, exposure of cells deficient in either the Ah receptor or cytochrome P-450 1A1 to phenolic antioxidants (e.g. tert-butylhydroquinone or 3,5-di-tert-butylcatechol) resulted in activation of gene transcription through the ARE.

In order to further define a structure-activity relationship for phenolic antioxidants, HepG2 cells transfected with an ARE-CAT construct were exposed to a series of polyhydroxylated benzene-ring compounds. Only those compounds containing a 1.2 or 1.4 substitution on the benzene ring were active as inducers through the ARE. A common feature of the phenolic antioxidants that activated transcription through the ARE was their ability to undergo or drive redox cycling with the potential to form superoxide anion and hydrogen peroxide. Our laboratory subsequently showed that hydrogen peroxide itself activated transcription of the GST Ya gene to the extent of the ARE (68).

Extensive deletion and point mutation analysis of the 41-nucleotide ARE showed that the sequence provided at least two transcriptional activities to the Ya subunit gene, basal and xenobiotic inducible. 5' deletion of the ARE sequence to nucleotide -699 abolished the basal level expression present in the complete ARE. The remaining sequence was still responsive to phenolic antioxidants and planar aromatic compounds. Similar deletions were carried out
from the 3′-end of the ARE. The remaining core sequence, 5′-GTC-GACAAAGC-3′, is known to be the minimal sequence required for inducible activity (66). Mutation analysis of the ARE revealed that any change in the TGAC of the core sequence abolished both basal and inducible activities. Mutations in either the G or C (or both) of the core sequence abolished only the inducible activity.

In vitro binding experiments and methylation interference and protection studies have shown that the ARE sequence binds with high affinity a trans-acting factor(s) (56, 57, 69). The factor(s) appears to bind to the major groove and involves contact with the Gp G dinucleotide and the G residue within the TGAC tetramer on the coding strand. The trans-acting factor(s) was shown to be present in nuclear extracts from untreated and tert-butylhydroquinone-induced cells as monitored by photochemical cross-linking experiments. The cross-linked protein appeared to be a heterodimer with subunit molecular mass of approximately 28,000 and 45,000 daltons (69).

Frilling et al. (70) have also identified two sequences in the 5′-flanking region of a mouse GST Ya subunit gene that shows significant sequence homology to the ARE sequence we identified in the rat GST Ya subunit gene. They have named the mouse sequence the electrophile-responsive element or EpRE. These authors have provided data suggesting that the EpRE is comprised of two non-overlapping AP-1 sites, similar to that shown for the rat GST γ promoter (43, 44). In gel mobility shift assays, utilizing in vitro translated c-jun and c-fos, Frilling et al. (71) described an interaction with the EpRE. In addition, they have shown transactivation of an EpRE-CAT construct by c-jun and c-fos in mouse embryonic F9 cells (71).

In contrast to the work of Frilling et al. (71) our laboratory has shown that in vitro translated c-jun and c-fos do not bind to the rat ARE sequence as judged by gel mobility shift assays. Furthermore, a consensus TRE sequence (AP-1 binding site) from the human collagenase gene (72) did not compete for the binding of the ARE with nuclear factors from the hepatoma cells. Therefore, the identity of the trans-acting factor(s) that interact with the rat ARE and activate transcription by reactive oxygen species is not a jun-fos heterodimer.

Firstly, the ability of reactive oxygen species to activate gene transcription through the ARE sequence is most likely part of a signal transduction pathway through which eukaryotic cells respond to oxidative stress. Activation of genes containing an ARE could lead to the induction of enzymes that protect the cells from endogenous and/or exogenous compounds that undergo redox cycling and form reactive oxygen species. Consistent with this hypothesis is the demonstration of a functional ARE sequence in the flanking regions of the rat (73) and human (74, 75) NADP+H-quinone reductase genes. This enzyme has been shown to be critical in protecting cells against reactive oxygen species due to redox cycling of endogenous and exogenous quinones (76).

Recently, the human homologues of the rat and mouse GST Ya subunit genes have been characterized (77–79). Although the human GST A1 and A2 genes have the same number of exons and introns as the rat and mouse genes, the 5′-flanking regions of the human genes are quite different in sequence. These data suggest that the human GST A1 and A2 genes may be regulated quite differently than the mouse or rat genes.

