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MARINE BIOTECHNOLOGY

Expressed Sequence Tag Analysis of Vanadocytes in a Vanadium-Rich Ascidian, *Ascidia sydneiensis samea*

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Abstract: Some species in the family Ascidiidae accumulate vanadium at concentrations in excess of 350 mM, which corresponds to about 10^7 times higher than that in seawater. In these species signet ring cells, with a single huge vacuole in which vanadium ion is contained, function as vanadium-accumulating cells, vanadocytes. To investigate the mechanism underlying this phenomenon, we performed an expressed sequence tag (EST) analysis of a complementary DNA library from vanadocytes of a vanadium-rich ascidian, *Ascidia sydneiensis samea*. We determined the nucleotide sequences of 1000 ESTs and performed a BLAST analysis against the SwissProt database. We found 93 clones of metal-related gene homologues, including the ferritin heavy subunit, hemocyanin, and metallothionein. Two ESTs, in particular, exhibited significant similarity to vanabins that have been extracted from *A. sydneiensis samea* blood cells as low molecular weight vanadium-binding proteins. We have named the genes encoding these ESTs *vanabin3* and *vanabin4*. Immobilized metal ion affinity chromatography revealed that these novel vanabin homologues bind vanadium(IV) ions.

Key words: EST, vanadium, vanabin, metal accumulation, ascidian, ferritin.

INTRODUCTION

Ascidians, also known as tunicates or sea squirts (Chordata, Urochordata, Ascidiacea), accumulate extremely high concentrations of vanadium (Henze, 1911; Michibata et al., 2003). In particular, species belonging to the family Ascidiidae are known to accumulate vanadium in excess of 350 mM, which corresponds to about 10^7 times the concentration of vanadium ion normally dissolved in seawater (Michibata et al., 1986). Vanadium ions accumulate in a vacuole within vanadocytes, a type of blood (coelomic) cell (Michibata et al., 1987; Ueki et al., 2002). The vanadium stored in the vacuoles is reduced from +5 oxidation state in the seawater to +4 and then +3 oxidation states (Kanamori and Michibata, 1994), and the vacuoles also contain high concentrations of protons and sulfate ions (Frank et al., 1986; Hirata and Michibata, 1991).

NADPH can reduce vanadium(V) to vanadium(IV) in vitro (Kanamori et al., 1999). Four types of enzyme that are related to the pentose phosphate pathway that produces NADPH are located in vanadocytes (Uyama et al., 1998a, 1998b, 1998c; Ueki et al., 2000). Furthermore, cDNAs for each of the vacuolar-type H^+-ATPase (V-ATPase) A, B, and

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C subunits, which are located on the vacuolar membranes of vanadocytes, have been isolated and analyzed. V-ATPase generates a proton-motive force that is thought to provide the energy for vanadium accumulation (Uyama et al., 1994; Ueki et al., 1998, 2001). In addition, 3 types of vanadium-binding protein, vanabins, with molecular masses of 12.5, 15, and 16 kDa, have been isolated (Kanda et al., 1997), and their cDNA sequences have been determined. The former 2 vanabins, vanabin1 and vanabin2, have been shown to bind vanadium(IV) (VO^{2+}) ions specifically (Ueki et al., 2003).

To clarify the unusual mechanism by which vanadium is accumulated and reduced, we require knowledge of genes other than those mentioned above. An expressed sequence tag (EST) analysis is a powerful tool to investigate cell and tissue function and the corresponding profiles of expressed genes. Gene expression profiles based on EST analysis of fertilized eggs, embryos, and several tissues have been reported on *Ciona intestinalis* (0.6 mM vanadium in blood cells) (Nishikata et al., 2001; Ogasawara et al., 2002; Satou et al., 2001; Takamura et al., 2001), and *Halocynthia roretzi* (0.01 mM) (Makabe et al., 2001). However, these are vanadium-poor ascidian species (Michibata et al., 1986). EST analysis on the blood cells of vanadium-rich ascidians (Michibata et al., 1986, 1991), such as *A. sydneiensis samea* (13 mM), *Phallusia mammillata* (20 mM), *A. ahodori* (60 mM, and *A. gemmata* (350 mM), have not yet been analyzed.

We previously performed EST analysis on whole blood cells from a vanadium-rich ascidian species, *A. sydneiensis samea* (Yamaguchi et al., 2002). Although various metal-related genes were found, no vanadium-related genes such as vanabins were found. Furthermore, the source of the library was not only vanadocytes but also included many other types of blood cells. Here, we report an extended EST analysis of the cDNA library from purified vanadocytes from *A. sydneiensis samea*. We sequenced 1000 cDNA clones (647 independent clones) obtained from the vanadocyte cDNA library. A BLAST search performed against protein sequences registered in the SwissProt database revealed additional metal-related proteins that were not found in the previous study, including novel vanabins.

Materials and Methods

Isolation of Vanadocytes from *A. sydneiensis samea*

Adult specimens of a vanadium-rich ascidian, *A. sydneiensis samea*, were collected near the Otsuchi Marine Research Center, part of the Ocean Research Institute of the University of Tokyo, in Otsuchi (Iwate Prefecture), Japan. Blood was drawn from each specimen by making an incision at the lower part of the tunic. To separate the blood cells from serum, whole blood was suspended in ASF-103 medium and NaCl (1:24 mixture of 5 M NaCl and ASF103 medium, Ajinomoto, Japan) to adjust for osmotic pressure against seawater, and centrifuged (140 *g*, 10 minutes, 4°C). The blood cells were resuspended in ASF-103 medium and NaCl containing 20% sucrose and then centrifuged (140 *g*, 10 minutes, 4°C) to remove the giant cells, which have a highly acidic content but no vanadium (Michibata et al., 1990). The remaining blood cells were suspended in 2 ml ASF-103 medium and NaCl.

