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Relation	

A novel vanadium transporter of the *Nramp* family expressed at the vacuole of vanadium-accumulating cells of the ascidian *Ascidia sydneiensis samea*

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Running title: A Novel Vanadium Transporter

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ABSTRACT

Background: Vanadium is an essential transition metal in biological systems. Several key proteins related to vanadium accumulation and its physiological function have been isolated, but no vanadium ion transporter has yet been identified.

Methods: We identified and cloned a member of the Nramp/DCT family of membrane metal transporters (*As*Nramp) from the ascidian *Ascidia sydneiensis samea*, which can accumulate extremely high levels of vanadium in the vacuoles of a type of blood cell called signet ring cells (also called vanadocytes). We performed immunological and biochemical experiments to examine its expression and transport function.

Results: Western blotting analysis showed that *As*Nramp was localized at the vacuolar membrane of vanadocytes. Using the *Xenopus* oocyte expression system, we showed that *As*Nramp transported VO^{2+} into the oocyte as pH-dependent manner above pH 6, while no significant activity was observed below pH 6. Kinetic parameters (K_m and V_{max}) of *As*Nramp-mediated VO^{2+} transport at pH 8.5 were 90 nM and 9.1 pmol/oocyte/h, respectively. A rat homolog, DCT1, did not transport VO^{2+} under the same conditions. Excess Fe^{2+}, Cu^{2+}, Mn^{2+} or Zn^{2+} inhibited the transport of VO^{2+}.

Conclusions: *As*Nramp was revealed to be a novel VO^{2+}/H^{+} antiporter, and we propose that *As*Nramp mediates vanadium accumulation coupled with the electrochemical gradient generated by vacuolar H^{+}-ATPase in vanadocytes.

General Significance: This is the first report of identification and functional analysis on a membrane transporter for vanadium ions.

Keywords

Membrane protein; Transport metals; Vanadium; Ascidian;

1. Introduction

Some ascidians, known as tunicates or sea squirts, belonging to the suborder Phlebobranchia (Chordata, Urochordata, Ascidiacea), accumulate extremely high levels of vanadium in vanadocytes, one of their approximately ten types of blood cell [1-4]. The vanadium concentration in vanadocytes can reach 350 mM [5, 6], corresponding to 10^7 times the level in seawater (35 nM). This is thought to be the highest rate of accumulation of a metal in any living organism.

In the sea water, vanadium is dissolved in the +5 oxidation state (HVO_4^{3-} or $H_2VO_4^{2-}$; V^V). During the accumulation process, V^V is reduced to the +3 oxidation state (V^{3+}; V^{III}) *via* the +4 oxidation state (VO^{2+}; V^{IV}) [7]. NADPH is a strong candidate to participate in the reduction of V^V to V^{IV}, as the enzymes involved in the pentose phosphate pathway are expressed exclusively in the cytoplasm of vanadocytes [8-11], and detailed *in vitro* studies have suggested that this reaction occurs with the assistance of several chelating substances [12, 13]. Vanabins, which are named as vanadium-binding proteins, isolated from the cytoplasm of vanadocytes as well as blood plasma are capable of binding as many as *ca*. 10–20 V^{IV} ions per molecule of protein, and are thought to play an important role in V^V-reduction, V^{IV}-transport, and V^{IV}-storage processes in the cytoplasm of vanadocytes [14-19].

The content of the vanadocyte vacuole is maintained at an extremely low pH reaching 1.9, and the concentration of vanadium is correlated with that of protons [5]. As vacuolar-type H^+-ATPase (V-ATPase) is localized on the vacuolar membrane of vanadocytes and V-ATPase maintains low pH in the vacuole of vanadocytes [20-22], we hypothesized that a proton gradient generated by V-ATPase provides the energy to transport vanadium across the vacuolar membrane. To assess this hypothesis, we first cloned a cDNA encoding a member of the Nramp (natural resistance-associated macrophage protein)/DCT1 family from vanadocytes of *Ascidia sydneiensis samea* because the Nramp/DCT1 family is known to transport a broad range of divalent cations (Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, and Pb^{2+}) across the membrane using a proton gradient as the motive force [23, 24]. We showed that the product of this gene, *As*Nramp, is localized on the vacuolar membrane and can operate as an antiporter of V^{IV} and H^+.

2. Materials and methods

2.1.Animals

Adults of the ascidian *A. sydneiensis samea* were collected at Yamada Bay, Iwate, Japan. Blood

was extracted and diluted with Ca^{2+}, Mg^{2+}-free artificial sea water (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). Blood cells were collected by centrifugation at 300 × *g* for 10 min at 4°C. Giant cells were removed by sucrose density gradient centrifugation, as this type of cell contains highly acidic materials that adversely affect protein and RNA extraction.

2.2. Molecular Cloning of the AsNramp Gene

We designed four degenerate primers from the conserved regions of known Nramp/DCT1 family metal transporter genes, DCT1AS1f: 5'-TTY WSN YTN MGN AAR YTN TGG GC-3', DCT1AS2f: 5'-TTY ACN GGN CCN GGN TTY YTN ATG-3', DCT1AS3r: 5'-GTD ATN ARN ACN CCN CCC CA-3', and DCT1AS4r: 5'-TGN CCN GCR TAN GTN CCN GTC AT-3'. RNAs were extracted from *A. sydneiensis samea* blood cells and cDNAs were synthesized from total RNA as previously [20]. The first-round PCR reaction was set up as follows: cDNAs corresponding to 150 ng of total RNAs, 200 pmol of each of the primers DCT1AS1f and DCT1AS4r, each dNTP at 0.2 mM, 1× reaction buffer, and 2.5 units of *Taq* DNA polymerase (TaKaRa, Inc.). The reaction volume was 50 μl After denaturation at 94°C for 2 min; 30 cycles of PCR were performed (94°C for 60 s, 45°C for 60 s, and 72°C for 120 s) followed by a final extension at 72°C for 6 min. After the reaction, 1-μl aliquots of the first-round reaction mixture were taken as the template for the second-round reaction. The second-round reaction was performed using the same amounts of primers DCT1AS2f and DCT1AS3r as in the first reaction, and PCR cycles were the same as those in the first round. The PCR products were separated by agarose gel electrophoresis. The band of the expected size (~350 bp) was excised and cloned into the pBluescript vector and its nucleotide sequence was determined by the dideoxy method using an ALFexpress DNA sequencer and Thermo Sequenase kit (GE healthcare). A cDNA library of *A. sydneiensis samea* blood cells [9] was screened with the cDNA fragment. Positive phages were rescreened until they were cloned. The cDNAs were excised *in vivo*, subcloned into pBluescript, and sequenced. Representative cDNA clone #1171 was used for further analysis. Amino acid sequences were compared by the neighbor-joining method [25] using ClustalW [26].

