Identification of Vanabin-interacting protein 1 (VIP1) from blood cells of the vanadium-rich ascidian *Ascidia sydneiensis samea*[§]

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Key words: vanadium, ascidian, metal-binding protein, protein-protein interaction

Abstract

Several species of ascidians, the so-called tunicates, accumulate extremely high levels of vanadium ions in their blood cells. We previously identified a family of vanadium-binding proteins, named Vanabins, from blood cells and blood plasma of a vanadium-rich ascidian, *Ascidia sydneiensis samea*. The 3-dimensional structure of Vanabin2, the predominant vanadium-binding protein in blood cells, has been revealed, and the vanadium-binding properties of Vanabin2 have been studied in detail. Here, we used Far Western blotting to identify a novel protein that interacts with Vanabin2 from a blood cell cDNA library. The protein, named Vanabin-interacting protein 1 (VIP1), was localized in the cytoplasm of signet ring cells and giant cells. Using a two-hybrid method, we revealed that VIP1 interacted with Vanabins 1, 2, 3, and 4 but not with Vanabin P. The N-terminal domain of VIP1 was shown to be important for the interaction. Further, Vanabin1 was found to interact with all of the other Vanabins. These results suggest that VIP1 and Vanabin1 act as metal chaperones or target proteins in vanadocytes.

1. Introduction

Ascidians in the phylum Chordata have been well known as vanadium-accumulating animals since the first report by Henze [1]. A recent genome analysis of the ascidian *Ciona intestinalis* has revealed the genes for the basic chordate body plan and functions as well as genes for lineage-specific innovations, including

cellulose metabolism and vanadium accumulation [2, 3]. The concentration of vanadium ions in seawater is 35 nM, whereas the concentration in the blood cells of several species of ascidians can approach 350 mM [4-6]. Vanadium ions are stored in the vacuoles of vanadium-accumulating cells called vanadocytes, which include signet ring cells and several other vacuolated cells [7, 8]. The vacuole is maintained at extremely low pH, and the pH is related to the vanadium concentration [6].

We have been examining how ascidians accumulate vanadium ions and have isolated several blood cell proteins that are likely involved in the vanadium-accumulation process of the vanadium-rich ascidian *Ascidia sydneiensis samea* [9-11]. Using anion exchange column chromatography and expressed sequence tags (EST) analysis, we isolated a family of vanadium-binding proteins, named Vanabin1 through 4, from vanadium-accumulating blood cells (vanadocytes) [12-14]. The Vanabins possess a conserved motif containing the consensus sequence {C}-{X_{2-5}}-{C} (X = any amino acid) and bind vanadium ions in the +4 oxidation state (VO^{2+}) as shown by the Hummel-Dreyer method and immobilized metal ion affinity column chromatography (IMAC) [13-15] . A nuclear magnetic resonance study has revealed the solution structure of Vanabin2 to be a novel bow-shaped conformation consisting of four α-helices connected by nine disulfide bonds [16]. On the same face of the molecule, vanadyl ions are exclusively localized and coordinated by amine nitrogens derived from amino acid residues such as lysines and arginines [16], as suggested by electron paramagnetic resonance results [17]. Another Vanabin homolog, designated VanabinP, was isolated from blood plasma (coelomic fluid) and also has the conserved motif found in the other

four Vanabins [18]. Five Vanabin orthologs were also identified in the vanadium-accumulating species *C. intestinalis* [3]. Thus, Vanabins constitute a unique gene family, which has thus far been found only in vanadium-accumulating ascidians.

Metal-binding proteins can interact with other metal-binding proteins and proteins such as membrane metal transporters, membrane anchor proteins, or metal-reducing/oxidizing proteins [19]. In the present study, we used the Far Western blotting method to obtain several proteins that interact with Vanabins. Among these, Vanabin interacting protein 1 (VIP1) was shown to be localized in the cytoplasm of vanadocytes and to clearly interact with Vanabins 1-4 but not VanabinP, based on two-hybrid analysis. The N-terminal domain of VIP1 was shown to be important for this interaction.

