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**Novel vanadium-binding proteins (Vanabins) identified in cDNA libraries and the genome
of the ascidian *Ciona intestinalis***

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Abstract

Ascidians, especially those belonging to the suborder Phlebobranchia, can accumulate high levels of vanadium. Vanadium-binding proteins (vanabins) were first isolated from a vanadium-accumulating ascidian, *Ascidia sydneiensis samea*, and then the vanabins were cloned, their expression was studied, and metal-binding assays were conducted. In order to unravel the mechanism of vanadium accumulation, we searched for vanabin-like genes in other animals, including other ascidians. A database search revealed five groups of cDNAs that encoded vanabin-like proteins in another ascidian, *Ciona intestinalis*. The genes encoding *C. intestinalis* vanabins, *CiVanabin1* to *CiVanabin5*, were clustered in an 8.4-kb genomic region. The direction of transcription of each gene was identical and each gene had a single intron. All the *C. intestinalis* vanabins were cysteine rich, and the repetitive pattern of cysteines closely resembled that of *A. sydneiensis samea* vanabins. Using immobilized metal ion affinity chromatography, we found that a recombinant protein of at least one of the *C. intestinalis* vanabins (*CiVanabin5*) bound to vanadium(IV) ions.

1. Introduction

Ascidians (so-called sea squirts) belong to the subphylum Urochordata, class Ascidiacea, and are sessile marine animals that dwell in shallow ocean waters worldwide. More than 2,300 species have been identified and they are classified into four suborders: Aplousobranchia, Phlebobranchia, Stolidobranchia, and Aspiculata. Of these, ascidians belonging to the suborder Phlebobranchia are known to accumulate high levels of the transition metal vanadium [1, 2]. The highest concentration of vanadium found in the blood cells of *Ascidia gemmata* [3] is about 10^7 times higher than that in sea water (350 mM vs. 35 nM) [4, 5]. However, there is a very wide range in the levels of vanadium accumulated, from 0.1 μ M to 350 mM. For example, the concentrations of vanadium found in blood cells of *Halocynthia roretzi*, *Ciona intestinalis*, *Ascidia sydneiensis samea*, and *A. gemmata* were 0.007, 0.6, 12.8, and 350 mM, respectively [1, 3]. These interspecies differences might be quite meaningful when considering the physiological roles played by vanadium in ascidians, although the roles remain unclear.

Recently, we identified three vanadium-binding proteins (vanabins), previously called vanadium-associated proteins [6], from the cytoplasm fraction of vanadium-containing blood cells (vanadocytes) of the vanadium-rich ascidian *A. sydneiensis samea*. They include two major proteins with apparent molecular weights of 12.5 and 15 kDa, and a minor 16-kDa protein. We subsequently cloned cDNAs encoding the two major vanabins, named vanabin1 and vanabin2 [7]. Vanabins are rich in charged residues and a conserved motif in both vanabins can be described using the consensus sequence {C}-{X₂₋₅}-{C}. Using recombinant proteins of these two distinct vanabins, we revealed that they bind to 10 or 20 vanadium(IV) [VO²⁺] ions with dissociation constants of 2.1×10^{-5} and 2.3×10^{-5} M, respectively [7]. An EPR study showed that vanabin2 can bind up to ~23.9 vanadium ions per molecule and most of the vanadium ions are in a mononuclear state and coordinated by amine nitrogen [8]. Furthermore, the analysis of expression sequence

tags (ESTs) in an *A. sydneiensis samea* blood cell cDNA library has already identified two other cDNAs that encode proteins that are closely related to vanabins (unpublished data). These results suggest that vanabins compose a unique gene family.

Therefore, we examined whether ascidian species other than *A. sydneiensis samea* that accumulate relatively high or low levels of vanadium have vanabin-like genes. In this article, we report the discovery of five groups of cDNAs encoding vanabin-like proteins in the ascidian *C. intestinalis*, which accumulates relatively low levels of vanadium [1]. Each group of cDNAs was encoded by five independent genes, *CiVanabin1* to *CiVanabin5*, which were aligned in the same orientation in a 8.4-kb genomic region. We also report the cloning, expression, and vanadium-binding ability of one of the *C. intestinalis* vanabins (*CiVanabin5*). The discovery of new vanabins and the genes encoding the vanabins in *C. intestinalis* will be an important clue as to why there is a very wide range in the vanadium levels accumulated among ascidian species.

