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Construction of a subtractive library from hexavalent chromium treated winter flounder (*Pseudopleuronectes americanus*) reveals alterations in non-selenium glutathione peroxidases

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Abstract

Chromium is released during several industrial processes and has accumulated in some estuarine areas. Its effects on mammals have been widely studied, but relatively little information is available on its effects on fish. Gene expression changes are useful biomarkers that can provide information about toxicant exposure and effects, as well as the health of an organism and its ability to adapt to its surroundings. Therefore, we investigated the effects of Cr(VI) on gene expression in the sediment dwelling fish, winter flounder (*Pseudopleuronectes americanus*). Winter flounder ranging from 300 to 360 g were injected i.p. with Cr(VI) as chromium oxide at 25 μg/kg chromium in 0.15N KCl. Twenty-four hours following injections, winter flounder were euthanized with MS-222 and the livers were excised. Half of the livers were used to make cytosol and the other half were used to isolate mRNA for subtractive hybridization. Subtractive clones obtained were spotted onto nylon filters, which revealed several genes with potentially altered expression due to Cr(VI), including an α class GST, 1-Cys peroxiredoxin (a non-selenium glutathione peroxidase), a P-450 2X subfamily member, two elongation factors (EF-1 gamma and EF-2), and complement component C3. Semi-quantitative RT-PCR was performed and confirmed that Cr(VI) down-regulated complement component C3, an EST, and two potential glutathione peroxidases, GSTA3 and 1-Cys peroxiredoxin. In addition, cytosolic GSH peroxidase activity was reduced, and silver stained SDS-PAGE gels from glutathione-affinity purified cytosol demonstrated that a 27.1 kDa GSH-binding protein was down-regulated greater than 50%. Taken together, Cr(VI) significantly altered the expression of several genes including two potential glutathione peroxidases in winter flounder.

Keywords: Flounder; GST; Gene expression; Chromium; Glutathione peroxidase; Complement component C3

1. Introduction

Chromium is found primarily as Cr(III), an essential trace metal, or Cr(VI), the more toxic form (Devi et al., 2001). Chromium is a major toxicant found at several estuarine Superfund sites around the US, including Shipyard Creek in Charleston, SC; Portsmouth Naval Shipyard, Kittery, ME; Pacific Sound Resources in Eliott Bay; and Puget Sound in Seattle, WA. At these sites, estuarine and marine organisms may be exposed to and accumulate chromium. Sediment dwelling or-

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ganisms are at higher risk because they are in direct contact with contaminated sediments and ingest other contaminated sediment dwelling organisms. Additionally, most metals, including chromium, tend to accumulate in the liver (Meador et al., 1995), especially if exposure is through an oral route (Witmer et al., 1989; Wilbur and Voytek, 1988). Winter flounder (*Pseudopleuronectes americanus*) are an excellent sentinel organism to study the effects of chromium on gene expression since they are associated with sediments, eat other sediment dwelling organisms and are in turn consumed by humans.

Chromium's toxicity is at least partially derived from its reduction from Cr(VI) to Cr(III) in the cell, which produces radicals (Wilbur and Voytek, 1988), and possibly depletes glutathione (GSH) (Hojo and Satomi, 1991). During Fenton type reactions, chromium ions are oxidized and reactive oxygen species (ROS) are generated that interact directly with macromolecules. Mao et al. (1995) have shown that reduction of Cr(VI), in the presence of ascorbate, reacted with *t*-butyl hydroperoxide in vitro and produced free radicals. Oxidative stress was induced by hexavalent chromium in the freshwater field crab (*Barytelphusa guerini*), as measured by increased lipid peroxidation, xanthine oxidase activity, and superoxide dismutase activity, indicating superoxide radical formation (Sridevi et al., 1998). Furthermore, increased production of malondialdehyde, an indicator of lipid peroxidation, was found in chromium treated mice (Rungby and Ernst, 1991). Electron spin resonance has demonstrated that the radical scavenger, ac-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone did not combine with the hydroxyl radical, but with fatty acid-derived radicals generated via lipid peroxidation in liver tissues following chromium treatment (Kadiiska et al., 1994). Taken together, this indicates that Cr(VI) can cause the formation of lipid hydroperoxides in vivo.

Organisms have a number of protective mechanisms against ROS including GSH, Vitamin C, superoxide dismutase (SOD), catalase, glutathione *S*-transferases (GSTs) and the glutathione peroxidases. These enzymes in combination catalyze the degradation of ROS to oxygen and water. Induction of antioxidant enzymes is intended to increase the detoxification of ROS. For example, a θ class GST gene (GSTA) in plaice (*Pleuronectes platessa*) has several antioxidant response elements (AREs) in its promoter region (Leaver et al., 1997). In addition, mouse and rat GST α class subunit genes and a rat GST π class gene are regulated by c-Fos and c-Jun, which in turn control GST basal activity and stimulate activity when animals are exposed to xenobiotics (Daniel, 1993).

Cr(VI) has also been shown to activate nuclear transcription factor kappa-B (NF-κB) (Chen et al., 1997; Ye et al., 1995; Shi et al., 1999), which can in turn induce transcription. However, Cr(VI) and its reduced product Cr(III) have also been shown to inhibit the binding of NF-κB to its promoter in vitro (Shumilla et al., 1998), probably through different mechanisms. Inhibition of NF-κB hinders GST π mediated protection of cells from ROS induced death (Yin et al., 2000). NF-κB controls the expression of a number of antioxidant enzymes such as Mn SOD (Das et al., 1995), GSTP1-1 (Xia et al., 1996), and xanthine oxidase (Xu et al., 1996), as well as several other genes including complement component C3 (Moon et al., 1999), and α1-antitrypsin (Ray et al., 1995) in mammals.

Alterations in gene expression are useful tools in that they estimate the potential cellular and physiological effects of a toxicant. Since there are relatively few sequenced and defined genes in flounder or other estuarine species of commercial and recreational significance, we performed suppressive subtractive hybridization to determine altered gene expression in winter flounder liver following Cr(VI)-treatment. This information can be used to produce useful and new biomarkers for Cr(VI) or metal exposure in estuarine organisms.