2. Materials and methods

2.1 Probes for Far Western blotting

Recombinant Vanabin2 protein was produced and purified as reported previously [13]. The LGF2 and Gal11 proteins were used as positive controls. Recombinant Vanabin2 was dialyzed three times against a greater than 100-fold volume of phosphate-buffered saline (PBS). The protein concentration was measured by the Bradford assay using a protein assay reagent (BioRad), and the protein solution was adjusted to 0.1 mg/ml. Recombinant Vanabin2 was biotinylated by incubation with a 40-fold molar excess of EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 3 h on ice. The

protein solution was then dialyzed three times against a greater than 100-fold volume of PBS.

To assess labeling efficiency, the biotin-labeled protein probe was spotted onto a nitrocellulose membrane. The membrane was incubated in 50 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.4 (TBST) containing 0.2% gelatin for 3 h, washed four times with TBST, and reacted with a 1:5000 dilution of streptavidin-horseradish peroxidase-conjugate (Amersham) for 1 h. After washing three times with TBST, the signals were detected using an ECL detection kit and HyperFilm-ECL (Amersham).

2.2 Screening of a cDNA library by Far Western blotting

A blood cell cDNA library of *A. sydneiensis samea* [20] was plated on NZYM-agar plates. The density of phage plaques was adjusted to about 3,000 per plate (140 × 100 mm). Nitrocellulose membranes were soaked in 10 mM IPTG for 10 min and dried. When the plaques reached the appropriate size, a pretreated nitrocellulose membrane was placed onto each plate and incubated at 37°C for 3 h. The membranes were removed, soaked in TBST containing 0.2% gelatin for 3 h, and reacted with 35 μg/ml biotinylated Vanabin2 protein probe at 4°C for 8 h. They were washed three times with TBST for 5 min each and then incubated in TBST containing streptavidin-horseradish peroxidase-conjugate (diluted 1:2000, Amersham) for 1 h. After three washes with TBST, the signals were detected using an ECL detection kit and HyperFilm-ECL (Amersham). Positive plaques were screened repeatedly until they were clonal. The cDNAs were excised as plasmids in vivo according to the manufacturer's

protocol (Stratagene) and the sequences were determined by the dideoxy-termination method. The 5′-flanking region was obtained by 5′-rapid amplification of cDNA ends (RACE) using phage DNA extracted from the same cDNA library as the template.

2.3 Two-hybrid experiments using E. coli *as a host*

A BacterioMatch two-hybrid system (Stratagene) was used to examine protein interactions in vivo. Each cDNA to be tested was inserted into either pBT or pTRG vector, which had been digested with *Eco*RI and *Xho*I. The plasmids derived from pBT or pTRG were amplified in LB medium containing chloramphenicol or tetracycline, respectively. Validation reporter strain XL1-blur MRF′ Kan cells were transformed with the cDNA-containing pBT and pTRG plasmids. The successful transformation of both plasmids into a cell would allow the cell to grow on non-selective medium (LB medium containing chloramphenicol and tetracycline). If the proteins encoded by the plasmids were to interact in a cell, the cell would grow and form a colony on selective medium (LB medium containing 3-amino-1, 2, 4-triazole, chloramphenicol, and tetracycline). Cells forming a colony on selective medium were transferred to dual-selective medium (LB medium containing streptomycin, 3-amino-1, 2, 4-triazole, chloramphenicol, and tetracycline). Colony PCR was performed to confirm the presence of two plasmids in the colony-forming cells.

2.4 Anti-VIP1 antibody

We synthesized a peptide that corresponded to the region between amino acids 159 and 175 of VIP1: CTKEGERSDNTTPLNGND. This peptide was conjugated by a carrier protein and injected into rabbits. After the sixth injection, blood was extracted, and the serum was used as antiserum.