2.3. Construction of Plasmids and In Vitro Synthesis of mRNAs for AsNramp and Rat DCT1

The coding region of *As*Nramp and rat DCT1 were amplified by Easy-A High-Fidelity PCR

cloning enzyme using specific primer sets with artificial restriction sites as follows: AsNramp-pHA-F1, 5'-AGA TCT GGA ACC ATG TCC TCT AAA G-3' and AsNramp-pHA-R1, 5'-GGA TCC TTC GTT AAT ACA TTC ATT TTC-3' for *As*Nramp (this study), and rDCT1-pHA-F1, 5'-AGA TCT ACC ATG GTG TTG GAT CCT GAA G-3' and rDCT1-pHA-R1, 5'-CTC GAG CCT TAG TAT TGC CAC CGC TG-3' for rat DCT1 (based on GenBank AF008439). The amplified fragments were digested with *Bgl*II and *Xho*I and ligated into the corresponding site of the pT7G HA C expression vector [27]. The nucleotide sequence was confirmed by the DNA Sequencing Service at the Natural Science Center for Basic Research and Development, Hiroshima University. The resulting plasmids (pT7G-AsSUL1-HA and pT7G-DCT1-HA, 10 μg each) were linearized with *Not*I. After incubation with 100 μg/ml Proteinase K, 0.5% SDS, and 5 mM EDTA at 56°C for 30 min, proteins were removed successively with Tris-saturated phenol and phenol–chloroform. Plasmid DNA was precipitated with ethanol and dissolved in 10 μl of TE buffer. Synthesis of mRNA was performed using a mMESSAGE mMACHINE kit (Ambion Life Technologies). The DNA template (1.0 μg) was mixed with ribonucleotide mixture, transcription buffer, and enzyme mix in a total volume of 20 ml. The mixture was incubated at 37°C for 3 h. Synthesized mRNAs were extracted with Tris-saturated phenol, phenol–chloroform, and chloroform successively, precipitated with ethanol, and dissolved in 13 μl of RNase-free water (~1 μg/ml). The actual concentration of mRNA was determined by spectrophotometry.

2.4. Metal solution and reducing agents

Vanadyl sulfate ($VOSO_4$, 99.5%, Wako Pure Chemical Industries) was dissolved at 10 mM in ultrapure water just before use. Iron chloride ($FeCl_2$), copper chloride, manganese chloride, zinc chloride and nickel chloride were also purchased from Wako, and were dissolved at 100 mM in ultrapure water.

2.5. Transport Assay for AsSUL1 and Rat DCT1 in Xenopus Oocytes

Ovaries were excised from *Xenopus laevis* females and treated with collagenase as described previously [28]. Oocytes at stage V or VI were collected in MBS buffer (8.8 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4 \cdot 7H_2O$, 0.33 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.41 mM $CaCl \cdot 2H_2O$), and synthetic mRNA was injected into each oocyte using a NANOJECT instrument (Drummond Scientific Co.). Oocytes were incubated at 18°C in MBS buffer supplemented with penicillin (10 U/ml) and streptomycin (10 μg/ml).

Uptake experiments were performed 4 days after injection of synthetic mRNAs. Uptake solution contained 100 mM NaCl or choline-Cl, 10 mM HEPES, 2 mM MES, 2 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, and the pH was adjusted by adding MES to pH 4.5 or 5.5 or Tris to pH 6.5, 7.5 and 8.5. Oocytes were settled at 20°C in fresh MBS buffer for at least 30 min before uptake experiments. They were then incubated in uptake solution containing appropriate concentrations of $VOSO_4$. In inhibition experiments, 50 molar excess of appropriate divalent cations were added together. When using iron chloride ($FeCl_2$), ascorbic acid was used at 4 molar excess to maintain reduced state of Fe^{II}. After incubation for appropriate times at 20°C, oocytes were washed five times with ice-cold uptake buffer without adding vanadium, and oocytes were put in separate tubes and homogenized in 1 N HNO_3. Total concentration of vanadium was determined by atomic absorption spectroscopy (AAS; Spectra AA-220Z; Varian Inc.).

2.6. Western blotting

Western blotting was performed to examine the expression of injected mRNAs in *Xenopus* oocytes and the subcellular localization of *As*Nramp in blood cells.

Oocytes were frozen in liquid nitrogen and homogenized in EB plus buffer (80 mM β-glycophosphate, 15 mM $MgCl_2$, 20 mM EGTA, pH 7.5) with protease inhibitors (2 mM PMSF, 0.1 mM Pepstatin A, 30 μg/ml Leupeptin). The supernatant was obtained by centrifugation at 15,000 rpm for 10 min at 4°C and used for SDS-PAGE and Western blotting. Anti-HA antibody (HA124; Nacalai Tesque, Inc.) was used at 1:10,000 dilution to detect *As*Nramp protein. HRP-conjugated anti-mouse IgG antibodies (PI-2000; Vector, Inc.) were used and the signals were detected with Chemi-Lumi-One reagents (Nacalai Tesque, Inc.).

A synthetic polypeptide (CLSRLKSSFSRLNE) corresponding to the C-terminal hydrophilic domain of *As*Nramp was prepared and conjugated with keyhole limpet hemocyanin. Aliquots of 15 μg of the peptide were injected intraperitoneally into 8-week-old female BALB/c mice. The injection was repeated five times at 2-week intervals. Three days after the last injection, blood was collected, allowed to stand at 37°C for 1 h, and incubated at 4°C for 12 h. The serum was removed from the clot, and any remaining insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant thus obtained was stored at –20°C and used as the anti-*As*Nramp antiserum. Fractionation of blood cell components and Western blotting were performed according to our previous report [29]. The antiserum was used for immunoblotting analysis and immunocytochemistry at 1:4000 and 1:1000

dilutions, respectively. The signals were detected as described for the anti-HA antibody.

3. Results

3.1. Cloning of an Ascidian Nramp Gene

We first amplified cDNA fragments related to an Nramp gene from the *A. sydneiensis samea* blood cell cDNA pool by nested PCR using two pairs of degenerate primers corresponding to conserved amino acid sequences of known Nramp family proteins. Using the PCR fragment as a probe, we then isolated a cDNA clone encoding a protein closely related to Nramp family proteins from the cDNA library of *A. sydneiensis samea* blood cells. This cDNA contained a single, long open reading frame of 1761 nucleotides, including the stop codon, which encoded a protein of 586 amino acids. The amino acid sequence deduced from the cDNA clone showed striking similarities to Nramp proteins from various organisms. The alignment of amino acid sequences predicted by cDNA cloning and human Nramp1 and Nramp2, and rat DCT1 proteins are shown in Fig. 1. The amino acid sequence showed 58.2%, 61.1% and 61.3% identity to human Nramp1 and Nramp2, and rat DCT1 sequences, respectively. The predicted amino acid sequence contained 12 putative transmembrane domains (TM1 – TM12) and a consensus transport motif (CTM), which exists in all Nramp family proteins. Therefore, we concluded that the cDNA clone corresponded to an Nramp homolog in *A. sydneiensis samea*, which we named *As*Nramp. Molecular phylogenetic analysis by the neighbor-joining method [25] did not significantly cluster this *As*Nramp homolog to either Nramp1 or Nramp2 group (Fig. 2). Another ascidian species *Ciona intestinalis* possess one ortholog of Nramp (CiCLSTR12032), which is clustered with *As*Nramp. No IRE-like sequence was found in its 3'-noncoding region.

