Identification of galectin I and thioredoxin peroxidase II as two arsenic-binding proteins in Chinese hamster ovary cells

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In this study, we report the identification of two arsenic-binding proteins from Chinese hamster ovary (CHO) cells. The crude extract derived from CHO and SA7 (arsenic-resistant CHO cells) was applied to a phenylarsine oxide–agarose affinity column, and after extensive washing, the absorbed proteins were eluted with buffers containing 20 mM 2-mercaptoethanol (2-ME) or dithiothreitol (DTT). Three differentially expressed proteins, galectin 1 (Gal-1; in the 2-ME-eluted fraction from CHO cells), glutathione S-transferase P-form (GST-P) and thioredoxin peroxidase II (TPX-II), respectively in the 2-ME- and DTT-eluted fractions from SA7 cells, were identified by partial amino acid sequence analysis after separation by SDS/PAGE. The GST-P protein has been previously shown to facilitate the excretion of sodium arsenite [As(III)] from SA7 cells. TPX II was detected predominately in SA7 cells [routinely cultured in As(III)-containing medium], but not in CHO or SA7N (a revertant of SA7 cells cultured in regular medium) cells. In contrast, Gal-1 was specifically identified in CHO and SA7N cells, but not in SA7 cells. The preferential expression of Gal-1 in CHO cells and TPX-II in SA7 cells was further illustrated by quantitative PCR analysis. The binding of Gal-1 and TPX-II with As(III) was further verified by both co-immunoprecipitation and co-elution of Gal-1 and TPX-II with As(III). It is suggested that Gal-1 and TPX-II are two proteins that serve as high-affinity binding sites for As(III) and thus both may be involved in the biological action of As(III).

Key words: antioxidant enzyme, arsenic resistance, phenylarsine oxide–agarose, sulphhydryl group, thiol group.

INTRODUCTION

Arsenic is a ubiquitous environmental contaminant, and exposure to arsenic remains of considerable importance in several areas today [1–3]. Chronic exposure to arsenic has been associated with liver damage, peripheral vascular disease and peripheral neuropathy [4]. Furthermore, exposure to excessive levels of arsenic has been correlated with an increased incidence of skin, lung and bladder cancers [1,2,5].

Inorganic trivalent arsenite [especially sodium arsenite; As(III)] and pentavalent arsenate [As(V)] are the major chemical species of arsenic that cause toxicological problems. As(III) is thought to be the most toxic form in Nature and has been suggested to act as a tumour promoter in the carcinogenic process [6]. Although As(V) is less toxic than As(III), numerous *in vivo* studies have revealed that As(V) is reduced to the more toxic form As(III) and then detoxified by methylation [7]. It has also been reported that when arsenic is administered to cells, it initially binds to cellular proteins before reduction or methylation can occur [7,8]. Therefore, the binding of arsenic to cellular proteins is a key determinant in arsenic metabolism [7]. There is evidence that arsenic binds to cellular proteins or enzymes in both animal tissues and *in vitro* cell cultures [8,9].

We have previously established an arsenic-resistant Chinese hamster ovary (CHO) cell line, SA7, which manifests higher levels of GSH and glutathione S-transferase P-form (GST-P) than parental cells [10,11]. Both GSH and GST-P confer As(III) resistance by facilitating the excretion of As(III) from SA7 cells [12,13]. Nevertheless, our previous study also revealed that As(III) efflux from SA7 cells is significantly inhibited by verapamil and cyclosporin A and the inhibitory effects are not due to a decrease in GSH levels or GST activity [12]. These results suggest that a pleiotropic response is associated with resistance of SA7 cells to As(III). These observations have prompted us to study further other cellular proteins modulated by As(III) in SA7 cells. To address this question, we used an arsenic affinity resin [phenylarsine oxide–agarose (PAO-agarose)] to isolate As(III)-modulated proteins from CHO and SA7 cells.

Though the mechanism of arsenic toxicity is not well characterized, arsenic has been shown to exert at least some of its toxic effects through interaction with thiol groups of proteins, thereby modulating the activities of key regulatory proteins, which could contribute to its carcinogenic process [6]. The PAO-agarose, with an arsine oxide moiety, was originally employed in the isolation of thiol compounds [14] and is therefore employed in this study to identify As(III)-binding proteins in CHO and SA7 cells.

Abbreviations used: CHO, Chinese hamster ovary; DMA^{III}, trivalent dimethylarsenic species; DTT, dithiothreitol; Gal-1, galectin-1; GST-P, glutathione S-transferase P-form; IPTG, isopropyl β-D-thiogalactoside; 2-ME, 2-mercaptoethanol; MMA^{III}, trivalent monomethylarsonous acid; Ni-NTA, Ni^{2+}-nitrilotriacetate; PAO-agarose, phenylarsine oxide–agarose; RT-PCR, reverse transcriptase PCR; SRB, sulphorhodamine B; TPX-II, thioredoxin peroxidase II.

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The nucleotide sequences reported here have been submitted to the GenBank™/EMBL Nucleotide Sequence Databases under the accession number AF221841.

EXPERIMENTAL

Chemical and cell culture

CHO and SA7N (a revertant of SA7) cells were cultured in McCoy's 5A medium supplemented with 10 % heat-inactivated fetal calf serum and antibiotics as described previously [10]. The cultures were maintained at 37 °C in a humidified gaseous phase of 95 % air and 5 % CO_2. SA7 cells were maintained in the same medium containing 30 μM As(III). As(III) was purchased from Sigma (St. Louis, MO, U.S.A.) and prepared freshly by dissolving in double-distilled water. The trivalent monomethylarsonous acid (MMA^{III}) and trivalent dimethylarsenic species (DMA^{III}) were prepared by bubbling monomethylarsonic acid ('MMA^{V}'; Chem Service, West Chester, PA, U.S.A.) and sodium dimethylarsonate (Merck) with SO_2 as described by Cullen et al. [15] and Goddard [16] respectively.

Preparation of cell extract

The exponentially grown CHO and SA7 cells were washed twice with PBS and harvested with a rubber policemen. After centrifugation at 259 ***g*** at 4 °C for 5 min, the cell pellets were re-suspended in PBS containing 2 % Triton for 15 min on ice. The cell suspensions were then briefly sonicated and centrifuged at 206 ***g*** for 5 min to remove cell debris. An aliquot of the cell extracts was subjected to PAO-agarose affinity column chromatography.

PAO-agarose affinity column chromatography

PAO-agarose ($\approx$ 12 ml) obtained from Sigma was placed in a 10 cm $\times$ 2.5 cm (inner diameter) column and washed extensively with 50 mM Tris buffer, pH 7.4 (buffer A), prior to use.