To separate vanadocytes from other blood cells, Percoll discontinuous density-gradient centrifugation was performed twice. Percoll (Amersham Biosciences Corp.) was dialyzed overnight against Ca^{2+} and Mg^{2+}-free artificial seawater (CMFASW: 460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 6 mM $NaHCO_3$, 5 mM HEPES, and 5 mM EDTA, at pH 7.0) and mixed with ASF-103 medium and NaCl to adjust the final concentration for separation. For the first separation a Percoll gradient containing 2 ml each of 20%, 40%, 60%, and 100% (w/v) Percoll solution was prepared in 10-ml centrifugation tubes. A suspension of blood cells (2 ml) was layered onto the surface of the discontinuous gradient, and the tubes were centrifuged (500 *g*, 20 minutes, 4°C). Vanadocytes were separated on the 40% Percoll layer; therefore, this fraction was collected, rinsed with ASF103 medium and NaCl and centrifuged (140 *g*, 10 minutes, 4°C). The rinsed vanadocyte fraction was resuspended in 2 ml ASF-103 medium and NaCl; the Percoll density-gradient centrifugation procedure was then repeated with 20%, 30%, 40%, and 50% (w/v) Percoll solution as stated above.

RNA Extraction and Construction of the Vanadocyte cDNA Library

Separated vanadocytes were suspended in a solution containing 4 M guanidine thiocyanate, 0.1% sodium N-lauryl sarcosinate, 5 mM EDTA, and 40 mM Tris-HCl at pH 7.0 and homogenized by ultrasonication. The homogenate was added to a solution of 50% cesium trifluoroacetate and 100 mM EDTA and centrifuged (100,000 *g*, 16 hours, 15°C) in an ultracentrifuge (model 70P72, Hitachi, Japan). Precipitated RNA was recovered and dissolved in sterilized RNase-

free water. A cDNA library was constructed from 30 μg total RNA using the Uni-Zap XR vector (Stratagene).

DNA Sequencing and BLAST Analysis

The recombinant λ-ZAPII vector was transferred into pBluescript SK^- plasmids by in vivo excision according to the manufacturer's instructions (Stratagene). We checked cDNA fragment insertion with direct polymerase chain reaction (PCR) and selected clones larger than 300 bp. The PCR mixture was denatured at 95°C for 2 minutes and then cycled 30 times with 3 pmol M13 forward and reverse primers at 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds, with a final 10-minute cycle at 72°C. After insertion were checked by electrophoresis, the second PCR was performed to amplify the template for direct sequencing using 1 μl of first PCR product and 1 pmol M13 forward primer. The second PCR product was used for sequencing by ThermoSequenase (Amersham Biosciences Corp.) with a modified T3 primer (5′-CCA TGA TTA CGC CAA GCT CGA AA-3′) and the DNA sequencer ALF ExpressII (Amersham Biosciences Corp.). For sequencing, PCR was carried out according to the manufacture's protocol. Using the program BLASTX (Altschul et al., 1997), a DNA sequence of approximately 500 bp from each cDNA clone was compared to sequences in the SwissProt database to identify related proteins. Several cDNA clones of particular interest were sequenced completely; cDNA with sequence similarity probability greater than 10^{-5} was classified as nonsimilar; sequences with a similarity probability less than 10^{-5} were classified as similar.

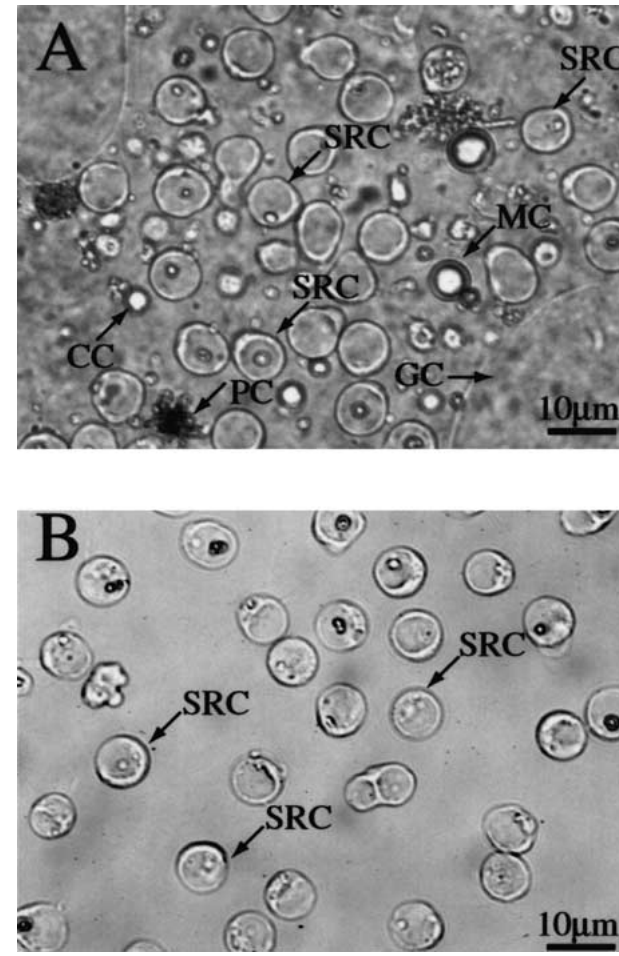

Figure 1. Purification of signet ring cells (vanadocytes) from *A. sydneiensis samea*. **A**: Whole blood cells. **B**: Vanadocytes purified by Percoll discontinuous density-gradient centrifugation. CC, compartment cell; GC, giant cell; MC, morula cell; PC, pigment cell; SRC, signet ring cell (vanadocyte). The proportion of intact vanadocytes was 91.1%.