Several amino acid residues had been revealed to be functionally important. For example, mutations in the first external loop (G119, D124, Q126 in rat DCT1) and E154 in TM3have been shown to affect uptake activity and specificity [30, 31]. Mutation in G216 in rat DCT1 (TM4) resulted in loss of iron uptake and caused anemia [32]. D117 (TM1), D223 (TM4) and E330 (TM7) in rat DCT1 have been revealed to be essential in yeast complementation assay. H267 and H272 (TM6) in rat DCT1 was identified to be important in pH regulation [33]. All of these essential residues were conserved in *As*Nramp (Fig. 1). Two potential N-glycosilation sites were also conserved, and it suggested that membrane topology is also conserved with other Nramps.

3.2. Subcellular Localization of AsNramp

In order to examine the sub-cellular localization of *As*Nramp within blood cells, we raised a polyclonal antibody against the C-terminal polypeptide of *As*Nramp. We performed Western blot analysis on blood cell homogenates fractionated by a stepwise centrifugal fractionation. The apparent molecular size of *As*Nramp was ca. 160 kDa (Fig. 3), which is higher than the calculated molecular weight (65.2 kDa). This could be due to glycosilation, as shown in oocyte experiments. V2C12 antibody [18] was used as a control for vacuolar localization. It detected a vacuolar membrane protein of ca. 130 kDa (Fig. 3), which was segregated in the same fraction as *As*Nramp. These results suggested that *As*Nramp was localized on the vacuolar membrane of the blood cells. Since the pH of the vacuole is extremely low in vanadium-rich ascidians, we expected that *As*Nramp could act as proton-dependent vanadium transporter.

3.3. Transport of VO^{2+} by AsNramp and Rat DCT1

No reports exist regarding the transport of vanadium by the Nramp/DCT1 family of metal transporters. We prepared *Xenopus* oocytes injected with *As*Nramp and rat DCT1 (rDCT1) for comparison. Non-injected eggs were used as controls. Expression of *As*Nramp and DCT1 was confirmed using anti-HA antibody (Fig. 4). The apparent sizes of *As*Nramp and rDCT1 ranged from 45 kDa to more than 100 kDa, while the predicted sizes were 65.2 and 61.3 kDa, respectively. Since treatment by trifluoromethanesulfonic acid (TFMS) decreased the extra bands (data not shown), this may have been due to glycosylation.

We first used an uptake buffer containing NaCl (100 mM NaCl, 10 mM HEPES, 2 mM MES, 2 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$), and found that control oocytes showed uptake activity for VO^{2+}, and the activity was higher at pH 5.5 than at pH 7.5 (Fig. 5A). Expression of *As*Nramp or rDCT1 had no significant effect on this basal activity under these conditions. This result suggested that *As*Nramp and rDCT1 could not transport VO^{2+} in the presence of sodium ions. Therefore, we used choline-Cl in place of NaCl, since choline can be used to maintain the osmolarity of the uptake buffer but do not compete with metal ions. As a result, *As*Nramp caused significant uptake activity in a buffer containing choline-Cl at pH 7.5. In contrast, transport activity in *As*Nramp-injected at pH 5.5 was slightly higher than control oocytes but the difference was not significant. rDCT1 injection did not cause any significant change in VO^{2+} uptake activity under any conditions. Thus, *As*Nramp could act as a VO^{2+} transporter at pH 7.5 in the absence of NaCl. In contrast, rDCT1 could not transport VO^{2+} under any of

the conditions examined.

As the Nramp/DCT family of proteins are known to be pH-dependent divalent cation transporters, the pH dependency of VO^{2+} transport by *As*Nramp was examined in detail (Fig. 5B). Uptake activity of control oocytes was higher at low pH. At pH 4.5 and 5.5, no significant uptake by *As*Nramp was observed. At pH 6.5–8.5, *As*Nramp transported VO^{2+} into oocytes significantly. A protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was used to confirm the relationship between VO^{2+}-transport and pH gradient across the oocyte membrane. As shown in Fig. 5C, addition of FCCP at 2.5 μM in the uptake buffer at pH8.5 neutralized the *As*Nramp-mediated transport of VO^{2+}. This experiment confirmed that VO^{2+}-transport by *As*Nramp was coupled with H^+.

The metal selectivity of *As*Nramp was assessed by adding excess concentration of metal ions in the transport assay (Fig. 6). In the presence of 50 molar excess of Cu^{2+}, Mn^{2+} and Zn^{2+}, VO^{2+}-transport by *As*Nramp was significantly suppressed ($p<0.01$), and the effect of Mn^{2+} was most significant. For experiments using 50 molar excess of Fe^{2+}, Ascorbic acid was used at 4 molar excess to maintain reduced state of Fe^{2+}. In control experiment with ascorbic acid but without Fe^{2+}, the uptake of VO^{2+} was reduced to about a half of control without ascorbic acid. The reason is unclear. When 50 molar excess of Fe^{2+} was added with ascorbic acid, the uptake of VO^{2+} was almost completely suppressed as compared to the control experiments with ascorbic acid but without Fe^{2+} ($p<0.05$). These results suggested that these divalent cations competed with VO^{2+}.

Kinetic assay for VO^{2+} transport by *As*Nramp was performed by changing the concentration of vanadium in the medium. At lower concentrations (up to 10 μM), the uptake rate appeared as Michaelis–Menten kinetics, but the uptake rate decreased in the range higher than 10 μM VO^{2+} (Fig. 7). The kinetic parameters (K_m and V_{max}) of *As*Nramp-mediated VO^{2+} transport at pH 8.5 were calculated to be 90 nM and 9.1 pmol/oocyte/h, respectively, at concentrations of VO^{2+} up to 10 μM.

4. Discussion

In this study, we first isolated a homolog of the Nramp/DCT1 divalent cation transporter family from the blood cells of the vanadium-rich ascidian *A. sydneiensis samea*. As the Nramp/DCT family consists of structurally and functionally well conserved proteins found in bacteria, fungi, animals, and plants, we hypothesized that an Nramp/DCT1 homolog exists in ascidians and acts as a divalent cation transporter. The present study indicated that *As*Nramp is localized in the vacuolar membrane of vanadocytes and acts as a VO^{2+}/H^{+} antiporter to accumulate vanadium in the vacuole using electrophysiological gradient of protons, which are accumulated by vacuolar-type H^{+}-ATPases (V-ATPases).