For Western blotting, blood cell homogenate was subjected to SDS-PAGE in a 10% acrylamide gel. To prepare blood cell homogenate, we separated signet ring cells from giant cells by centrifuging at 1,000×g in Ca^{2+}- and Mg^{2+}-free artificial seawater (CMFASW) containing 20% sucrose. The resulting pellet was homogenized in 500mM Tris-HCl (pH8.0). The homogenate was centrifuged at 1,100×g for 5min at 4°C . This pellet contain the cytoplasmic membrane and nuclear fraction. The supernatant was centrifuged 11,000×g for 10 min at 4°C , and the pellet contain vacuolar-membrane fraction. The remaining supernatant comprised the cytoplasmic-protein. The proteins were transferred to a nitrocellulose membrane by the semi-dry transfer method using blotting buffer (1% glycine, 0.3% Tris base, 0.02% SDS) for 3 h at 200 mA. The membrane was washed once with 1× TEN buffer (25 mM Tris, 1 mM EDTA, 150 mM NaCl, pH7.4) at room temperature (RT) for 10 min, and then blocked with 1× TEN plus 1% BSA for 1 h at RT. The membrane was incubated at 4°C for 16 h with the anti-VIP1 antiserum diluted 1:500 in 1× TEN plus 1% BSA, washed twice with 1× TEN plus 0.05% Tween 20 at RT for 10 min, and then incubated at RT for 1 h with HRP-conjugated anti-rabbit IgG antibody (Amersham) diluted 1:2500 in 1× TEN plus 0.05% Tween 20. After eight washes with 1× TEN plus 0.05% Tween 20 at RT for 15 min each, the membrane was soaked in ECL detection reagent (Amersham, England) and observed by a

luminoimage analyzer (FAS-1000, TOYOBO). For the markers of fractionation, V2C12 monoclonal antibody that recognize 130kDa vacuolar membrane protein [21], and S4D5 monoclonal antibody that recognize 6-phosphogluconate dehydrogenase [20], were used at 1:100 and 1:5000 dilution, respectively.

Immunocytochemistry studies were performed essentially as described previously [21]. The collected blood was suspended in CMFASW containing 460 mM NaCl, 9 mM KCl, 32 mM Na_2SO_4, 6 mM $NaHCO_3$, 5 mM HEPES, and 5 mM EDTA (pH 7.0), and centrifuged at 130 × *g* for 10 min. The separated blood cells were resuspended in CMFASW and applied to 3-aminopropyl-trithoxy-silane-coated coverslips for 5 min. All of the above procedures were carried out at 4°C. The coverslips were soaked in 10% formalin-CMFASW (10% formalin plus 0.005% Triton X-100 in CMFASW) for 5 min, and sequentially washed with CMFASW, 1× PBS plus 150 mM NaCl, and 1× PBS for 10 min each. To reduce non-specific background, the samples were treated with 1× TEN plus 2% BSA at RT for 30 min. The coverslips were immersed in a 1:50 dilution of anti-VIP1 antiserum at RT for 1 h and then washed three times with 1× PBS. The washed coverslips were immersed in a 1:50 dilution of fluorescein-labeled goat anti-rabbit IgG (AP307F, Chemicon, Millipore) at RT for 1 h. To remove excess antibody, the samples were washed six times with 1× PBS at RT for 15 min. The coverslips were mounted with 1× PBS and sealed with nail polish. Samples were observed under a spinning disc confocal microscope (CSU10; Yokogawa Electric Co., Japan) in combination with an inverted microscope (IX-71; Olympus Optical Co., Ltd., Japan) and a CoolSNAP-Pro camera (Media Cybernetics, USA) [22].

