A 100-kDa Antigen Recognized by a Newly Prepared Monoclonal Antibody Specific to the Vanadocytes of the Vanadium-Rich Ascidian, Ascidia sydneiensis samea, is Glycogen Phosphorylase

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A 100-kDa Antigen Recognized by a Newly Prepared Monoclonal Antibody Specific to the Vanadocytes of the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea*, is Glycogen Phosphorylase

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ABSTRACT—Ascidians have the unusual physiological ability to accumulate high levels of vanadium and reduce it to the +3 oxidation state (V^{III}) in vanadocytes, the vanadium-containing blood cells. We are characterizing several polypeptides specific to vanadocytes that may participate in this. This study revealed that a 100-kDa antigen, recognized by a newly prepared monoclonal antibody, S8E4, is exclusively localized in vanadocytes, and identified the antigen as glycogen phosphorylase (EC 2.4.1.1) by sequencing the encoded cDNA. Since two enzymes, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44), both in the pentose phosphate pathway, have already been identified in vanadocytes, at least three enzymes involved in carbohydrate metabolism are localized in vanadocytes in huge amounts.

INTRODUCTION

Ascidian species, commonly known as tunicates, selectively accumulate high levels of vanadium in their blood cells (coelomic cells), especially ascidians belonging to the suborder Phlebobranchia. The highest recorded concentration of accumulated vanadium is 350 mM, which is 10^7 times its concentration in seawater (Michibata *et al*., 1991). Almost all of the vanadium ions accumulated are reduced to the +3 oxidation state (V^{III}) via the +4 oxidation state (V^{IV}) in the blood cells (Hirata and Michibata, 1991). Studies of this phenomenon are summarized in two recent review articles (Michibata and Kanamori, 1998; Michibata *et al*., 1998).

Ascidian blood cells can be grouped into six categories on the basis of their morphology: hemoblasts, lymphocytes, leukocytes, vacuolated cells, pigment cells, and nephrocytes (Wright, 1981). The vacuolated cells can be further divided into at least four different types: morula cells, signet ring cells, compartment cells, and small compartment cells (Kaneko *et al*., 1995; Wuchiyama and Michibata, 1995). Of these, the signet ring cells have been identified as the vanadocytes, which contain high levels of vanadium, sulfate ions, and protons in their vacuoles and are thought to play a central role in the accumulation of vanadium (Michibata *et al*., 1987; Michibata *et al*., 1991; Kanamori and Michibata, 1994; Uyama *et al*., 1994). We are characterizing several polypeptides specific to vanadocytes that may participate in the accumulation and reduction of vanadium, with the ultimate goal of elucidating this unusual physiological function in ascidians. So far, large amounts of two different polypeptides, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44), both in the pentose phosphate pathway, have been found to be localized in vanadocytes (Uyama *et al*., 1998a, b).

In this experiment, we discovered that a 100-kDa antigen recognized by a newly prepared monoclonal antibody, S8E4, specific to vanadocytes, is glycogen phosphorylase (EC 2.4.1.1), an enzyme that catalyzes the phosphorolysis of glycogen to produce glucose 1-phosphate. The glucose 1-phosphate is interconverted into glucose 6-phosphate, the initial substrate in both the pentose phosphate and the Embden-Meyerhof pathways.

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MATERIALS AND METHODS

Ascidians

Specimens of the vanadium-rich ascidian, *Ascidia sydneiensis samea*, were collected in the vicinity of the Asamushi Marine Biological Station of Tohoku University at Asamushi, Aomori Prefecture, and the Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo, Otsuchi, Iwate Prefecture, Japan. The ascidians were maintained in an aquarium that contained circulating natural seawater at 18°C.

Preparation of monoclonal antibodies

Ascidian blood was centrifuged at 300 × g for 10 min to separate the blood cells from the serum. The blood cells were suspended in Ca^{2+}- and Mg^{2+}-free artificial seawater containing 0.2 M sucrose, 368 mM NaCl, 7.2 mM KCl, 26.4 mM Na_2SO_4, 4.8 mM $NaHCO_3$, and 4 mM HEPES, at pH 7.0, to prevent clotting and were centrifuged at 100 × g for 10 min at 4°C. The centrifuged blood cells formed two layers. The upper layer, consisting of a subpopulation of giant cells predominantly, was discarded. The lower one, consisting of signet ring cells (vanadocytes), morula cells, compartment cells, and pigment cells, was used to prepare antigens.

An aliquot of 200 mg wet weight of blood cells was homogenized in 6 ml of 0.2 M Tris-HCl buffer (pH 8.0) containing protease inhibitors [leupeptin, pepstatin A, chymostatin, phenylmethylsulfonyl fluoride (PMSF), each at a concentration of 10 μg/ml], using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 × g for 10 min. The supernatant was further centrifuged at 100,000 × g for 1 hr to obtain the microsome fraction. The precipitate was suspended in phosphate buffered saline (PBS), which consisted of 136.9 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.2. An aliquot of 0.5 ml of the suspension, which contained approximately 250 μg protein, was mixed with Freund's complete/incomplete adjuvant and injected intraperitoneally into female BALB/c mice. Monoclonal antibody was prepared in the same manner as described previously (Uyama *et al.*, 1991).