Preparation of Recombinant Protein and Purification of Vanabin Homologues

Isolated vanabin genes were used to examine vanadium ion binding. To construct plasmids for the expression of fusion proteins with maltose binding protein (MBP), cDNA fragments of vanabin homologues spanning the coding region of 2 novel vanabin homologues, vanabin3 and vanabin4, were amplified by PCR using specific primer sets to which the following artificial restriction sites had been added: vanabin3 forward/reverse primer, 5′-GGA ATT CTA CCC TGA TGT GAT TGC-3′/5′-GGT CGA CTC AAA ATC GAA GAT GAC A-3′; vanabin4 forward/reverse primer, 5′-GGA ATT CAT GGA TTC ATG CAA AAC-3′/5′-GGT CGA CTT AAT GAC AAT TTT-3′. The amplified fragments were digested with *Eco*RI and *Sal*I and then ligated into the corresponding site of pMAL-c2X (New England BioLabs Inc.). The vanabin coding regions were ligated to the Tac promoter and the coding region for MBP of the vector to produce a fusion protein. The plasmid was introduced into a primary bacterial host, *Escherichia coli* DH5α and then transferred to the BL21 strain. An overnight culture of noninduced BL21 cells bearing vanabin-expressing plasmids was diluted 1:10 in LB medium containing 50 μg/ml ampicillin and 0.25 mM IPTG (Wako, Japan) and then cultured at 37°C for 8 hours. Bacterial cells were collected by centrifugation (2,500 *g*, 10 minutes, 4°C) and sonicated in amylose resin column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, 10 mM mercaptoethanol). The fusion protein was purified

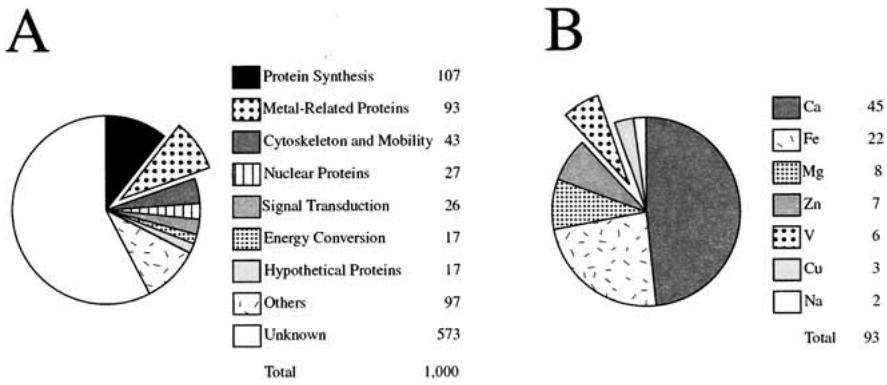

Figure 2. **A**: Summary of 1000 expressed sequence tag (EST) clones obtained from the vanadocyte cDNA library of *A. sydneiensis samea*. Clones were classified into 9 categories; 427 clones were similar to proteins registered in the SwissProt database; no similar proteins were detected for the remaining 573 clones. **B**: Metal-related EST clones classified according to related metals. Six of the vanabin homologues were placed in the vanadium category.

by a chromatography with an amylose resin column according to the manufacturer's protocol (New England BioLabs Inc.). The purities of fusion proteins were assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were measured with a protein assay (BioRad Laboratories Inc.) using bovine serum albumin (Pierce Inc.) as a standard.

To cut off recombinant vanabin4 from the fusion proteins, proteins were digested with 1:200 (wt/wt) factor Xa (Haematologic Technologies Inc.) for 16 hours at 30°C in elution buffer for amylose resin column. Vanabin4 was separated by DEAE-Sephacel anion exchange chromatography in 50 mM Tris-HCl (pH 8.5).

Vanadium Binding Assay with Immobilized Metal Ion Affinity Chromatography

Proteins (50 μg/250 μl) were incubated with 1 mM EDTA for 1 hour at 4°C and then dialyzed against a phosphate buffer (20 mM phosphate, pH 7.4, 100 mM NaCl) overnight at 4°C. For immobilized metal ion affinity chromatography (IMAC) preparation, 250 μl of slurry (Chelating Sepharose Fast Row, Amersham Biosciences Corp.) was washed in 1 ml of milli-Q water, then centrifuged (600 *g*, 2 minutes, 4°C), and the supernatant was removed. This process was repeated 4 times. Vanadium was immobilized by adding 1 ml of 100 mM $VOSO_4$ (99.9% pure, Wako, Japan) to the slurry and shaking the solution for 30 minutes at room temperature (RT). Vanadium-immobilized resin was washed in milli-Q water (4 times) and the phosphate buffer (2 times) in the manner described above. Dialyzed proteins were added to the vanadium-immobilized resin and shaken for 30 minutes at RT. After centrifugation the supernatant was kept as a nonbound fraction, and the resin was washed twice in column buffer. For elution we used 250 μl column buffer sequentially at pH 5.5, pH 3.5, and with 50 mM EDTA. Eluted proteins were analyzed by SDS-PAGE.

Results and Discussion

In this study we constructed a cDNA library from vanadocytes purified by Percoll density gradient centrifugation from blood cells (approx. 1 *g* wet weight) without giant cells from about 30 adult *A. sydeneiensis samea* specimens. About 250 mg of vanadocytes was recovered (91.1% purity; Figure 1) and used for RNA extraction and cDNA library construction. About 4.0×10^6 primary recombinant clones were used.