2. Materials and methods

2.1. Treatment of winter flounder with Cr(VI)

All studies were carried out according to NIH guidelines for humane use of research animals and were pre-approved by the Mount Desert Island Biological Laboratory (MDIBL) Animal Care and Use Committee. Male winter flounder weighing between 300 and 360 g were captured in the Gulf of Maine and transported to MDIBL in Salsbury Cove, ME. Flounder were housed in 2348 gal tanks for 1 week until being transferred to individual 10 gal tanks and acclimated for 24 h prior to treatment. Three control flounder were

injected intraperitoneally (i.p.) with 100 μl 0.15N KCl and three flounder were injected with 25 μg/kg of Cr(VI) as chromium trioxide (Sigma, St. Louis, MO) dissolved in 0.15N KCl. Twenty-four hours following injection, the winter flounder were euthanized with MS-222 (Sigma, St. Louis, MO) and livers were excised.

2.2. Sample preparation and affinity chromatography

Excised livers were cut into slices with sterile scissors and approximately half of each liver was placed in TriReagent (Sigma, St. Louis, MO). Livers were frozen at −80 °C until RNA was extracted according to previously published protocols (Chomczynski and Sacchi, 1987). RNA was treated with DNAse I (Clontech, Palo Alto, CA). The other half of the liver was used to produce cytosol and microsomes according to previously published methods (Vander Hoeven and Coon, 1974), and protein concentrations were determined according to Bradford (1976). Glutathione-binding proteins were purified by glutathione-sepharose affinity chromatography from approximately 4.5 mg (300–350 μl) of pooled cytosolic protein (Baldwin and LeBlanc, 1996) with modifications. Specifically, glutathione-sepharose columns were obtained from Sigma (St. Louis, MO), equilibrated and washed after addition of pooled cytosol from control or treated fish with 10 mM potassium phosphate (KPi) buffer, pH 7.4, prior to elution with 10 mM KPi buffer containing 7.0 mM glutathione. Protein concentrations of GSH-binding proteins were determined spectrophotometrically at 260 nm.

2.3. Production of subtractive clones for macroarray preparation and hybridization

Isolation of mRNA from the liver RNA was performed using the Qiagen Oligotex Poly A RNA Purification Kit™ (Qiagen, Valencia, CA) and resulting mRNA from the treatment groups were pooled. Differentially expressed sequences between the control and Cr(VI) treated groups were isolated using the Clontech PCR-Select™ cDNA Subtraction Kit (Palo Alto, CA). Subtractive hybridization was performed in two directions in order to produce clones both induced and down-regulated by chromium. At the end of the procedure, subtracted sequences were inserted into plasmid pCR® 2.1 and transfected into competent INVαF *E. coli* (Invitrogen, Carlsbad, CA). Bacterial clones were isolated and plasmids were extracted from the bacteria using the QiaPrep® Spin MiniPrep Kit (Qiagen). Genes isolated during this procedure were amplified with primers made to linkers added during the subtractive hybridization procedure (forward: 5′-TCGAGCGGCCGCCCGGGCAGGT-3′, reverse: 5′AGCGTGGTCGCGGCCGAGGT-3′). Equal amounts of PCR product and 0.6N NaOH were mixed and spotted on nitrocellulose membranes to make 96-well arrays. Blots were then neutralized for 2–4 min in 0.5 M Tris–HCl, pH 7.5, washed with water and then UV cross-linked (SpectroLinker XL-1000, Spectronics Corp., Wesbury, NY).

Probes were prepared from either forward and reverse subtracted products from the subtractive hybridization procedure, or from individual flounder (Jin et al., 1997). Briefly, 100 ng dsDNA was denatured for 8 min at 95 °C, and then 5 μl [^{33}P]dATP (Perkin-Elmer Life Sciences Inc., Boston, MA), 3 μl reaction buffer, 2 μl random primer mix, and 1 μl Klenow enzyme (Clontech Laboratories, Palo Alto, CA) was added and incubated for 30 min at 37 °C. At the end of the incubation, 5 μl of stop solution was added and the probe was purified using the ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were prehybridized for 1 h in 5 ml of ExpressHyb (Clontech Laboratories) in the presence of 0.5 mg of sheared salmon sperm DNA and then hybridized overnight at 72 °C in the presence of the probes. Blots were washed four times at 68 °C with a low stringency wash (2× SSC, 0.5% SDS) and twice with a high stringency wash (0.2× SSC, 0.5% SDS) and then exposed to X-ray film for 5–7 days with an intensifying screen at −80 °C. Genes that showed differential expression following the subtractive arrays were selected and sequenced at the University of Maine DNA Sequencing Facility (Orono, ME). Genes expressed from arrays performed with individual control and Cr(VI)-treated fish RNA were quantified on an EPI-Chemi Darkroom with LabWorks™ analysis software (UVP Laboratory Products, Upland, CA). Statistical analysis was performed using Mann–Whitney *U*-test on StatView® software (SAS Institute Inc., Cary, NC). A *P*-value of ≤ 0.05 was regarded as significant.