3. Results

3.1 Identification of novel proteins interacting with Vanabin2

LGF2 and Gal11 were prepared for use as controls on the Far Western blot. Recombinant LGF2 was synthesized as a fusion protein with maltose-binding protein (MBP). The fusion protein was purified by amylose resin column chromatography, and LGF2 was excised using Factor Xa (Fig. 1A). After dialysis against PBS, LGF2 was biotinylated by EZ-Link Sulfo-NHS-LC-biotin reagent. For Gal11, *E. coli* cells were transformed with pTRG-Gal11, and the expression of Gal11 was induced by IPTG. Cell extracts were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was reacted with the biotinylated LGF2, and LGF2 was detected by an avidin-HRP conjugate. The LGF2 probe clearly detected the Gal11 protein band on a Far Western blot (Fig. 1B), indicating that a biotinylated probe can recognize an interacting protein under these conditions. We used this protocol to screen for proteins that interact with Vanabin2.

Vanabin2 recombinant protein was synthesized as a fusion protein with MBP, excised using Factor Xa, purified, and biotinylated for use as a probe. Approximately 10,000 plaques from a blood cell cDNA library of *A. sydneiensis samea* were screened with the Vanabin2 probe, and we obtained two independent cDNA clones. One of these clones, which we named Vanabin-interacting protein 1 (VIP1), appeared to contain an almost full-length cDNA fragment and was analyzed in the present study. The VIP1

cDNA was 1,246 bp in length and encoded a putative 308-amino acid protein (Fig. 2). The deduced VIP1 protein sequence showed no significant similarity to known proteins in public databases. Secondary structural analyses suggested that the region between amino acids 213 and 232 is a hydrophobic helix domain.

To confirm the protein interaction between VIP1 and Vanabin2, we performed two-hybrid experiments in *E. coli* cells. The interactions between VIP1 and other Vanabins, as well as the interactions among Vanabins, were also assessed by the same method. The results are summarized in Table 1. VIP1 interacted with Vanabins 1-4 but not with VanabinP. Among the Vanabins, Vanabin1 interacted with itself and with Vanabins 2, 3, and 4. No interaction was detected between the Vanabins or VIP1 and vector-derived proteins.

3.2 Localization of VIP1

More than 10 types of blood cells have been distinguished in *A. sydneiensis samea* on the basis of morphology. These include hemoblasts, lymphocytes, leukocytes, pigment cells, morula cells, signet ring cells, compartment cells, small compartment cells, and giant cells [23-26]. Of these, signet ring cells are the vanadium-accumulating cells, also known as vanadocytes, and generally account for more than 70% of the total blood cells [6-8].

To examine the localization of VIP1, we prepared anti-VIP1 antiserum using a synthetic peptide corresponding to the N-terminal partial amino acid sequence of VIP1. The antiserum detected 38-kDa and 55-kDa proteins on a Western blot of blood cell

extracts of *A. sydneiensis samea* (Fig. 3). Although we used several methods for identifying posttranslational modifications such as glycosylation about 55-kDa protein, we did not detect any evidence of modifications. The 5′-RACE analysis of VIP1 mRNA did not yield a longer cDNA. By immunocytochemistry, the antiserum detected the VIP1 protein in the cytoplasm of signet ring cells and giant cells (Figs. 4). No detectable protein was present in the other blood cells. Note that some pigment cells showed autonomous fluorescence.

4. Discussion

Vanabins, the vanadium-binding proteins, are thought to play a major role in vanadium accumulation in ascidians. However, the details of their functions are still unknown. In this study, we identified a novel protein from a blood cell cDNA library of *A. sydneiensis samea* and showed that it interacts with Vanabin2 on Far Western blots. We named this protein Vanabin-interacting protein 1 (VIP1).

We used a two-hybrid method to examine the interactions of VIP1 with the Vanabins and found that VIP1 interacts with all four cytoplasmic Vanabins, i.e., Vanabins 1-4, suggesting that VIP1 functions in the cytoplasm of vanadocytes. The fact that VanabinP, which is localized in the blood plasma, did not interact with VIP1 supports this idea. Structural modeling of Vanabins 1, 3, and 4 based on the NMR data of Vanabin2 resulted in fairly homologous structures, whereas the modeled structure of VanabinP differed from that of Vanabin2, especially in the long loop domain between the third and

fourth helices (Fig.5). The root mean square deviation value of main chain atoms among Vanabin2 and VanabinP was 2.04 angstrom, which was about twice larger than those for Vanabins 1, 3 and 4 (1.14, 0.89 and 1.16 angstrom, respectively). This structural difference may account for the lack of a detectable interaction between VanabinP and VIP1.