2. Materials and methods

2.1. Cloning and production of recombinant *CiVanabin5*

The cDNAs for *C. intestinalis* vanabins were provided by Dr. N. Satoh and Dr. Y. Satou (Dept. of Zoology, Graduate School of Science, Kyoto University, Japan). We prepared plasmid DNA, and the DNA sequences were determined using an ALF Express II DNA sequencer (Amersham Pharmacia Biotech).

The cDNA region corresponding to the putative mature cysteine-rich region of *CiVanabin5* was amplified by PCR using specific primer sets containing restriction sites. The forward and reverse primers were 5'-GGA ATT CAA CAA GAA ACT TGG-3' and 5'-GGT CGA CTT ATT CTG TTT CGC C-3', respectively, which correspond to N-K-K-L-G and E-T-E-stop.

Before performing PCR, a computer simulation was done using Amplify software. The PCR mixture contained plasmid DNA (60 ng), 50 pmol of each primer, 10 mM dNTPs, 1 × PCR buffer, and rTaq DNA polymerase (TaKaRa, Inc.) in a reaction volume of 50 µl. The PCR conditions were as follows: 94°C for 2 min (hot start), 30 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 90 sec, and a final extension at 72°C for 5 min.

The PCR-amplified fragments of the expected length were gel purified and directly cloned into pBluescript vector at the *EcoRV* site with T-overhangs. Plasmid DNA was prepared and the correct insertion was verified by DNA sequencing. The plasmid DNA was then digested with *EcoRI* and *SalI*, and ligated into the corresponding site of the pMAL-c expression vector (New England Biolabs Inc.). This vector has a *lac* promoter and the region encoding *E. coli* maltose binding protein (MBP), to which the *CiVanabin5* coding region was ligated to produce a fusion protein. The plasmid DNA was introduced into *E. coli* TB1 strain. An insert check was performed by PCR using primers MAL-F and MAL-R, which have the sequences 5'-TCG AGG GAA GGA TTT CAG-3' and 5'-CAA GCT TGC CTG CAG GTC-3', respectively. The correct insertion was verified again by DNA sequencing.

An overnight culture of non-induced *E. coli* cells bearing the plasmid was diluted 1 : 10 (v/v) in LB medium containing 50 µg/ml ampicillin. The cells were cultured at 37°C for 6 h after adding 0.5 mM IPTG. The cultured cells were sedimented by centrifugation at 10,000×g, for 30 sec, at 4°C, and then resuspended in 1× lysis buffer containing 4 M urea. After sonication, the sample was dialyzed against 1× low salt column buffer, and centrifuged at 10,000×g, for 10 min at 4°C, to remove the insoluble fraction. The supernatant was subjected to amylose resin column chromatography and the fusion protein was prepared according to the manufacturer's instructions (New England Biolabs Inc.). The concentration of the fusion protein was measured with Bio Rad protein assay reagent (Bio Rad Laboratories Inc.). FactorXa (1 : 100, w/w) was added to the fusion protein and incubated at 25°C for 16 h to cut the junction between MBP and the vanabin. A

small aliquot from each chromatography and digestion step was analyzed by SDS-PAGE on a 15% acrylamide gel.

The plasmid for *A. sydneiensis samea* vanabin2 used in this study was the same one used in our previous study [7]. The pMAL-c plasmid containing the putative mature region of vanabin2 had been introduced into *E. coli* TB1 strain. The cells were cultured and the recombinant protein was obtained using the same procedure as described for *CiVanabin5* above.