All chromatographic steps were carried out at 4 °C. The clear supernatants (12 mg of protein) were diluted with PBS to 2 mg/ml and applied to the PAO-agarose column pre-equilibrated with buffer A. The column was washed with buffer A at 1 ml/min. Afterwards the column was washed further with 2 M NaCl in buffer A to remove the non-specific binding proteins. Until the absorbance at 280 nm of eluate had decreased to the baseline, the specific binding proteins were sequentially eluted with a 20 mM 2-mercaptoethanol (2-ME) and 20 mM dithiothreitol (DTT) in buffer A. The eluted proteins were concentrated and de-salted with Amicon ultrafiltration cells using a 3 kDa cut-off membrane (Amicon, Beverly, MA, U.S.A.). The concentrated proteins were stored at -20 °C.

SDS/PAGE and amino acid sequence analysis

Proteins in the eluted fraction were quantified spectrophotometrically [17], and separated by SDS/PAGE (12.5 % gel) [18]. The isolated proteins on the gel were either visualized directly with Commassie Brilliant Blue dye staining or electroblotted on to PVDF membranes (Pall Ultrafine Filtration Co., Glen Cove, NY, U.S.A.) with a semi-dry blotting system. After staining and de-staining, protein bands of interest were excised from the membrane and sequenced using a Procise model 491 sequencer (Applied Biosystems, Foster City, CA, U.S.A.) for automated cycles of Edman degradation.

Alternatively, proteins from the affinity column were dissolved in SDS sample buffer containing 3.5 % SDS and boiled for 3 min. The mixture was alkylated in 40 mM iodoacetamide at room temperature for 20 min in the dark. The proteins were then separated by SDS/PAGE (12.5 % gel) and visualized by the reverse staining protocol of Fernandez Patron et al. [19]. Protein bands of interest were digested *in situ* with *Achromobacter* protease I (Wako, Osaka, Japan) at 42 °C overnight in 100 mM $(NH_4)_2CO_3$, pH 9.2, according to the general outline of Rosenfeld et al. [20]. The resulting peptides were extracted sequentially from the gel with 0.1 % trifluoroacetic acid, 30 % acetonitrile/0.1 % trifluoroacetic acid and 60 % acetonitrile/0.1 % trifluoroacetic acid. The solutions were combined and the volume reduced *in vacuo* to less than 50 μl prior to HPLC separation on a Vydac C_{18} reversed-phase column (1 mm $\times$ 250 mm). Peptides were eluted from the column using a linear acetonitrile gradient at a flow rate of 28 μl/min and the elution profile was monitored at 210 nm. Fractions were collected and spotted on to PVDF membranes for automated cycles of Edman degradation.

Reverse transcriptase PCR (RT-PCR) and cDNA sequencing

mRNA was isolated from CHO and SA7 cells respectively using TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.), and suspended in 10 μl of DEPC (diethyl pyrocarbonate)-treated water. We subjected 1 μg of mRNA to reverse transcription and PCR using Superscript RnaseH$^-$ Reverse Transcriptase and *Taq* DNA Polymerase (Gibco, Grand Island, NY, U.S.A.). On the basis of the reported cDNA sequence of galectin-1 (Gal-1), two primers were synthesized: an upstream primer, 5′-ATGGCCTGTGGTCTGGTCGC-3′, and a downstream primer, 5′-TCACTCAAAGGCCACGCACT-3′. The primers for thioredoxin peroxidase II (TPX-II) were deduced from rat and mouse: upstream primer, 5′-ATGTCTTCAGGAAATGCAAAA-3′, and downstream primer, 5′-TCACTTCTGCTTAGAGAAATA-3′. Amplification was carried out with 30 cycles at 94 °C for 1 min (denaturation), 52 °C for 1 min (annealing) and 72 °C for 2 min (extension) for Gal-1; similar procedures were used to amplify TPX-II except that the temperature for annealing was 49 °C. The PCR products were resolved by 1.6 % agarose gel electrophoresis. The correct single PCR products (408 bp for Gal-1 and 600 bp for TPX-II) were gel-purified and, respectively, cloned together with isopropyl β-D-thiogalactoside (IPTG) and 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal') markers into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). Two Gal-1- and three TPX-II-positive plaques were cloned and nucleotide sequences of these clones were determined by a model 373 ABI Prism auto-sequencer (Applied Biosystems).

For quantitative PCR analysis, the cDNA products of reverse transcription from CHO and SA7 cells were serially diluted and acted as templates for PCR using Gal-1 and TPX-II primers, respectively, as described above.

Binding assay

To demonstrate further the binding of Gal-1 and TPX II with As(III), we performed the binding assay using co-immunoprecipitation and co-elution of Gal-1 and TPX-II with As(III). For the co-immunoprecipitation assay, CHO and SA7 cells in an exponential growth phase were treated with various concentrations of As(III) for 2 h. At the end of the treatment, cells were washed with PBS three times and harvested by trypsinization. An aliquot of cells (5×10^6) was homogenized by sonication. Afterwards, antibodies for Gal-1 (a kind gift from Professor John L. Wang, Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A.) or TPX II (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were added to the CHO and SA7 cell extracts respectively and allowed to react for 1 h at room temperature. The extract was then mixed with Protein A–agarose (50 μl/reaction) and incubated for a further 1 h. The extract was

then centrifuged at 180 ***g*** for 5 min and the supernatant discarded. After being washed with PBS three times, the Protein A-precipitated Gal-1 and TPX-II were subjected to arsenic content determination using an atomic absorption spectrophotometer equipped with a hydride formation system (Perkin Elmer AA100-FIAS-100; Norwalk, CT, U.S.A.) according to the methods of Wang and Lee [13]. In brief, after digesting with concentrated nitric acid (HNO_3), the arsenicals were converted to arsines by reaction with sodium borohydrate ($NaBH_4$). Arsines were then flushed by nitrogen gas to a quartz cell for atomization and detected by an arsenic photomultiplier tube at 193.7 nm. The reliability of the assay was calibrated by spiking samples with known amounts of reference standards (stock solution, 1 g/l H_3AsO_4 in 0.5 M HNO_3) from Merck with coefficients of variation between 5 and 10 %. T7-*Taq* monoclonal antibody (Novagen catalogue number 69999-3) or catechol 2,3-dioxygenase rabbit polyclonal antiserum (a kind gift from Professor Shirly Huang, Department of Life Science, National Central University, Taoyuan, Taiwan, Republic of China) was included as the negative control.

