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Localization of Vanabins, Vanadium-Binding Proteins, in the Blood Cells of the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea*

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Some species of the family Ascidiidae accumulate vanadium in concentrations in excess of 350 mM, which is about 10^7-fold higher than the concentration of vanadium in seawater. In these species, signet ring cells with a single large vacuole in which vanadium ions are contained function as vanadium-accumulating cells. These have been termed vanadocytes. We recently isolated five vanadium-binding proteins, which we named Vanabin1, Vanabin2, Vanabin3, Vanabin4, and VanabinP, from vanadocytes of the vanadium-rich ascidian *Ascidia sydneiensis samea*. In this study, we analyzed localization of the Vanabins in the blood cells of *A. sydneiensis samea* using monoclonal antibodies and confocal microscopy. The Vanabin1 and Vanabin2 proteins were found in the cytoplasm and/or in some organelles of vanadocytes. Vanabin3 was also detected in the cytoplasm, while Vanabin4 was found exclusively in the cytoplasmic membrane.

Key words: vanadium, Vanabin, metal accumulation, ascidian

INTRODUCTION

Some ascidian species (Chordata, Urochordata, Ascidiacea) accumulate high levels of vanadium in their vanadocytes (Michibata *et al.*, 1987; Uyama *et al.*, 1991; Ueki *et al.*, 2002), which are vanadium-containing blood cells. The vanadium concentration in vanadocytes may be as high as 350 mM, which is 10^7-fold higher than the vanadium concentration in seawater (Henze, 1911; Michibata *et al.*, 2003). Vanadium ions are accumulated in the large vanadocyte vacuoles, in which almost all the vanadium ions are reduced to the +3 oxidation state *via* the +4 oxidation state, and seem to exist as free ions (Hirata and Michibata, 1991). These vacuoles also contain high levels of sulfate ions (Kanamori and Michibata, 1994) and are maintained at extremely low pH levels by vacuolar-type H^+-ATPase (Michibata *et al.*, 1991; Uyama *et al.*, 1994).

From the vanadocytes of the vanadium-rich ascidian *Ascidia sydneiensis samea*, we recently isolated proteins that are probably involved in the process of vanadium accumulation (Kanda *et al.*, 1997; Uyama *et al.*, 1998a, b, c; Ueki *et al.*, 2001, 2003a, b; Trivedi *et al.*, 2003). Of these proteins, the Vanabins, which were originally called vanadium-associated proteins (VAPs) (Kanda *et al.*, 1997), have attracted attention. To date, five Vanabin homologs, Vanabin1, Vanabin2, Vanabin3, Vanabin4, and VanabinP, have been isolated from *A. sydneiensis samea* (Ueki *et al.*, 2003a; Yamaguchi *et al.*, 2004; Yoshihara *et al.*, 2005). All of these proteins have conserved motifs characterized by the consensus sequence {C}–{X_{2-5}}–{C}, and they are rich in positively charged residues, such as lysine and arginine.

Three-dimensional structural analysis using nuclear magnetic resonance (NMR) has revealed that Vanabin2 has a novel bow-shaped conformation consisting of four α-helices connected by nine disulfide bonds (Hamada *et al.*, 2005). Vanabin1 and Vanabin2 can bind 10 and 20 V(IV) ions with dissociation constants (Kd) of 2.1×10^{-5} M and 2.3×10^{-5} M, respectively (Ueki *et al.*, 2003a). This binding to V(IV) ions is significantly inhibited by a 10 molar excess of Cu(II) ions, but is only weakly inhibited by the presence of Mg(II) or Mo(V) ions (Ueki *et al.*, 2003a). Vanabin3 and Vanabin4, discovered by EST analysis, have been shown by immobilized metal affinity chromatography (IMAC) also to bind V(IV) ions (Yamaguchi *et al.*, 2004). VanabinP, which is localized in the blood plasma but is not detectable in other

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tissues or blood cells, was found to bind a maximum of 13 V(IV) ions per molecule with a Kd of 2.8×10^{-5} M (Yoshihara *et al.*, 2005). Therefore, the Vanabin family should provide some clues as to the process of selective accumulation of vanadium in ascidians.