The 308 amino acids encoded by the VIP1 cDNA include a hydrophobic region (Fig. 2). We first considered this region to be a transmembrane domain and constructed N- and C-terminal halves in order to separately examine the protein interactions of each. As shown in Table 1, the N-terminal domain was important for the interaction with Vanabins 1-4. However, Western Blotting and immunocytochemistry revealed that VIP1 is localized in the cytoplasm of signet ring cells. Thus it may be that VIP1 acts as a molecular partner for Vanabins 1-4 in the cytoplasm of signet ring cells, via its N-terminal domain. It is not clear whether VIP1 can also bind vanadium. If VIP1 were to bind vanadium ions, it may also act to transfer vanadium from or to Vanabins. The nature of 55-kDa protein detected in Western blotting is unclear. This could correspond to a protein similar to VIP1, post-translational modification of VIP1 or a longer variant of VIP1, although we could not identify such a gene product. The possibility that 55-kDa protein is robust homodimer of 38-kDa protein is less likely because there was no interaction between VIP1s (Table 1).

By combining the Vanabins, we found that Vanabin1 can interact with the other three cytoplasmic Vanabins, suggesting the possibility that Vanabin1 functions as a metal chaperone to transfer vanadium ions from or to Vanabins 2, 3, and 4. We are currently

investigating the vanadium transfer among Vanabins.

In our present working model (Fig. 6), vanadium ions in the +5 oxidation state [V(V)] are transported into the cytoplasm of signet ring cells where they are reduced to the +4 oxidation state [V(IV)]. The chemical species V(IV) would readily precipitate at physiological pH, and Vanabins 1-4 act as V(IV) carrier proteins in the cytoplasm. VIP1 isolated in this study is a cytoplasmic protein that can interact with Vanabins 1-4. A previous immunological study showed that Vanabins 1-3 are uniformly distributed in the cytoplasm of signet ring cells and that Vanabin4 is loosely attached to the cytoplasmic membrane [21]. In our study, VIP1 was uniformly distributed in the cytoplasm, which may reflect the distribution of Vanabins 1-4. We propose that VIP1 acts as a molecular partner to assist the sub-cellular localization of Vanabins 1-4, although future experiments to distinguish the distribution of each Vanabin are necessary. We also hypothesize that VIP1 helps to transfer vanadium from or to metal transporters by binding to both Vanabins and transporters. Identifying and characterizing more proteins that interact with VIP1 or Vanabins will further elucidate their functions, and an analysis of these protein networks would help to explain the mechanism of vanadium accumulation in ascidians.