2.4. RT-PCR confirmation of differential expression

RT-PCR was used to confirm differential gene expression. Reverse transcription was performed to create cDNA using 200 units Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT) (Promega Corporation, Madison, WI), a 10 mM dNTP mixture, and 0.05 μg random hexamers. Gene specific primers were made and RT-PCR was performed with primers as indicated below: 1-Cys peroxiredoxin (1-Cys PRX) (non-selenium glutathione peroxidase) forward (5′-GGATGGATTGCCCCTCACC-3′) and reverse primers (5′-CACCGTTGGGGAAGAGAGC-3′) with an annealing temperature of 66 °C; GSTA3 forward (5′-GATCTCCGTCACTGAGGAG-3′) and reverse (5′-GGCTGGAAAAGTTGTGCTGAG-3′) annealing temperature of 58 °C; GSTA4 forward (5′-GGTACCAGATGTTCAACAG-3′) and reverse (5′-CAAGAGGAAGTCGCAGCC-3′) annealing temperature of 51 °C; complement component C3 forward (5′-GTGGGCGTCTTACAGCCAGC-3′) and reverse (5′-CCTACAGTGTGGATAGCTC-3′) annealing temperature of 52 °C; elongation factor 2 (EF-2) forward (5′-CGCCGTCGCTGGGACACCC-3′) and reverse (5′-GCAGGAATTAGGCATGAGCC-3′) annealing temperature of 63 °C; 3-29 forward (5′-GTATG-CAGCCCACGGTG-3′) and reverse (5′-CGCAGTGT-TTATCACATCC-3′) annealing temperature of 57 °C; 3-32 forward (5′-CCCCTAATCTATATCAATAC-3′) and reverse (5′-GCAAACTGAGACAAAGTG-3′) annealing temperature of 47 °C; P-450 2X forward (5′-TAGGCCATGAGGTAGAGGAAAG-3′) and reverse (5′-GAGGAGCACAAGAAAACCAGAG-3′) annealing temperature of 52 °C; elongation factor-1 (EF-1) gamma forward (5′-CTCCAACGAGGACAC-ACTG-3′) and reverse (5′-CACCATGGTCTTGCAC-TCC-3′) annealing temperature of 60 °C; and β-actin forward (5′-GCACAGCTGGAGCAGAGG-3′) and reverse (5′-GCAACACGGAGCTCGTTG-3′) annealing temperature 63 °C. All reactions were cycled at 95 °C for 30 s, the appropriate annealing temperature for 30 s, and 72 °C for 45 s. 1-Cys PRX and GSTA3 were amplified for 39 cycles. Complement component C3 was amplified for 25 cycles, EF-2 and GSTA4, 35 cycles; EF-1, 25 cycles; β-actin, 32 cycles; 3-29, 40 cycles; 3-32, 27 cycles; and P-450 2X for 40 cycles. The PCR products were separated in a 1.6% agarose gel, stained with ethidium bromide and viewed, analyzed and quantified densitometrically using the EPI-Chemi Darkroom and LabWorks™ Analysis Software (UVP, Upland, CA). Densitometric units for each gene were divided by the housekeeping gene, β-actin. Statistical analysis was performed using Mann–Whitney *U*-test on StatView® software. A *P*-value of ≤ 0.05 was regarded as significant.

2.5. Electrophoresis and silver staining

Proteins purified via GSH-sepharose chromatography were separated (50 μg) by polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gels as described elsewhere (Laemmli, 1970). Proteins were visualized by silver staining (Bio-Rad, Hercules, CA). Initial silver stain development took approximately 5 min, and individual proteins were quantified every 20 s following initial development from the silver stained gels using the LabWorks™ program and the EPI-Chemi Darkroom. Molecular weights of the GST subunits were determined by co-electrophoresis with molecular weight standards (Bio-Rad, Hercules, CA).

Two-dimensional electrophoresis was also performed on affinity purified GSTs according to previously published protocols (O'Farrell, 1975). Isoelectric focusing was carried out in a glass tube of inner diameter 2.0 mm using 2.0% pH 3.5–10 ampholines (Amersham Pharmacia Biotech, Piscataway, NJ) for 9600 Vh. The enclosed tube gel pH gradient plot for this set of ampholines was determined with a surface pH electrode. A train of 20 carbamylated creatine phosphokinase (CPK) isoelectric point (p*I*) markers was added to the sample. The isoelectric points of each of the charge forms for conditions of 9 M urea at room temperature have been determined and vary from 5.75 to 7.54. After equilibration for 10 min in buffer (10% glycerol, 2.3% SDS and 0.0625 M Tris, pH 6.8), the tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (0.75 mm thick), and SDS gel electrophoresis was carried out. Molecular weight standards were added to a well in the agarose that sealed the tube gel to the slab gel. Proteins were visualized by silver staining.

2.6. GSH levels and enzyme assays

GSH levels were measured according to previously published protocols (Anderson, 1985). GST

activity was measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid, 3,4-dichloronitrobenzene (DCNB), and cumene hydroperoxide (Sigma Chemical Co., St. Louis, MO) according to Habig and Jakoby (1981). All GST enzyme assays were conducted at 25 °C and spectrophotometric changes measured every 15 s for 5 min. Unpaired *t*-tests were performed with StatView® software. A *P*-value of ≤ 0.05 was regarded as significant.

3. Results

3.1. Subtractive hybridization and subtractive arrays

Subtractive hybridization was used to determine changes in gene expression in flounder liver following treatment with hexavalent chromium, from which two subtractive libraries were developed. One library contained transcripts after subtracting Cr(VI)-treated flounder liver RNA from control liver RNA (down-regulated by Cr(VI), and the other library contained transcripts after subtracting control liver RNA from Cr(VI)-treated flounder liver RNA (genes up-regulated by Cr(VI). One-hundred and ninety-two clones were dotted onto two distinct sets of 96-well membranes. Differential expression was determined using probes from the reverse and forward subtracted products developed from the subtractive hybridization procedure. These subtractive arrays are not quantitative since probes are obtained from the subtracted products and not individual fish. However, qualitative differences can be determined from these arrays. Genes that appeared differentially expressed in the subtracted array were sequenced and subjected to a BLAST search to determine identity (Table 1). A total of 29 genes were found to be differentially expressed, including an α class GST (GSTA3), 1-Cys peroxiredoxin, EF-1 gamma, EF-2, a carboxypeptidase and a P450 2X family member.

Most translation and nucleotide searches were in agreement as to the putative identity of the clones. However, nucleotide searches did not recognize a potential match for carboxypeptidase or mitochondrial elongation factor G1 (EF-G1). Furthermore, both EF-G1 and α hemoglobin β were submitted as ESTs to GenBank because there were significant discrepancies in alignment between the sequenced flounder clones and other known sequences, such as stop codons within the sequence, indicating that these genes are potential pseudogenes (Moos and Gallwitz, 1982).