The genomes of vertebrates possess two orthologs of the Nramp/DCT1 protein family in their genome. From two ascidians, *A. sydneiensis samea* and *Ciona intestinalis*, we identified only one ortholog in each species. Molecular phylogenetic analysis did not significantly cluster the *As*Nramp to either Nramp1 or Nramp2, while *As*Nramp and its homolog in *C. intestinalis* were clearly clustered (Fig. 2). This suggested that gene duplication occurred in the ancestor of vertebrates.

Western blotting analysis using antiserum against *As*Nramp indicated that *As*Nramp is localized on the vacuolar membrane of blood cells in *A. sydneiensis samea* (Fig. 3). This is reminiscent of Nramp1 in humans and rodents. In mammals, Nramp1 is expressed predominantly in macrophages and monocytes, and regulates intracellular pathogen proliferation and inflammatory responses [34]. Tissue localization study indicated that Nramp1 is present in lysosomes/late endosomes in macrophages [35]. In contrast, Nramp2 is expressed in most tissues and acts as an iron uptake transporter operating at the endosome membrane with the transferrin system [36]. In addition to such ubiquitous expression and function, the expression of Nramp2 is highest at the brush border membrane of the intestine and acts as a transferrin-independent iron uptake transporter [37, 38]. It is necessary to examine in future study whether *As*Nramp also functions in the intestine or other tissues.

The direction of transport and metal selectivity is well studied for mammalian Nramps. Nramp2 is reported to be a symporter for proton/divalent cations that uses a proton electrochemical gradient as an energy source. Transport study using *Xenopus* oocytes or mammalian cell lines indicated that Nramp2 transports a broad range of divalent cations, such as Fe^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, Cu^{2+}, and Co^{2+}, but not Ca^{2+} or Mg^{2+}, in a pH-dependent manner [38]. In contrast, the direction of proton/metal transport by Nramp1 is controversial. One study used *Xenopus* oocytes to examine the metal transport by Nramp1 and revealed that Nramp1 acts as an antiporter for proton and divalent cations Fe^{2+}, Zn^{2+}, and Mn^{2+} [39]. The

present study indicated that *As*Nramp acts as a proton/VO^{2+} antiporter (Fig. 5). We examined the effect of excess divalent ions on the transport of VO^{2+} and found that Fe^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} inhibited at certain degrees (Fig. 6). This result suggested that *As*Nramp can also transport several divalent cations as the same manner. It is desirable to compare the structure of metal binding site(s), the site(s) to specify metal selectivity and proton coupling in *As*Nramp and other Nramps, but three dimensional structure of any Nramp proteins have not been determined yet. So far, spontaneous and artificial mutant studies in mammals and yeast suggested several amino acid residues that are important for metal transport and proton coupling, and at least the amino acid residues that are known to be important in mammals and yeast were conserved in *As*Nramp (Fig. 1). Further functional analysis should be performed to identify amino acid residues that are unique to *As*Nramp and responsible for VO^{2+} transport, or those common to human Nramp1 and *As*Nramp that could be responsible for antiport of protons and divalent cations.

Kinetic analysis of *As*Nramp indicated that the uptake rate appeared to fit Michaelis-Menten kinetics at low concentrations up to 10 μM VO^{2+} (Fig. 7). If we compare the K_m value with those for other metals, the apparent K_m for VO^{2+} obtained by *As*Nramp transport assay (90 nM) was one or several orders smaller than those obtained for Fe^{2+} (2.2 μM) [38, 40], Cd^{2+} (1.0 μM) [41], and Zn^{2+} (562 nM) [39]. This result suggested that *As*Nramp could act as a high-affinity VO^{2+} transporter.

Vanadium transport by Nramp has not been reported in any other organism. Since we found that rat DCT1 cannot transport VO^{2+} under the present assay conditions, the vanadium transport could be a unique feature of Nramp in vanadium-rich ascidians. It is necessary to examine other Nramp/DCT homologs from additional organisms including other vanadium-rich ascidians to examine whether they can transport vanadium or not. In addition, we found that *Xenopus* oocytes can accumulate VO^{2+} at low pH (Fig. 5A), which indicated that a proton/VO^{2+} symporter is expressed in the oocytes, although its identity is not clear.

When we consider the accumulation process, V^V is a major chemical species in the external environment and probably in coelomic fluid, transported into the cytoplasm, and reduced to V^{III} *via* V^{IV} [7]. A question remains regarding the identity of the transporter for V^V. Phosphate transporter is a candidate transporter for V^V (HVO_4^{3-} or $H_2VO_4^{2-}$), the chemical features of which resemble those of phosphate anions (HPO_4^{3-} or $H_2PO_4^{2-}$), and we are currently attempting to identify homologs of NaPi- or PiT-type phosphate transporters expressed in vanadocytes. Such anion transporters may also act in the first step of vanadium uptake in the branchial sac or the intestine.

In our model, V^V is readily reduced to V^{IV} and V^{IV} is stabilized by Vanabins, which act as vanadium reductase and vanadium chaperone, in the cytoplasm (Fig. 8) [19, 42]. Then, V^{IV} (VO^{2+}) is

transported into the vacuole by a cation transporter, *As*Nramp, as suggested in the present study. The results of the present study indicated that *As*Nramp is a VO^{2+}/H^{+} antiporter expressed on the vacuolar membrane of vanadocytes. These findings supported the proposed model that proton electrochemical gradient generated by V-ATPase is the driving force for VO^{2+} transport from the cytoplasm into the vacuole (Fig. 8). We reported previously that the vacuole of vanadocytes contains high concentrations of protons and sulfate ions [5, 43, 44]. V-ATPase is expressed on the vacuolar membrane of the vanadocytes [22] and a functional sulfate transporter *As*SUL1 was isolated from vanadocytes [44]. Most of the vanadium ions that accumulate in the vanadocytes are in the +3 oxidation state (V^{3+}; V^{III}) and are stably stored in the acidic vacuole, where sulfate ions act as counterions.

Several other pathways may exist that act together (Fig. 8). Another possible mechanism for V^{IV} transport into the vacuole is driven by P_{1B}-type ATPase, *As*HMA1, which we have already cloned from blood cells of *A. sydneiensis samea* and examined its metal selectivity and transport activity (Ueki *et al*., unpublished data). In addition, some portion of vanadium is circulated as V^{IV} in coelomic fluid and transported by a cation transporter on the plasma membrane of vanadocytes, as we have previously identified a vanadium-binding protein VanabinP that can bind and stabilize V^{IV} in the coelomic fluid,

The accumulation, reduction, and transport of vanadium ions should be tightly linked. The analysis of vanadium transporters should provide more detailed information regarding the vanadium transport pathway that underlies the extraordinarily high level of vanadium accumulation in ascidians. The physiological function of accumulated vanadium ions in reduced forms, V^{IV} or V^{III}, must be exerted through redox-coupled reactions. The study of vanadium assimilation in ascidians should also provide fundamental insight into the physiological roles of vanadium not only in ascidians but also in mammals, in which vanadium is an essential element.