For the co-elution assay, full-length Gal-1 and TPX-II cDNAs were inserted respectively into pQE vectors to generate a plasmid encoding either Gal-1 or TPX-II, with a 6× His tag at the C-terminus. The Gal-1 and TPX-II constructs were then expressed in *Escherichia coli*. The expression of Gal-1 and TPX-II were induced with 1 mM IPTG in the presence of 100 μM As(III) for 4 h. The His-tagged Gal-1 or TPX-II was then purified to homogeneity using Ni^{2+}-nitrilotriacetate (Ni-NTA) affinity chromatography according to the manufacturer's instructions (Qiagen) and subjected to arsenic content determination with an atomic absorption spectrophotometer as described above. The control plasmid used in this experiment was glycine RNA synthetase cDNA constructed and expressed in the pQE vector (a kind gift from Professor Duke Wang, Department of Life Science, National Central University, Taiwan).

Cell growth assay

Cell viability was assayed using the sulphorhodamine B (SRB) staining method in Gal-1- or TPX-II-overexpressing mammalian cells [21]. Briefly, full-length cDNA of Gal-1 and TPX-II was inserted into the *Eco*RI cloning site of the vector pcDNA3.1 and transfected into 3T3 cells using Lipofectamine according to the manufacturer's instructions (Life Technologies). The transfected cells (3T3-Gal and 3T3-TPX) were routinely cultured in medium containing 400 μg/ml G418. For survival assay, the cells were treated with drug for 24 h and allowed further growth in drug-free medium for 24 h. The cells were washed twice with PBS and fixed at 4 °C for 1 h with 1 ml of 10 % trichloroacetic acid. The cells were then washed three times with distilled water and stained with 1 ml of 0.4 % SRB for 1 h. The dishes were then washed with 1 % acetic acid, air-dried and the protein-bound SRB was dissolved in 1 ml of 10 % Tris solution (pH 10.5). The absorbance was determined at 565 nm.

RESULTS

PAO-agarose chromatography of arsenic-modulated proteins

Arsenic has been shown to exert at least some of its toxic effects through interaction with thiol groups of proteins. The mechanism of arsenic carcinogenicity has been attributed to its binding to key regulatory proteins and inhibiting their functions [6]. As proposed by many other reports, As(III) induces a number of stress proteins [22,23]. Our experimental results also indicate that several proteins are differentially altered by As(III) treatment in CHO or SA7 cells (Figure 1). To further identify proteins having affinity for As(III), the cell extract of CHO and SA7 cells was chromatographically resolved by PAO-agarose. Figure 2 shows the SDS/PAGE profiles of protein fractions eluted from PAO-agarose chromatography. Indeed, many proteins from CHO and SA7 cells were retained respectively by the arsenic affinity column. Only three unique proteins, however, with molecular masses of 14, 22 and 23 kDa, were apparently expressed differentially in either CHO or SA7 cells and retained by the PAO-agarose affinity column. The 14 and 23 kDa proteins, overexpressed in CHO and SA7 cells respectively, were eluted from the PAO-agarose affinity column using 2-ME. The 22 kDa protein eluted using DTT or 2-ME was differentially expressed only in SA7 cells.

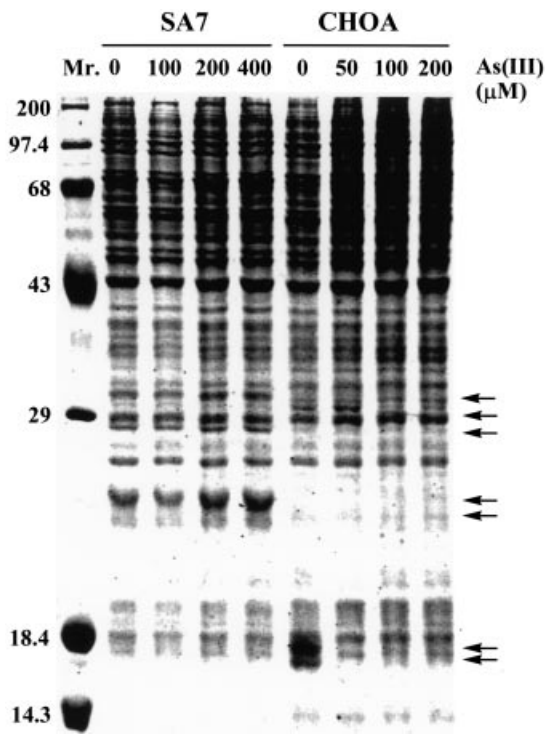

Figure 1 Typical SDS/PAGE profiles of proteins from CHO and SA7 cells treated with As(III)

CHO (CHOA) and SA7 cells were treated with As(III) (0–400 μM) for 2 h. Protein composition (30 μg of cellular proteins/lane) was assessed by SDS/PAGE (12.5% gel). Arrows indicate the positions of proteins modified by As(III) treatment.

Amino acid sequence analysis

Amino acid sequencing suggested that the 23 kDa protein, which was overexpressed in SA7 cells, was the GST-P protein. However, since N-terminal amino acid blockage prevented direct amino acid sequencing of the 14 and 22 kDa proteins, we resorted to determination of the internal partial amino acid sequence of the proteins subsequent to pre-alkylation and digestion. After HPLC separation of the *Achromobacter* protease I-digested peptides (Figure 3), two internal peptides of each respective protein were extracted and subjected to partial amino acid sequence analysis. On the basis of the partial amino acid sequence, the 14 kDa protein differentially expressed in CHO and SA7N (a revertant of SA7 cells) was identified as Gal-1. The 22 and 23 kDa proteins

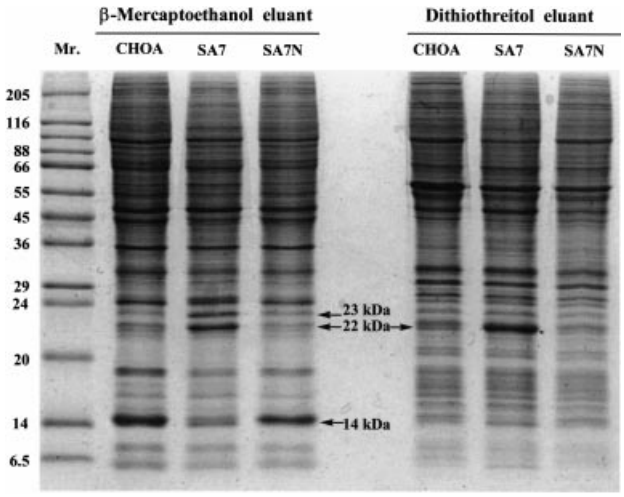

Figure 2 SDS/PAGE profiles of protein fractions obtained by PAO-agarose affinity chromatography

The clear extract from CHO (CHOA), SA7 and SA7N cells was applied to the PAO-agarose affinity column as described in the Experimental section. After extensive washing, the specific binding proteins were sequentially eluted with 20 mM 2-ME and DTT in buffer A. Protein fractions were concentrated and desalted by ultrafiltration, and an aliquot containing 2.5 μg of protein was separated by SDS/PAGE mini-gel (12.5%). Arrows indicate the positions of proteins preferentially expressed in CHO, SA7 or SA7N cells.

