Differential gene regulation by V^{IV} and V^{V} ions in the branchial sac, intestine, and blood cells of a vanadium-rich ascidian, *Ciona intestinalis*

Satoshi Kume • Tatsuya Ueki • Hiroki Matsuoka • Mayuko Hamada • Nori Satoh • Hitoshi Michibata

S. Kume • T. Ueki (E-mail) • H. Matsuoka • Hitoshi Michibata

Molecular Physiology Laboratory, Department of Biological Science, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan

E-mail: ueki@hiroshima-u.ac.jp

Mayuko Hamada • Nori Satoh

Marine Genomics Unit, Okinawa Institute of Science and Technology Graduate University, 1919-1 Tancha, Onna-son, Kunigami-gun, Okinawa 904-0495, Japan

Corresponding author: Tatsuya Ueki, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan. Tel/Fax: +81 82 424 7437; E-mail: ueki@hiroshima-u.ac.jp

Abstract

Ascidians are hyperaccumulators that have been studied in detail. Proteins and genes involved in the accumulation process have been identified, but regulation of gene expression related to vanadium accumulation remains unknown. To gain insights into the regulation of gene expression by vanadium in a genome-wide manner, we performed a comprehensive study on the effect of excess vanadium ions on a vanadium-rich ascidian, *Ciona intestinalis*, using a microarray. RT-PCR and enzyme activity assay were performed from the perspective of redox and accumulation of metal ions in each tissue. Glutathione metabolism-related proteins were significantly up-regulated by V^{IV} treatment. Several genes involved in the transport of vanadium and protons, such as Nramp and V-ATPase, were significantly up-regulated by V^{IV} treatment. We observed significant up-regulation of glutathione synthesis and degradation pathways in the intestine and branchial sac. In blood cells, expression of *Ci*-Vanabin4, glutathione reductase activity, glutathione levels, and vanadium concentration increased after V^{IV} treatment. V^{IV} treatment induced significant changes related to vanadium exclusion, seclusion, and redox pathways in the intestine and branchial sac. It also induced an enhancement of the vanadium reduction and accumulation cascade in blood cells. These differential responses in each tissue in the presence of excess vanadium ions suggest that vanadium accumulation and reduction may have regulatory functions. This is the first report on the gene regulation by the treatment of vanadium-rich ascidians with excess vanadium ions. It provided much information for the mechanism of regulation of gene expression related to vanadium accumulation.

Keywords *Ascidian • Vanadium • Microarray • Gene regulation • Glutathione*

Introduction

Hyperaccumulation of vanadium in ascidians (sea squirts and tunicates) was first reported in the early 20^{th} century (Henze 1911). Since the discovery that vanadate (V^{V}) acts as a specific inhibitor of Na^{+}/K^{+}-ATPase (Cantley et al. 1977), and because vanadium compounds have insulin-mimicking properties (Heyliger et al. 1985; Meyerovitch et al. 1987), interest in vanadium has increased. Recent studies on several vanadium haloperoxidases in marine algae and vanadium-containing nitrogenases in *Azotobacter* have increased our understanding of vanadium.

Studies performed in the 1980s measured vanadium levels in many ascidian species (particularly the family Ascididae) and revealed that they accumulate high levels of vanadium in blood cells (Michibata et

al. 1991; Michibata et al. 1986). The vanadium concentration in *Ascidia gemmata* blood cells reaches a maximum at 350 mM, corresponding to a 10^7-fold increase compared to seawater, where vanadium is dissolved at a concentration of 35 nM (Michibata et al. 1991). This is thought to be the highest accumulation factor of metal ions in any living organism.

Redox properties of vanadium in ascidians have also been studied under physiological conditions, where vanadium ions are limited to the +3 (V^{III}), +4 (V^{IV}), and +5 (V^{V}) oxidation states (Boas and Pessoa 1987). In natural seawater environments, vanadium exists in an oxidized state of V^{V}. When V^{V} in seawater is assimilated into ascidians, it is first reduced to V^{IV} in vanadocytes (vanadium-accumulating blood cells) and then stored in their vacuoles, where V^{IV} is finally reduced to V^{III}, which exists as complex cations such as $[V^{III}(H_2O)_6]^{3+}$ and $[V^{III}(H_2O)_5(SO_4)]^{+}$ (Frank et al. 1994; Frank et al. 1999). Therefore, reducing agents must participate in the accumulation of vanadium in vanadocytes. Ascidians are a model organism for studying the mechanisms of accumulation and redox of metal ions.

Proteins involved in vanadium-accumulation and redox processes have been isolated from several vanadium-rich ascidians (*A. gemmata*, *A. sydneiensis samea*, and *Ciona intestinalis*), such as vacuolar-type H^{+}-ATPase (Ueki et al. 1998; Ueki et al. 2001; Uyama et al. 1994), chloride channel (Ueki et al. 2003), enzymes of the pentose phosphate pathway (Ueki et al. 2000; Uyama et al. 1998; Uyama et al. 1998; Uyama et al. 1998), glutathione transferase (Yoshinaga et al. 2007; Yoshinaga et al. 2006), Vanabins (Kanda et al. 1997; Samino et al. 2011; Trivedi et al. 2003; Ueki et al. 2003; Ueki et al. 2008; Yamaguchi et al. 2006; Yamaguchi et al. 2004; Yoshihara et al. 2005), and VBP-129 (Michibata et al. 2007). Structural and biochemical analyses have been conducted on these proteins, mostly on Vanabin2 of *A. sydneiensis samea*, which has a unique bow-shaped structure with nine disulfide bonds (Hamada et al. 2005) and is a novel vanadium reductase (Kawakami et al. 2009). Despite such extensive analyses on the structure and functions of each gene product, the regulation of gene expression related to vanadium accumulation is largely unknown.

Specifically, one model for studying genome-wide transcriptional regulation is a species of ascidian, *C. intestinalis*. The draft genome of *C. intestinalis* contains approximately 16,000 protein-coding genes, and cDNA for transcripts of 13,464 genes have been characterized and compiled as the "*Ciona intestinalis* Gene Collection Release I" (Dehal et al. 2002; Satou et al. 2003; Satou et al. 2002). This species is known to accumulate vanadium at 0.6 mM in its blood cells (Michibata et al. 1986) and can be a model organism for studying vanadium accumulation.

This study was designed to identify genes regulated by excess vanadium ions using an oligonucleotide-based cDNA microarray (Sasaki and Satoh 2007) in *C. intestinalis*. Vanadium ions generally exist as V^{V} and V^{IV} ions under physiological conditions. Thus, we treated *C. intestinalis* with different valences of vanadium ions (V^{IV} or V^{V}) and compared the expression profile of each gene to controlled and untreated specimens. Several genes involved in the transport of vanadium and protons were significantly up-regulated by V^{IV} treatment in a whole body assay. It was also found that the expression of *Ci*-Vanabin1, 3, 4, and 5 were affected by both vanadium treatment and glutathione metabolism-related proteins, and were significantly up-regulated by V^{IV} treatment. These responses were

examined in detail using RT-PCR and measurement of glutathione reductase activity from the perspective of redox and accumulation of metal ions in each tissue. We discuss changes in gene expression and enzyme activity, as well as vanadium and GSH concentrations, in relation to vanadium exclusion, seclusion, and redox pathways.

Materials and methods

Animals and reagents

Adult individuals of *Ciona intestinalis* were cultivated at the Maizuru Fisheries Research Station, Kyoto University, Maizuru Bay, Kyoto, Japan. Individuals at 0.8-cm to 7-cm in length were transported to Hiroshima University, and kept in an artificial sea water (ASW; 3.5% Marine Art SF1 in distilled water (DW), Tomita Pharmaceutical, Japan) at 18°C until use. They were fed with a micro algae *Chaetocerus gracilis* which was commercially available from Nisshin Marinetech, Japan.

Vanadyl sulfate (V^{IV}; $VOSO_4 \cdot nH_2O$, n=3–4), sodium orthovanadate (V^{IV}; Na_3VO_4) and iminodiacetic acid (IDA) were purchased from Wako Pure Chemical Industries Inc., Japan. IDA was dissolved in deionized water (DW) at 50 mM. Vanadyl sulfate was dissolved in distilled water containing IDA at a ratio of 1:1 because they form a very stable complex around neutral pH. Its pH was adjusted to be 7.0 and the volume was set to give the final concentration of 10 mM of each vanadyl and IDA. This V^{IV} stock solution was prepared just before use. V^{V} solution was prepared by dissolving orthovanadate in DW at slightly more than 10 mM. After adding hydrogen chloride and heating at 65°C repeatedly, pH was adjusted to 7.0. DW was added to make 10 mM V^{V}, and finally incubated at 37°C for overnight to let the solution colorless. This V^{V} stock solution can be stored at room temperature.