Both the interactions and functions of Vanabins remain unclear, although it may be inferred that Vanabins carry vanadium ions in a manner similar to metallo-chaperones (Michibata *et al.*, 2003). As a step towards elucidating these issues, the localizations of these proteins must be clarified. In the present study, we analyzed the localization of Vanabins in the blood cells of *A. sydneiensis samea* using monoclonal antibodies against individual recombinant Vanabins, confocal microscopy, and Western blot (WB) analysis. We found Vanabin1 and Vanabin2 localized in the cytoplasm and/or some organelle of vanadocytes. We also detected Vanabin3 in the cytoplasm, but found Vanabin4 exclusively in the vanadocyte cytoplasmic membrane.

MATERIALS AND METHODS

Preparation of anti-Vanabin monoclonal antibodies

The cDNA fragments that encode the putative mature Vanabin1, Vanabin2, Vanabin3, and Vanabin4 proteins were amplified by PCR using specific primers with adapted restriction enzyme sites. The following forward and reverse primers were used: for *Vanabin1*, 5'-GGAATTCGGCCCAGGCTGCAAA-3' and 5'-CGTCGACTCACACACAATTCAA-3'; for *Vanabin2*, 5'-GGAATTCGCTCCGGTGGATTGC-3' and 5'-CGTCGACTCACTTGCAGTTTGTC-3'; for *Vanabin3*, 5'-GGAATTCTACCCTGATGTGATTGC-3' and 5'-GGTCGACTCAAAATCGAAGATGACA-3'; and for *Vanabin4*, 5'-GGAATTCATGGATTCATGCAAAAC-3' and 5'-GGTCGACTTAATGACAATTTT-3'. Recombinant Vanabin proteins were prepared as described in our previous paper (Yamaguchi *et al.*, 2004).

To generate antibodies against Vanabins 1 to 4, 50 μg of each purified recombinant Vanabin was injected intraperitoneally into 8-week-old female BALB/c mice. Since it proved difficult to purify recombinant Vanabin3 due to precipitation, 100 μg of the recombinant protein fused with the maltose-binding protein derived from plasmid vector pMAL-c2x (New England BioLabs, USA) was injected. The mice were euthanized three or four days after the last injection. Blood samples were collected immediately, incubated at 37°C for 1 h, and allowed to stand at 4°C for 16 h. Serum samples were obtained as polyclonal antibodies by centrifugation at 10,000×*g* for 10 min at 4°C, and the samples were stored at –20°C. Spleen cells from the sacrificed mice were fused with myeloma cells using the PEG method (Galfre *et al.*, 1977). In order to produce a single colony, the hybridomas were cultured in HAT plus ASF-103 medium (Ajinomoto, Japan) supplemented with 3% fetal calf serum (FCS) (Uyama *et al.*, 1991). The hybridoma culture supernatants were used as the monoclonal antibody solutions.

To make acetone powder for the antibody-absorption test, recombinant Vanabin4-producing *E. coli* cells in 50 ml cultures were collected and sonicated in 250 μl of Milli-Q water. About 1 ml of cold acetone was added, and the sample was incubated on ice for 30 minutes. It was centrifuged at 10,000×*g* for 10 min at 4°C and the supernatant was removed. The pellet was washed with ice-cold acetone and spun twice. The washed pellet was spread out, ground into a fine powder on a sheet of filter paper, and air-dried.

WB analysis

WB analysis was performed to verify the specificity of each monoclonal antibody. Aliquots of each recombinant Vanabin and blood-cell homogenate were dissolved in sample buffer at a concentration of 1 μg protein/ml and subjected to SDS-PAGE in a 15% acrylamide gel. Protocols for the preparation of the recombinant proteins and blood-cell homogenates were described in our previous paper (Yamaguchi *et al.*, 2004). In order to maintain Vanabin3 (about 15 kDa in size) in the soluble form, it was excised from the MBP-Vanabin3 fusion protein by treatment for less than 10 h with a low concentration of Vanabin3 (500 μg/ml) and Factor Xa (2.5 μg/ml). The proteins were transferred from SDS-PAGE gel to a nitrocellulose membrane by the wet transfer method using blotting buffer (1% glycine, 0.3% Tris base, 0.02% SDS) for 2.5 h at 400 mA. The membrane was washed twice with 1× TEN buffer (25 mM Tris base at pH 7.4, 1 mM EDTA, 150 mM NaCl) at RT for 10 min, and then blocked with 1×TEN plus 1% BSA for 1 h at RT. The washed membrane was incubated at 4°C for 16 h with the anti-Vanabin monoclonal antibody (supernatant of the cell culture) diluted 1:1,000 in 1× TEN plus 1% BSA. The membrane was 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-mouse IgG (H+L) monoclonal antibody diluted 1:2,000 in 1× TEN plus 0.05% Tween-20. After eight washes with 1×TEN plus 0.05% Tween-20 at RT for 30 min, the membrane was soaked in ECL detection reagents (Amersham, England) and exposed to Hyperfilm-ECL.