Western blot analysis

In order to identify the antigen recognized by S8E4 monoclonal antibody, Western blot analysis was performed as described previously (Uyama *et al.*, 1997, 1998b). In brief, samples containing approximately 30 μg protein were dissolved in a sample dissociation buffer solution consisting of 62.5 mM Tris-HCl at pH 6.8, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 2.3% (w/v) SDS. The dissolved sample was electrophoresed by 10% uniform SDS-PAGE and subsequently subjected to Western blot analysis to detect the antigen recognized by the monoclonal antibody S8E4. The antigen-antibody reaction was visualized by ECL Western blotting detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Immunoscreening the cDNA library

The cDNA library, prepared using the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA) as described previously (Uyama *et al.*, 1998b), was screened using S8E4 monoclonal antibody as a probe. The one positive clone obtained was purified by two rounds of screening and subcloned by *in vivo* excision in accordance with the protocol provided by Stratagene. The resulting cDNA was inserted into the *Eco*RI-*Xho*I site of the plasmid vector pBluescript SK(–). The plasmid clone, which contained the 3 kbp-cDNA of the S8E4 antigen gene, was sequenced on both strands using the dideoxy chain-termination

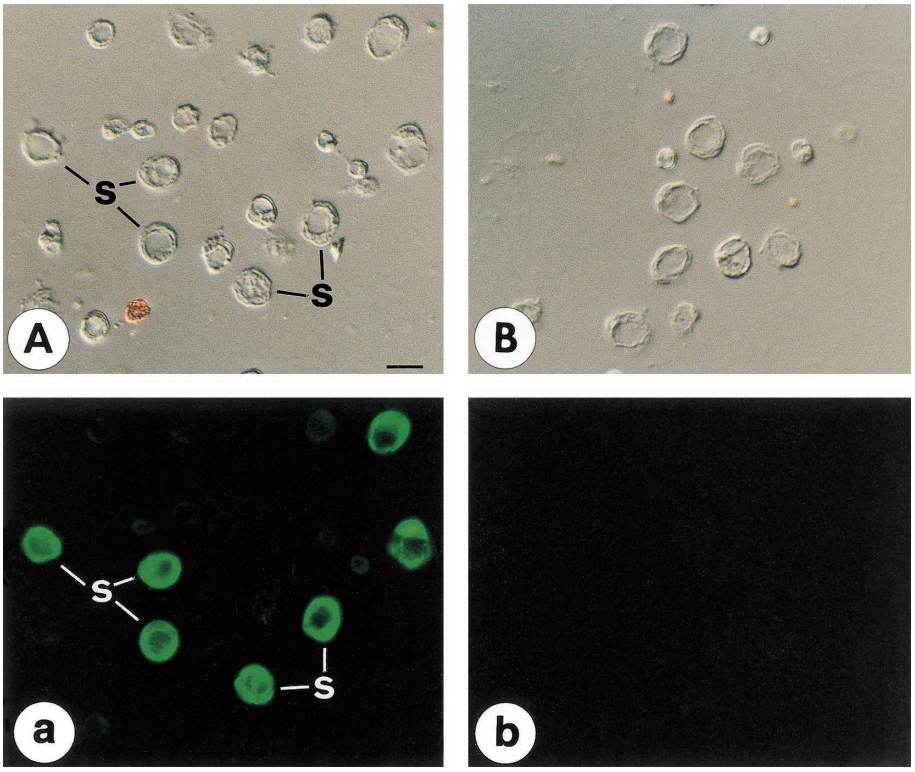

Fig. 1. Immunocytological detection of S8E4 monoclonal antibody in blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. The blood cells shown in panels **A** and **a** were reacted with S8E4. The blood cells in panels **B** and **b** were reacted with nonimmune mouse serum as a negative control. The upper (**A** and **B**) and lower (**a** and **b**) panels were visualized by Nomarski differential-interference and fluorescence microscopy, respectively. Vanadocytes (signet ring cells) were exclusively recognized by S8E4 and fluoresced with FITC. No immunoreactivity was observed in the other types of blood cells. Morula cells faintly emitted autofluorescence. s, vanadocytes (signet ring cells). Scale bar indicates 10 μm.

method with a ThermoSequenase Kit for the ALFexpress DNA sequencer (Amersham Pharmacia Biotech) using Cy5 labeled primers and resolving the samples on denaturing 6% polyacrylamide gels.