The nucleotide sequences of a 1000 cDNA clones randomly selected from the vanadocyte cDNA library were compared to sequences in the SwissProt database. Of the 1000 clones, the sequences of 427 were similar and those of 573 were nonsimilar to known proteins. As shown in Figure 2(A), cDNAs with sequence similarity were placed in one of the following 8 categories, according to the function of the gene: protein synthesis (107 clones), metal-related proteins (93 clones), cytoskeleton and motility (43 clones), nuclear proteins (27 clones), signal transduction (26 clones), energy conversion (17 clones), hypothetical proteins (17 clones), and others (97 clones). Ninety-three metal-related clones were further subdivided into 7 categories (Figure 2B). Similar proteins, with similarity scores and frequencies for each EST, are presented in Table 1.

The heavy subunit of ferritin, an iron storage protein, was found in 15 clones. The nucleotide sequences did not match completely, but the few differences among the clones are likely due to PCR errors or variation among individuals. Therefore, we believe that these nucleotide sequences are encoded by a single gene locus. A representative se-

Table 1. Metal-Related ESTs Identified from the Vanadocytes of *Ascidia sydneiensis samea*[a]

EST no.	Metal	Closest protein	Species	Prob.	Freq.
Asy-sig-009	Ca	Thrombospondin 1 precursor	Mouse	1e-12	4
Asy-sig-047	Ca	Fibrillin 1 precursor	Mouse	1e-08	25
Asy-sig-083	Ca	Calmodulin	Coral	2e-51	
Asy-sig-087	Ca	Microfibril-associated glycoprotein 4.	Human	1e-18	
Asy-sig-095	Ca	Annexin A13	Human	1e-29	
Asy-sig-105	Ca	Fibulin-1 precursor	Chicken	8e-21	
Asy-sig-130	Ca	45-kDa Calcium-binding protein precursor	Mouse	2e-39	
Asy-sig-141	Ca	Fibrillin 2 precursor	Human	4e-34	
Asy-sig-227	Ca	Calreticulin precursor	Human	1e-26	
Asy-sig-252	Ca	Clathrin light chain B	Rat	6e-30	
Asy-sig-342	Ca	Integrin α9 precursor	Human	3e-16	
Asy-sig-382	Ca	Calcineurin B subunit isoform 1	Mouse	3e-13	
Asy-sig-466	Ca	Annexin A13	Dog	2e-33	2
Asy-sig-481	Ca	Protein-glutamine glutamyltransferase	Human	2e-14	
Asy-sig-678	Ca	Programed cell death protein 6	Human	2e-20	
Asy-sig-758	Ca	Annexin V	Human	1e-12	
Asy-sig-966	Ca	Integrin α1	Human	8e-06	
Asy-sig-119	Fe	Cytochrome *c* oxidase polypeptide I	Fish	2e-78	3
Asy-sig-145	Fe	Soma ferritin	Chicken	5e-26	15
Asy-sig-242	Fe	Adrenodoxin	Sheep	3e-27	
Asy-sig-564	Fe	Arachidonate 5-lipoxygenase	Human	2e-30	
Asy-Sig-731	Fe	Ubiquinol-cytochrome *c* reductase	Rat	8e-28	
Asy-sig-790	Fe	Cytochrome B-245 light chain	Bovine	2e-14	
Asy-sig-062	Mg	Long-chain-fatty-acid-CoA ligase 2	Mouse	3e-26	2
Asy-sig-185	Mg	Pyruvate kinase, M2 isozyme	Rat	1e-55	
Asy-sig-353	Mg	Glutathione reductase precursor	Human	2e-20	
Asy-sig-475	Mg	Matrix metalloproteinase-14 precursor	Human	2e-18	3
Asy-sig-650	Mg	Protein phosphatase 2C δ isoform	Mouse	1e-13	
Asy-sig-074	Zn	Geranylgeranyl transferase type II	Human	5e-27	
Asy-sig-516	Zn	Zinc-finger protein zpr1	Yeast	7e-15	
Asy-sig-533	Zn	ADAM-TS 1 precursor	Mouse	2e-07	2
Asy-sig-654	Zn	DnaJ homolog subfamily B member 12	Mouse	2e-14	
Asy-sig-734	Zn	Phosphate regulating endopeptidase	Human	4e-27	
Asy-sig-931	Zn	PDZ and LIM domain protein 1	Rat	4e-16	
Asy-sig-298	**V**	**Vanabin 1**	Ascidian	1e-71	4
Asy-sig-322	**V**	**Novel vanabin (vanabin3)**	Ascidian	5e-14	
Asy-sig-972	**V**	**Novel vanabin (vanabin4)**	Ascidian	5e-46	
Asy-sig-715	Cu	Metallothionein	Fish	3e-07	2
Asy-sig-903	Cu	Hemocyanin G-type, units ODA to ODG.	Octopus	9e-19	
Asy-sig-245	Na	Amiloride-sensitive brain sodium channel	Rat	4e-23	
Asy-sig-411	Na	Na/K-transporting ATPase α1 chain	Human	2e-62	

[a]Using the program BLASTX, EST sequences were compared with the SwissProt database to identify related proteins without vanabins. Novel vanabins were compared with known vanabins by a private BLASTX program. The EST number and results of the homology search (closest protein name, species, probability, and frequency) are shown.

quence is shown in Figure 3. Ferritin consists of 24 subunits of 2 types—namely, heavy and light subunits—which form a shell-like structure with a hollow interior (Aisen et al., 1999). Although we also found a ferritin H-subunit gene