Vanadium treatment and RNA extraction for microarray

V^{IV} or V^{V} stock solution were diluted by ASW to the final concentration of 1 mM, respectively, and put into plastic dish with about 20 individuals of *C. intestinalis* (about 0.8-cm in length). Control individuals were put in ASW without the addition of vanadium. After incubating at 18°C for 24 hours, ascidians were collected from each dish and frozen at -80°C.

Each frozen samples were homogenized in 4 ml of guanidium thiocyanate solution (4 M guanidium thiocyanate, 50 mM sodium citrate, 0.5% sarcosyl and 1% 2-mercaptoethanol, pH 7.0). Sodium acetate (final 0.2 M), citrate buffer-saturated phenol (4 ml) and chloroform-isoamyl alcohol (0.8 ml) were added

to the homogenate. After mixing briefly, the mixture was kept on ice for 15 minutes and centrifuged at 10,000×g for 15 minutes at 4°C. The supernatant was moved into a new tube, and the RNA was precipitated by equal volume of 2-propanol. Recovered RNA was once washed with 75% ethanol, dried up and dissolved in diethylpyrocarbonate (DEPC)-treated water. RNA was dispensed into small aliquots and stored at -80°C until use.

The concentration of RNA was determined by spectrophotometry, and the quality was checked by agarose gel electrophoresis with ethidium bromide staining. The RNA was treated with DNaseI (TaKaRa Bio Inc., Japan) to remove contaminating DNA, and recovered by phenol extraction and ethanol precipitation. The quality of RNA was checked by RNA 6000 Nano assay and Agilent 2100 bioanalyzer (Agilent Technologies).

Microarray analysis

cRNA was synthesized using 300 ng of purified RNA from control or V treated samples of *C. intestinalis* (about 0.8 cm) and using Agilent low RNA input fluorescent linear amplification kit. Efficiency of incorporation of Cy3- and Cy5-labeled nucleotides was assessed by a spectrophotometer (Nanodrop ND-3300, Thermo Fisher Scientific Inc.).

Oligonucleotide-based microarray version 2 for *C. intestinalis* was used (Sasaki and Satoh 2007), which conveys 44,290 spots including 39,523 gene-specific probes that represent 19,964 genes predicted from cDNA and EST sequence information as well as genome information from the U.S. Department of Energy Joint Genome Institute (DOE-JGI) version 1 assembly. Up-to-date information is available at Kyoto University (http://hoya.zool.kyoto-u.ac.jp/SearchGenomekh.html) and DOE-JGI (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html). Two sets of 1-μg cRNA from two samples (i.e., control vs. V^{IV}-treated ones or control vs. V^{V}-treated ones) were used for competitive hybridization. Hybridization and signal detection were done at 60°C for 17 hours according to the manufacturer's protocol.

Vanadium treatment for RT-PCR, enzyme activity and measurement of vanadium

Three adult individuals (5 to 7 cm in length) of *C. intestinalis* were kept in 500-ml glass beaker in which 1 mM V^{IV} and 1 mM V^{V} ASW were filled, respectively, in each set of experiment. As a control, the individuals were kept in ASW without adding excess vanadium in the same manner. After the incubating at 18°C for 24 hours, each individuals were dissected and their blood cells were extracted, suspended in Ca^{2+}, Mg^{2+}-free artificial sea water (CMFASW; 460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 5 mM HEPES, 6 mM $NaHCO_3$, 5 mM EDTA, pH 7.0) and centrifuged at 1,500×g for 10 min at 4 °C. The

supernatant fraction including the blood plasma was removed. The pelleted cells at the bottom, which contained vanadocytes, were washed with CMFASW and collected by centrifugation at 1,500×g for 10 min at 4 °C. The other tissues were dissected and washed twice in CMFASW. These materials were submitted for RT-PCR analysis, measurement of enzyme activities, and quantification of vanadium and glutathione (GSH) contents as follows.

RT-PCR analysis

RNA was extracted from whole body or dissected tissues as described above. RT-PCR analyses were performed as follows: 50 ng of total RNA was reverse-transcribed using ReverTra Ace (Toyobo Co., Ltd., Japan). The primer pairs used in this study are listed in Table 1. PCR was performed with 5 μM each primer and 2.5 U of Taq DNA polymerase (New England BioLabs, UK) according to the manufacturer's instructions. One cycle of PCR was carried out for 60 sec at 94°C, for 30 sec at 60°C, and 60 sec at 72°C serially, and this cycle was repeated 35 times. The amplified products were analyzed by electrophoresis in a 1.5% agarose gel. The DNA bands were stained with ethidium bromide and visualized under UV light.

Measurement of the glutathione reductase activity in tissues

Dissected tissues were homogenized in five times homogenizing buffer (200 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 7.2) on ice. The homogenate was centrifuged at 20,600 × *g* for 20 min at 4°C. The supernatant was kept at -80°C for the assay.

The glutathione reductase (GR) enzyme activity was determined by NADPH consumption following reduction of GSSG to GSH, according to Racker et al. (Racker 1955). The GR reaction mixture contained 1 mM EDTA, 0.1 M Na_3PO_4 (pH 7.6), 1 mM GSSG, and 0.1 mM NADH. The reaction was initiated by adding 50 μg protein extract, and the absorbance at 340 nm was monitored at 25°C. The specific activity is expressed as ΔA_{340}/min/mg protein.

Quantification of vanadium and glutathione in tissues

For vanadium measurement, dissected tissues were homogenized in 10 times the volume of 0.1 N nitric acid and centrifuged at 10,000 × *g* for 10 min. Vanadium concentration in the supernatant was determined by atomic absorption spectrometry (AA-220Z, Agilent Technologies).

The quantity of GSH in blood cells was determined according to BIOXYTECH GSH-400™ (Percipio Biosciences, Inc.). The GSH quantification method in blood cells that were extracted using the

above method was performed following the product protocol. Blood cells were collected and homogenized in four times the volume of ice-cold 5% metaphosphoric acid (MPA) working solution and centrifuged at 3,000 × *g* for 10 min at 4°C. The upper clear aqueous layer was kept at 0–4°C for approximately 1 h.

Results

Microarray analysis of gene expression profiles in *C. intestinalis* treated with excess vanadium

To examine changes in the gene expression profile after treatment with excess vanadium ions, approximately 20 individuals of *C. intestinalis* (about 0.8 cm in length) were incubated in artificial seawater containing either 1 mM $VOSO_4$ (V^{IV}) or 1 mM Na_3VO_4 (V^{V}) for 24 h at 18°C. Controlled specimens were incubated in artificial seawater without vanadium ions at the same time and temperature. Total RNA was extracted from vanadium-treated and control specimens, labeled with Cy3 or Cy5, and hybridized to oligonucleotide-based microarray slides (ver. 2) (Sasaki and Satoh 2007).

The ratio of fluorescence intensities was calculated using the V^{IV} treated and control probes (V^{IV}:C), as well as the V^{V} treated and control probes (V^{V}:C). Reproducibility was confirmed by two hybridization experiments with swapped dyes. The spots showing reproducible results were chosen for further analysis. Spots that had contradictory results between dye-swap experiments were omitted.

Figure 1 shows a scatter plot of the relationship between V^{IV}:C and V^{V}:C for each spot. The R^2 value for all the spots was -1.1. Among the 39,523 gene-specific probes on the microarray slide, we calculated the number of spots showing a V^{IV}:C or V^{V}:C ratio greater than 1.5 or smaller than 0.66 in a reproducible manner (Table 2). These threshold values were determined according to the detection limit of the microarray hybridization and scanning system. The number of spots where intensity increased in both V^{IV} and V^{V} treated individuals was 550, and the number with decreased intensities in both treated individuals was 820. In contrast, reverse effects were observed for only 29 and 50 spots. This suggests that the overall changes in gene expression were similar between V^{IV} and V^{V} treated individuals.

Significantly increased or decreased genes are listed in Table 3. Each microarray spot is mapped to genome and EST databases, and linked to a cluster of EST clones derived from the same genes. Therefore, we used the cluster ID (CLSTR) to specify each gene. In some cases, cluster ID was not assigned to the spot and the ID was given as an EST clone number (e.g., ciem849o16). The most significantly up-regulated genes were CLSTR02666 (no hits) and CLSTR35308 (molecule against microbe) for the V^{IV} and V^{V} treatments, respectively. Heat-shock protein 70 (CLSTR33198) and molecule against microbes A

(CLSTR33501 and 31913) were up-regulated by V^{V}, but not significantly by V^{IV}, which suggests that V^{V} induced some stress response.