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Figure Legends

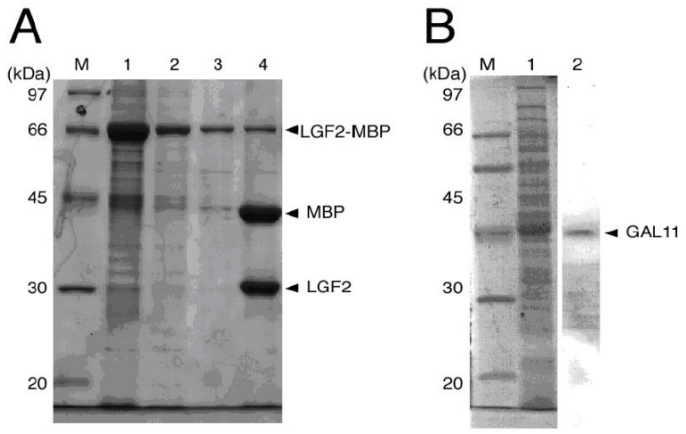

Fig.1. Far Western blot analysis of LGF2 and GAL11 proteins. (A) Synthesis and purification of recombinant LGF2 protein in *Escherichia coli*. Proteins were separated by SDS-PAGE and stained by Coomassie Brilliant Blue. Lane M, molecular weight marker; lane 1, soluble fraction of *E. coli* cells expressing LGF2-MBP fusion protein; lane 2, flow-through fraction from the affinity column; lane 3, purified LGF2-MBP fusion protein; lane 4, LGF2 and MBP proteins digested by Factor Xa. LGF2 was further purified by diethylaminoethyl (DEAE) anion exchange chromatography and labeled with biotin for Far Western blotting (not shown). (B) Far Western blotting. Lane M, marker; lane 1, total cell lysate of *E. coli* expressing GAL11; lane 2, Far Western blot of GAL11 detected by LGF2 probe.

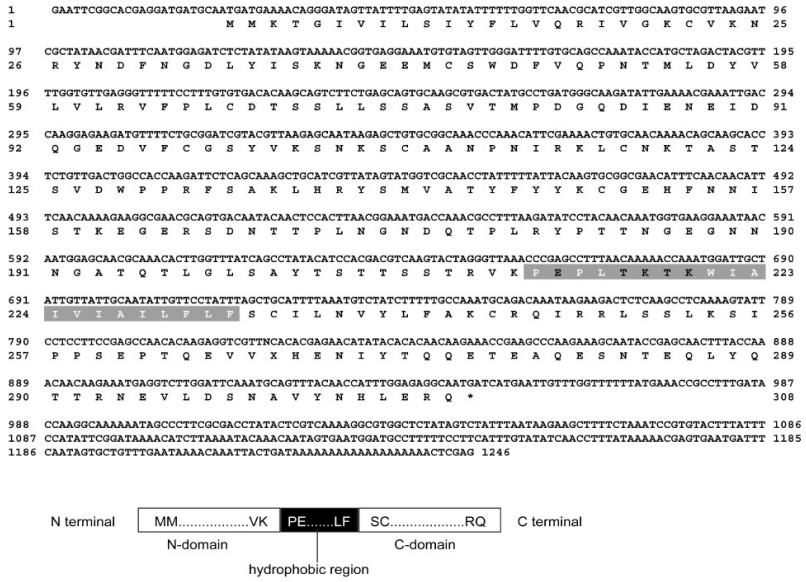

Fig. 2. Nucleotide and amino acid sequences of Vanabin-interacting protein 1 (VIP1) isolated from the vanadium-rich ascidian *Ascidia sydneiensis samea*. The VIP1 cDNA was 1,246 bp in length and encoded a putative 308-amino acid protein with a hydrophobic region (amino acids 213 to 232). No similar protein was found in public databases.