Immunocytological detection of Vanabins

Adult specimens of *A. sydneiensis samea* were collected near the International Coastal Research Center, Ocean Research Institute, The University of Tokyo, Otsuchi, Iwate Prefecture, Japan. The collected blood was suspended in Ca^{2+}- and Mg^{2+}-free artificial seawater (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 140×*g* for 10 min. The separated blood cells were resuspended in CMFASW and applied to 3-aminopropyltrithoxy-silane-coated cover slips for 5 min. All of the above procedures were carried out at 4°C. The cover slips were soaked in 5% formalin-CMFASW (5% formalin plus 0.001% Triton X-100 in CMFASW) for 5 min, and sequentially washed with CMFASW, 1× TEN plus 150 mM NaCl, and 1×TEN for 10 min each. To reduce non-specific background, the samples were treated with 1× TEN plus 1% BSA at RT for 30 min. The cover slips were then immersed in a dilution of each monoclonal antibody at RT for 1 h, and then washed three times with 1× TEN. The washed cover slips were immersed in a dilution of FITC-conjugated anti-mouse IgG antibody (Organon Teknika, Netherlands) at RT for 30 min. To remove excess antibody, the samples were washed four times with 1× PBS at RT for 30 min. The cover slips were mounted with 1× PBS and sealed with nail polish. Samples were observed by spinning-disc confocal microscopy (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) (Tadakuma *et al.*, 2001). Monoclonal antibody S4D5, which specifically recognizes 6-phosphogluconate dehydrogenase (6PGDH) (Uyama *et al.*, 1991), was used as a specific marker for the cytoplasmic compartment of vanadocytes. A 1:100 dilution of wheat germ agglutinin (WGA) conjugated to fluorescein (FLK-2100; Vector Inc., UK) was used as a marker of the cytoplasmic membrane.

To examine whether EDTA or vanadium ions affects the localization of Vanabin4, we incubated blood cells in different conditions: CMFASW with or without EDTA, CMFASW containing 10, 100 and 1,000 μM of $VOSO_4$, CMFASW containing 10, 100 and 1,000 μM of Na_3VO_4, plasma of *A. sydneiensis samea*. Blood cells were incubated at RT for 10 min. $VOSO_4$ was used as free ions or chelated by iminodiacetic acid (IDA) in a 1:1 ratio. Na_3VO_4 was used as free monomeric ions. Incubated blood cells were immediately fixed by EDTA-free 5% formalin-CMFASW.

Preparation of monoclonal antibodies that recognize the vacuolar membrane

To prepare an antibody specific for the vacuolar membrane of the vanadocytes of *A. sydneiensis samea*, blood was drawn from a