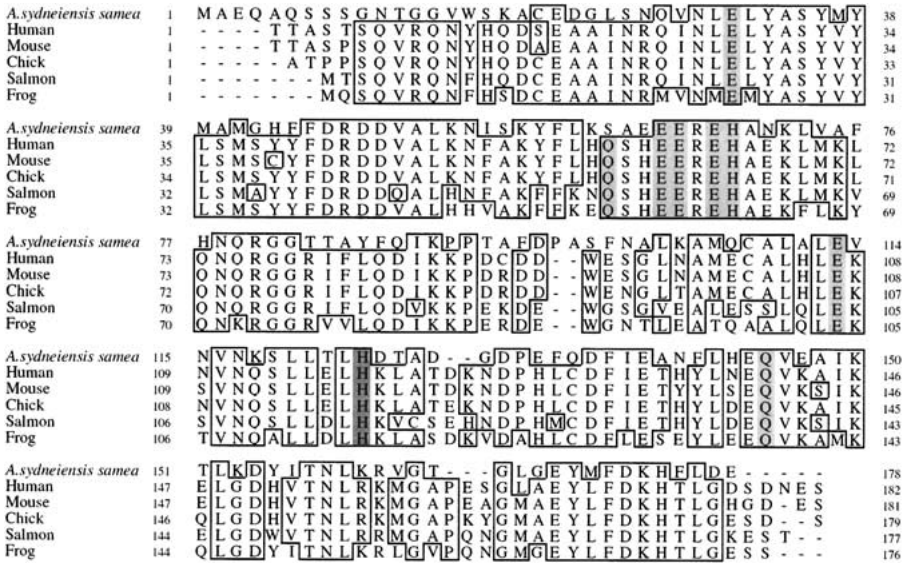

Figure 3. Comparison of the ferritin heavy subunit homologue sequence from *A. sydneiensis samea* with the ferritin heavy subunit of human (SwissProt accession number P02794), mouse (P09528), chicken (P08267), salmon (P49946), and frog (P49948). Conserved residues are boxed and putative iron-binding residues are shaded light gray. Histidine residues (reported to be a vanadium-binding site in the human ferritin heavy subunit) are shaded dark gray.

with a high frequency in the present study as well as in a previous study (Yamaguchi et al., 2002), the L subunit was not found. Several studies have revealed that vanadium is incorporated into ferritin (Sabbioni et al., 1980; Sabbioni and Marafante, 1981; De Cremer et al., 2002), and site-directed mutagenesis studies have suggested that His-118 in human ferritin heavy subunit is a vanadium-binding site (Grady et al., 2000). A histidine residue corresponding to the vanadium-binding site is also conserved in the ascidian ferritin heavy subunit. Collectively, these observations suggest that ferritin might play a role in the storage of vanadium in vanadocytes.

Hemocyanin, a large copper-containing respiratory protein found in arthropods and mollusks (Markl and Declcer, 1992; van Holde and Miller, 1995), was identified in our EST analysis as Asy-sig-903. Other oxygen-carrying proteins, such as hemoglobin, myoglobin, hemerythin, and chlorocruorin, were not found. EST and genome analysis of *Ciona intestinalis* have demonstrated that several types of hemocyanin subunit are expressed in the blood cells of this species (Dehal et al., 2002). These results suggested that hemocyanin may be a respiration protein in ascidians. All oxygen-carrying proteins have metal ions (ferric or cupric ions) to capture oxygen molecules. Following the observation that vanadium accumulates in ascidian blood cells, vanadium has been considered an oxygen-carrying cofactor in addition to copper and iron (Carlisle, 1968). Although several hypotheses have been proposed that support this contention, the ascidian oxygen-carrying molecules and the putative role of vanadium in vivo have not yet been confirmed (Michibata, 1996). To elucidate the role of vanadium, it needs to be ascertained whether vanadium is included in the native form of hemocyanin. In addition, whether hemocyanin functions as an oxygen-carrying protein needs to be analyzed.

In this study we identified 4 ESTs for vanabin1 and 2 novel vanabin genes, but homologues for vanabin2 were not found. Novel vanabins, which we have named vanabin3 and vanabin4, each had 18 conserved cysteines, in common with previously described vanabins, and the intervals of cysteine residues were highly conserved (Figure 4A). An identity matrix of the cysteine-rich regions of each of the vanabins is shown in Table 2. Comparison revealed that vanabin4 is most similar to vanabin2. The amino acid contents for the vanabins are shown in Figure 4(B). Vanabins have higher lysine contents, which have been suggested by ESR (electron spin resonance) studies to be binding sites for vanadium(IV) ions (Fukui et al., 2003). In vanabin3 there are between 3 and 7.5 times as many arginine residues as in other vanabins, but fewer lysine residues. The differences between vanabin2, vanabin3, and vanabin4 might be related to their different abilities to bind vanadium ions.

To examine vanadium-binding ability, we performed IMAC for vanabin2, vanabin3, and vanabin4, using *E. coli* MBP as a control (Figure 5). Vanabin3 was applied to IMAC as a fusion protein, because it precipitated after factor Xa digestion and was difficult to resolve, even with an 8 M urea solution. As a result, vanabin2, vanabin3, and vanabin4 were bound to the resin via VO^{2+} and eluted by EDTA, whereas MBP did not bind to the resin. The majority of vanabin2 and