Expression profile of genes related to accumulation and reduction of vanadium

The effects of V^{IV} or V^{V} treatment on several gene categories, which are known to or expected to play a role in vanadium accumulation and reduction, are summarized in Table 4.

The genome of *C. intestinalis* contains five genes for vanadium-binding proteins (Vanabins), which are unique to vanadium-rich ascidians (Trivedi et al. 2003). In the microarray analysis, the expression of *Ci*-Vanabin1, 3, and 5 increased after treatment with V^{IV} and V^{V}. V^{V} treatment decreased the expression of *Ci*-Vanabin4, while V^{IV} treatment did not affect it significantly. *Ci*-Vanabin2 signals were not sufficient for analysis.

In our previous work on *A. sydneiensis samea*, vacuolar-type H^{+}-ATPase (V-ATPase) was expressed in vanadocytes and was found to play a role in vacuole acidification (Ueki et al. 1998; Ueki et al. 2001; Uyama et al. 1994). The electrochemical gradient of protons between the cytoplasm and the vacuole is the motive force and is used to accumulate vanadium ions in the vacuole by the heavy metal transporter Nramp (Ueki et al. 2011). In this study, we found that 10 out of 12 V-ATPase subunits were significantly up-regulated by V^{IV} treatment, while six were up-regulated by V^{V} treatment (Table 4). No subunit was down-regulated by either treatment, which suggests that excess vanadium induces V-ATPase expression.

The expression profiles of five membrane heavy metal transporters, including Nramp and eight redox-related proteins, are summarized in Table 4. In most cases, expression of the transporters was significantly up-regulated by both V^{IV} and V^{V} treatment. None of the transporters were down-regulated.

The expression of some redox-related proteins was up-regulated by V^{IV}, such as glutaredoxin (CLSTR02251), thioredoxin reductase (CLSTR08610), peroxiredoxin (CLSTR00138), and sulfiredoxin (CLSTR12272). Peroxiredoxin and sulfiredoxin were also up-regulated, but glutaredoxin was down-regulated, by V^{V}. These proteins reduce or oxidize proteins and/or metal ions by competing with specific factors. For instance, glutaredoxin reduces arsenate to arsenite conversion by ArsC, GSH, and NADPH (Gladysheva et al. 1994), and the ferredoxin/thioredoxin system regulates fundamental processes via thiol-disulfide exchange reactions (Balmer et al. 2006).

On of the enzymes to produce NADPH in the pentose phosphate pathway is 6PGDH (CLSTR32174) which is exclusively localized in vanadocytes of *Ascidia sydneiensis samea* (Uyama et al. 1998). Expression of 6PGDH was also up-regulated by V^{IV} treatment.

In addition, expression of phytochelatin synthase (CLSTR08515), which synthesizes phytochelatin, acts as a transporter for glutathione conjugates, and associates with plant heavy-metal-binding peptides such as glutathione (Grill et al. 1989), MRP (CLSTR07189), and MDR (CLSTR12664) (Table 4) was also up-regulated by V^{IV} treatment. Thus, the expression level of these proteins related to metal ion transport or reduction of oxidized proteins and metal ions was affected by vanadium.

RT-PCR analysis of glutathione-related proteins in whole animal samples

Genes related to glutathione metabolism involved in vanadium accumulation and reduction were up- or down- regulated significantly (Table 4). Therefore, we focused on the changes in expression of glutathione-related proteins. RT-PCR was performed to confirm the expression changes in glutathione-related genes in whole animal samples (Fig. 2). The results were consistent with microarray analysis, and are summarized on the pathway scheme (Fig. 3). Overall, V^{IV} treatment was more effective against glutathione metabolism than V^{V} treatment, as shown by both the microarray data values and the band strength in RT-PCR. V^{IV} treatment increased the expression of six genes out of the seven examined, while GPX (CLSTR10702) decreased them. This indicates that V^{IV} treatment accelerated both the synthesis and degradation of glutathione in the glutathione metabolic pathway. Inversely, V^{V} treatment down-regulated GPX and GST (CLSTR30138) and up-regulated GCLM (CLSTR04033) and GR (CLSTR04792) (Table 4). GCL is the rate-limiting enzyme in GSH synthesis, and GR reduces GSSG to GSH, which could increase the GSH/GSSG ratio.

RT-PCR analysis of glutathione-related proteins and *Ci*-Vanabins

Our recent work indicated that Vanabin2 acts as a vanadium reductase in a cascade composed of NADPH, GR, GSH, Vanabin2, and V^{V} *in vitro* (Kawakami et al. 2009). As discussed in the previous sections, we have evaluated the expression changes of 6PGDH, GR, GCLM, GCLC, and *Ci*-Vanabins by vanadium treatment in whole body samples (Table 4, Figs. 2 and 3).

To examine the tissue-specific regulation of glutathione-related genes, we treated mature individuals of *C. intestinalis* (ca. 5 to 7 cm in length) using the same conditions as in the microarray analysis. RNA was extracted from each tissue and reverse transcribed to perform RT-PCR analysis. The results are summarized in Figure 4A. Contrary to our expectations, gene expressions in blood cells, which are the final tissue for vanadium accumulation, were not significantly affected. Instead, there were significant changes in the gene expression profiles of the intestine and branchial sac, both of which are the first tissues to import vanadium ions from the outer environment. After V^{IV} treatment, expression of GCLM, GCLC (CLSTR07652), and γ-GT (CLSTR10178) were up-regulated in the branchial sac. This was also observed for GCLM and γ-GT in the intestine. This indicates that glutathione turnover was accelerated in the branchial sac and intestine. In muscle cells, no significant changes were observed and expression of GPX was not significantly affected in any tissue. Expression of GST was also constant among all tissues, except for blood cells, which gave highly variable signals in repeated experiments (data not shown).

In addition, we also performed RT-PCR analysis on *Ci*-Vanabins in the same manner (Fig. 4B). For

blood cells, among the five genes, four Vanabins were detected by RT-PCR. The expression of *Ci*-Vanabin4 was enhanced by both V^{IV} and V^{V} treatment. The expression of five Vanabins in other tissues was not changed by vanadium treatment (data not shown). Thus, expression of *Ci*-Vanabin4 in blood cells was significantly affected by both V^{IV} and V^{V} treatment.

Analysis of GR activity

To identify biochemical changes in these tissues that may reflect the changes in gene expression for glutathione metabolism-related enzymes, we analyzed GR activity, which is involved in the vanadium reduction cascade (Kawakami et al. 2009), in muscle, intestine, the branchial sac, and blood cells (Fig. 5). Consistent with the RT-PCR analysis, GR activity increased after V^{IV} treatment in the branchial sac. In blood cells, GR activity clearly increased after V^{IV} treatment, although the RT-PCR results indicated no significant change in GR expression. The increase in GR activity may be related to the vanadium reduction cascade, and is discussed below.

Vanadium and glutathione content in each tissue

In normal seawater, vanadium concentration in each tissue is strictly regulated because exposure to excess vanadium ions can affect the gene expression profile, and thus result in changes in the vanadium concentration and redox state of each tissue. To examine the effect of exposure to excess V^{IV} or V^{V} ions, vanadium concentrations were determined (these are summarized in Fig. 6). Vanadium in muscle and intestine significantly increased after both V^{IV} and V^{V} treatment. Only V^{IV} treatment significantly increased vanadium concentration in the branchial sac and blood cells. Vanadium concentration in blood cells significantly increased by approximately 6.8-fold after V^{IV} treatment.

GSH levels were determined in blood cells (Fig. 6) because GSH plays a role in the vanadium reduction cascade. The GSH concentrations in control blood cells were 1.25 mM. The values increased approximately 1.2-fold after V^{IV} treatment, but did not change after V^{V} treatment.

Discussion

We performed the first microarray analysis on a vanadium-accumulating ascidian treated with excess vanadium to examine changes in the gene expression profile in response to excess vanadium ions. Vanadium ions generally exist as V^{V} and V^{IV} ions under physiological conditions. Thus, we evaluated

both chemical species and compared the results. The whole body analysis by microarray and RT-PCR identified the overall changes in the gene expression profile as well as significant changes in glutathione-related pathways. Detailed RT-PCR and enzyme assays of each tissue provided evidence for the possible regulatory mechanism of vanadium accumulation and reduction.