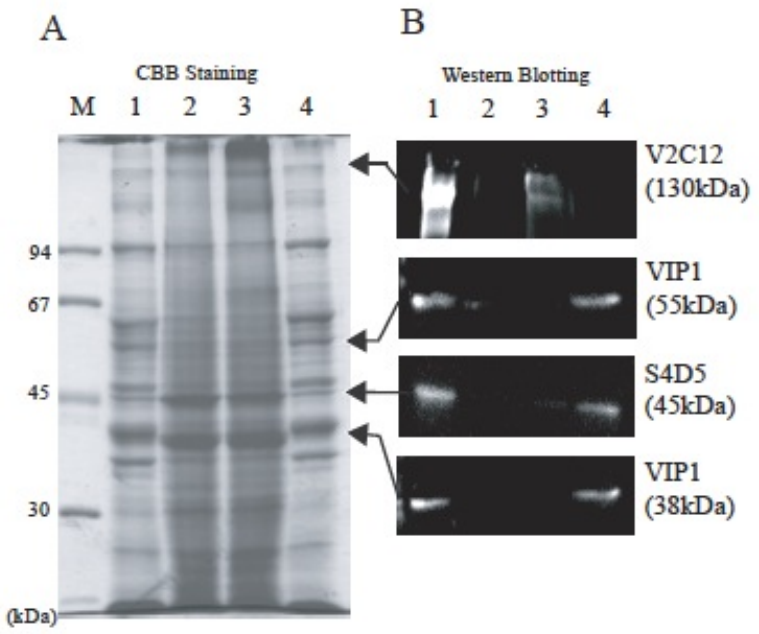

Fig. 3. Expression of VIP1 protein identified by anti-VIP1 antiserum on a Western blot after fractionation. Samples were loaded and separated using 10% SDS-PAGE gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (A), or the protein bands were transferred onto a nitrocellulose membrane and reacted with anti-VIP1 antiserum (B). Lane M, Molecular weight marker. Lane 1, homogenate blood cells without giant cells; Lane 2, pellet obtained by centrifugation at 1,100×g; Lane 3, pellet obtained by centrifugation at 11,000×g; Lane 4, soluble protein. V2C12 recognizes ascidian 130-kDa vacuolar membrane protein; S4D5 recognizes 6-phosphogluconate dehydrogenase (45 kDa). Note that VIP1 signals (38 kDa and 55 kDa) were detected in homogenate and soluble fraction.

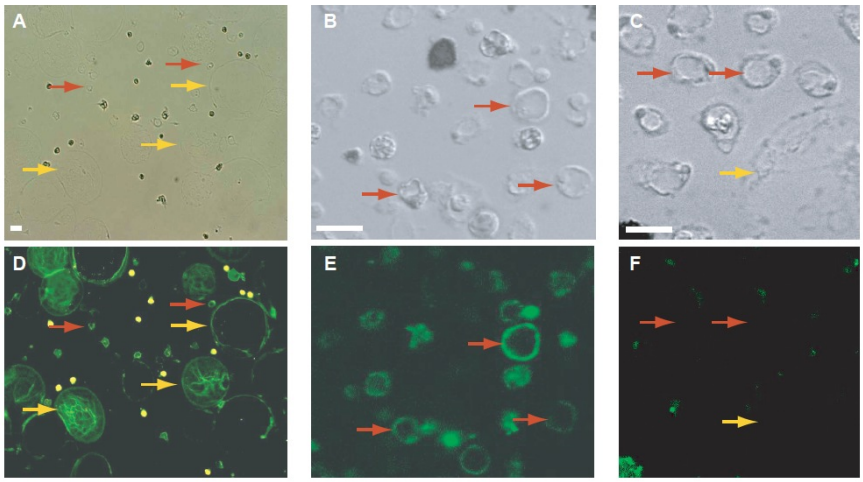

Fig. 4. Immunocytochemistry of blood cells of *Ascidia sydneiensis samea* using anti-VIP1 polyclonal antiserum. The cells were fixed and reacted with (D, E) or without (F) the anti-VIP1 antiserum. The cells were then reacted with FITC-conjugated secondary antibody and observed by Nomarski microscopy (A, B, C) or fluorescence microscopy (D, E, F). Fluorescence microscopic observations were made using an ordinary fluorescence microscope (D) or a confocal microscope (E, F). Note that staining was detected in the signet ring cells (red arrows), also known as vanadocytes, and giant cells (yellow arrows), but not in the other blood cells such as morula cells or pigment cells. Some pigment cells show yellowish autonomous fluorescence. Scale bar, 20 μm.