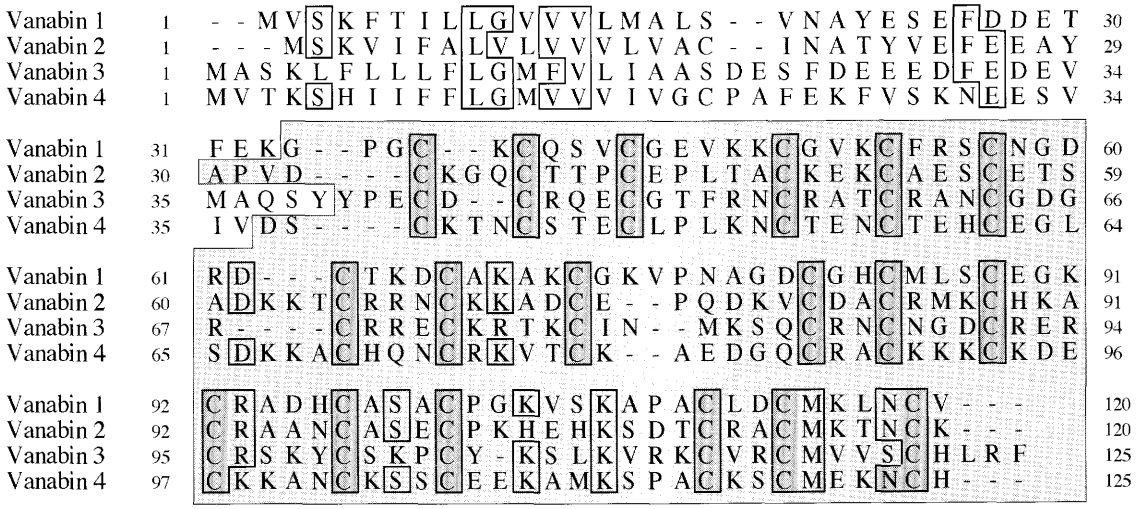

Fig. 1. Alignment of the amino acid sequences of Vanabin1, Vanabin2, Vanabin3, and Vanabin4. Conserved residues are boxed, and the eighteen cysteine residues in the Vanabins are indicated by dark shading. Light shading indicates the protein region that was used as the antigen in the preparation of monoclonal antibodies.

specimen and suspended in CMFASW, and the suspension was centrifuged at 300×*g* for 10 min at 4°C. The separated blood cells were resuspended in CMFASW containing 20% sucrose and centrifuged at 1,500×*g* for 10 min at 4°C, to remove giant cells. The resulting pellet was washed twice with CMFASW and homogenized in 200 mM Tris-HCl (pH 8.0). The homogenate was centrifuged at 1,000×*g* for 10 min at 4°C. This pellet comprised the cytoplasmic-membrane and nuclear fraction. The supernatant was centrifuged at 10,000×*g* for 1 h at 4°C, and the pellet was retained as the mitochondrial fraction. The supernatant was re-centrifuged at 100,000×*g* for 1 h at 4°C, and the resulting pellet comprised the vacuolar-membrane fraction. The remaining supernatant comprised the cytoplasmic-protein fraction.

These fractionated samples were used as antigens. Injections of the individual fractions into mice were repeated 4–6 times at intervals of 2 weeks, to boost the titers of antibodies. The mice were euthanized three or four days after the final injection. The murine spleen cells were treated according to the protocol of Uyama *et al.* (1991), as described in the previous section, to obtain hybridoma cell lines. Immunocytological screening detected four cell lines, V1E4, V2C12, V2D7, and V3F3, that produced monoclonal antibodies that recognized the vacuolar membranes of signet ring cells.

Localization analysis of Vanabins by WB analysis

Ascidian blood cells were homogenized and fractionated, as described above. Each fraction was subjected to SDS-PAGE and WB analysis. The V2-3D03 and V4-1F10 antibodies were used to analyze Vanabin localizations. A 1:1000 dilution of WGA conjugated to fluorescein was used as a marker of the cytoplasmic membrane in WB analyses. The V2C12 (this study) and anti-V-ATPase subunit B antibodies (Uyama *et al.*, 1994) were used at 1:100 dilutions as vacuolar-membrane markers of vanadocytes. The anti-Nup153 (R-15) antibody (1:100 dilution; Santa Cruz Biotechnology, USA) was used as a nuclear marker in the WB analyses.

RESULTS AND DISCUSSION

An alignment of the amino acid sequences of Vanabin1, Vanabin2, Vanabin3, and Vanabin4 are shown in Fig. 1. Each recombinant Vanabin was injected into mice as an antigen for the generation of monoclonal antibodies. As a result, about 2,000 hybridoma cell lines that produced monoclonal antibodies against Vanabins 1–4 were obtained in this study. Although almost all of these antibodies cross-reacted with all the Vanabins in ELISA and WB analyses, several monoclonal antibodies specific for a single Vanabin were obtained (Table 1). The monoclonal antibodies recognizing recombinant Vanabin2, Vanabin3, and Vanabin4 were designated as V2-3D03, V3-2F05, and V4-1F10, respectively. Each of these antibodies recognized a single band corresponding to one Vanabin in the blood cell homogenate (Fig. 2C, D, E). The anti-Vanabin1 antibody, designated as V1-2C06, recognized not only Vanabin1 but also cross-reacted weakly with Vanabin3 and Vanabin4 (Fig. 2B). These monoclonal antibodies were used for the immunological detection of Vanabins in vanadocytes.