(overexpressed in SA7 cells, but not CHO or SA7N cells) were identified as TPX-II and GST-P respectively (Table 1).

Quantitative PCR analysis of the expression of Gal-1 and TPX-II

To confirm further the differential expression of Gal-1 and TPX-II in CHO and SA7 cells, the mRNA of both cell lines was isolated and quantified by reverse transcription and PCR using synthesized primers corresponding to the partial sequences of Gal-1 and TPX-II respectively. As shown in Figure 4, the PCR reaction using primers from Gal-1 produced a single 408 bp product with a higher level of expression in CHO cells than in SA7 cells (Figure 4A). In contrast, by using primers for TPX-II, a single 600 bp product was expressed at significantly higher levels in SA7 cells (Figure 4B).

In order to understand how As(III) modulates Gal-1 and TPX-II expression, a time course experiment was conducted. The CHO and SA7N cells were treated with 30 μM As(III) and the total RNA was isolated prior to RT-PCR analysis using Gal-1 and TPX-II primers respectively. As shown in Figure 5(A), the expression of Gal-1 in CHO cells was suppressed significantly after 72 h incubation with 30 μM As(III). In contrast, the level of TPX-II began to increase at 12 h and reached a plateau around 72 h after the treatment of SA7N cells with 30 μM As(III) (Figure 5B).

Cloning of Gal-1 and TPX-II cDNAs

The 408 and 600 bp PCR products, suspected to be Gal-1 and TPX-II, were prepared for sequencing by their respective cloning into the pGEM-T Easy vector. We selected two Gal-1 and three TPX-II clones from each PCR product and sequenced the cDNA inserts from both forward (+) and reverse (−) orientations. The 408 bp cDNA sequence proved to be identical to the published Gal-1 sequence [24]. The 600 bp cDNA sequence, however, was not found in the GCG data bank, but instead were 94.5, 93.7 and 91.5% homologous with the TPX-II sequences of rat, mouse and human respectively (Figure 6A).

Figure 6(B) shows an alignment of the deduced amino acid sequence of TPX-II from CHO cells with that of TPX-II from human, mouse and rat. Although the amino acid sequence of TPX-II from CHO cells shows high homology with other species, there exists one striking difference at position 30. This was a

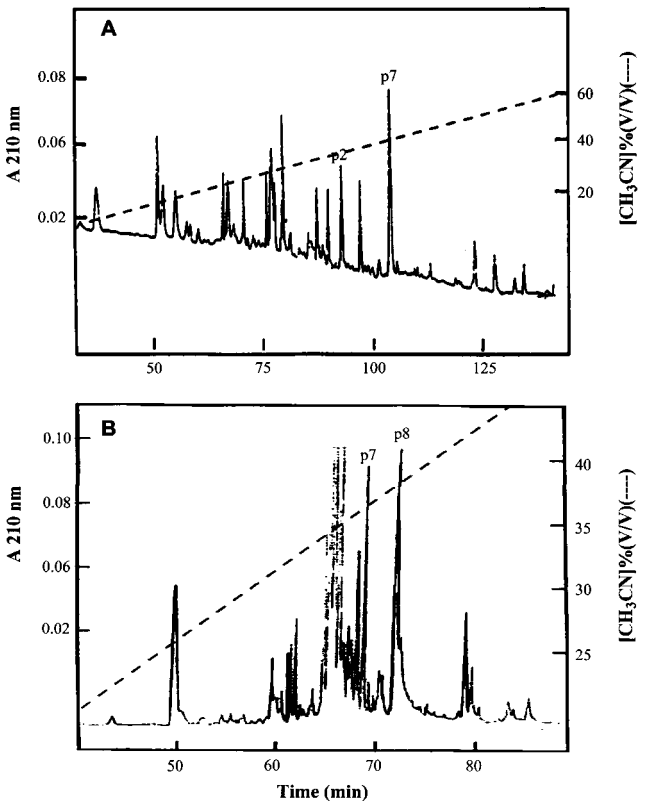

Figure 3 Reversed-phase HPLC analysis of peptides derived from the 22 and 14 kDa proteins

The proteins from PAO-agarose affinity column were subjected to pre-alkylation and then SDS/PAGE separation. The 22 and 14 kDa bands were digested *in situ* with *Achromobacter* protease I and the resulting peptides were separated by reversed-phase HPLC as described in the Experimental section. Peptide elution was monitored by measurement of A_{210}. Selected peaks from (**A**) 22 kDa protein (corresponding to peaks 2 and 7 in Table 1) and (**B**) 14 kDa protein (corresponding to peaks 7 and 8 in Table 1) are indicated. CH_3CN, acetonitrile.

Table 1 Identification of the 14, 22 and 25 kDa protein by microsequencing

After separating the digested peptide by reversed-phase HPLC, the selected peaks were collected and subjected to peptide sequencing.

Protein (and HPLC peaks)	Peptide sequence	Amino acids	Modification
14 kDa protein (Gal-1)			N-terminal block
Peak 7	RLNMEAINYM	112–121	
	DSNNLCLHFNPRFNAH	38–54	
Peak 8	FPNRLNMEAI	109–117	
22 kDa protein (TPX-II)			N-terminal block
Peak 2	RTIAQDGY	110–116	
Peak 7	LNCQVIGASVDSHF	69–82	
23 kDa protein (GST-P)			–
	PPYTIVYFPVRGRCEAMRILL	2–21	