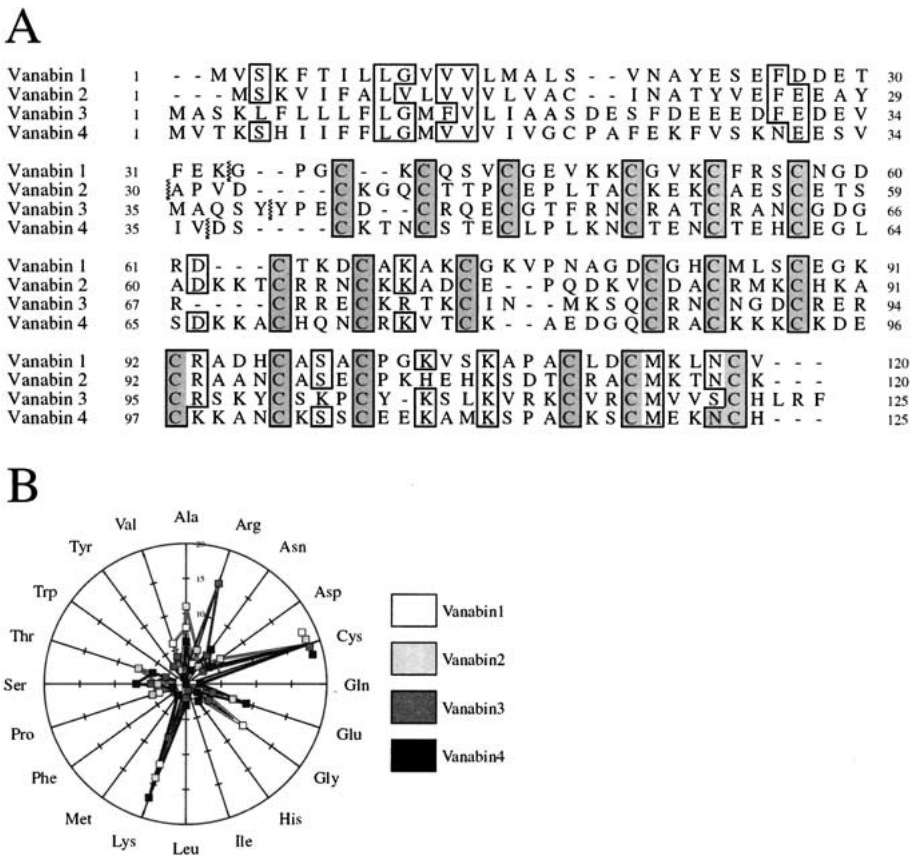

Figure 4. **A**: Amino acid sequences of vanabin1, vanabin2, and the novel vanabins, vanabin3 and vanabin4 (deduced from their cDNA clones, Asy-sig-322 and Asy-sig-972—see text for details). Conserved residues are boxed and cysteine residues are shaded. The wavy lines indicate the N-termini of mature vanabin1 and vanabin2 and initial residues for recombinant proteins from vanabin3 and vanabin4. **B**: Amino acid contents of vanabin1 (white), vanabin2 (gray), vanabin3 (dark gray), and vanabin4 (black).

Table 2. Identity Matrix Between Vanabins in Cysteine-rich Region

	Vanabin 1	Vanabin 2	Vanabin 3
Vanabin 1			
Vanabin 2	41%		
Vanabin 3	29%	31%	
Vanabin 4	37%	47%	29%

[a]Ratio of identity was calculated by PSI-BLAST.

vanabin4 bound to the vanadium-chelating resin. However, only a small proportion of vanabin3 did; this difference could be due to structural interference of MBP fused to vanabin3 or a low binding ability of vanabin3 itself. The binding abilities of vanabin2 and vanabin4 to VO^{2+} ion were not changed when they were used for IMAC as the fusion protein (data not shown). These results suggest that vanabin3 and vanabin4 can bind to vanadium(IV) immobilized in resin at least. We need to confirm the localization, metal selectivity, binding constant, and interactions with other proteins to elucidate the roles of vanabin3 and vanabin4 in the *A. sydneiensis samea* vanadocytes.

Metallothioneins are proteins that bind copper, cadmium, zinc, and other metals (Kägi, 1993). In the present study the EST clones Asy-sig-715 and Asy-sig-997 were similar to metallothioneins. In particular, the N-terminal protein sequences were identical to the cadmium-binding protein extracted from another ascidian species, *Pyura stolonifera* (Figure 6) (Liebrich et al., 1995). The fact that both the vanabins and metallothionein homologues were found in vanadocytes is particularly interesting. More information about ascidian metallothioneins will be useful to determine the function of vanabins and metallothioneins in the accumulation of vanadium.

In conclusion, this study has revealed several interesting genes that are expressed in vanadocytes, such as the ferritin H subunit, hemocyanin subunit, and metallothionein. Furthermore, 2 novel vanabins were identified, the gene products of which might be involved in accumulation and reduction of vanadium. In particular, it was determined that vanabin3 and vanabin4 are able to bind VO^{2+} ions. More detailed analysis of each of these genes will facilitate the elucidation of the vanadium accumulation system in ascidians.

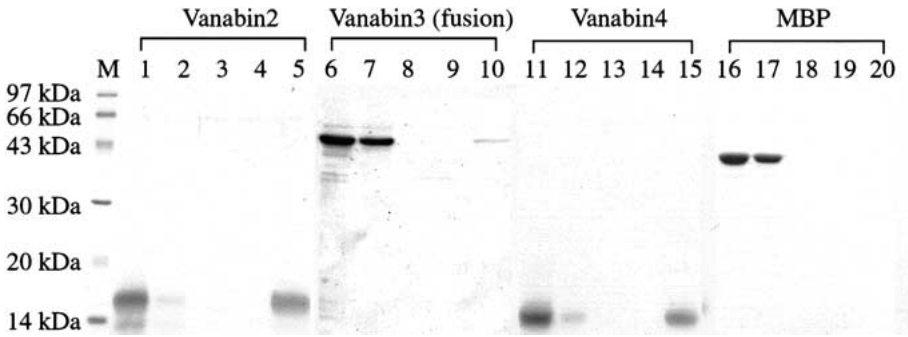

Figure 5. Assay of binding ability of vanabin2, vanabin3, vanabin4, and MBP to VO^{2+} ions by immobilized metal ion affinity chromatography (IMAC). Lane M, low molecular weight marker; lanes 1–5, vanabin2; 6–10, vanabin3; 11–15, vanabin4; 16–20, MBP. Lanes 1, 6, 11, and 16: prior to binding assay. Lanes 2, 7, 12, and 17: unbound fraction. Lanes 3, 8, 13, and 18: fraction eluted at pH 5.5. Lanes 4, 9, 14, and 19: fraction eluted at pH 3.5. Lanes 5, 10, 15, and 20: fraction eluted with 50 mM EDTA.