One of the genes sequenced, a putative GSTA3, provided BLAST matches similar to several genes in the α class. Therefore, protein alignments were done to evaluate this gene's subclass (Fig. 1, Table 2). Fig. 1 reveals that the flounder GST is most identical and similar to the chicken's GSTCL3, which is an α class GST. It is also very similar to the mouse GSTA3, which suggests that it is most likely a GSTA3 subclass member. Blast scores also reveal that rat GSTA5 shows high similarity to the flounder GSTA3, and rat

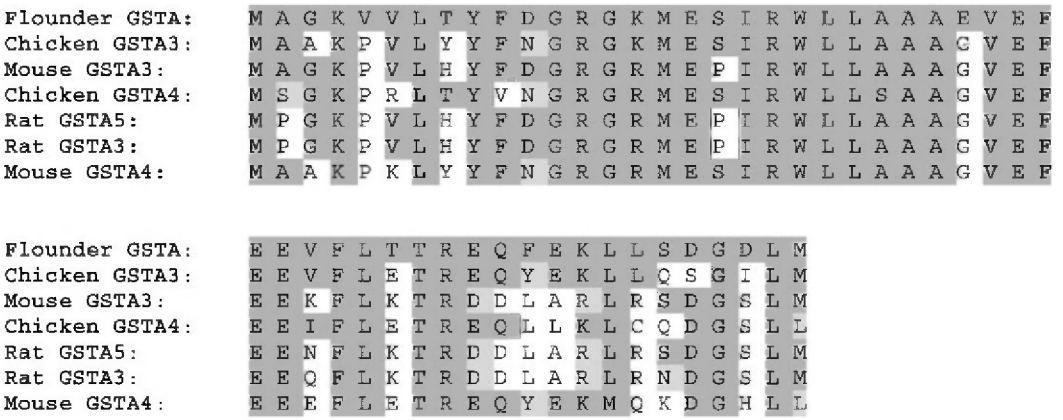

Fig. 1. Protein translation and clustal alignment of GST α genes from various species compared to winter flounder. Dark gray indicates identity and light gray indicates similarity to the winter flounder sequence within the chicken, rat and mouse GST α class genes.

Table 1
Differential expression of genes on the subtractive arrays

Sample	Gene similarity	*E*-value (*n*)[a]	*E*-value (*P*)[b]	Accession no.
3-1	Carboxypeptidase B	NS[c]	9.00E−06	AY225096
3-8	EST			CB074834
3-11	EST			CB74827
3-13	Glutathione *S*-transferase A3	2.00E−16	2.00E−14	AY156727
3-14	1-Cys peroxiredoxin	2.00E−10	4.00E−30	AY156726
3-17	Glucose 6 phosphate dehydrogenase	7.00E−13	5.00E−40	AY225097
3-22	EST			CB074828
3-23	EST			CB074843
3-25	EST (EF-G1)	NS	2.00E−22	CB074836
3-26	EST			CB074829
3-29	EST			CB074832
3-30	EST			CB074830
3-32	EST			CB074825
3-33	EST			CB074831
3-34	EST			CB074840
3-36	Elongation factor-2	3.00E−52	2.00E−35	AY225098
3-39	EST			CB074841
3-42	Cytochrome P450 2X	NS	1.80E−34[d]	AY225104
3-43	Serine proteinase inhibitor	2.00E−06	2.00E−10	CB074846
3-46	C-type lectin domain	4.00E−10	2.00E−40	AY225103
3-58	Complement component C3	0.00E+00	E−123	AY225099
3-60	EST			CB074833
3-79	EST			CB074837
4-9	EST			CB074383
4-12	EST (α hemoglobin B)	2.00E−11	8.00E−26	CB074845
4-29	EST			CB074842
4-41	EST			CB074835
4-50	Elongation factor-1 gamma	1.00E−35	3.00E−52	AY225101
4-55	EST			CB074844

Above are the genes that were found differentially expressed on the subtractive arrays and their GenBank accession no.

[a] *E*-values based on nucleotide–nucleotide blast on GenBank (blastn).

[b] *E*-values based on nucleotide–protein blast (tblastx) on GenBank.

[c] *E*-value of no significant value on GenBank.

[d] *E*-value calculations based on the blastx program to Fugu set up as an experimental service on the P-450 database (http://132.192.64.52/p450.html) by Rob Edwards and David Nelson.

GSTA5 is orthologous to mouse GSTA3 (Eaton and Bammler, 1999). However, chicken GSTCL4 shows greater similarity to flounder GSTA3 than rat GSTA5, and both rat GSTA3 and mouse GSTA4 show high similarity to the flounder GSTA3. Thus flounder GSTA3 is not definitively a GSTA3. At this time we have designated this gene as an α class GST subfamily member that is most likely a GSTA3. Recently an EST from winter flounder has been putatively identified as an α class GST with high homology to GSTA4 (GenBank accession no. CF195645). Therefore, to avoid discrepancies we will call clone 3-13, GSTA3.

3.2. Confirmation of gene expression changes

Hybridization to the arrays were performed using RNA from individual fish. These are not as sensitive as the subtractive arrays and therefore several genes with low expression levels that were differentially expressed in the earlier hybridizations (Table 1) did not appear on this array. However, these arrays are quantitative (Chan et al., 1997). The genes that were statistically significantly down-regulated include ESTs 3-26, 3-29, 3-30, 3-32 and 3-33 (Table 3).