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Figure legends

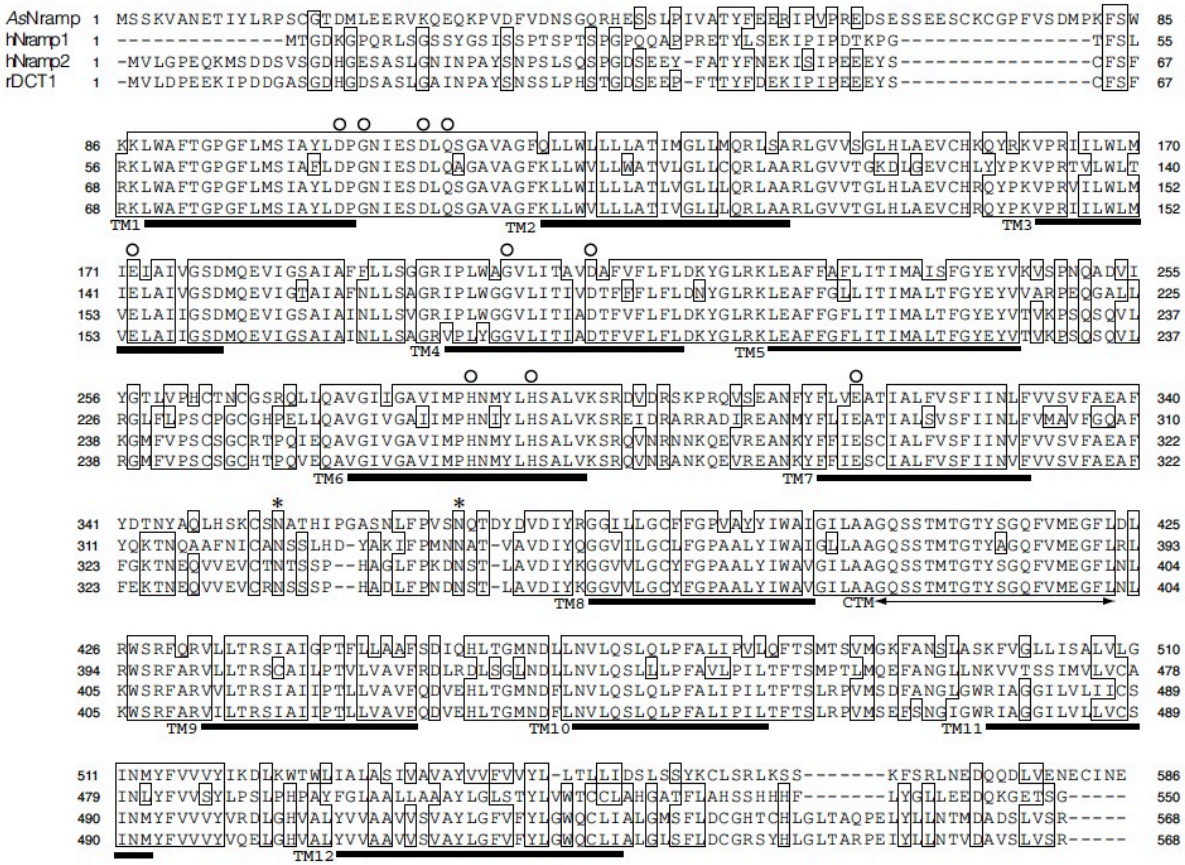

Fig. 1. Amino acid sequence alignment of an Nramp homolog of *Ascidia sydneiensis samea* (*As*Nramp), human Nramp1 (hNramp1), Nramp2 (hNramp2) and rat DCT1 (rDCT1). Identical amino acid residues are boxed. Twelve putative transmembrane domains are shaded (TM1 to TM12). Numbers indicate amino acid residues for each sequence. Open circles over each amino acid residue indicate identical ones that have been reported to be functionally essential or important. Asterisks indicate the position of possible N-glycosilation sites. Conserved transport motif is indicated by a double-headed arrow (CTM).

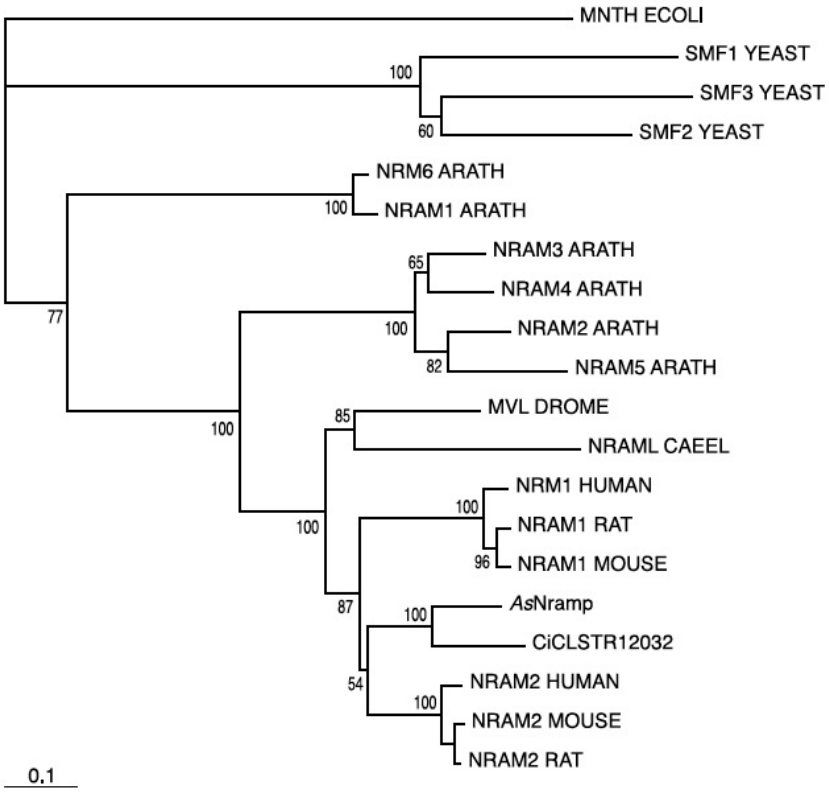

Fig. 2. Phylogenetic tree using the neighbor-joining method based on amino acid sequences of Nramp family proteins. Two ascidian species (*As*Nramp and CiCLSTR12032, from *Ascidia sydneiensis samea* and *Ciona intestinalis*, respectively) were compared with homologs from representative organisms Labels are taken from SwissProt database IDs (ECOLI, *Escherichia coli*; YEAST, *Saccharomyces cerevisiae*; ARATH, *Arabidopsis thaliana*; DROME, *Drosphila melanogaster*; CAEEL, *Caenorhabditis elegans*; HUMAN, *Homo sapiens*; RAT, *Rattus norvegicus*; MOUSE, *Mus musculus*). The scale bar indicates 0.1 amino acid substitutions per site. Numbers at each branch indicate the percentage of times that a node was supported in 1000 bootstrap pseudoreplicates. Note that two ascidian species were clearly clustered, but they were not significantly clustered to either Nramp1 or Nramp2.