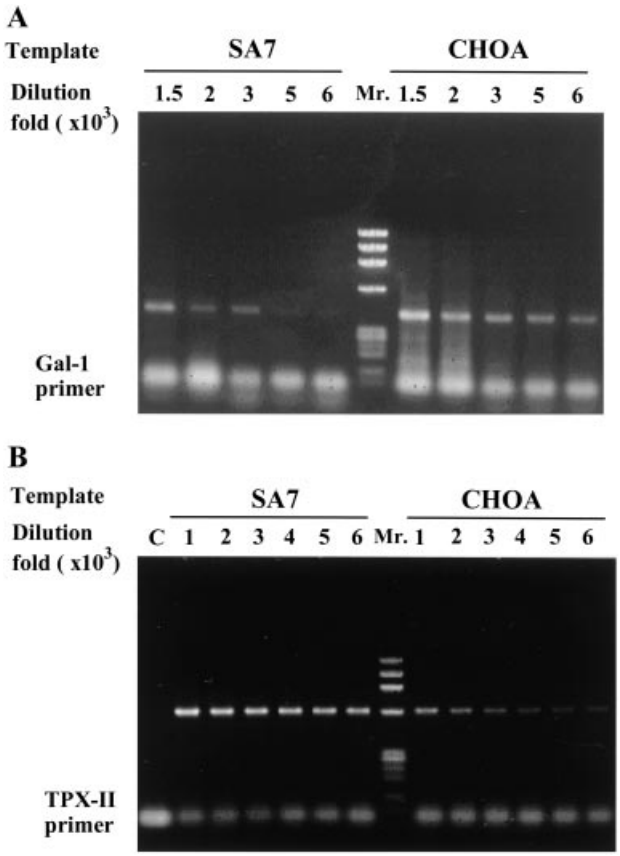

Figure 4 Quantitative PCR analysis of the expression of Gal-1 and TPX-II in CHO and SA7 cells

cDNA were synthesized with reverse transcription from 1 μg of mRNA of CHO (CHOA) and SA7 cells respectively. The cDNA of CHO and SA7 cells were then serially diluted as templates for PCR amplification, as described in the Experimental section using (**A**) Gal-1 primer or (**B**) TPX-II primer. Lane C, control (only primer was added to the PCR reaction).

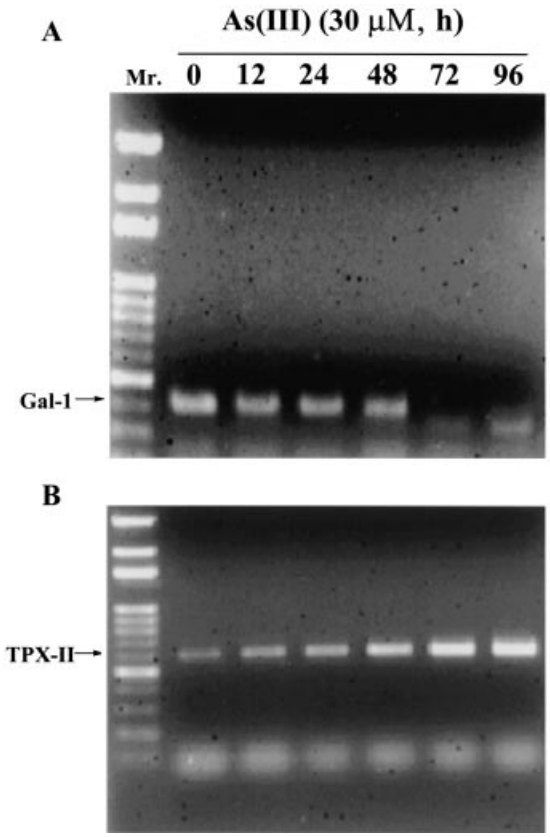

Figure 5 Time course effects of As(III) on the expression of Gal-1 and TPX-II in CHO and SA7N cells

Total RNA isolated from As(III)-treated CHO (**A**) or SA7N (**B**) cells were subjected to RT-PCR analysis using Gal-1 (**A**) and TPX-II (**B**) primers respectively. PCR products were analysed on a 1.6% (w/v) agarose gel.

cysteine residue, not present in the other species' amino acid sequences that were examined in this study.

Binding of Gal I and TPX II with As(III)

Figure 7 illustrates the binding of Gal-1 and TPX-II with As(III) *in vivo*. After treatment of CHO and SA7 cells with various concentrations of As(III), the cellular Gal-1 or TPX-II proteins were immunoprecipitated with Gal-1 (Figure 7A) or TPX-II (Figure 7B) antibodies and Protein A–agarose respectively. The immunocomplexes of Protein A–Gal-1 or –TPX-II were subjected to arsenic content determination. The result shows that cellular levels of Gal-1- and/or TPX-II-bound arsenic increased concomitantly in a dose-dependent fashion, in relation to increased As(III) within the dose-range used in this study. In contrast, the Gal-1 or TPX-II in the control culture, which did not have antibodies added, bound no arsenic. Protein-bound arsenic was also not detected in the negative controls when T7-*Taq* monoclonal antibody or catechol 2,3-dioxygenase antiserum was used in place of Gal-1 or TPX-II antibody.

Figure 8 further reveals the binding of recombinant Gal-1 and TPX-II to As(III). The expression of recombinant Gal-1 and/or TPX-II in *E. coli* followed induction by IPTG in the presence of As(III). Elution of Gal-1 (Figure 8A) and TPX-II (Figure 8B) from a Ni-NTA affinity column resulted in the co-elution of As(III). Inference from these data would indicate that As(III) interacts with Gal-1 and TPX-II in the molar ratios 1:1.5 and 1:1 for As(III)/Gal-1 and As(III)/TPX-II respectively. No free or protein-bound arsenic was detected in the washing fraction. The bacterially expressed glycine RNA synthetase (with a 6× His tag) bound no arsenic, as revealed by Ni-NTA affinity chromatography (results not shown), indicating that As(III) bound specifically to Gal-1 and TPX-II.

Effect of Gal-1 and TPX-II on arsenic toxicity

To clarify further the role of Gal-1 and TPX-II in arsenic toxicity, we transfected 3T3 cells with vector control or cDNA encoding hamster Gal-1 and TPX-II protein. As shown in Figure 9(A), the transfection induced a significant increase in Gal-1 or TPX-II (3T3-Gal and 3T3-TPX) protein levels compared with sham-transfected cells (3T3-V) and parental cells (3T3). The results in Figure 9(B) indicate that 3T3-Gal and 3T3-TPX were more sensitive to As(III) treatment compared with 3T3-V. TPX-II functions as an antioxidant enzyme and is up-regulated in SA7 cells. Figure 9(C) shows that SA7 cells were indeed more resistant to H_2O_2 treatment, whereas 3T3-TPX still shows more vulnerability to H_2O_2 treatment.