Semi-quantitative RT-PCR was also performed to confirm differential expression of select genes iso-

Table 2
Amino acid similarity and identity of winter flounder GSTA translations to other subfamily members in different species

Protein	Similarity (%)	Identity (%)	*E*-value[a]	Accession no.
Chicken GSTCL3	83	80	2.0E−14	M38219
Mouse GSTA3	77	72	4.0E−13	M73483
Chicken GSTCL4	79	68	1.0E−12	AF133251
Rat GSTA5	75	70	2.0E−12	X78847
Rat GSTA3	75	68	2.0E−12	X78848
Mouse GSTA4	79	70	3.0E−12	NM_010357

[a] *E*-value calculations based on the tblastx program in GenBank.

lated during subtractive hybridization, as well as GSTA4 (Fig. 2). GSTA4 was examined because it is in the same GST family as GSTA3 and was recently cloned in subtractive hybridization experiments with pollution exposed winter flounder. The graphs in Fig. 2 indicate that 3-29, complement component C3, 1-Cys PRX (a non-selenium GSH peroxidase), and GSTA3 were all significantly down-regulated. Complement component C3 is an acute phase protein (Moon et al., 1999), and both GSTA3 and 1-Cys PRX are potential glutathione peroxidases (Fisher et al., 1999; Martinez-Lara et al., 2002; Yang et al., 2002). EF-1 was the only up-regulated gene, but this observation was not significant because of a fish that did not respond to Cr(VI)-treatments (Fig. 2). Similarly, RT-PCR indicated that 3-32 was not down-regulated, while individual arrays had indicated that 3-32 was significantly down-regulated (Table 3). Both confirmatory techniques indicate that individual fish demonstrate high variability in the expression of this gene (Fig. 2, Table 3).

Table 3
Differential expression of genes of individual chromium-treated winter flounder following array analysis

Sample	Control ± S.E.	Cr(VI) ± S.E.
3-26	100 ± 40.0	32.7 ± 3.1
3-29	100 ± 29.4	29.0 ± 23.1
3-30	100 ± 30.5	28.1 ± 5.6
3-32	100 ± 61.1	14.1 ± 9.8
3-33	100 ± 44.7	13.8 ± 3.6

Probes were made from individual flounder liver RNA samples and hybridized to the subtraction arrays. Arrays were analyzed with LabWorks™ densitometry to determine differential expression. Data are shown as percentage of control ± S.E. ($n = 3$) for genes with P-values <0.05. Data were analyzed statistically using Mann–Whitney U-test.

3.3. Silver staining and densitometry of GSH-affinity purified proteins

We were interested in examining GSH-binding proteins and GSH peroxidase activity further because two of the genes demonstrating differential expression are potential glutathione peroxidases (GSTA and 1-Cys PRX). To determine if differential expression of RNA translated to differential expression of protein, cytosols were run through a GSH-affinity column to purify GSH-binding proteins, including GSTs. Silver stained SDS-PAGE gels demonstrated that several GSH-binding proteins were captured by the column. Two of these proteins at 27.1 and 25.2 kDa were highly expressed (Fig. 3) and have similar molecular weights as GST subunits from other teleost species (Pham et al., 2002; George et al., 1989). The molecular weight of 1-Cys PRX in fish is unknown, but is 24.9 kDa in mouse (GenBank O08709). Comparison between purified pooled samples from control and chromium-treated cytosols by densitometry indicated that the protein at 27.1 kDa had been decreased in the chromium-treated samples (Fig. 3A and B). In contrast, another GSH-affinity purified protein band at 25.2 kDa was not altered by chromium treatment (Fig. 3A and C).

Two-dimensional gel electrophoresis was performed with affinity purified cytosol from control flounder liver samples to confirm the size of the GSTs, and determine the pI of the down-regulated band of GSTs at 27.1 kDa. The two-dimensional silver stained gel indicates the presence of several GSTs with two distinct MW bands of approximately 27.1 and 25.2 kDa. The bulk of GSTs at 25.2 kDa had pIs between 5.4 and 7.1 (Fig. 4). The GSTs with a MW of 27.1 had pIs that varied between