2.2. Metal-binding assay using immobilized metal ion affinity chromatography

Chelating Sepharose Fast Flow Resin (250 μ l; Amersham Pharmacia Biotech) was added to an Eppendorf tube and washed twice with deionized water (DW). After each wash, the resin was re-sedimented by centrifugation at 600 \times g, for 2 min at RT, and the supernatant was discarded. Vanadium solution (1 ml of 0.1 M VO_2^{+} -n H_2O , n=3~4, in DW) was added to the resin and rotated for 15 min at RT. The resin was re-sedimented by centrifugation at 600 \times g, for 2 min at RT, and the supernatant was removed. The resin was washed four times with DW in order to remove the unbound excess vanadium ions. After each wash, the tube was centrifuged at 600 \times g, for 2 min at RT, and the supernatant was removed. The resin was then equilibrated by washing twice with start buffer (100 mM NaCl, 20 mM NaH_2PO_4 , pH 7.5). After each wash, centrifugation was repeated under the same conditions as described above and the supernatant was removed.

The FactorXa-digested protein was dialyzed overnight against start buffer and the insoluble fraction was removed by centrifuging at 10,000 \times g, for 10 min at 4°C. One milliliter of supernatant was added to the resin and it was then rotated for 30 min at RT. The resin was re-sedimented by centrifugation at 600 \times g, for 2 min at RT. The supernatant was stored at 4°C for SDS-PAGE analysis. The resin was washed twice with 1 ml start buffer. After each wash, the mixture was centrifuged under the same conditions as described above and the supernatant was

stored for SDS-PAGE analysis. The following elution buffers were used in successive steps in order to elute the recombinant protein bound to the vanadium ions immobilized on the resin: (1) 200 mM NaCl, 20 mM NaH₂PO₄, pH 7.5; (2) 300 mM NaCl, 20 mM NaH₂PO₄, pH 7.5; (3) 400 mM NaCl, 20 mM NaH₂PO₄, pH 7.5; (4) 500 mM NaCl, 20 mM NaH₂PO₄, pH 7.5; and (5) 50 mM EDTA, pH 8.0. One milliliter of elution buffer was applied to the resin and rotated for 30 min at RT. After centrifuging at 600×g, for 2 min at RT, the supernatant was stored for SDS-PAGE analysis. This process was repeated for each elution buffer in successive steps.

3. Results

A search of the EST database [9-16] of an ascidian, *Ciona intestinalis*, using vanabin1 and vanabin2 of *Ascidia sydneiensis samea* [7] with the program TBLASTN revealed five groups of cDNAs encoding vanabin-like proteins. Each group of cDNAs shared an identical nucleotide sequence, and the assembled nucleotide sequence of each group encoded an independent vanabin-like protein. By comparing the cDNA data and the *C. intestinalis* genome sequence [17], we found that these five groups of cDNAs were encoded by five independent genes and there were no other vanabin-like genes or pseudogenes in the genome. We named the five genes encoding group 1 to 5 vanabin-like proteins as *CiVanabin1* to *CiVanabin5*, respectively. The five genes were clustered in an 8.4-kb genomic region in scaffold 20. The genome map of the *C. intestinalis* vanabins is shown in Fig. 1. It was also evident that the direction of transcription of each gene was identical, and each gene had a single intron in very similar positions, between cysteines 6 and 7 (Figs. 1 and 2).

Table 1 shows the number of ESTs corresponding to the five *C. intestinalis* vanabin genes in libraries for twelve different developmental stages and tissues (data obtained from <http://ghost.zool.kyoto-u.ac.jp/>). Of the 239,086 ESTs examined, 362 ESTs were found to be

encoded by the five vanabin genes. Of the six different tissues examined, the most ESTs (78) were found in the endostyle for CiVanabin4, which was also prominent in the neural complex and heart. In contrast, only five ESTs were found in blood cells, of which three belonged to CiVanabin3 and two belonged to CiVanabin1. CiVanabin5 was expressed in all developmental stages from egg to young adult, while the others were found at specific developmental stages. Of the six developmental stages examined, the most ESTs (79) were found in the young adult stage for CiVanabin4.