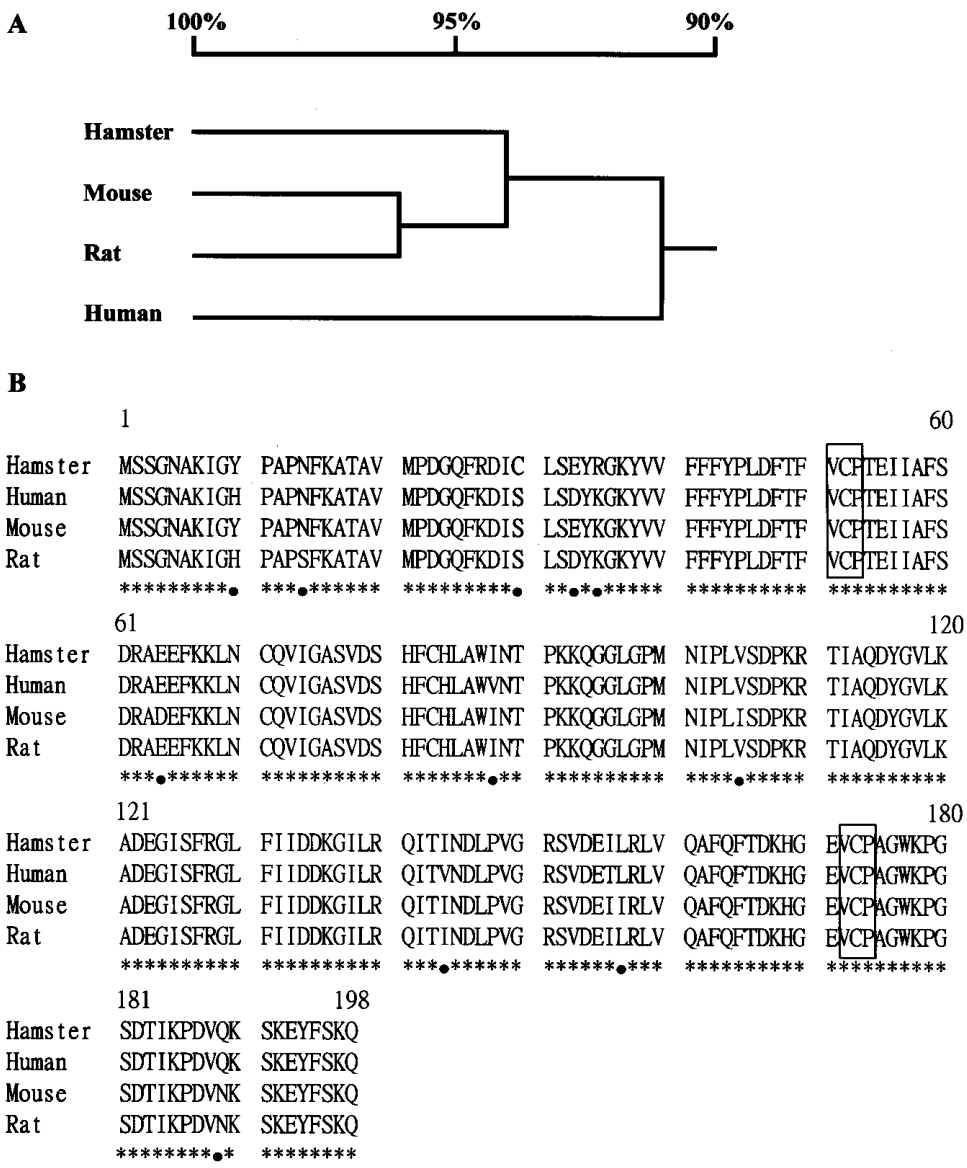

Figure 6 Phylogeny of TPX-II and its homology

(**A**) Cluster analysis of the homology of CHO, rat, mouse and human TPX-II genes. The tree was generated by the DNASTAR program using the nucleotide sequence of TPX-II. (**B**) Alignment of deduced amino acid sequences of CHO cell TPX-II with that of rat, mouse and human. Asterisks indicate the homologous regions between species and the conserved VCP sequence areas are boxed. Black spots indicate non-homologous sequence. These two conserved VCP regions contain essential Cys residues, which form an intermolecular disulphide bond. In addition, Cys[30] (bold) is unique to hamster TPX-II as compared with other species.

DISCUSSION

Arsenic is a unique human carcinogen without substantial evidence for carcinogenicitiy in animals. Arsenic toxicity has been proposed to result from its affinity for the thiol groups of proteins [6]. In fact, arsenic has been shown to bind to a variety of cellular proteins and other macromolecular constituents of tissues [7,8]. In this study, SDS/PAGE profiles indeed show numerous proteins retained by a PAO-agarose matrix with an arsine moiety (Figure 2). To identify the proteins modulated by As(III) treatment, the differentially expressed proteins in either CHO cells (14 kDa proteins) or SA7 cells (22 and 23 kDa proteins) were selected for further investigation. By partial amino acid sequencing, the 14 kDa protein down-regulated in SA7 cells [regularly cultured in 30 μM As(III)-containing medium] was identified as Gal-1, whereas the 22 and 23 kDa proteins up-regulated by As(III) in SA7 cells were identified as TPX-II and GST-P respectively. GST-P had previously been shown to overexpress and facilitate the efflux of As(III) in SA7 cells [11,13]. The association, however, of Gal-1 and TPX-II with As(III) has not been reported previously.

The binding specificity between Gal I, TPX-II and As(III) was further verified by the co-immunoprecipitation and co-elution of arsenic-bound Gal-1 and TPX-II (Figures 7 and 8). Although a number of studies have attempted to isolate arsenic-binding proteins, no specific proteins from mammalian tissues have been clearly identified and demonstrated to bind with As(III). Bogdan et al. [7] isolated three proteins with molecular masses of 100, 450

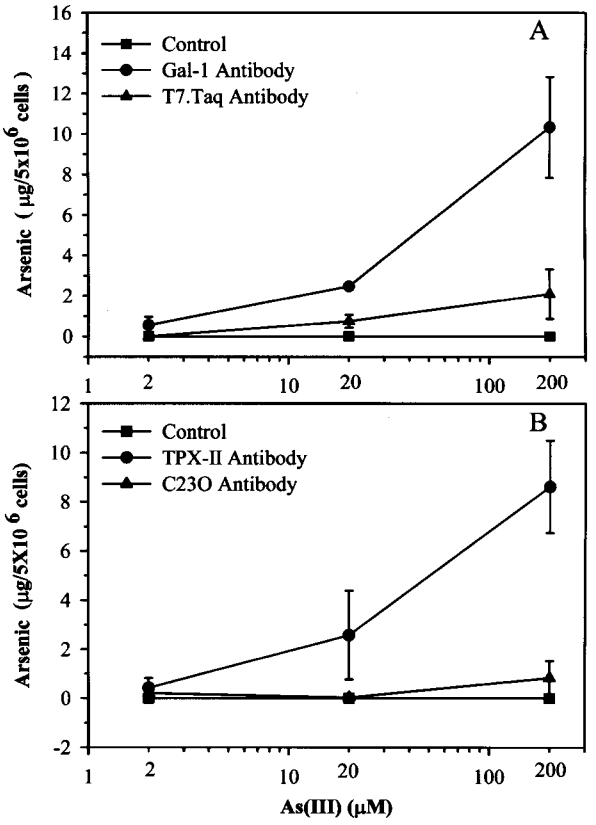

Figure 7 Binding of Gal-1 (A) and TPX-II (B) with As(III) in mammalian cells

CHO (**A**) and SA7 (**B**) cells were treated with varying concentrations of As(III) for 2 h and, thereafter, crude cell extracts were subjected to immunoprecipitation with Gal-1 (**A**) or TPX-II (**B**) antibodies as described in the Experimental section. The arsenic contents of the immunocomplex were then analysed with an atomic absorption spectrophotometer.