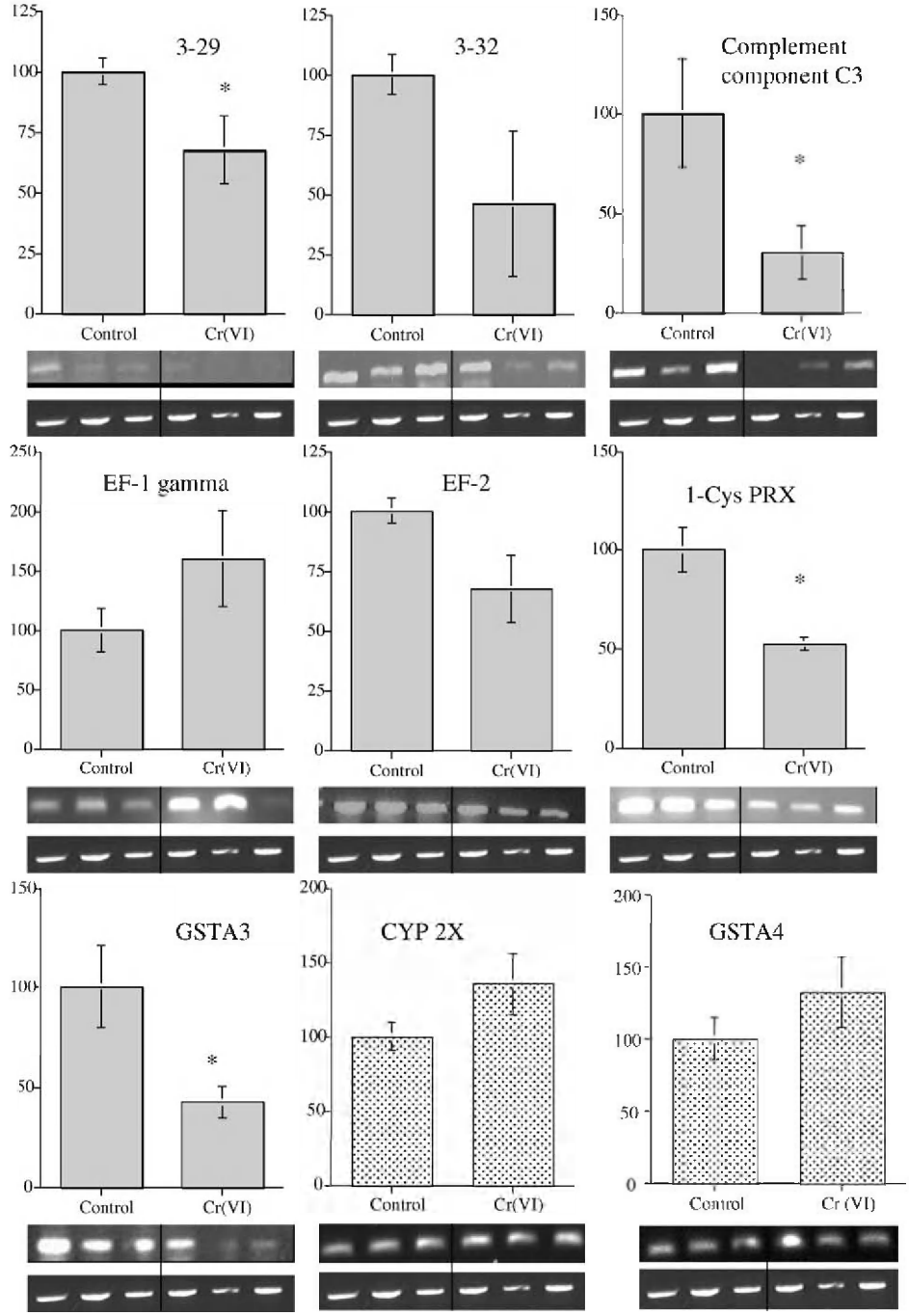

Fig. 2. Confirmation of differential expression by semi-quantitative RT-PCR. RT-PCR was performed as described in Section 2, and bands quantified using LabWorks™. Densitometry was taken of the bands on the gel using LabWorks™ analysis software. Ethidium bromide stained RT-PCR gels are shown below the graphs, with the first three bands being controls and the second three Cr(VI)-treated flounder. The first row of bands is the gene in question and the second row is a PCR of β-actin, our housekeeping gene. Data is expressed as mean relative density ± S.E. ($n = 3$) relative to β-actin expression. An asterisk indicates statistical significance at $P < 0.05$ by Mann–Whitney U-test.

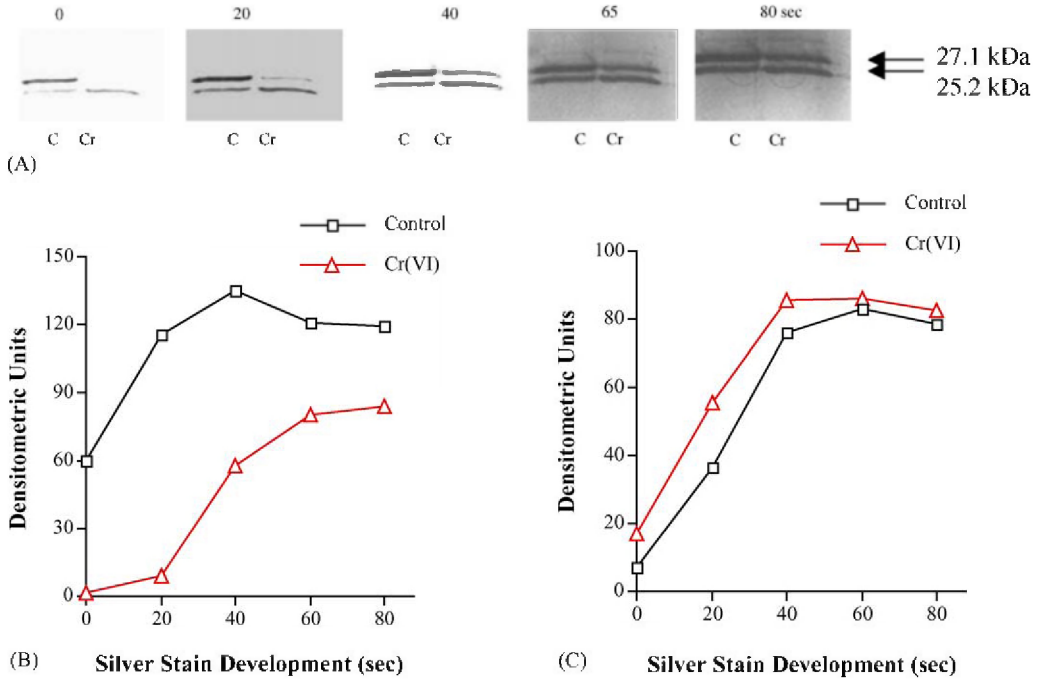

Fig. 3. Quantitation of GSH-binding proteins from control and Cr(VI)-treated winter flounder. Cytosolic proteins were partially purified through a GSH affinity column, separated by SDS-PAGE, and visualized via silver staining (A). Initial silver stain development took approximately 5 min, and individual proteins were quantified every 20 s following initial development as described in Section 2. Molecular weights of the GST subunits were determined by co-electrophoresis with molecular weight standards (Bio-Rad, Hercules, CA). Graphical representation of quantified GSH-binding proteins at (B) 27.1 kDa and (C) 25.2 kDa are below. Squares represent controls and triangles represent Cr(VI)-treated proteins.