Glutathione S-Transferrases as Therapeutic Targets in Disease

Asthma—Leukotrienes and peptidoleukotrienes, such as LTD₄, have been shown to be important in the pathogenesis of diseases such as human bronchial asthma. The leukotrienes are derived from arachidonic acid that is released within the cell from the sn-2 position of membrane phospholipids probably through the action of cytochrome P450-dependent cyclooxygenase. The leukotriene-δ₅-lipoxygenase, Leukotriene A₄, is further metabolized via one of two possible pathways. It is either hydrated by the cytosolic enzyme LTA₄ hydrolase producing LTD₄ or it is conjugated to reduced glutathione by a membrane-bound enzyme to form the sulfidopeptide leukotriene C₄. The enzyme that conjugates GSH to LTA₄ is leukotriene C₄ (LTC₄) synthase, a novel glutathione S-transferase (82, 83).

Recently, Nicholson et al. (84, 85) have succeeded in purifying leukotriene C₄ synthase from differentiated U937 cells and the human monocyte cell line THP-1. The final enzyme preparation was greater than 10,000-fold purified and contained three polyepitides of molecular masses of 37.1, 24.5, and 18.0 kDa. The 18-kDa polypeptide was thought to be associated with enzymatic activity since it could be specifically labeled by a radiiodinatated leukotriene C₄ photofluorophy probe.

Since peptidoleukotrienes such as LTD₄ have been shown to be involved in diseases such as human bronchial asthma, the inhibition of the synthesis of LTC₄ or development of specific antagonists to LTD₄ (which is derived from LTC₄ by removal of γ-glutamyl transpeptidase) receptors represents two new and important therapeutic approaches to the treatment of asthma. In fact, LTD₄ receptor antagonists such as MK571, MK579, and ICI 204,219 as well as inhibitors of leukotriene synthesis (e.g. Zilueton®, ICI D2218, and MK 886) have either entered or have been in clinical trials for some time (86, 87). Many of these compounds have shown efficacy in human bronchial asthma. Since the data to date suggest that the leukotriene C₄ synthase is a unique glutathione S-transferase required for the production of peptidoleukotrienes, it should be considered an important therapeutic target for the inhibition of the production of peptidolipid leukotrienes.

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GSKs in Anti-cancer Drug Resistance—One mechanism that has been proposed to explain anti-cancer drug resistance is the increased and/or differential expression of one or more of the GST isozymes in resistant cells compared with normal cells. Although many examples of increased GST activity and resistance have been reported, we have chosen just a few examples to discuss.

During the development of an Adriamycin-resistant human breast cancer cell line (AdRMC 7), Cowan and coworkers (88) have observed that the resistant cells showed a marked increase in GST activity (45-fold) compared with the wild type cells. More than 90% of the increased activity was associated with GST π, which is not found normally in wild type cells.

Moscow et al. (89) overexpressed the human GST π cDNA into drug-sensitive MCF-7 cell human breast cancer cells, which usually have low amounts of GST activity and do not express the GST π isozyme. The GST activity of the transfected cells was increased as much as 15-fold over that in the wild type MCF-7 cells. The transfected cells were more resistant (1.3–4.1-fold) to benzo[a]pyrene and its toxic metabolite benzo[a]pyrene-anti-7,8-dihydrodiol-9,10-epoxide than the wild type cells. However, the transfected cells were not more resistant to adriamycin, melphalan, or cisplatin. Therefore, based on these data it appears that increased expression of GST π, in addition to the changes that occur from other intracellular changes, produces a modest level of protection from the cytotoxic effects of some lipophilic carcinogens but does not markedly contribute to resistance against doxorubicin, melphalan, or cisplatin.

Puchalski and Fahl (90) have expressed GST π, GST 1–1, and GST 3–3 enzymes in cultured mammalian cells and have demonstrated that GST 1–1 confers the greatest cellular resistance to chlorambucil and melphalan (1.3–2.9-fold), whereas GST 3–3 conferred the greatest increase in resistance to cisplatin (1.5-fold). The resistance values were statistically significant and agree with values seen clinically.

These results suggest that overexpression of specific GSTs in mammalian cells can lead to biological resistance to alkylating agents that are used in cancer chemotherapy. However, they do not establish that elevated GST activity in resistant cells correlates with high rates of cephalosporin of the parent drug to glutathione. Several reports have shown that such a situation could exist. Three glutathione adducts of melphalan have been identified in reactions catalyzed by rabbit or human microsomal GSTs and several cytochrome P450 (91, 92). Clearly more of these studies must be carried out to determine the importance of GSTs in drug resistance. Nonetheless, these data are suggestive that therapeutic strategies aimed at inhibiting specific GSTs might be useful in extending the efficacy of certain anti-cancer drugs.

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