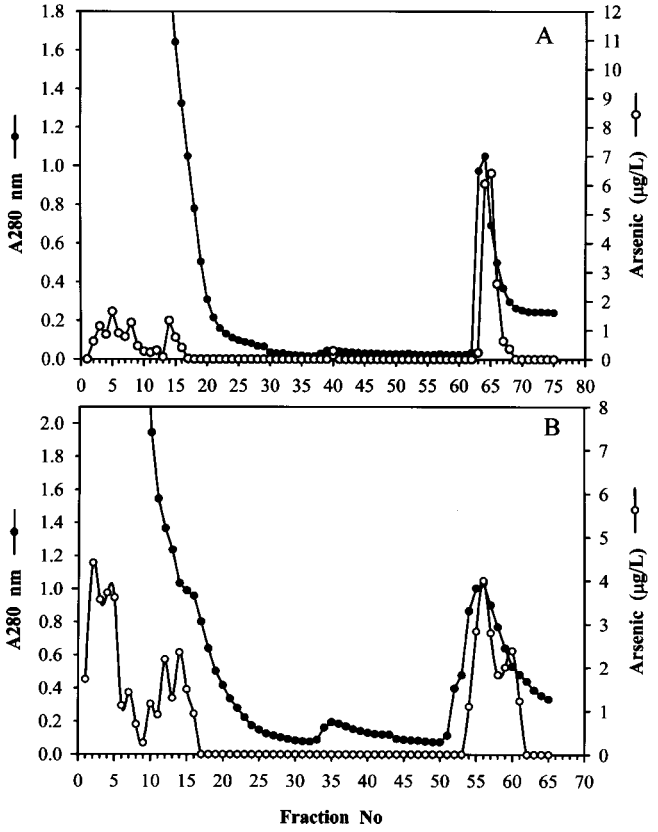

Figure 8 Binding of recombinant Gal-1 (A) and TPX-II (B) to As(III)

Gal-1- or TPX-II-transfected *E. coli* were incubated in the presence of IPTG and As(III) (100 μM) for 4 h. After centrifugation and removal of bacterial debris, the clear extract from *E. coli* was applied to an Ni-NTA affinity column. After extensive washing, the recombinant Gal-1 or TPX-II proteins were eluted with 150 mM imidazole. Protein fractions of 1 ml were collected and assayed for arsenic content as described in the Experimental section.

and >2000 kDa, which strongly bound radiolabelled As(III), but the protein species were not clearly identified. Recently, Menzel et al. [9] also employed arsenical-based affinity chromatography to identify 'tubulin' and 'actin' as two arsenic-binding proteins; however, this assumption was made solely on the basis of their molecular masses. Our present results show that only Gal-1- or TPX-II-bound arsenic dose-dependently increases with As(III) concentration administrated to cells (Figure 7). These results suggest that the interaction of Gal-1 and TPX-II with As(III) involves specific binding *in vivo*.

The affinity for thiol groups of proteins has long been suspected as the underlying toxic mechanism of As(III) [6]. Moreover, PAO-agarose affinity columns with an arsine oxide moiety were originally designed for separation of proteins containing vicinal dithiol groups [14]. Members of the peroxidase family of mammalian proteins can be divided into two subgroups: peroxidase I (1-Cys) and peroxidase II (2-Cys), the latter of which contains two conserved cysteines that correspond to Cys^{47} and Cys^{170} of yeast peroxidation protein. The Cys^{47}-SH group is the primary site of oxidation by H_2O_2 [25]. Typically, TPX exists in an oxidized form (either homodimers or heterodimers) through disulphide bonds formed between the intermolecular conserved cysteines. Dimers of TPX are reduced by thioredoxin to monomers. Monomeric TPX then scavenges oxidants and becomes dimeric again [26]. In contrast, although monomer Gal-1 contains six cysteines, it forms a non-covalent homodimer. Therefore, Gal-1 has six free thiol groups and is inactive in the absence of reducing agents [27].

It has been proposed that As(III) may interact with closely spaced cysteine residues on critical cellular proteins and the arsenic-binding motif of these proteins may involve three thiol groups arranged 3–6 Å (1 Å $\equiv$ 0.1 nm) apart by the tertiary structure of the proteins [28]. From crystallographic data [29,30], although Cys^{16} and Cys^{88} are separated by 72 amino acids along the Gal-1 primary sequence, they are brought together within 6.75 Å of each other in the folded conformation of the protein. Indeed, Inagaki et al. [31] have analysed the structure of Gal-1 under non-reducing conditions and found that oxidized Gal-1 forms intramolecular disulphide bonds between Cys^{16} and Cys^{88}. In contrast, the smallest distance (6.87 Å) between Cys in TPX-II exists between Cys^{82} on one monomer and Cys^{82} on the other monomer in the tertiary structure of dimeric TPX-II. These thiol groups could be potential candidates for As(III) binding. However, further work is needed to clarify this suspicion. Interestingly, TPX-II amino acid sequence from CHO cells contains unique Cys^{30} (Figure 6), which is usually a serine residue in other species' TPX-II [32]. The role of this cysteine residue as it relates to As(III) toxicity is presently unknown.