Various blood cell types, such as signet ring cells, morula cells, pigment cells, giant cells, amoebocytes, small compartment cells, hemoblasts, lymphocytes, and leukocytes, were distinguished morphologically in *A. sydneiensis samea* (Wuchiyama and Michibata, 1995). Signet ring cells were identified as vanadocytes containing high levels of vanadium (Michibata *et al.*, 1987; Ueki *et al.*, 2002). Therefore, vanadocytes (signet ring cells) were subjected to indirect immunofluorescence staining. Immunostaining with the V2-3D03 and V3-2F05 monoclonal antibodies revealed Vanabin2 and Vanabin3 to be localized in the cytoplasm of vanadocytes (Fig. 3H, J). Immunostaining with the V1-2C06 antibody, which recognizes Vanabin1 and other Vanabins, gave positive signals in the cytoplasmic compartment of the signet ring cells (Fig. 3F), which indicates that Vanabin1 localizes to the cytoplasm of vanadocytes. The V4-1F10 monoclonal antibody detected Vanabin4 localized in the cytoplasmic membrane of signet ring cells (Fig. 3L). In addition, immunocytological staining with polyclonal anti-

Table 1. List of monoclonal anti-Vanabin antibodies, created in this study, that are specific for Vanabins 1-4.

Antigen	Name of Monoclonal Antibody			
Vanabin1	V1-2C06			
Vanabin2	V2-3A01	V2-3B03	V2-3D03	V2-3G03
Vanabin3	V3-1E03	V3-2F05		
Vanabin4	V4-1F10			

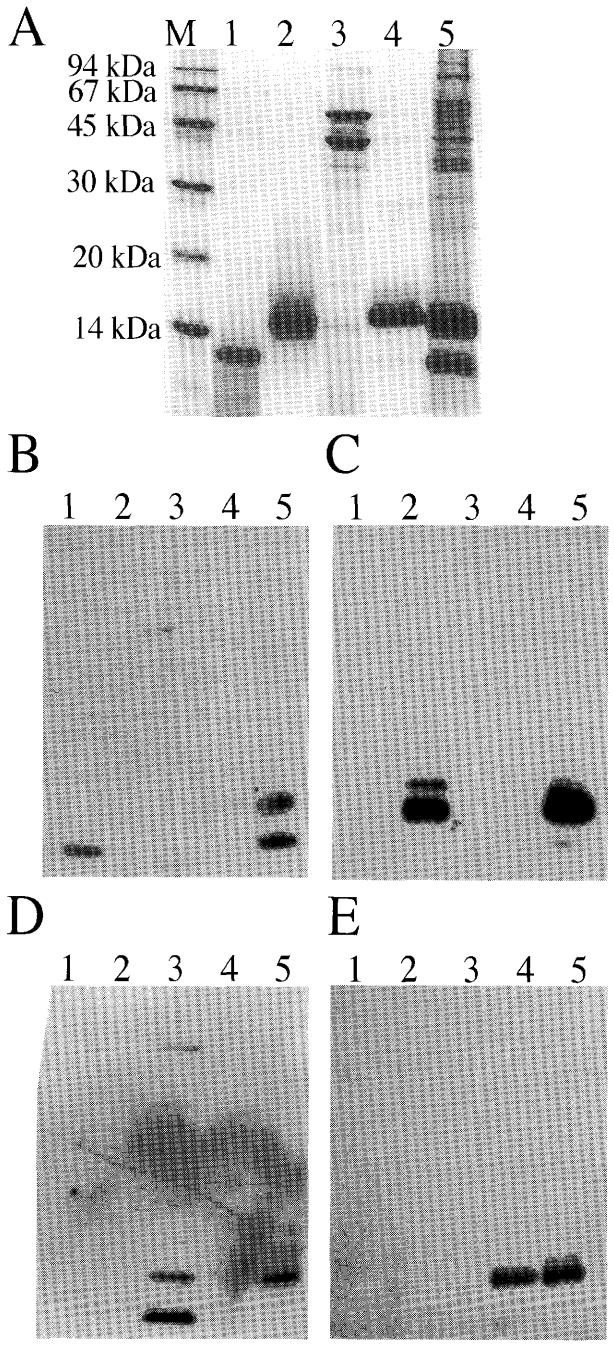

Fig. 2. Specificity of monoclonal antibodies for each Vanabin. **Panel A** is stained with CBB. **Panels B, C, D, and E** were reacted with V1-2C06, V2-3D03, V3-2F05 and V4-1F10, respectively. **M,** low-molecular-size marker; **lane 1,** purified recombinant Vanabin1; **lane 2,** purified recombinant Vanabin2; **lane 3,** MBP-Vanabin3 fusion protein and digested Vanabin3; **lane 4,** purified recombinant Vanabin4; **lane 5,** homogenate of blood cells of *A. sydneiensis samea.* Vanabin3 was not digested completely or purified due to its instability. The sizes of the MBP-Vanabin3, MBP, and Vanabin3 proteins are 55 kDa, 40 kDa, and 15 kDa, respectively. In lane 5 of panel B, the lower band is Vanabin1 and the upper band is Vanabin3 and/or Vanabin4.