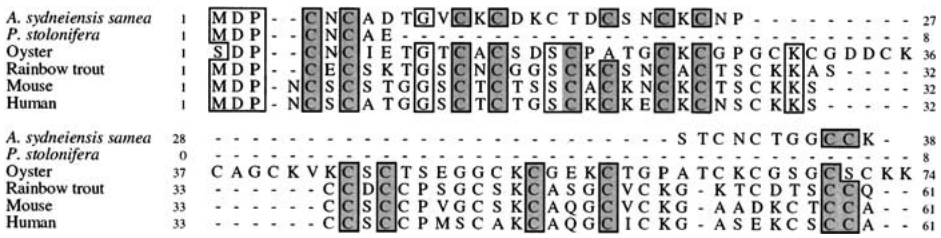

Figure 6. Comparison of amino acid sequences of metallothionein homologues from *A. sydneiensis samea*, *P. stolonifera* (not registered), oyster (P23038), rainbow trout (P09862), mouse (P02798), and human (P04731). Boxes and shaded regions

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References

Aisen, P., Wessling-Resnick, M., and Leibold, E.A. (1999). Iron metabolism. *Bioinorg Chem* 3:200–206.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.

Carlisle, D.B. (1968). Vanadium and other metals in ascidians. *Proc R Soc Lond B Biol Sci* 171:31–42.

De Cremer, K., Van Hulle, M., Chery, C., Cornelis, R., Strijckmans, K., Dams, R., Lameire, N., and Vanholder, R. (2002). Fractionation of vanadium complexes in serum, packed cells and tissues of Wistar rats by means of gel filtration and anion-exchange chromatography. *J Biol Inorg Chem* 7:884–890.

Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D.M., Harafuji, N, Hastings, KEM, HoI, Ho, I, Hotta, K., Huang, W., Kawashima, T., Lemaire, P., Martinez, D., Meinertzhagen, I.A., Necula, S., Nonaka, M., Putnam, N., Rash, S., Saiga, H., Satake, M., Terry, A., Yamada, L., Wang, H.G., Awazu, S., Azumi, K., Boore, J., Branno, M., Chin-bow, S., De Santis, R., Doyle, S., Francino, P., Keys, D.N., Haga, S., Hayashi, H., Hino, K., Imai, K.S., Inaba, K., Kano, S., Kobayashi, K., Kobayashi, M., Lee, B.I., Makabe, K.W., Manohar, C., Matassi, G., Medina, M., Mochizuki, Y., Mount, S., Morishita, T., Miura, S., Nakayama, A., Nishizaka, S., Nomoto, H., Ohta, F., Oishi, K., Rigoutsos, I., Sano, M., Sasaki, A., Sasakura, Y., Shoguchi, E., Shin-I, T., Spagnuolo, A., Stainier, D., Suzuki, M.M., Tassy, O., Takatori, N., Tokuoka, M., Yagi, K., Yoshizaki, F., Wada, S., Zhang, C., Hyatt, P.D., Larimer, F., Detter, C., Doggett, N., Glavina, T., Hawkins, T., Richardson, P., Lucas, S., Kohara, Y., Levine, M., Satoh, N., and Rokhsar, D.S. (2002). The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* 298:2157–2167.

Frank, P., Carlson, R.M.K., and Hodgson, K.O. (1986). Vanadyl ion EPR as a non-invasive probe of pH in intact vanadocytes from *Ascidia ceratodes*. *Inorg Chem* 25:470–478.

Fukui, K., Ueki, T., Ohya, H., and Michibata, H. (2003). Vanadium-binding protein in a vanadium-rich ascidian *Ascidia sydneiensis samea*: CW and pulsed EPR studies. *J Am Chem Soc* 125:6352–6353.

Grady, J.K., Shao, J., Arosio, P., Santambrogio, P., and Chasteen, N.D. (2000). Vanadyl(IV) binding to mammalian ferritins: an EPR study aided by site-directed mutagenesis. *J Inorg Biochem* 80:107–113.

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

Hirata, J., and Michibata, H. (1991). Valency of vanadium in the vanadocytes of *Ascidia gemmata* separated by density-gradient centrifugation. *J Exp Zool* 257:160–165.

Kägi, J.H.R. (1993). In: *Metallothionein III*, Suzuki, K.Y., Imura, N., and Kimura, M. (Eds.). Basel, Switzerland: Birkhäuser, pp 29–55.

Kanamori, K., and Michibata, H. (1994). Raman spectroscopic study of the vanadium sulfate in blood cell homogenate of the ascidian, *Ascidia gemmata*. *J Mar Biol Assoc UK* 74:279–286.

Kanamori, K., Sakurai, K., Kinoshita, M., Uyama, T., Ueki, T., and Michibata, H. (1999). Direct reduction from vanadium(V) to vanadium(IV) by NADPH in the presence of EDTA. A consideration of the reduction and accumulation of vanadium in the ascidian blood cells. *J Inorg Biochem* 77:157–161.

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

Liebrich, W., Brown, AC., and Botes, D.P. (1995). Cadmium-binding proteins from a tunicate, *Pyura stolonifera*. *Comp Biochem Physiol* 112C:35–42.