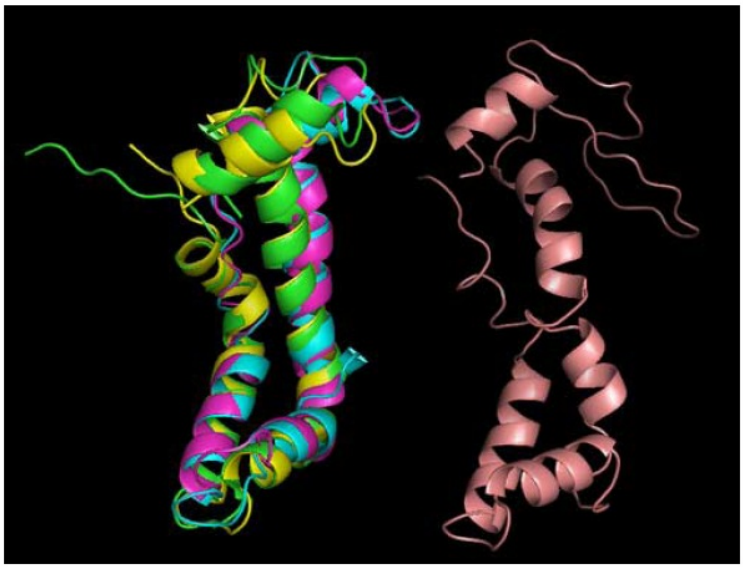

Fig. 5. Homology modeling of Vanabins from *Ascidia sydneiensis samea*. Blue, Vanabin1; Green, Vanabin2; Purple, Vanabin3; Yellow, Vanabin4; Brown, VanabinP. Structures were calculated by Modeller software 8v1 (http://salilab.org/modeller/) based on NMR data of Vanabin2 [16], and pixelated by MacPyMol software. Vanabins 1-4 are superimposed to give a minimum root mean square deviation value. Helix numbers determined for Vanabin2, and N- and C-termini for Vanabins 2, 3 and P are indicated, while those for Vanabins 1 and 4 are omitted for simplicity. Note that VanabinP has a long loop domain (arrow).

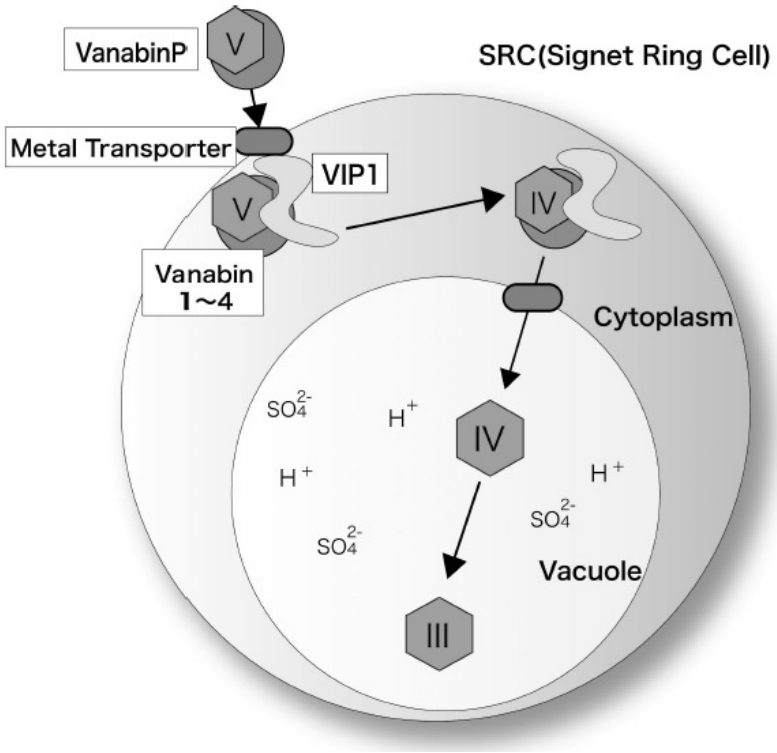

Fig. 6. Model for vanadium transport in vanadocytes of ascidians. Vanadium in the +5 oxidation state is carried by VanabinP and transported into signet ring cells via a metal transporter. The vanadium is then bound by Vanabins 1-4 and moved into the vacuole in the +3 oxidation state. VIP1 may help to localize Vanabins 1-4 or to transfer vanadium to metal transporters by binding to the Vanabins as well as metal transporters.