The predicted amino acid sequences of the five *C. intestinalis* vanabins are highly conserved and were similar to the *A. sydneyensis samea* vanabins (Fig. 2). All the vanabins were cysteine rich and the intervals between the cysteine residues were very regular. The intervals between cysteines 6-7, 9-10, and 15-16 were less well conserved. In CiVanabin3, an additional cysteine was located between cysteines 5-6. Of note, CiVanabin1 and CiVanabin2 had long C-terminal domains, which were rich in aspartic acid and glutamic acid residues. They also had relatively long N-terminal domains, and appeared to have diverged from the other vanabins of *C. intestinalis* and *A. sydneyensis samea*.

The identity of the amino acid residues in the cysteine-rich region among vanabins from *A. sydneyensis samea* and *C. intestinalis* is shown in Table 2. The identity was calculated for the region from conserved cysteine 1 through 18, excluding the 18 conserved cysteine residues themselves. The highest identity (26%) was between CiVanabin1 and CiVanabin2, whereas the lowest identity (8%) was between CiVanabin4 and CiVanabin5. The identity between vanabin2 and each of CiVanabin1, CiVanabin2, and CiVanabin5 was relatively high (24%).

To examine whether the *C. intestinalis* vanabins can bind to vanadium, we performed a metal-binding assay using a batch immobilized metal ion affinity chromatography (IMAC) method. It is evident from Fig. 3A that recombinant CiVanabin5 protein bound to vanadium ions in the +4 oxidation state (vanadium(IV), [VO²⁺]) immobilized on the sepharose resin. The bound

vanabin was then eluted most strongly using elution buffer containing 400 mM NaCl. In contrast, the vanabin2 of *A. sydneiensis samea*, which bound to vanadium(IV) ions immobilized on the sepharose resin, was eluted most strongly by the elution buffer containing 50 mM EDTA (Fig. 3B). This suggests that the binding of recombinant vanabin2 to vanadium(IV) is stronger than that of recombinant CiVanabin5.

4. Discussion

In this study, we discovered five genes in the ascidian *C. intestinalis* that encode proteins homologous to vanabins, which we previously isolated from *A. sydneiensis samea* [6, 7]. Although we have identified low-molecular-weight proteins that react with anti-vanabin polyclonal and monoclonal antibody in several ascidian species [18] and a polychaete worm [19], this is the first study to clone vanabin genes from organisms other than *A. sydneiensis samea*.

The frequency of ESTs for one gene in each developmental stage and in each adult tissue roughly reflects the level of mRNA expression. Contrary to our expectations, *Ciona intestinalis* vanabin transcripts are not abundant in blood cells judged from the frequency of EST clones (Table 1). An exhaustive EST analysis of blood cells by Dr. Satoh's laboratory identified only five clones from two groups of vanabins (*CiVanabin1* and *CiVanabin3*) out of 28,596 ESTs. Instead, the greatest frequency was observed in the endostyle for *CiVanabin4*; the frequency reached 3% of the total ESTs determined from this tissue. Moreover, in the entire body of young adults, *CiVanabin4* transcripts comprised 0.27% of the total ESTs. The function of *CiVanabin4* in the endostyle will be the subject of future studies.

Comparing the EST and genome databases of *C. intestinalis*, we found that the five groups of vanabins were encoded by five independent genes. All five genes possess a single intron in the middle of their coding region, at very similar positions. Moreover, the five genes are

aligned in the same orientation as their transcription. These facts suggest that the five genes were derived from one ancestral gene by tandem gene duplication. We do not know how many vanabin genes exist in the *A. sydneiensis samea* genome and where they are located in the genome. As of yet, we have not identified vanabin homologues from organisms other than these two ascidian species in public DNA and protein databases. Limiting genomic research in this field will leave fundamental questions unanswered.