The results of the present study also suggest that overexpression of Gal-1 renders 3T3 cells more sensitive to As(III) treatment

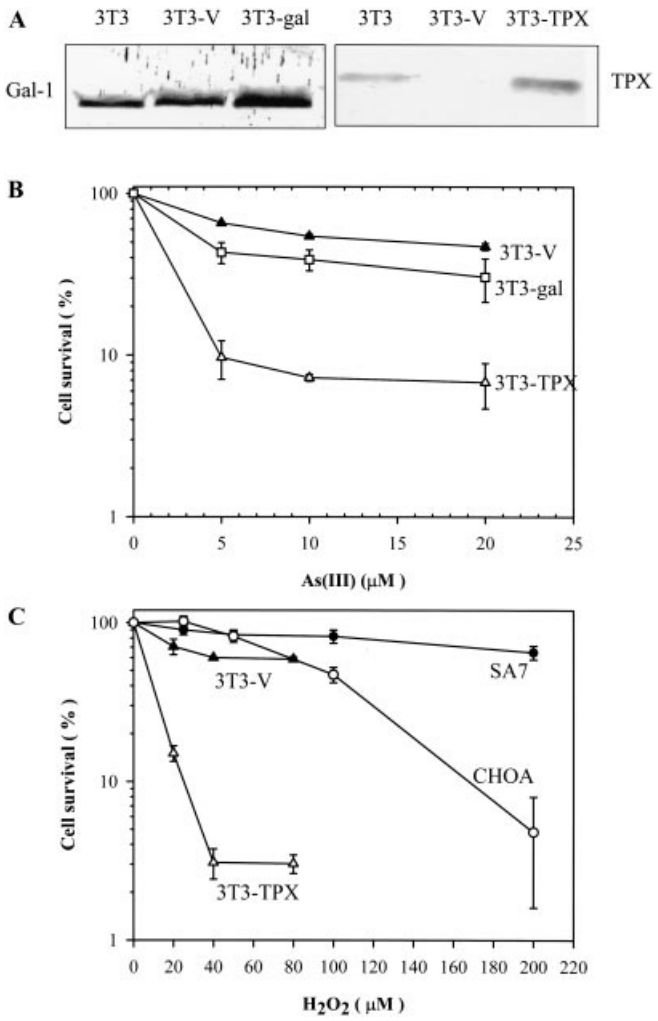

Figure 9 Effect of Gal-1 and TPX-II expression on cell growth

The transduced cells 3T3-Gal and 3T3-TPX, sham-transfected cells (3T3-V) and parental cell (3T3) were assessed for overexpression of Gal-1 or TPX-II proteins by using Western blotting analysis (**A**) or exposed to various doses of As(III) (**B**) or H_2O_2 (**C**) for 24 h and further incubated in fresh medium for another 24 h. At the end of treatment, the cells were subjected to survival assay by using SRB staining as described in the Experimental section.

(Figure 9B). These results indicate that Gal-1 acts as a negative mediator for cell growth in response to As(III) and may explain why Gal-1 is down-regulated in arsenic-resistant SA7 cells. TPX-II is a newly identified antioxidant protein that helps to protect cells against oxidative stress, which has recently been implicated in arsenic-mediated cellular injury [33,34]. To our surprise, the 3T3-TPX cells were also more sensitive to As(III) and H_2O_2 treatment (Figures 9B and 9C). A recent study has shown that TPX-II transduction decreases the GSH level in 3T3 cells and shows differing effects on cell viability [35]. Furthermore, in this study the authors conclude that TPX can either increase or decrease intracellular oxidative stress depending on the cell type or experimental conditions [35]. Our related study has shown that TPX-II overexpression in NB4 cells (acute promyelocytic leukaemia cells sensitive to arsenic trioxide) reduces the rate of H_2O_2 generation by 28 % ($P < 0.01$) relative to vector-infected control cells when exposed to arsenic trioxide (results not shown). The CHO cells are also indeed more susceptible to the deleterious effects of H_2O_2 than the arsenic-resistant SA7 cells in which TPX-II was up regulated (Figure 9C).

Galectins are a family of proteins that are involved in the regulation of cell adhesion and immune function as well as proliferation and apoptosis [36]. There are now at least 10 known galectins derived from animal cells. Although the most extensive studies have been performed on Gal-1 and Gal-3, the function of both galectins still remains largly unknown [37]. Gal-1 has been shown to function as a human cell growth inhibitor, while it is mitogenic at lower concentration [38]. In the study by Inagaki et al. [31], the oxidized Gal-1 contained three intramolecular disulphide bonds and exhibited distinct axonal regeneration activity, but lacked lectin activity. Furthermore, the activity of oxidized Gal-1 was observed at concentrations (pg/ml) substantially lower than those at which the lectin effects of Gal-1 are exhibited in neuronal cells *in vitro* (> ng/ml) [39]. As(III) binding to Gal-1 via thiol groups is likely to cause the conversion of Gal-1 into an oxidized form and disturb the normal function of Gal-1. The down-regulation of Gal-1 after exposure to As(III) might help cells to avoid the detrimental effect of oxidized Gal-1. However, further study is needed to resolve this question.

TPX is a member of a family of proteins initially identified from yeast and rat, and found to be important in protecting glutamate synthetase oxidation by a metal ion-catalysed reaction [40]. Oxidative stress has long been proposed to be involved in metal carcinogensis [41]. As(III)-induced oxidative damage has also been intensively studied in recent years [33,42]. To cope with potentially destructive oxidative stress induced by external stimuli, cells have evolved antioxidant defenses. The major cellular reducing agents (antioxidants) include glutathione, glutaredoxin and thioredoxin [43]. Thioredoxin provides reducing equivalents for numerous critical cellular enzymes, such as TPX. Recent studies suggest that arsenical treatment results in inhibition of thioredoxin reductase [44]. Inhibition of thioredoxin reductase could reduce the availability of thioredoxin needed to reduce oxidized TPX and may induce cells to synthesize *de novo* TPX, as shown in arsenic-resistant SA7 cells. The up-regulation of TPX-II may imply a potential physiological role for TPX-II in the protection of cells from death by oxidative damage elicited by As(III) treatment.

In vivo methylation has long been proposed as a detoxication pathway of inorganic arsenic [7]; however, it has been shown recently that MMA^{III}, an intermediate in inorganic arsenic methylation, is more toxic than inorganic arsenic [44,45]. We therefore examined the effect of trivalent methylated arsenic (MMA^{III} and DMA^{III}) on the expression of Gal-1 and TPX-II. The results show that both MMA^{III} and DMA^{III} are indeed more toxic to cells than As(III) (results not shown). However, DMA^{III} has no effect on the expression of Gal-1 and TPX-II as estimated by Western blotting; MMA^{III} also has no effect on the expression of Gal-1, but slightly enhances the expression of TPX-II at low dose (1 μM; results not shown). These results suggest that the more potent trivalent methylated arsenic species (MMA^{III} and DMA^{III}) may exert their toxic effect through a mechanism that is distinct from that of inorganic arsenics.

Gal-1 plays an important role in cell proliferation and apoptosis; TPX-II functions as an antioxidant enzyme. No association of Gal-1 and TPX-II with As(III) has previously been reported. Our present study suggests that As(III) treatment results in inhibition and up-regulation of Gal-1 and TPX-II, respectively, in CHO cells. Furthermore, both Gal-1 and TPX-II at least act as arsenic-binding proteins in these cells. The mechanism of As(III)-mediated expression of Gal-1 and TPX-II are the subject of current study in our laboratory.

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