Vanabin4 antibody or FITC-conjugated WGA as a cytoplasmic-membrane marker revealed a similar staining pattern (Fig. 3N, P). Furthermore, antibody-absorption experiments showed that this antibody recognized Vanabin4 specifically (Fig. 3R). These results indicated that of the four Vanabins, only Vanabin4 localizes to the cytoplasmic membrane.

To confirm the immunolocalization findings, WB analyses were performed with anti-6-PGDH monoclonal antibody (S4D5), anti-Nup153, and WGA as markers of the cytoplasm, nucleus, and cytoplasmic membrane, respectively. The anti-V-ATPase subunit B antibody (Uyama *et al.*, 1994) was used as a vacuolar membrane marker. The V2C12 antibody was also used as a vacuolar membrane marker, although the target molecule has not been identified. Vanabin4 was detected in the supernatant obtained by centrifugation at 100,000×*g*, *i.e.*, the cytoplasmic fraction (Fig. 4). Vanabins 1 and 3 were found in the cytoplasmic fraction (data not shown). These results are inconsistent with the immunological detection of Vanabin4 in whole-mount signet ring cells. It is possible that Vanabin4 had been weakly associated with cytoplasmic membrane or cortex and was released by homogenization.

Since Vanabin4 must be exposed in the homogenization step to the vacuolar contents of vanadocytes, including an excess of vanadium ions, sulfate ions and protons, we thought that the localization of Vanabin4 might be regulated by these factors. To examine the effect of vanadium ions on the localization of vanabin4, we incubated blood cells in CMFASW containing several concentrations of vanadium ions prior to immunocytological analysis. We also tested the possibility that the inconsistent results were an artifact due to the presence of EDTA, which is a strong chelator of vanadium ions, by comparing Vanabin4 localization with or without EDTA in CMFASW. In addition, we substituted CMFASW with plasma of *A. sydneiensis samea* in order to mimic the natural environment of blood cells. None of these three treatments affected the localization of Vanabin4 detected by immunocytological analysis. To clarify the properties of Vanabin4, we must identify the Vanabin4-binding factor in the cytoplasmic membrane of vanadocytes.

Vanadium dissolved in seawater is taken into the ascidian body *via* the branchial sac or alimentary canal, transferred to the coelomic fluid (blood plasma), and stored in the vacuoles of vanadocytes at concentrations in excess of 350 mM, which is about 10^7-fold the concentration of vanadium in seawater (Michibata *et al.*, 2003). In the process of vanadium accumulation, Vanabins may function as metal carriers, so-called metallo-chaperones, rather than as proteins involved in metal storage or detoxification. However, both the pathway of vanadium accumulation in ascidians and the functional interactions among Vanabins remain to be elucidated. In this sense, it is essential to define the localization of Vanabins, not only within vanadocytes but also within the accumulation pathways.

We previously isolated five different Vanabins that are likely to be involved in vanadium-accumulation processes in *A. sydneiensis samea*. Vanabin1 and Vanabin2 localize to vanadocytes (Wuchiyama *et al.*, 1997), whereas VanabinP is found in the blood plasma (Yoshihara *et al.*, 2005). The present study has followed up initial results on the localization of members of the Vanabin family. Confocal microscopy revealed that Vanabin1 and Vanabin2 localized to the cytoplasm and/or some organelle of vanadocytes, which is in accordance with the previous study (Wuchiyama *et al.*, 1997). Vanabin3 was also detected in the vanadocyte cytoplasm. However, Vanabin4 was found by confocal micros-