Using a batch IMAC method, we examined the vanadium-binding ability of one of the *C. intestinalis* vanabins (CiVanabin5), since CiVanabin5 shared the highest similarity with vanabin2 of *A. sydneiensis samea* in the first round of the EST database search. IMAC allows the qualitative analysis of the interaction between metals and proteins. Our metal-binding assay revealed that the recombinant CiVanabin5 bound to vanadium(IV) [VO²⁺] ions, and the vanabin was then eluted most strongly by the elution buffer containing 400 mM NaCl. We also performed IMAC analysis for vanabin2 of *A. sydneiensis samea* under the same conditions. Unlike CiVanabin5, the recombinant vanabin2 of *A. sydneiensis samea*, which bound to vanadium(IV), was eluted most strongly by elution buffer containing 50 mM EDTA. Since NaCl is thought to be a weaker competitor than EDTA, this suggests that the binding of CiVanabin5 is weaker than the binding of vanabin2 of *A. sydneiensis samea*. In this context, it is significant that the vanadium concentrations in the blood cells of *C. intestinalis* and *A. sydneiensis samea* are 0.6 and 12.8 mM, respectively. One probable explanation for the lower level of vanadium in *C. intestinalis* is the weaker binding ability of its vanabin. We are performing similar analysis for the other four *C. intestinalis* vanabins, especially the vanabins expressed in blood cells (*CiVanabin1* and *CiVanabin3*), as well as experiments to determine the dissociation constants and maximum number of vanadium ions per protein, such as using Hummel-Dreyer's method and ESR titration experiments [8]. Using Hummel-Dreyer's method, we previously found that vanabin1 and vanabin2 of *A. sydneiensis samea* bind 10 and 20 vanadium(IV) ions with dissociation constants of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively [7].

Previously, we identified proteins similar to vanabin in several ascidians and a polychaete that accumulate vanadium using anti-vanabin antibodies. A monoclonal antibody, F8DH, against *A. sydneyensis samea* vanabin2 and a related 16-kDa protein showed high, moderate, and low reactivity in blood cells of *A. sydneyensis samea*, *Ascidia ahodori*, and *Ciona intestinalis*, respectively [18]. We have also reported that the polychaete *Pseudopotamilla ocellata* possesses antigens recognized by a polyclonal antibody against vanabins [19]. Since Henze's discovery of high levels of vanadium in ascidian blood cells [2], several marine animals have been reported to be vanadium accumulators, including holothurians and nudibranchs [20-23], but *Pseudopotamilla ocellata* is the only undoubted non-ascidian vanadium accumulating animal [24]. This species contains vanadium in the branchial crown at a concentration of 1.75 mg vanadium/g wet weight [24], which is comparable with that in the blood cells of ascidians [1]. Although the family Polychaete is distant from the family Ascidiidae phylogenetically, it might have a mechanism of vanadium-accumulation similar to that in ascidians.

Vanabins are thought to play a key role as a vanadium carrier protein in the cytoplasm of vanadium-accumulating cells, and the analysis of the genes encoding the vanabins in *C. intestinalis* will provide important clues to resolve the reason for the different levels of vanadium accumulation. We have revealed that most EPR-active VO^{2+} ions bound to vanabins coordinate amine nitrogen ligands, suggesting that almost all amine nitrogens are used in vanadium-saturated vanabins [8]. The number of lysines, which account for most of the amine nitrogen, varies in each vanabin. For example, *A. sydneyensis samea* vanabin1 and vanabin2 have 12 and 13 lysines within the conserved region (83 and 86 amino acids) between cysteines 1 and 18, respectively. For the *C. intestinalis* vanabins, CiVanabin1 to CiVanabin5 have 4 lysines out of 92 conserved amino acid residues, 11 out of 92, 7 out of 95, 6 out of 101, and 11 out of 91, respectively. These differences might affect the binding number and dissociation constant of vanadium ions to each protein directly or indirectly. A three-dimensional structural analysis of vanabins that is in progress will provide more information on how these relatively small proteins can bind such a

large number of metal ions.

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Table 1. A list of EST clones for *Ciona intestinalis* vanabins. Data are obtained from <http://ghost.zool.kyoto-u.ac.jp/> on February 22, 2003.