Regarding the overall changes in the expression profile determined by microarray on whole body specimens, we found that 550 spots were significantly up-regulated and 820 spots were significantly down-regulated among the 39,523 gene-specific probes (Table 2). Reverse effects were observed for only a small number of spots and the overall changes in gene expression were similar between the V^{IV} and V^{V} treatments. The most significantly up-regulated genes were stress-related genes, such as heat-shock protein and anti-microbe protein (Table 3). Heat-shock protein 70 (CLSTR33198) is a cytoplasmic protein in the DnaK subfamily, and the only heat-inducible protein in *C. intestinalis* (Fujikawa et al. 2009). This gene is up-regulated by stress, such as the presence of reactive oxygen species, which can be generated by V^{V} and V^{IV} ions in the cytoplasm (Capella et al. 2007). Due to antimicrobial proteins being sequestered in the vacuoles of blood cells and induced after immune challenge (Fedders et al. 2008), the induction of antimicrobial protein was also inferred to be a stress response. V^{V} may participate in NADPH oxidation leading to O_2^- and H_2O_2, whereas V^{IV} may react with H_2O_2 to generate V^{V} and the OH^- radical, or directly produce peroxovanadium compounds (Capella et al. 2007). These reactive oxygen species could induce the stress-response genes observed in our experiment.

We predicted that the genes responsible for the import, export, and reduction of vanadium ions must be induced or repressed. Therefore, we first examined genes related to vanadium accumulation. As summarized in Table 4, V^{V} and V^{IV} treatments had different effects on the gene expression of known vanadium-related genes, such as Vanabins, vacuolar-type H^+-ATPase, glutathione-related proteins, heavy metal transporters, and redox-related proteins in whole body specimens.

Regarding the vanadium/proton antiport system, we previously reported that a specific inhibitor of the V-ATPases, which generate a proton-motive force by hydrolyzing ATP, inhibited the proton pump in the vacuoles of the vanadocyte and neutralized the contents of vacuoles (Uyama et al. 1994). Therefore, we proposed that the electrochemical gradient generated by V-ATPase is linked to the accumulation of vanadium. Furthermore, the Nramp family of divalent cation transporters is a vanadium/proton antiporter on the vacuolar membrane of vanadocytes (Ueki et al. 2011). In this study, genes encoding subunits of V-ATPase were up-regulated by both V^{V} and V^{IV} treatment, and the expression of Nramp was significantly up-regulated by V^{IV} in whole body specimens (Table 4). These results suggest that excess V^{IV} ions induced the expression of vanadium transport machinery using a proton-motive force to sequester vanadium ions in specific *C. intestinalis* tissue, resulting in increased vanadium levels in each tissue. Thus, vanadium may be imported into vacuoles of the blood cells, in which vanadium concentrations are increased.

Another possible vanadium transport system is the GS-X pump system. This system includes at least two types of transporters (MRP and MDR) and is linked to glutathione metabolism (Fig. 3). V^{IV} treatment induced the expression of GCLC, GCLM, GS, and GR, and suppressed GPX in whole body specimens.

V^{IV} treatment also induced γ-GT and GST. This change should result in the rapid synthesis of GSH and the acceleration of GS-X conjugation (Figs. 2 and 3). Because two GS-X pumps (MRP and MDR) were also up-regulated by V^{IV} treatment in the same specimens (Table 4), this treatment resulted in the export of glutathione conjugates by GS-X pumps. Among the tissues examined, V^{IV} treatment-related up-regulation of GCLM and γ-GT expression was observed in the branchial sac and intestine (Fig. 4A). This suggests that the GS-X transport pathway was up-regulated after V^{IV} treatment in these tissues, which may interact with the outer environment in the presence of excess vanadium. GS-X pumps may play a role in maintaining the homeostasis of the intracellular vanadium concentration through exclusion or seclusion. The vanadyl-glutathione conjugate could be a target of the exclusion or seclusion pathway (Dessi et al. 1993; Pessoa et al. 2002). In this scheme, the activation of γ-GT by V^{IV} treatment seems contradictory. Mehdi et al. reported that γ-GT on the vacuolar membrane stimulates the YCF1 transporter in yeast (Mehdi et al. 2001), and that Cd activates γ-GT and glutathione transferase 2 in yeast. This activation is necessary for the regulation of cytoplasmic Cd levels and the recycling of glutamate and regeneration of GSH (Adamis et al. 2009). Therefore, it is possible that the up-regulation of γ-GT could accelerate the GS-X transport pathway.

In addition to the two transport systems discussed above, several studies have suggested that changes in the cellular status may affect vanadium reduction. First, vanadium levels were significantly increased by exposure to excess V^{IV} ions (Fig. 6). This change was also observed in blood cells, which are the final destination tissue of vanadium accumulation in ascidians. Moreover, RT-PCR analysis and enzyme assays on *C. intestinalis* blood cells indicated that *Ci*-Vanabin4 was significantly up-regulated (Fig. 4B) and that GR activity was induced by V^{IV} treatment (Fig. 5). We also found that GSH levels were slightly increased by V^{IV} compared to the control and V^{V} treated specimens (Fig. 6). This indicates that V^{IV} treatment enhanced vanadium reduction and the accumulation cascade in *C. intestinalis* blood cells, as suggested in our previous study on *A. sydneiensis samea* (Kawakami et al. 2009). However, this is contradictory to the reduction pathway of V^{V} in the outer environment. We could not determine the concentration and valence of vanadium in blood plasma because its concentration was too low. If vanadium levels in blood plasma are increased and exist as V^{V}, they should enhance V^{V} uptake and reduction in blood cells. Thus, it is necessary to examine whether the vanadium reduction cascade is enhanced, repressed, or not affected when isolated blood cells are treated with excess vanadium ions or an inhibitor of GSH synthesis.

C. intestinalis treatment with excess vanadium ions was used to identify genes regulated by vanadium ions. We found that the expression of V-ATPase subunits and Nramp were significantly up-regulated by V^{IV} treatment using a whole body assay. The relationship between the proton motive force and vanadium accumulation across the vacuolar membrane was also supported by changes in vanadium levels and the gene expression profile. We identified up- and down-regulated genes, among which the enzymes in the glutathione metabolic pathway were most significantly affected by vanadium levels. These changes were observed in the branchial sac and intestine. Protein expression in the vanadium reduction cascade also increased after vanadium treatment in whole body samples. These responses were

examined in detail by RT-PCR and measurement of glutathione reductase activity in each tissue. As a result, V^{IV} treatment induced GR activity, GSH concentration, the expression of *Ci*-Vanabin4 and vanadium concentration in blood cells. In the future, it will be necessary to perform the inhibition studies described above or direct mutagenesis of genes that respond to excess vanadium ions. These studies will provide insights into the accumulation and reduction pathway of vanadium in ascidians. The metabolism of vanadium ions in biological systems is still not well understood and vanadium-rich ascidians can be used as a model to study this system.

Acknowledgments We thank Ms. K. Hirayama and Dr. Yutaka Satou at Kyoto University and the staff at the Maizuru Fisheries Research Station, Kyoto University, Maizuru Bay, Kyoto, Japan, for providing *C. intestinalis*, through the National Bio-Resource Project (NBRP) of the MEXT, Japan. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#21570077 to H. M. and #20570070 to T. U.) and a grant from the Toray Science Foundation (03-4402 to T. U.).

References

Adamis PD, Mannarino SC, Eleutherio EC (2009) Glutathione and gamma-glutamyl transferases are involved in the formation of cadmium-glutathione complex. FEBS Lett 583: 1489-1492

Balmer Y, Vensel WH, Cai N, Manieri W, Schurmann P, et al. (2006) A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts. Proc Natl Acad Sci U S A 103: 2988-2993

Boas LV, Pessoa JC (1987) Comprehensive Coordination Chemistry, vol. 2. In: (ed). Pergamon Press, Oxford. pp453–583

Cantley J, L. C. , Josephson L, Warner R, Yanagisawa M, Lechene C, et al. (1977) Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle. J Biol Chem 252: 7421-7423

Capella MAM, Valente RC, Capella LS (2007) Cytotoxity of vanadim compounds. In: MA Alves (ed). Vanadium BiochemistryResearch Signpost, Kerala. pp323-339

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, et al. (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298: 2157-2167

Dessi A, Micera G, Sanna D (1993) EPR investigation of the oxovanadium(IV) complexes formed by the tripeptide glutathione and some related ligands in aqueous solution. J Inorg Biochem 52: 275-286

Fedders H, Michalek M, Grotzinger J, Leippe M (2008) An exceptional salt-tolerant antimicrobial peptide derived from a novel gene family of haemocytes of the marine invertebrate Ciona intestinalis.