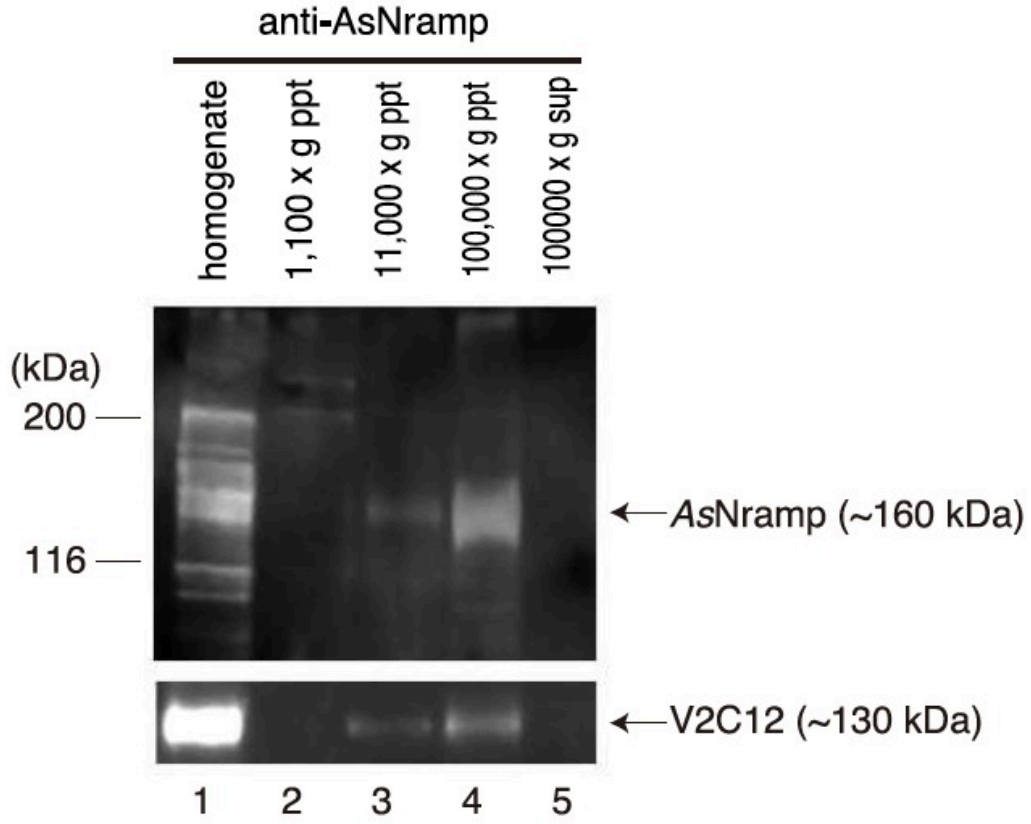

Fig. 3. Expression of *As*Nramp protein identified by anti-*As*Nramp antiserum on a Western blot after fractionation. Samples were separated by 8% SDS-PAGE. After electrophoresis, the gels were transferred onto nitrocellulose membranes and reacted with 1:4000 dilution of anti-*As*Nramp antiserum. Lane 1, homogenate blood cells without giant cells; lane 2, pellet obtained by centrifugation at 1100 × *g*; lane 3, pellet obtained by centrifugation at 11,000 × *g*; lane 4, pellet obtained by centrifugation at 100,000 × *g*; lane 5, soluble protein. V2C12 recognizes a 130-kDa vacuolar membrane protein. Note that an *As*Nramp signal (~160 kDa) was detected in the vacuolar membrane fraction (lane 4).

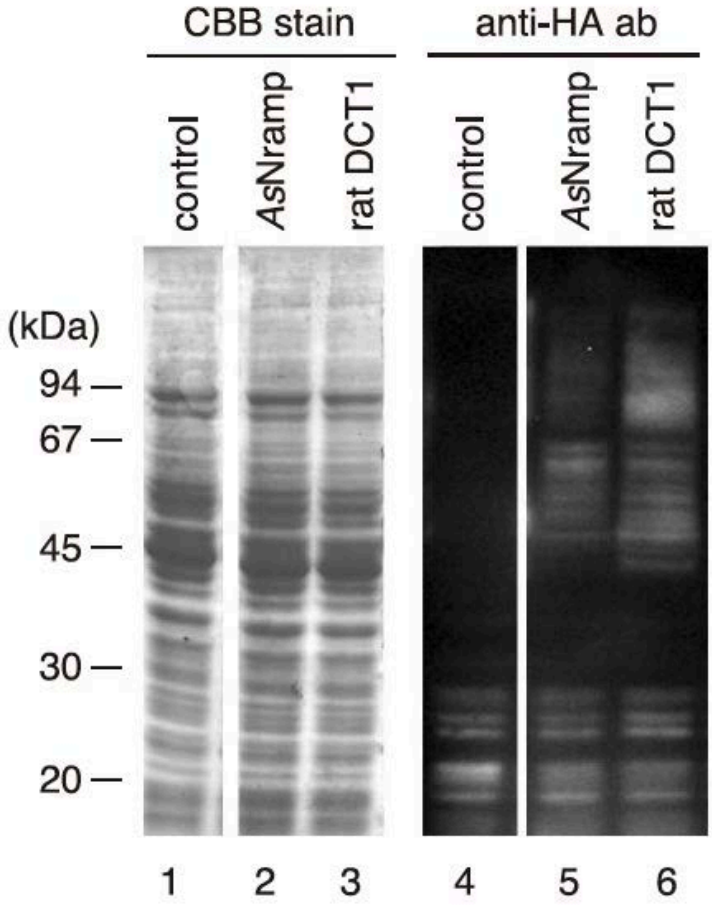

Fig. 4. Western blotting analysis of *Xenopus* oocytes injected with *AsNramp* or rat *DCT1* mRNA. After SDS-PAGE, the gels were either stained with Coomassie Brilliant Blue (lanes 1–3), or the protein bands were transferred onto nitrocellulose membranes and probed with anti-HA antibody (lanes 4–6). Lanes 1 and 4, extract from control *Xenopus* oocytes; lanes 2 and 5, extract from oocytes injected with *AsNramp* mRNA; lanes 3 and 6, extract from oocytes injected with rat *DCT1* mRNA. Note the broad signal from 45 kDa to more than 100 kDa detected only in oocytes injected with *AsNramp* or rat *DCT1* mRNA (lanes 5 and 6).