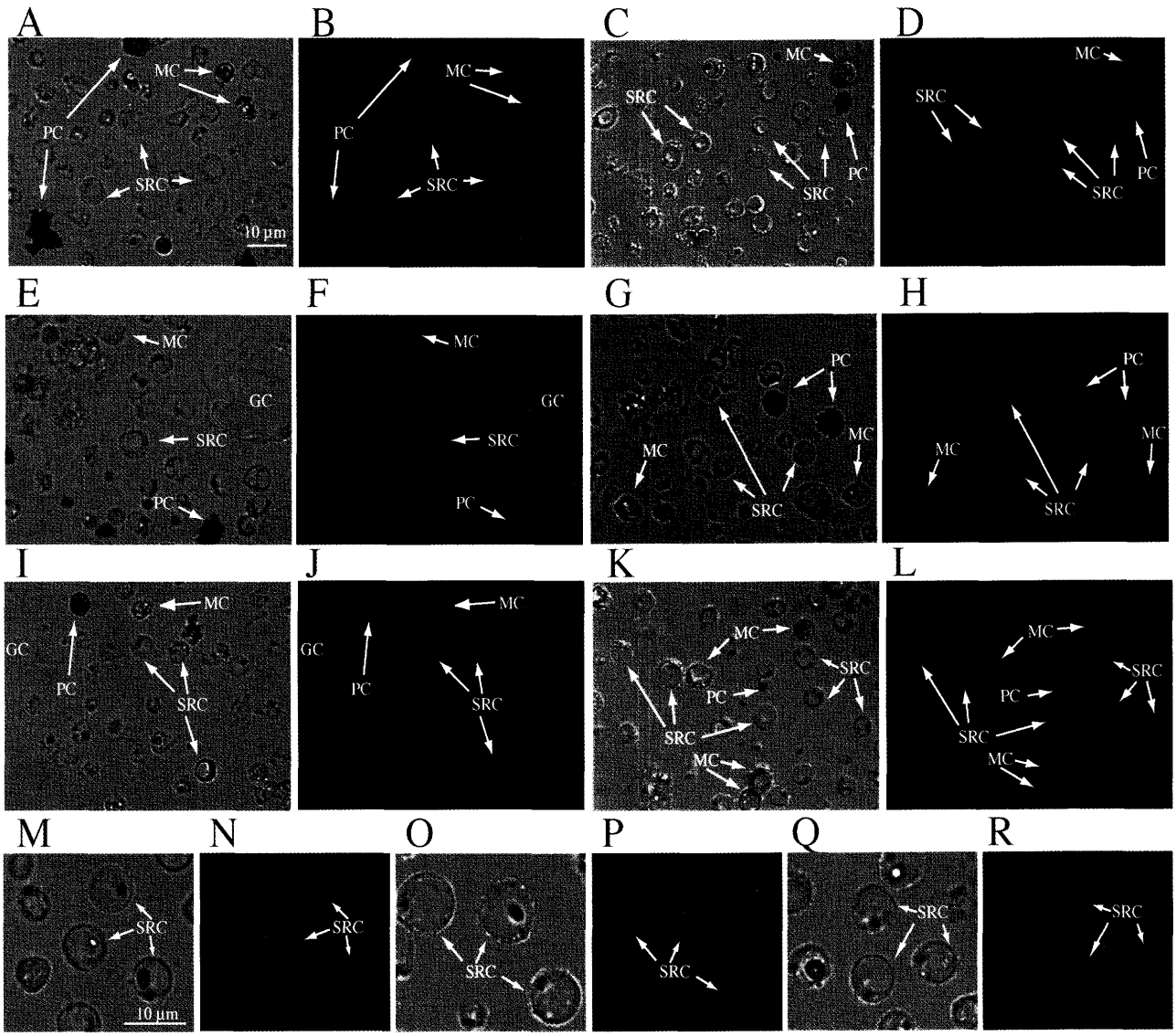

Fig. 3. Immunological detection of Vanabin1, Vanabin2, Vanabin3, and Vanabin4. A, C, E, G, I, K, M, O, and Q, Nomarski differential-interference; B, D, F, H, J, L, N, P, and R, fluorescence confocal micrographs; **A and B,** *negative control* (FITC-labeled anti-mouse IgG only); **C and D,** positive control (S4D5, a marker of the cytoplasm of signet ring cells); **E and F,** anti-Vanabin1 antibody V1-2C06; **G and H,** anti-Vanabin2 antibody V2-3D03; **I and J,** anti-Vanabin3 antibody V3-2F05; **K and L,** anti-Vanabin4 antibody V4-1F10; **M and N,** anti-Vanabin4 polyclonal antibody; **O and P,** WGA staining as a cytoplasmic membrane marker; **Q and R,** anti-Vanabin4 antibody-absorption. SRC, signet ring cell; MC, morula cell; PC, pigment cell; GC, giant cell.

copy to localize exclusively to the cytoplasmic membrane of vanadocytes (Fig. 3L), which conflicts with the results of the WB analysis of fractionated blood cells (Fig. 4). This apparent discrepancy cannot be explained at present. We will examine in detail the localization shift of Vanabin4, which should clarify the roles of the Vanabins and the vanadium accumulation system in vanadocytes.