Biochem J 416: 65-75

Frank P, Hedman B, Carlson RMK, Hodgson KO (1994) Interaction of vanadium ansd sulfate in blood cells from the tunicate *Ascidia seratodes*: observations using X-ray absorption edge structures and EPR spectroscopies. Inorg Chem 33: 3794-3803

Frank P, Hedman B, Hodgson KO (1999) Sulfur allocation and vanadium – sulfate interactioins in whole blood cells from the tunicate *Ascidia seratodes*, investigated using X-ray absorption spectrometry. Inorg Chem 38: 260-270

Fujikawa T, Munakata T, Kondo SI, Satoh N, Wada S (2009) Stress response in the ascidian Ciona intestinalis: transcriptional profiling of genes for the heat shock protein 70 chaperone system under heat stress and endoplasmic reticulum stress. Cell Stress Chaperones

Gladysheva TB, Oden KL, Rosen BP (1994) Properties of the arsenate reductase of plasmid R773. Biochemistry 33: 7288-7293

Grill E, Loffler S, Winnacker EL, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific gamma-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proc Natl Acad Sci U S A 86: 6838-6842

Hamada T, Asanuma M, Ueki T, Hayashi F, Kobayashi N, et al. (2005) Solution structure of Vanabin2, a vanadium(IV)-binding protein from the vanadium-rich ascidian *Ascidia sydneiensis samea*. J Am Chem Soc 127: 4216-4222

Henze M (1911) Untersuchungen über das Blut der Ascidien. I. Mitteilung. Die Vanadiumverbindung der Blutkörperchen. Hoppe-Seyler's Z Physiol Chem 72: 494-501

Heyliger CE, Tahiliani AG, McNeill JH (1985) Effect of vanadate on elevated blood glucose and depressed cardiac performance of diabetic rats. Science 227: 1474-1477

Kanda T, Nose Y, Wuchiyama J, Uyama T, Moriyama Y, et al. (1997) Identification of a vanadium-associated protein from the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 14: 37-42

Kawakami N, Ueki T, Amata Y, Kanamori K, Matsuo K, et al. (2009) A novel vanadium reductase, Vanabin2, forms a possible cascade involved in electron transfer. Biochim Biophys Acta 1794: 674-679

Mehdi K, Thierie J, Penninckx MJ (2001) gamma-Glutamyl transpeptidase in the yeast Saccharomyces cerevisiae and its role in the vacuolar transport and metabolism of glutathione. Biochem J 359: 631-637

Meyerovitch J, Farfel Z, Sack J, Shechter Y (1987) Oral administration of vanadate normalizes blood glucose levels in streptozotocin-treated rats. Characterization and mode of action. J Biol Chem 262: 6658-6662

Michibata H, Iwata Y, Hirata J (1991) Isolation of highly acidic and vanadium-containing blood cells from among several types of blood cell from Ascidiidae species by density-gradient centrifugation. J Exp Zool 257: 306-313

Michibata H, Terada T, Anada N, Yamakawa K, Numakunai T (1986) The accumulation and

distribution of vanadium, iron, and manganese in some solitary ascidians. Biol Bull 171: 672-681

Michibata H, Yoshinaga M, Yoshihara M, Kawakami N, Yamaguchi N, et al. (2007) Genes and proteins involved in vanadium accumulation by ascidians. In: K Kustin, J Costa-Pessoa and DC Crans (ed). Vanadium the Versatile metalOxford University Press, Oxford. pp264-280

Pessoa JC, Tomaz I, Kiss T, Kiss E, Buglyo P (2002) The systems V(IV)O(2+)-glutathione and related ligands: a potentiometric and spectroscopic study. J Biol Inorg Chem 7: 225-240

Racker E (1955) Glutathione reductase from bakers' yeast and beef liver. J Biol Chem 217: 855-865

Samino S, Michibata H, Ueki T (2011) Identification of a Novel Vanadium-binding Protein by EST Analysis on the Most Vanadium-rich Ascidian, Ascidia gemmata. Mar Biotechnol (NY)

Sasaki A, Satoh N (2007) Effects of 5-aza-2'-deoxycytidine on the gene expression profile during embryogenesis of the ascidian *Ciona intestinalis*: A microarray analysis. Zool Sci 24: 648-655

Satou Y, Kawashima T, Kohara Y, Satoh N (2003) Large scale EST analyses in Ciona intestinalis: its application as Northern blot analyses. Dev Genes Evol 213: 314-318

Satou Y, Yamada L, Mochizuki Y, Takatori N, Kawashima T, et al. (2002) A cDNA resource from the basal chordate *Ciona intestinalis*. Genesis 33: 153-154

Trivedi S, Ueki T, Yamaguchi N, Michibata H (2003) Novel vanadium-binding proteins (vanabins) identified in cDNA libraries and the genome of the ascidian *Ciona intestinalis*. Biochim Biophys Acta 1630: 64-70

Ueki T, Adachi T, Kawano S, Aoshima M, Yamaguchi N, et al. (2003) Vanadium-binding proteins (vanabins) from a vanadium-rich ascidian *Ascidia sydneiensis samea*. Biochim Biophys Acta 1626: 43-50

Ueki T, Furuno N, Michibata H (2011) A novel vanadium transporter of the *Nramp* family expressed at the vacuole of vanadium-accumulating cells of the ascidian *Ascidia sydneiensis samea*. Biochim Biophys Acta 1810: 457-464

Ueki T, Satake M, Kamino K, Michibata H (2008) Sequence variation of Vanabin2-like vanadium-binding proteins in blood cells of the vanadium-accumulating ascidian *Ascidia sydneiensis samea*. Biochim Biophys Acta 1780: 1010-1015

Ueki T, Uyama T, Kanamori K, Michibata H (1998) Isolation of cDNAs encoding subunits *A* and *B* of the vacuolar-type ATPase from the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 15: 823-829

Ueki T, Uyama T, Kanamori K, Michibata H (2001) Subunit C of the vacuolar-type ATPase from the vanadium-rich ascidian *Ascidia sydneiensis samea* rescued the pH sensitivity of yeast *vma5* mutants. Mar Biotechnol 3: 316-321

Ueki T, Uyama T, Yamamoto K, Kanamori K, Michibata H (2000) Exclusive expression of transketolase in the vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Biochim Biophys Acta 1494: 83-90

Ueki T, Yamaguchi N, Michibata H (2003) Chloride channel in vanadocytes of a vanadium-rich

ascidian *Ascidia sydneiensis samea*. Comp Biochem Physiol B Biochem Mol Biol 136: 91-98

Uyama T, Kinoshita T, Takahashi H, Satoh N, Kanamori K, et al. (1998) 6-Phosphogluconate dehydrogenase is a 45-kDa antigen recognized by S4D5, a monoclonal antibody specific to vanadocytes in the vanadium-rich ascidian, *Ascidia sydneiensis samea*. J Biochem 124: 377-382

Uyama T, Moriyama Y, Futai M, Michibata H (1994) Immunological detection of a vacuolar-type H^+-ATPase in the vanadocytes of the ascidian *Ascidia sydneiensis samea*. J Exp Zool 270: 148-154

Uyama T, Ueki T, Suhama Y, Kanamori K, Michibata H (1998) A 100-kDa antigen recognized by a newly prepared monoclonal antibody specific to the vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*, is glycogen phosphorylase. Zool Sci 15: 815-821

Uyama T, Yamamoto K, Kanamori K, Michibata H (1998) Glucose-6-phosphate dehydrogenase in the pentose phosphate pathway is localized in vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 15: 441-446

Yamaguchi N, Amakawa Y, Yamada H, Ueki T, Michibata H (2006) Localization of Vanabins, vanadium-binding proteins, in the blood cells of the vanadium-rich ascidian *Ascidia sydneiensis samea*. Zool Sci 23: 909-915

Yamaguchi N, Kamino K, Ueki T, Michibata H (2004) Expressed sequence tag analysis of vanadocytes in a vanadium-rich ascidian, *Ascidia sydneiensis samea*. Mar Biotechnol (NY) 6: 165-174

Yoshihara M, Ueki T, Watanabe T, Yamaguchi N, Kamino K, et al. (2005) VanabinP, a novel vanadium-binding protein in the blood plasma of an ascidian, *Ascidia sydneiensis samea*. Biochim Biophys Acta 1730: 206-214

Yoshinaga M, Ueki T, Michibata H (2007) Metal binding ability of glutathione transferases conserved between two animal species, the vanadium-rich ascidian *Ascidia sydneiensis samea* and the schistosome *Schistosoma japonicum*. Biochim Biophys Acta 1770: 1413-1418