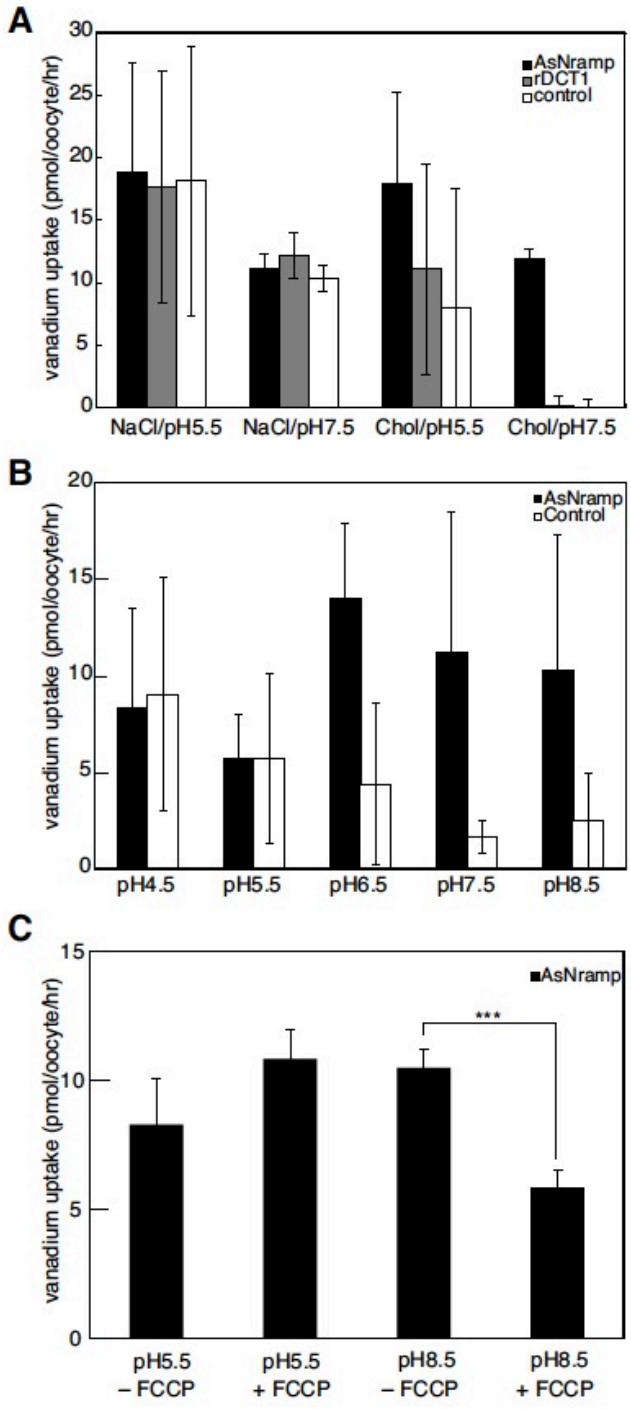

Fig. 5. Specificity of vanadium uptake by *As*Nramp and rat DCT1 in *Xenopus* oocytes. Oocytes were injected with 30–36 ng of *As*Nramp (solid bars) or rat DCT1 (gray bars) mRNA per oocyte and incubated for 4 days in MBS buffer before the uptake experiment. Control oocytes (open bars) were prepared at the same time and incubated in MBS buffer. A) VO^{2+} uptake was examined in a basal buffer

(10 mM HEPES, 2 mM MES, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) supplemented with 100 mM sodium chloride (NaCl) or choline chloride (Chol), and at pH 5.5 and 7.5, as indicated, at 20°C. The initial VO^{2+} concentration was 10.5 μM. Bars indicate means ± SD (n = 8). B) VO^{2+} uptake was examined in basal buffer (10 mM HEPES, 2 mM MES, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) supplemented with 100 mM choline chloride at 20°C. The initial VO^{2+} concentration was 10.5 μM. Bars indicate means ± SD (n = 5). C) The inhibition of VO^{2+}-uptake by protonophore FCCP. VO^{2+} uptake was examined in basal buffer (10 mM HEPES, 2 mM MES, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 5.5 or 8.5) supplemented with 100 mM choline chloride at 20°C. The initial VO^{2+} concentration was 10.5 μM. FCCP was added at 2.5 μM where indicated. Bars indicate means ± SE (n = 12). ***, P<0.001.

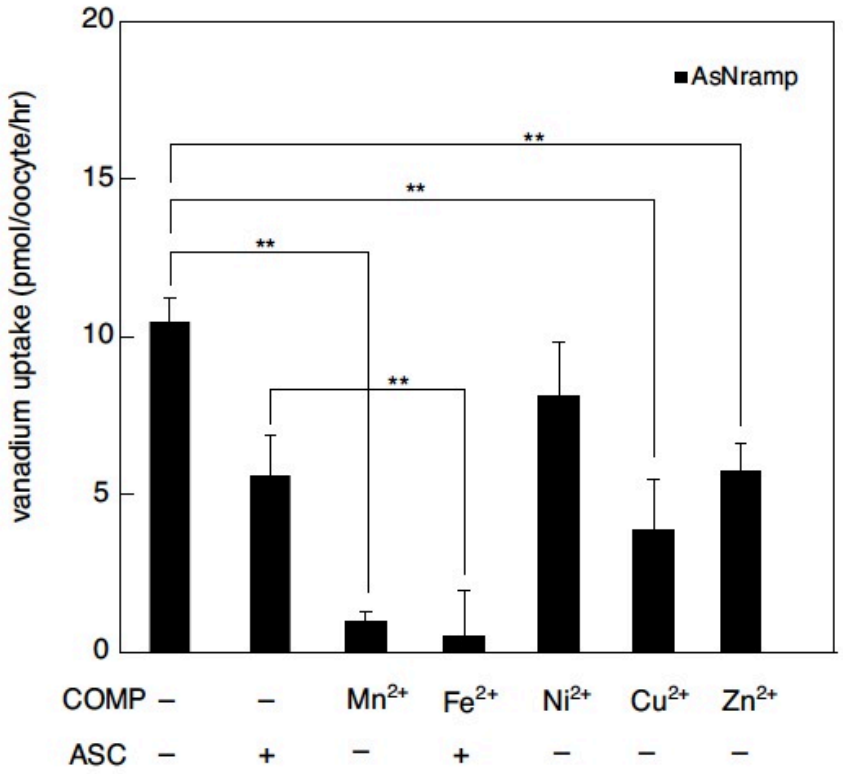

Fig. 6. The inhibition of VO^{2+}-uptake by *As*Nramp by excess divalent cations. Oocytes were injected with 32 ng of *As*Nramp mRNA per oocyte and incubated for 4 days before the uptake experiment. VO^{2+} uptake was examined in basal buffer (10 mM HEPES, 2 mM MES, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 8.5) supplemented with 100 mM choline chloride at 20°C. The initial VO^{2+} concentration was 10.5 μM, and the concentration of competitive divalent cations (COMP) were 525 μM (50 molar excess). Ascorbic acid (ASC) was added at 2.1 mM (4 molar excess) for control and Fe^{2+}. Bars indicate means ± SE (n = 10–12). *, P<0.05; **, P<0.01.