Makabe, K.W., Kawashima, T., Kawashima, S., Minokawa, T., Adachi, A., Kawamura, H., Ishikawa, H., Yasuda, R., Yamamoto, H., Kondoh, K., Arioka, S., Sasakura, Y., Kobayashi, A., Yagi, K., Shojima, K., Kondoh, Y., Kido, S., Tsujinami, M., Nishimura, N., Takahashi, M., Nakamura, T., Kanehisa, M., Ogasawara, M., Nishikata, T., and Nishida, H. (2001). Large-scale cDNA analysis of the maternal genetic information in the egg of *Halocynthia roretzi* for a gene expression catalog for ascidian development. *Development* 128:2555–2567.

Markl, J., and Decker, H. (1992). Molecular structure of the arthropod hemocyaninis. In: *Adv Comp Environ Physiol 13; Blood and Tissue Oxygen Carriers*, Mangum, C.P. (Ed.). Germany: Heidelberg: Springer, pp 325–376.

Michibata, H. (1996). The mechanism of accumulation of vanadium by ascidians: some progress toward an understanding of this unusual phenomenon. *Zool Sci* 13:489–502.

Michibata, H., Hirata, J., Uesaka, M., Numakunai, T., and Sakurai, H. (1987). Separation of vanadocytes: determination and characterization of vanadium ion in the separated blood cells of ascidian, *Ascidia ahodori*. *J Exp Zool* 244:33–38.

Michibata, H., Iwata, Y., and 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., and Numakunai, T. (1986). The accumulation and distribution of vanadium, iron, and manganese in some solitary ascidians. *Biol Bull* 171:672–681.

Michibata, H., Uyama, T., and Hirata, J. (1990). Vanadium containing cells (vanadocytes) show no fluorescence due to the tunichrome in the ascidian *Ascidia sydneiensis samea*. *Zool Sci* 7:55–61.

Michibata, H., Yamaguchi, N., Uyama, T., and Ueki, T. (2003). Molecular biological approaches to the accumulation and reduction of vanadium by ascidians. *Coordination Chem Rev* 234:41–51.

Nishikata, T., Yamada, L., Mochizuki, Y., Satou, Y., Shin-I, T., Kohara, Y., and Satoh, N. (2001). Profiles of maternal expressed genes in fertilized eggs of *Ciona intestinalis*. *Dev Biol* 238:315–33l.

Ogasawara, M., Sasaki, A., Metoki, H., Shin-I, T., Kohara, Y., Satoh, N., and Satou, Y. (2002). Gene expression profiles in young adult *Ciona intestinalis*. *Dev Genes Evol* 212:173–185.

Sabbioni, E., Rade, J., and Bertolero, F. (1980). Relationships between iron and vanadium metabolism: the exchange of vanadium between transferrin and ferritin. *J Inorg Biochem* 12:307–315.

Sabbioni, E., and Marafante, E. (1981). Relations between iron and vanadium metabolism: in vivo incorporation of vanadium into iron proteins of the rat. *J Toxicol Environ Health* 8:419–429.

Satou, Y., Takatori, N., Yamada, L., Mochizuki, Y., Hamaguchi, M., Ishikawa, H., Chiba, S., Imai, K., Kano, S., Murakami, D.S., Nakayama, A., Nishino, A., Sasakura, Y., Satoh, G., Shimotori, T., Shin-I, T., Shoguchi, E., Suzuki, M.M., Takada, N., Utsumi, N., Yoshida, N., Saiga, H., Kohara, Y., and Satoh, N. (2001). Gene expression profiles in *Ciona intestinalis* tailbud embryos. *Dev Biol* 238:315–331.

Takamura, K., Oka, N., Akagi, A., Okamoto, K., Okada, T., Fukuoka, T., Hogaki, A., Naito, D., Oobayashi, Y., and Satoh, N. (2001). EST analysis of genes that are expressed in the neural complex of *Ciona intestinalis* adults. *Zool Sci* 18:1231–1236.

Ueki, T., Takemoto, K., Fayard, B., Salomé, M., Yamamoto, A., Kihara, H., Susini, J., Scippa, S., Uyama, T., and Michibata, H. (2002). Scanning x-ray microscopy of living and freeze-dried

blood cells in two vanadium-rich ascidian species, *Phallusia mammillata* and *Ascidia sydneiensis samea*. *Zool Sci* 19:27–35.

Ueki, T., Uyama, T., Kanamori, K., and 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., Yamamoto, K., Kanamori, K., and 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., Uyama, T., Kanamori, K., and Michibata, H. (2001). Subunit C of the vacuolar-type ATPase from the vanadium-rich ascidian *Ascidia sydneiensis samea* rescued the pH sensitivity of yeast ma5 mutants. *Mar Biotechnol* 3:316–321.

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

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

Uyama, T., Moriyama, Y., Futai, M., and 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., Takahashi, H., Suhama, Y., Kanamori, K., and Michibata, H. (1998b). 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., and Michibata, H. (1998c). Glucose-6-phosphate dehydrogenase in the pentose phosphate pathway is localized in vanadocytes of vanadium-rich ascidian, *Ascidia sydneiensis samea*. *Zool Sci* 15:441–446.

van Holde, K.E., and Miller, K.I. (1995). Hemocyanins. *Adv Prot Chem* 47:1–81.

Yamaguchi, N., Togi, A., Ueki, T., Uyama, T., and Michibata, H. (2002). Expressed sequence tag analysis of blood cells in the vanadium-rich ascidian, *A. sydneiensis samea*—a survey of genes for metal accumulation. *Zool Sci* 19:1001–1008.