Group of ESTs	1	2	3	4	5	
cluster ID	06625	13989	04798	02771	00859	
Gene	<i>CiVanabin1</i>	<i>CiVanabin2</i>	<i>CiVanabin3</i>	<i>CiVanabin4</i>	<i>CiVanabin5</i>	
tissue or stage						total ESTs
eggs	0	0	0	0	5	29,444
cleaving embryos	0	1	0	0	24	26,796
gastrula & neurula	0	0	0	0	4	23,475
tailbud embryos	2	2	0	4	9	31,209
larvae	9	0	4	5	10	24,532
young adults	36	0	6	79	20	29,138
gonad	1	0	1	0	7	16,239
testis	1	0	1	0	0	4,717
endostyle	0	0	0	78	0	2,497
neural complex	2	0	0	20	1	10,029
heart	7	0	4	14	0	12,414
blood cell	2	0	3	0	0	28,596
total	60	3	19	200	80	239,086

Table 2. Identity of amino acid residues in cysteine-rich region among vanabins from *Ascidia sydneiensis samea* (As) and *Ciona intestinalis* (Ci). Identity was calculated excluding the conserved 18 cysteine residues.

		As		Ci			
		vanabin1	vanabin2	CiVanabin1	CiVanabin2	CiVanabin3	CiVanabin4
As	vanabin1						
	vanabin2	24%					
	CiVanabin1	18%	24%				
	CiVanabin2	15%	24%	26%			
Ci	CiVanabin3	10%	15%	15%	10%		
	CiVanabin4	14%	13%	11%	14%	17%	
	CiVanabin5	15%	24%	17%	14%	14%	8%

Fig. 2. The alignment of the amino acid sequences of vanabins from two ascidian species: *Ascidia sydneiensis samea* (vanabin1 and vanabin2) and *Ciona intestinalis* (CiVanabin1 to CiVanabin5). The 18 cysteine residues conserved among the seven vanabins are boxed and numbered. Other conserved amino acids are also boxed. The positions of the introns are indicated by triangles. The N-terminus of vanabin1 and vanabin2 determined by the Edman degradation method, as well as the N-terminus of recombinant CiVanabin5 used in this study, are indicated by arrows.

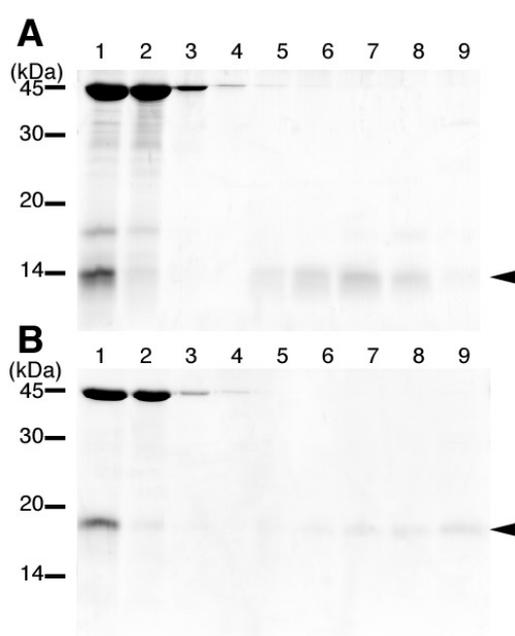


Fig. 3. Analysis of the metal-binding ability of (A) CiVanabin5 and (B) *Ascidia sydneiensis samea* vanabin2. The metal binding experiment was performed using 100 mM NaCl, 20 mM NaH₂PO₄, pH 7.5. Lane 1, recombinant proteins digested by FactorXa (a mixture of vanabin and maltose binding protein). Lane 2, proteins that do not bind to vanadium(IV) [VO²⁺] ions immobilized on the resin. Lanes 3 and 4, proteins in sequential fractions. Lanes 5-8, proteins eluted with 200 mM NaCl (lane 5), 300 mM NaCl (lane 6), 400 mM NaCl (lane 7), and 500 mM NaCl (lane 8). Lane 9, proteins eluted with 50 mM EDTA. The arrows indicate vanabins.