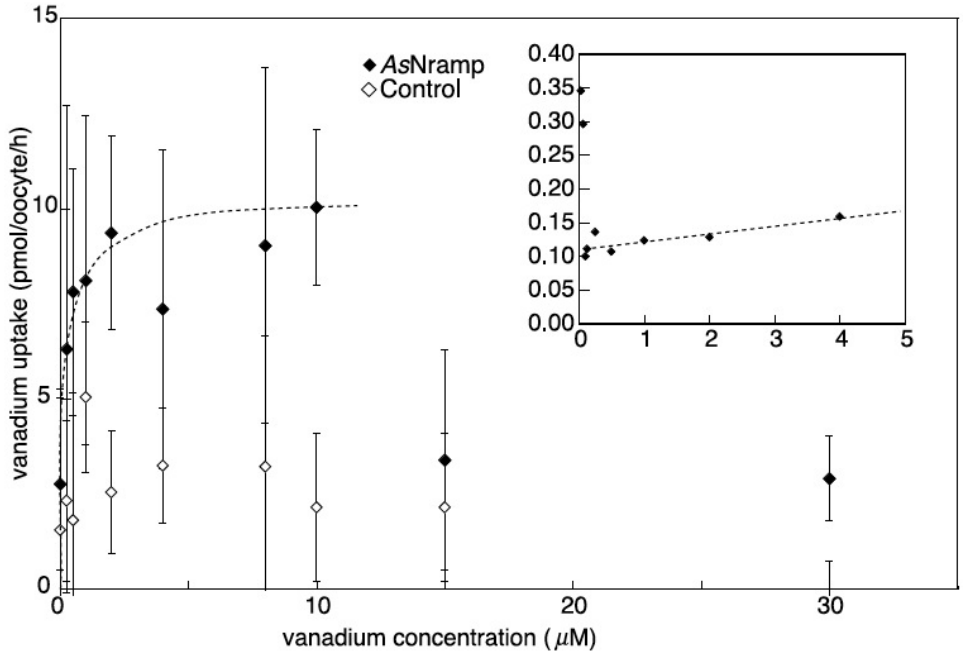

Fig. 7. Kinetic properties of vanadium uptake by *As*Nramp. Vanadium uptake was measured with increasing concentrations of VO^{2+} in uptake buffer (10 mM HEPES, 2 mM MES, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 8.5) containing 100 mM choline chloride. Each data point represents the mean value ± SD (n = 5). Calculated from the data between 0 and 10 μM, the K_m and V_{max} of *As*Nramp were 90 nM and 9.1 pmol/oocyte/h, respectively, under these conditions (dotted lines).

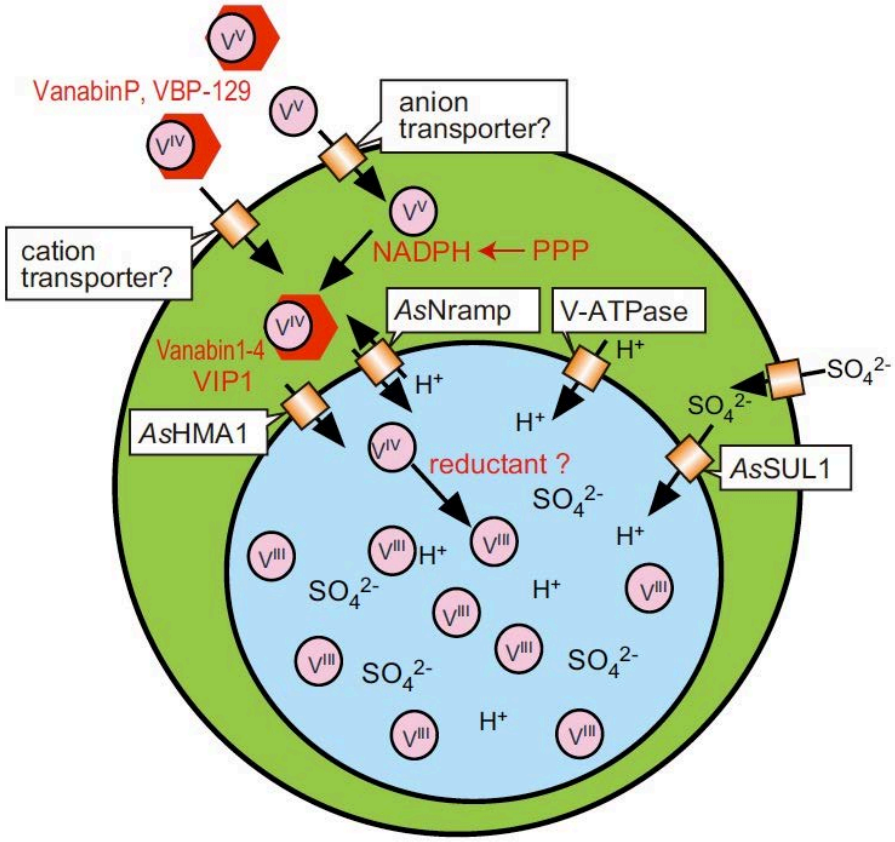

Fig. 8. A model for vanadium transport in vanadocytes of the vanadium-rich ascidian *Ascidia sydneiensis samea*. Vanadium ions are transported by an anion transporter (for V^{V}) or cation transporter (for V^{IV}) into the cytoplasm of vanadocytes. V^{V} is reduced to V^{IV} through an electron transfer cascade from the electron donor, NADPH, *via* glutathione reductase, glutathione, and Vanabin2 [19]. The vacuole content is acidified by V-ATPase, and *As*SUL1 accumulates sulfate ions. P_{1B}-type ATPase, *As*HMA1, and/or a proton/V^{IV} antiporter, *As*Nramp, transports V^{IV} into the vacuole. V^{IV} is reduced to V^{III} by an as yet unknown reductant and stabilized in acidic solution, in which sulfate ions exist as counterions for V^{III}. Reproduced from [45] with permission from the copyright holders.