The more than 2,300 ascidian species are classified into four suborders: Aplousobranchia, Phlebobranchia, Stolidobranchia, and Aspiculata. Species in the suborder Phlebobranchia are known to accumulate high levels of vanadium (Michibata *et al.*, 1986), although the level of accumulated vanadium varies greatly among species. The blood cells of *Ciona intestinalis,* a well-known model for embryological studies and for which the draft genome sequences have been revealed (Dehal *et al.*, 2002), contain vanadium ions at a concentration of 0.6 mM (Michibata *et al.*, 1986). In this species, five Vanabin homologues (CiVanabin1-5) have been identified from the draft genome database and confirmed by IMAC (immobilized metal ion affinity chromatography) to have vanadium-binding activity (Trivedi *et al.*, 2003). Vanabin homologues have also been found in *C. savignyi* (unpublished data). Therefore, Vanabins occur in several vanadium-containing ascidian species. Attempts to elucidate the localization of Vanabin family members and to understand the interactions among Vanabins are expected to provide additional information on the unusual accumulation of vanadium by this group of marine organisms.

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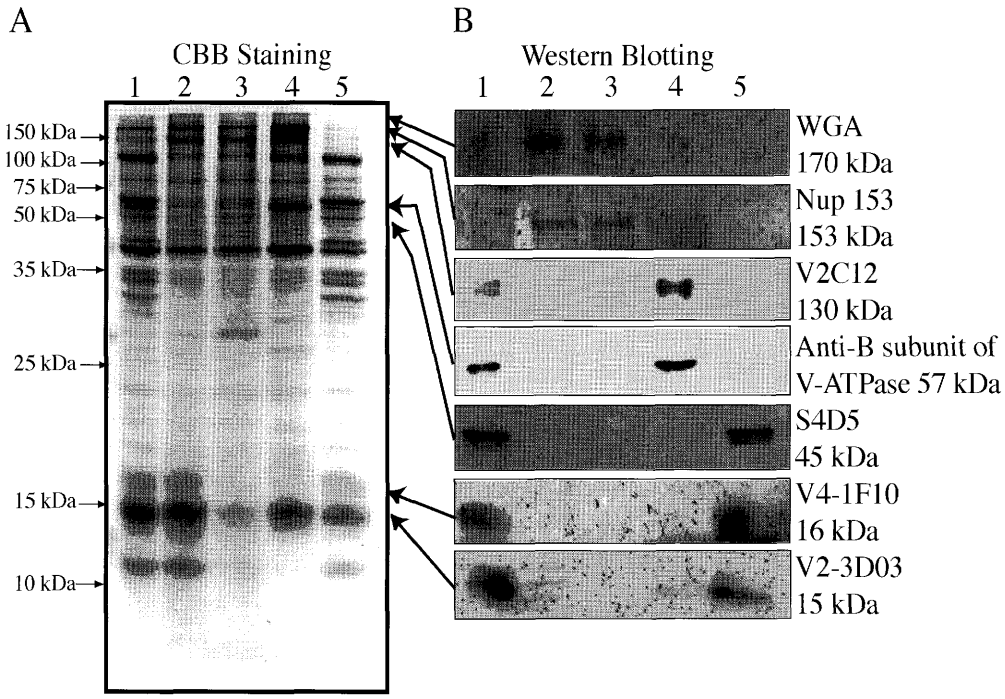

Fig. 4. Localization of Vanabin4 demonstrated by WB analysis. Blood cells of *A. sydneiensis samea* were homogenized and fractionated, as described in Materials and Methods. **A,** CBB staining; **B,** western blotting; **lane 1,** homogenate of blood cells; **lane 2,** pellet obtained by centrifugation at 1,000×*g*; **lane 3,** pellet obtained by centrifugation at 10,000 × *g*; **lane 4,** pellet obtained by centrifugation at 100,000×*g*; **lane 5,** soluble protein. WGA recognizes the sugar chain of the 170-kDa cytoplasmic-membrane protein; Nup 153 recognizes the 153-kDa nuclear-fraction marker; V2C12 recognizes the ascidian 130-kDa vacuolar-membrane protein; Anti-B subunit of V-ATPase recognizes the B subunit of ascidian V-ATPase; S4D5 recognizes 6-phosphogluconate dehydrogenase; V4-1F10 recognizes Vanabin4; and V2-3D03 recognizes Vanabin2.

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