Yoshinaga M, Ueki T, Yamaguchi N, Kamino K, Michibata H (2006) Glutathione transferases with vanadium-binding activity isolated from the vanadium-rich ascidian *Ascidia sydneiensis samea*. Biochim Biophys Acta 1760: 495-503

Figure Legends

Fig. 1

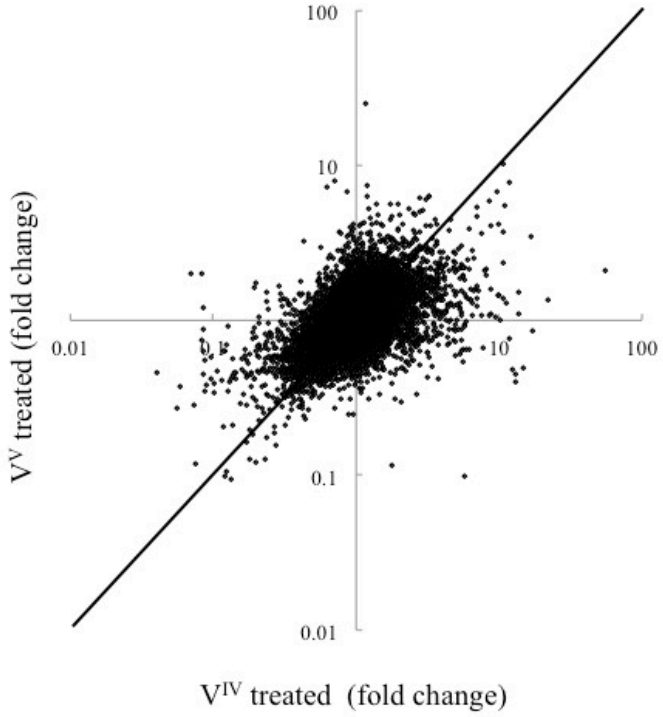

Fig. 1. Scatter plot of fluorescent signals for all spots (excluding the negative and positive controls) from a microarray analysis of *C. intestinalis* specimens treated with 1 mM V^V or V^{IV} for 24 h. Plots indicate the average value for each spot from dye-swap experiments for V^V treated:control (vertical axis) and V^{IV} treated:control (horizontal axis), respectively. The spots that gave contradictory signals by the same treatment in dye-swap experiments were omitted. The oblique line indicates that the signals from the V^{IV} treated:control and the V^V treated:control are equal.

Fig. 2

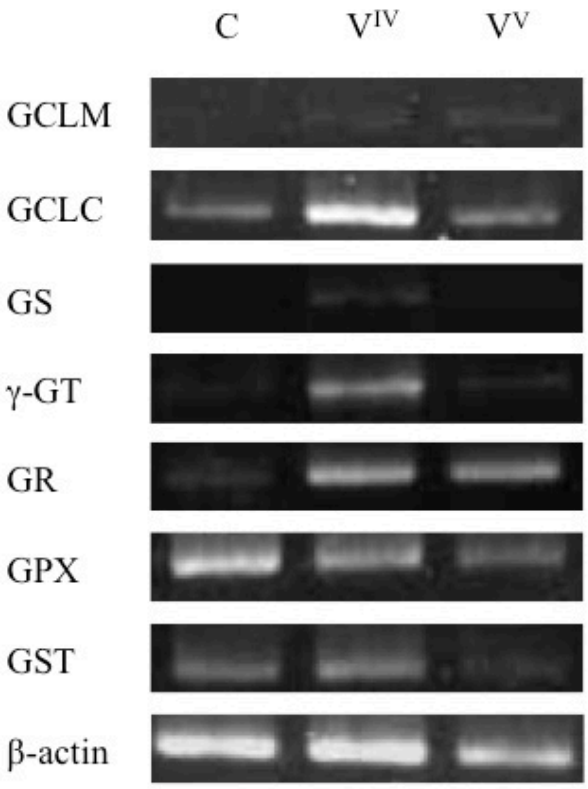

Fig. 2. RT-PCR analysis of genes in the glutathione pathway for whole body samples of *C. intestinalis*. RNA was extracted from specimens treated with V^{IV} at 1 mM for 24 h (V^{IV}) or V^{V} at 1 mM for 24 h (V^{V}) or untreated controls (C). Reverse-transcribed cDNAs were used for PCR by each gene-specific primer set, as listed in Table 1, and the PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The β-actin expression levels are shown as a control. In this analysis, we used the same RNAs as in the microarray analysis. Representative data for at least two repetitive assays are shown.

Fig. 3

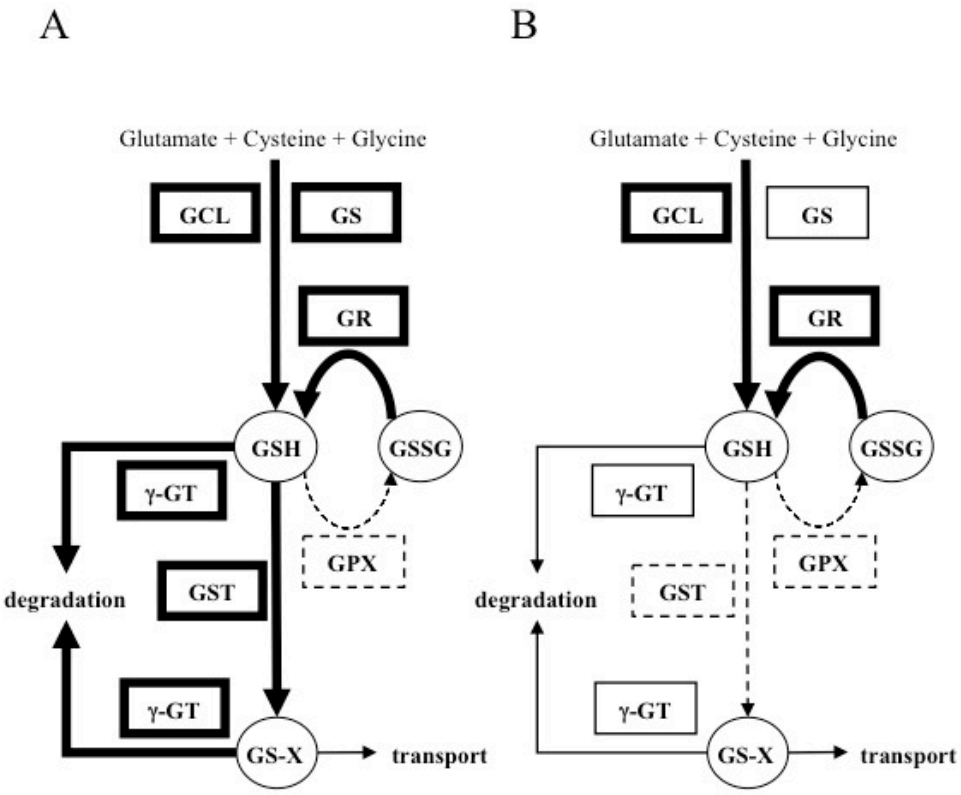

Fig. 3. Summary of the changes in gene expression related to glutathione metabolism. A) V^{IV} treatment, B) V^{V} treatment. Thick lines indicate up-regulated genes, while dotted lines indicate down-regulated genes. V^{IV} treatment was more effective against glutathione metabolism than V^{V} treatment. GSH is synthesized from glutamate, cysteine, and glycine by GCL (EC:6.3.2.2) and GS (EC:6.3.2.3). γ-GT (EC:2.3.2.2) degrades GSH. GR (EC 1.8.1.7) reduces GSSG to GSH, and GPX (EC:1.11.1.9) oxidizes GSH to GSSG. GSH is conjugated to a xenobiotic substance by GST (EC:2.5.1.18) to form a complex (GS-X), which is transported by MDR or MRP.