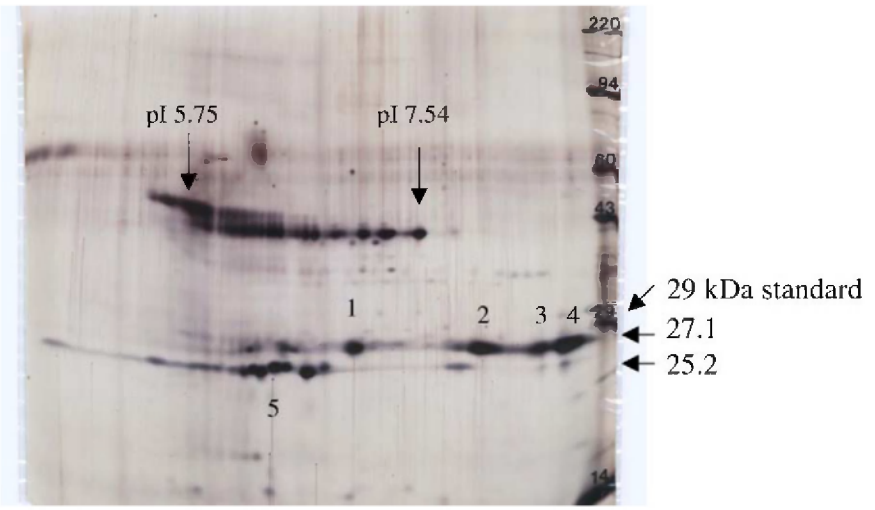

Fig. 4. Two-dimensional gel electrophoresis of GSH-affinity purified proteins. GSH-binding proteins were separated by two-dimensional gel electrophoresis and silver stained. Proteins at 27.1 and 25.2 kDa separated into several distinct bands. The 27.1 kDa band produced primarily basic GSH-binding proteins (bands 2–4) with the exception of the neutral protein (band 1). The 25.2 kDa proteins were primarily neutral to slightly acidic (band 5).

7.26 and 9.1 kDa (Fig. 4). Bands 2–4 all had p*I*s greater than 8. It is possible that the bands at MW 27.1 kDa represent distinct α class GSTs. GSTA3 and GSTA4 from a number of species, as well as plaice θ class GSTs, are basic proteins. The theoretical p*I* of GSTA3 proteins from chicken, mouse, rat and human range from 8.42 to 9.06 (GenBank accession nos. are provided in Table 2), while θ class GSTs from plaice (X63761, X95200) are slightly less basic (p*I* 8.29) based on the ProtParam program from Expasy (http://www.us.expasy.org). Therefore, many of the silver stained proteins down-regulated at 27.1 kDa have p*I*s consistent with α class GSTs.

3.4. Total GSH, GSH peroxidase and GST activities in Cr(VI)-injected flounder

Total GSH levels were determined following Cr(VI)-treatment as a potential indicator of oxidative stress. GSH levels increased 44% in livers of Cr(VI)-treated flounder (Table 4). However, this increase was not significant. In addition, several different GST assays were performed to determine if there were substantial reductions in GST and GSH peroxidase activity. GST activity using CDNB as the substrate was reduced 19%, but not in a significant manner (Table 4). CDNB is a substrate often used as an indicator of overall GST activity. GST specific activity measured using ethacrynic acid and DCNB as substrates were low compared to cumene hydroperoxide and CDNB, and were unaffected by Cr(VI)-treatment in flounder liver (data not shown).

GSH-dependent peroxidation is performed primarily by α class GSTs, and both selenium and non-selenium glutathione peroxidases in mammals (Yang et al., 2002). θ Class GSTs also perform GSH-dependent lipid peroxidation in plaice (Martinez-Lara et al., 2002). GSH-dependent peroxidation using cumene hydroperoxide as the substrate, was decreased modestly (18%) but significantly (Table 4). Additionally, GSH-dependent peroxidation and CDNB conjugation was measured in the GSH-affinity purified proteins and both activities were decreased in a similar fashion to the individual samples, with cumene hydroperoxide activity decreasing 20% and CDNB conjugation decreasing 12% (data not shown). This work suggests that down-regulation of 1-Cys PRX and GSTA3 decreased overall GSH-dependent peroxidation in liver of Cr(VI)-treated flounder.

Table 4
Modulation of GST activity in winter flounder liver following Cr(VI)-treatment

Enzyme assay	Control	Cr(VI)-treated
CDNB[a]	323 ± 72	259 ± 77
Cumene hydroperoxide[a]	46.7 ± 0.9	38.3 ± 3.1*
Glutathione (GSH)[b]	10.9 ± 1.3	15.7 ± 3.4

All data are presented as mean ± S.E. ($n = 2$–3).

* Indicates a statistical difference from control using Student's *t*-test ($P < 0.05$).

[a] GST activity measurements are presented as nmol/min mg protein.

[b] GSH levels are presented as nmol GSH/μg protein.

4. Discussion

Hexavalent chromium treatment altered the expression of several genes in winter flounder liver, including some ESTs (Table 1, Fig. 2). Similar gene expression profiles were found in male European flounder (*Platichthys flesus*) collected from polluted sites with high concentrations of metals. Four genes were down-regulated in European flounder including EF-2, EF-1, and complement component C3 (Williams et al., 2003).

Many of the genes altered in winter flounder are altered in other species by ROS or involved in the reduction of oxygen radicals, including GSTA3, 1-Cys PRX, complement component C3 and some elongation factors (Collard et al., 1998; Bols et al., 2001; Daniel, 1993; Chen et al., 2000; Patel et al., 2002; Fisher et al., 1999). The generation of ROS can alter gene expression by activating transcription factors that bind the antioxidant response element, or activate NF-κB transcriptional activity, and Cr(VI) has been shown to increase NF-κB and AP-1 activity in cells (Kaltreider et al., 1999). 1-Cys PRX 5′ flanking region revealed consensus binding sequences for c-Jun, c-Myc and Sp1 (Lee et al., 1999). Some α class GSTs are induced through the ARE (Zhu et al., 2001; Daniel, 1993), and a rat GSTA3 has AP-1, ARE, and NF-κB transcription factor binding sites in its regulatory region (Fotouhi-Ardakani and Batist, 1999). A θ class GST, termed GSTA, in plaice is also under the control of the ARE (Leaver et al., 1997), and the pro-oxidant

ethoxyquin increased GST activity with CDNB as the substrate while decreasing GST activity towards (+/−)-anti-benzo[a]pyrene-trans-7,8-dihydrodiol-9, 10-epoxide (BPDE) in brown bullhead (Henson et al., 2001). Furthermore, complement component C3 is induced by IL-1β activation of NF-κB in Caco-2 (human intestinal epithelial) cells (Moon et al., 1999). Interestingly, IL-1β also down-regulates α class GSTs in Caco-2 cells. However, there is no evidence that this is mediated by NF-κB and may instead be regulated by hepatic nuclear factor 1 (HNF-1) (Romero et al., 2002).