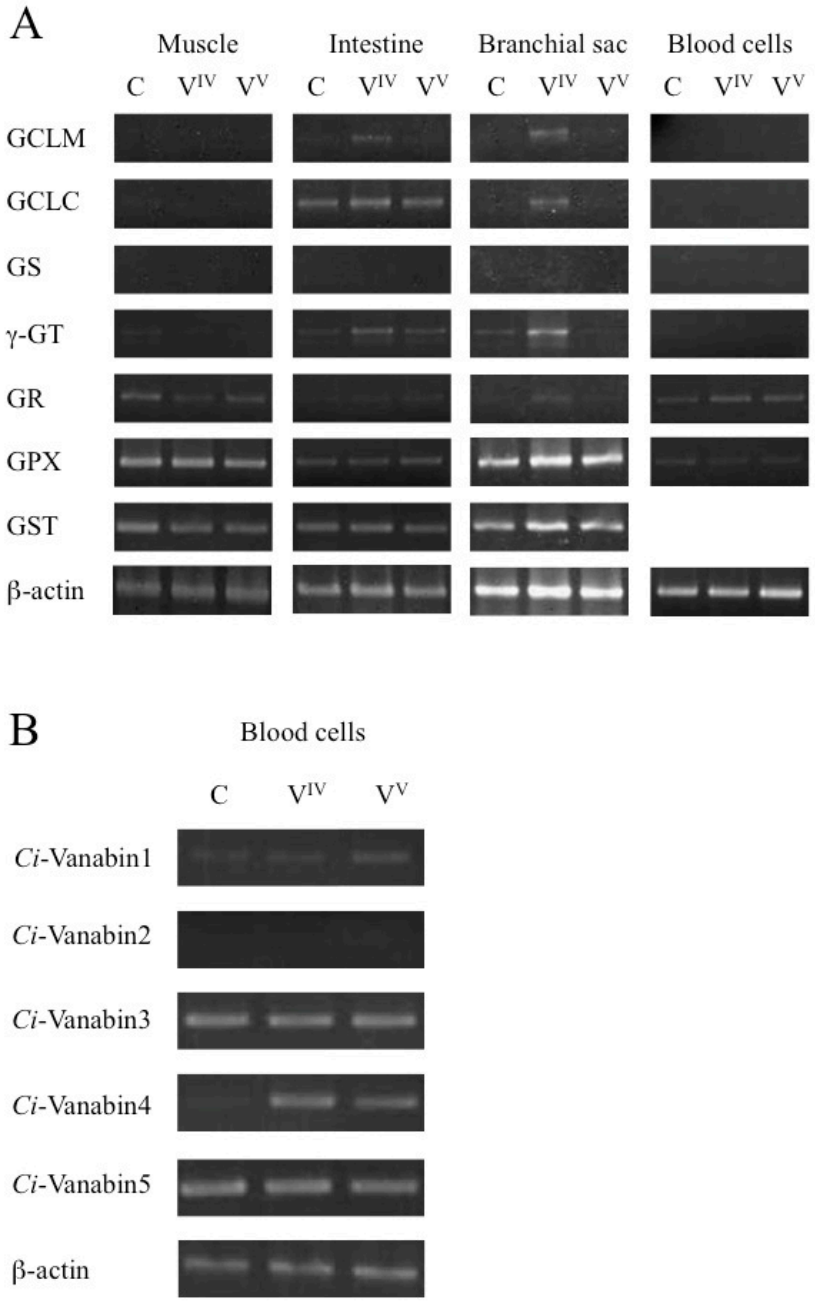

Fig. 4. RT-PCR analysis of each *C. intestinalis* tissue. RNA was extracted from specimens treated with V^{IV} at 1 mM for 24 h (V^{IV}), V^{V} at 1 mM for 24 h (V^{V}), or untreated controls (C). Reverse-transcribed cDNAs were used for PCR by each gene-specific primer set, as listed in Table 1, and the PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. A) Expression of glutathione-related proteins in each tissue, B) expression of *Ci*-Vanabins in blood cells. The expression levels of β-actin are shown as a control. Representative data for at least three repetitive assays are shown.

Fig. 5

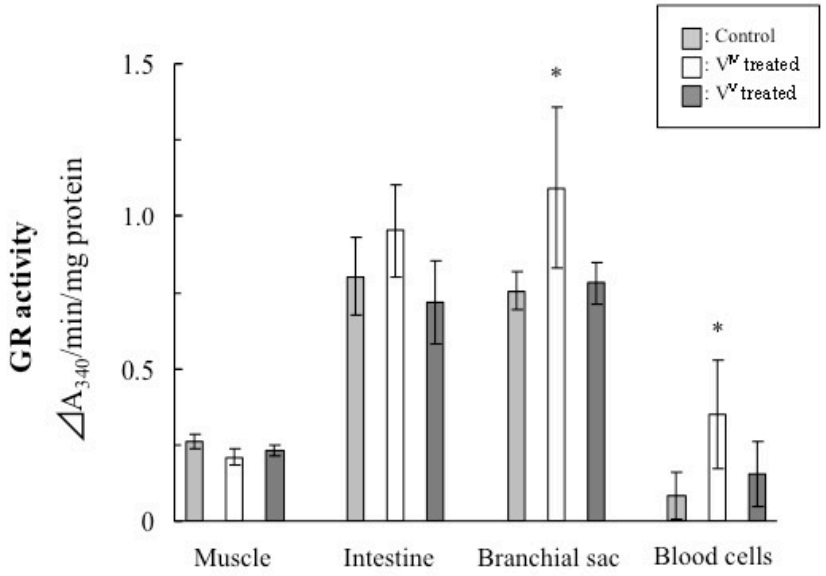

Fig. 5. The GR activity of each *C. intestinalis* tissue. Total protein was extracted from specimens treated with V^{IV} at 1 mM for 24 h (V^{IV} treated), with V^{V} at 1 mM for 24 h (V^{V} treated), or untreated controls (Control). GR activity was examined as described in the Experimental Procedures. The activity is shown as the change in absorbance due to NADPH consumption per total protein. Results are expressed as the mean ± SD from four independent experiments. *, $P < 0.05$ compared to controls.

Fig. 6

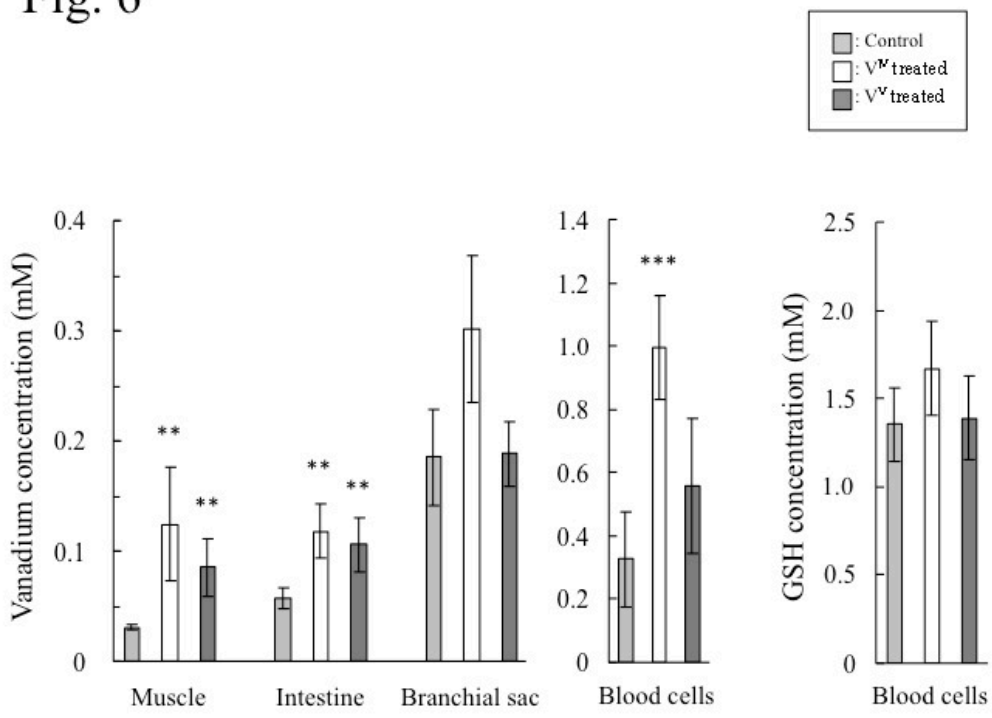

Fig. 6. Vanadium and GSH content in tissues from control and vanadium-treated *C. intestinalis* specimens. Each tissue was collected from specimens treated with V^{IV} at 1 mM for 24 h (V^{IV} treated), with V^{V} at 1 mM for 24 h (V^{V} treated), or untreated controls (Control). Vanadium and GSH concentrations were examined as described in the Experimental Procedures. Results are expressed as the mean ± SD from at least four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to controls.

Table1. Primer sequences for conventional RT-PCR

Cluster ID	Putative gene name	primer sense primer antisense	PCR product (bp)
07652r1	Glutamate-cysteine ligase catalytic subunit	5'-CATTATGTTGGCCCTGAGTG-3' 5'-CTCAGAGATGCTGCATCTTG-3'	885
04033r1	Glutamate-cysteine ligase modifier subunit	5'-GGCGTGGCTAATTGAAAATG-3' 5'-CACATTAAACCCTATGCACC-3'	883
31686r1	Glutathione synthetase	5'-CCCAACAAACAAGCACTGGG-3' 5'-CTGACAGGTTAAACACTGCCAC-3'	1006
10178r1	Gamma-glutamyltranspeptidase 1 precursor	5'-GTATTGGTGGTGGATTCTTC-3' 5'-GGTAACACAACAATGGCTTG-3'	1426
04792r1	Glutathione reductase	5'-CTATGCACCTGTTTGTGACG-3' 5'-CGTCCCACATGATAGTAAAC-3'	784
10702r1	Glutathione peroxidase precursor	5'-GTTCTTCAAGGACGATCTAC-3' 5'-CACTGCGAATGACGTAAAAC-3'	686
30138r1	Glutathione S-transferase II	5'-GACTGGATCACGAAATTAAG-3' 5'-GAAATCATGAAGTTCTTCCG-3'	458
06625r1	*Ci*-Vanabin1	5'-CACATCAAGAATATGGTCGC-3' 5'-CTGAGTATCTTCTTCAGTTGG-3'	427
13989r1	*Ci*-Vanabin2	5'-GGAACCGGGAATGAATAAAG-3' 5'-GGAACCTTCACTTGGTTTTG-3'	420
04798r1	*Ci*-Vanabin3	5'-GCTGCTGTTGTGTTTGTAAC-3' 5'-GCAGTTGTTCATTAGACACC-3'	398
02771r1	*Ci*-Vanabin4	5'-CATCATGGAGGATTGATGGG-3' 5'-GAACGCATCCACTTAGTTGC-3'	321
00859r1	*Ci*-Vanabin5	5'-GTAAGCTTGATGGTTGTCGC-3' 5'-GCATCGCTTGTTCTTCTTTGG-3'	317
00046r1	β-actin	5'-GTGCTTTCATTGTACGCTTCTGGTC-3' 5'-CGGCGATTCCAGGGAACATAG-3'	517

Table 2. Summary of the number of spots that showed the significant change of V^{IV}:control or V^{V}:control ratio

	V(IV)↑ [a] V(V)↑	V(IV)↑ V(V)↑ [c]	V(IV)↑ V(V)↓ [b]	V(IV)↓ V(V)↓	V(IV)↓ V(V)↓	V(IV)↓ V(V)↓	V(IV)↓ V(V)↓	V(IV)↓ V(V)↓
Spots	550	1170	50	29	1024	820	813	406

[a] V^{IV} treated:Control or V^{V} treated:Control ratio greater than 1.5.

[b] V^{IV} treated:Control or V^{V} treated:Control ratio smaller than 0.66.

[c] V^{IV} treated:Control or V^{V} treated:Control between 0.66 and 1.5.

Table 3. Description of significant induced or repressed genes by V^{IV} or V^{V}

Cluster ID	EST sequence similarities (BLASTX results)	fold change [a]
V^{IV} up		
02666r1	No hits found	55.590
15657r1	Hedgling	23.442
05750r1	Scavenger receptor Cys-rich protein	17.378
03311r1	No hits found	16.596
16980r1	Mannose-binding-like lectin	14.622
14505r1	Sodium-dependent proline transporter	13.948
30272r1	No hits found	13.646
13715r1	Rhamnose binding lectin	13.646
16551r1	Putative coagulation serine protease	13.183
05827r1	Zinc finger protein	11.749
V^{IV} down		
ciem849o16	Metallo-beta-lactamase family protein	0.041
15769r1	Alkyl sulfatase or beta-lactamase	0.056
00087r1	No hits found	0.058
12566r1	Polydomain protein-like	0.069
05759r1	Epidermal growth factor receptor	0.073
35118r1	Secreted protein	0.073
06819r1	Cubilin	0.075
33011r1	Vwa1 protein	0.083
12566r1	Polydomain protein-like	0.084
35074r1	Mannose receptor C1	0.085
V^{V} up		
35308r1	Molecule against microbes A	25.132
33198r1	Heat shock protein 70	10.144
33501r1	Molecule against microbes A	7.999
05827r1	Zinc finger protein	7.751
31913r1	Molecule against microbes A	7.443
30247r1	Putative uncharacterized protein	6.749
15395r1	E3 ubiquitin-protein ligase HACE1	6.700
13526r1	Transcription factor protein	6.303
30247r1	No hits found	5.914
01216r1	Ankyrin repeat and SOCS box protein 2	5.658
V^{V} down		
31898r1	mRNA, endostyle-specific	0.094
32728r1	Lipopolysaccharide-binding protein	0.098
15550r1	Sodium-dependent proline transporter	0.099
03538r1	Predicted protein	0.104
15550r1	Sodium-dependent proline transporter	0.106
06819r1	Cubilin	0.119
10261r1	mRNA, endostyle-specific	0.122
10808r1	glucose transporter	0.156
15810r1	Putative uncharacterized protein	0.164
ciem806h18	Putative uncharacterized protein	0.176

[a] Fold changes are indicated as the average of V^{IV}:C or V^{V}:C ratio for dye-swap experiments.

Table 4. Induction or repression of gene expression for several categories of proteins

Cluster ID	EST sequence similarities (BLASTX results)	V^{IV} treatrd Fold change [a]	V^{V} treatrd Fold change [a]
***Ci*-Vanabin**			
06625r1	*Ci*-Vanabin1	1.626	1.600
13989r1	*Ci*-Vanabin2	N.D.	N.D.
04798r1	*Ci*-Vanabin3	1.854	1.969
02771r1	*Ci*-Vanabin4	0.783	0.465
00859r1	*Ci*-Vanabin5	1.930	1.776
Vacuolar H^+-ATPase			
13148r1	Vacuolar H^+-ATPase V0 sector, subunit a	1.432	1.258
01661r1	Vacuolar H^+-ATPase V0 sector, subunits c/c'	1.607	1.061
02915r1	Vacuolar H^+-ATPase V0 sector, subunit c''	1.266	1.025
36592r1	Vacuolar H^+-ATPase V0 sector, subunit d	1.693	1.247
00203r1	Vacuolar H^+-ATPase V1 sector, subunit A	2.181	1.664
10226r1	Vacuolar H^+-ATPase V1 sector, subunit B	2.101	1.633
03693r1	Vacuolar H^+-ATPase V1 sector, subunit C	1.514	1.543
04491r1	Vacuolar H^+-ATPase V1 sector, subunit D	1.600	2.113
06374r1	Vacuolar H^+-ATPase V1 sector, subunit E	1.817	1.100
06857r1	Vacuolar H^+-ATPase V1 sector, subunit F	5.096	2.056
00005r1	Vacuolar H^+-ATPase V1 sector, subunit G	2.043	1.180
12728r1	Vacuolar H^+-ATPase V1 sector, subunit H	1.897	1.852
Glutathione related protein			
07652r1	Glutamate-cysteine ligase catalytic subunit (GCLC)	1.413	0.917
04033r1	Glutamate-cysteine ligase modifier subunit (GCLM)	2.234	2.153
31686r1	Glutathione synthetase (GS)	3.099	0.860
10178r1	Gamma-glutamyltranspeptidase 1 precursor (GGT)	2.718	0.742
04792r1	Glutathione reductase (GR)	1.532	1.263
10702r1	Glutathione peroxidase precursor (GPX)	0.510	0.441
30138r1	Glutathione S-transferase II (GST)	2.791	0.337
08515r1	Phytochelatin synthase	1.518	1.040
07189r1	Multidrug resistance-associated protein (MRP)	3.493	1.116
12664r1	Multidrug resistance protein 1a (MDR)	2.496	1.053
Heavy metal related transporter			
11242r1	Heavy metal exporter HMT1, ABC superfamily	2.404	1.521
11447r1	Zinc transporter (SLC39A7)	2.103	1.507
12032r1	Mn^{2+} and Fe^{2+} transporters of the NRAMP family	1.874	1.409
15864r1	Iron transporter (SLC40A1)	1.056	1.693
ciem812e12	Cation transport regulator-like protein 1 (CHAC1)	3.899	1.103
Redox related protein			
02251r1	Glutaredoxin	2.213	0.685
07147r1	Thioredoxin domain-containing protein 1	1.279	1.921
34064r1	Thioredoxin domain-containing protein 3 homolog	0.461	0.603
08610r1	Thioredoxin reductase 1	2.827	1.457
00138r1	Peroxiredoxin	4.104	2.386
02869r1	Peroxiredoxin-6	0.568	0.632
12272r1	Sulfiredoxin-1	3.342	1.663
32174r1	6-phosphogluconate dehydrogenase (6PGDH)	1.563	1.227

[a] Fold changes are indicated as the average of V^{IV}:C or V^{V}:C ratio for dye-swap experiments.