Down-regulation of these genes may also suggest that short-term exposure to chromium blocked AP-1 or NF-κB from binding their respective promoter regions. Cr(VI) and Cr(III) at 1 and 0.5 mM respectively, directly inhibited NF-κB binding to its DNA-binding sequence (Shumilla et al., 1998). It is interesting to speculate that acute exposure to chromium interferes with transcriptional controls because induction of GSH peroxidases would be considered protective.

Cr(VI) has been shown to induce GSH Prx in other studies and different tissues. GSH Prx mRNA levels were induced by 300 μM potassium dichromate in human lung epithelial cells (A549) 4.33-fold (Ye and Shi, 2001), and GSH Prx activity was increased slightly by Cr(VI) in cultured lymphocytes at 10 μg/ml (Geetha et al., 2002). In contrast, other studies in A549 cells at 5–200 μM have shown no changes in GSH Prx levels (Dubrovskaya and Wetterhahn, 1998). Furthermore, studies in rats treated with potassium dichromate showed decreased GSH Prx activity and decreased GST activity in the rat intestine (Sengupta et al., 1990), and 1 mM potassium dichromate decreased GST activity in isolated hepatocytes (Ueno et al., 1989). We are unaware of any studies demonstrating an increase in GST activity or GSH Prx activity in liver tissue by Cr(VI).

Overall, the expression data suggests that much of the toxicity of Cr(VI) in winter flounder is due to either its reduction to Cr(III) and other chromium species, or redox cycling between different forms of chromium. This is consistent with previous observations that demonstrate chromium's effects on lipid peroxidation and the formation of free radicals (Mao et al., 1995; Sridevi et al., 1998; Rungby and Ernst, 1991; Kadiiska et al., 1994). However, evidence indicates that in mammals some of Cr(VI)'s toxicity may be due to its direct effects, or its reduced form, Cr(III) cross-linking with DNA or proteins (Wilbur and Voytek, 1988). Other research indicates that 8-hydroxyguanosine adduct formation is caused by a combination of radical production and Cr(III), and does not occur unless both hydrogen peroxide and Cr(III) are provided (Tsou et al., 1996). Chromium-induced protein cross-linking may not cause alterations in gene expression, and we did not find changes in DNA repair enzymes. However, since several ESTs were altered (Table 1), it is possible that Cr(VI) at low doses has other mechanisms of toxicity in winter flounder that cannot be inferred based on our current data.

Of particular interest are the two potential non-selenium glutathione peroxidases (GSTA3 and 1-Cys PRX) that have potential roles in cellular antioxidant defense systems. Members of the α class of GSTs have high glutathione peroxidase activity (Yang et al., 2002; Martinez-Lara et al., 2002), and both α class GSTs and the 1-Cys PRX are involved in the metabolism of phospholipid hydroperoxides (Fisher et al., 1999). The 1-Cys PRX is also known as peroxiredoxin 6, anti-oxidant protein 2 (AOP-2), and a calcium-independent phospholipase A2, and to our knowledge this is the first time that a 1-Cys PRX family member has been cloned and sequenced in a fish species. Transcript levels of both of these proteins were reduced approximately 50% in winter flounder liver following acute treatment with chromium (Fig. 2). Furthermore, a GSH-binding protein of approximately 27.1 kDa was significantly reduced (Fig. 3). The molecular weight of this protein is consistent with the molecular weight of GSTs from several aquatic species (Baldwin and LeBlanc, 1996; Fisher et al., 1999; George et al., 1989; Henson et al., 2001; Pham et al., 2002; Leaver et al., 1997). Two-dimensional electrophoresis demonstrated that the p*I* of the proteins at 27.1 kDa were basic, which is consistent with α class GSTs, but not with the neutral to acidic peroxiredoxins. Thus, the 27.1 kDa proteins probably represent α class GSTs, but it cannot be ruled out that some bands may be θ class GSTs.

In addition, the down-regulation of non-selenium GSH peroxidases reduced total cytosolic GST activity with cumene hydroperoxide as the substrate by 18%

(Table 4). The non-selenium GSH peroxidases account for only a portion of the total GSH peroxidase activity in an organ, and are species and organ dependent (Ketterer, 1988). For example, GSTA1–GSTA3 accounted for approximately half of the phospholipid hydroperoxide activity of the liver of mouse and rat liver extracts of 28,000 × *g* supernatant fractions (Yang et al., 2002). Thus, an 18% decrease in cumene hydroperoxide activity may represent a significant decrease in glutathione peroxidase activity if only a couple of the GSH peroxidases have been down-regulated. This may have significant biological effects on the ability of this fish to defend itself against oxidative damage. Alternatively, reduced glutathione peroxidase activity may also indicate a need to increase lipid peroxidation, prostaglandin and leukotriene signaling, and thus increase inflammatory responses or possibly mitogenic responses (Lagarde et al., 1997). This may indicate the importance of inflammatory responses in the acute effects of chromium since the expression of complement component C3, an acute phase reactant was also decreased (Fig. 2).

The α class GST with which our partial sequence aligns best is GSTCL3 in chicken and GSTA3 mouse. However, there is also similarity to GSTA4 and GSTA5 class members (Fig. 1, Table 2). Flounder may have fewer α class GSTs than mammalian species and thus some of its α class GSTs may be difficult to compare phylogenetically. At this time we recognize this flounder GST as an α class member with higher similarity to GSTA3 from mammals than with other α class GSTs.

In conclusion, Cr(VI) altered the expression of several genes including some ESTs. DNA sequencing and semi-quantitative RT-PCR confirmed that an EST, complement component C3, and two non-selenium GSH peroxidases (GSTA and 1-Cys PRX) were down-regulated. The down-regulation of the GSH peroxidases was further established by quantifying proteins purified through a GSH affinity chromatography column and by measuring cumene hydroperoxide activity, indicating that Cr(VI) can have significant effects on genes regulated by ROS and genes that regulate ROS. We anticipate using this information to identify and validate useful biomarkers for exposure to Cr(VI), or other metals and toxicants that may cause oxidative stress in estuarine organisms.

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