RESULTS

Immunological detection of S8E4

As shown in Fig. 1, the newly prepared monoclonal antibody, designated S8E4, specifically recognized signet ring cells, which are the so-called vanadocytes. No immunoreactivity was observed in blood cells other than the vanadocytes, although *A. sydneiensis samea* has about ten types of blood cells. After the homogenate was subjected to SDS-PAGE, many proteins were visualized with Coomassie Brilliant Blue staining (Fig. 2, lane 1). Of these proteins, Western blot analysis showed that S8E4 monoclonal antibody clearly recognized a 100-kDa band (Fig. 2, lane 2).

cDNA cloning and sequence analysis

One cDNA clone was isolated as the result of screening a cDNA library prepared from ascidian blood cells for the gene encoding the 100-kDa antigen, using S8E4 monoclonal antibody as a probe. The insert, which contained a full-length cDNA designated *asgp*, was subcloned into the plasmid vector pBluescript SK(–) using the sequencing strategy shown in Fig. 3. Consequently, *asgp* was found to include 116 bp of the 5' untranslated region, a 2,598 bp open reading frame (ORF), and 327 bp of the 3' untranslated sequence, as shown in Fig. 4. The ORF encoded an 865 amino acid protein. A search of the SwissProt sequence database for proteins similar to *asgp* detected matches with glycogen phosphorylase (GP) for both the nucleotide and amino acid sequences. The match was closest for the amino acid sequence. The 865 amino acid protein shares 71.4%, 70.9%, and 69.6% identity with GP derived from the liver, brain, and muscle of the rat, respectively (Schiebel *et al*., 1992; Hudson *et al*., 1993). The calculated molecular mass of the predicted protein was 99 kDa, which

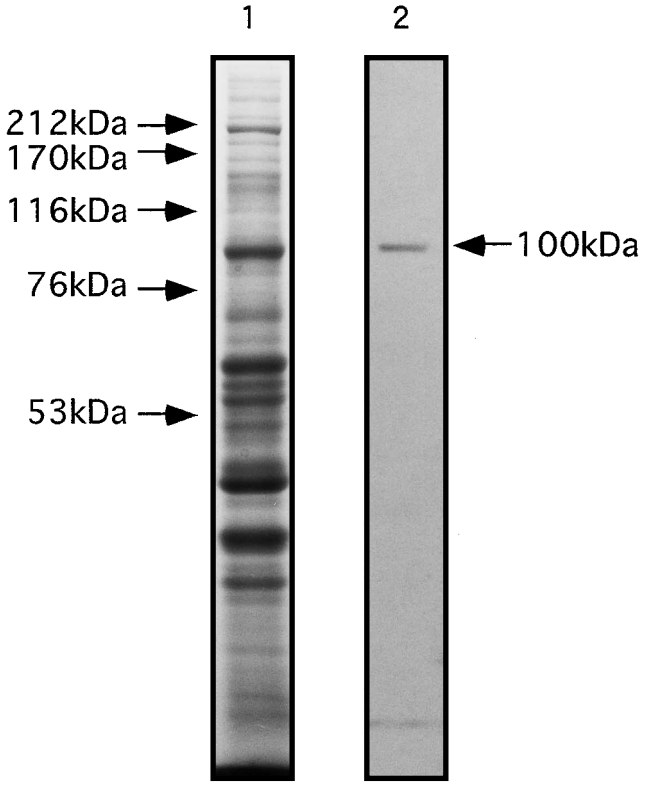

Fig. 2. SDS-PAGE and Western blot analysis. Blood cells of *A. sydneiensis samea* were homogenized and separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue (lane 1). The separated proteins were blotted onto nitrocellulose paper and reacted with S8E4 monoclonal antibody (lane 2). A positive band corresponding to a 100-kDa protein was observed. Lane 1, homogenate of blood cells; Lane 2, Western blot analysis with S8E4.

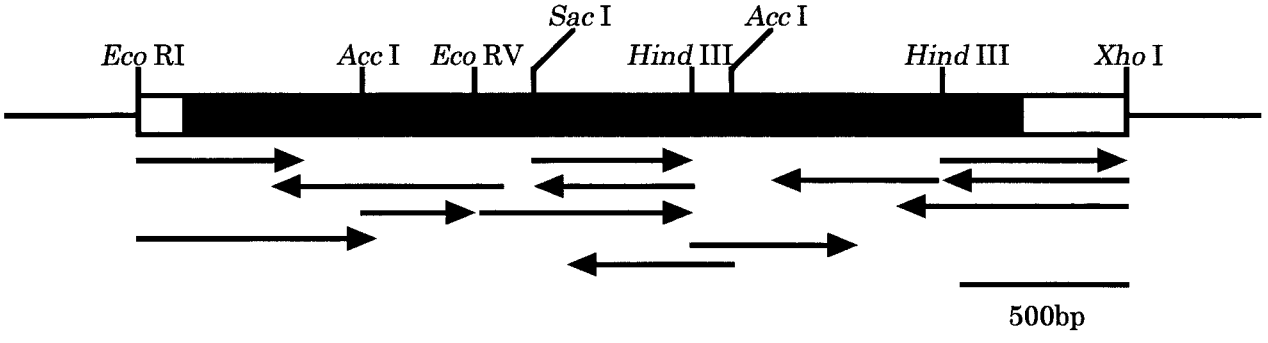

Fig. 3. Subcloning and nucleotide sequencing strategy for the cDNA encoding the 100-kDa antigen recognized by S8E4 monoclonal antibody. A diagram illustrating the structure of a cDNA clone *asgp* encoding the 100-kDa cDNA. *asgp* cDNA (3 kbp) was inserted in pBluescript SK(–) between the *Eco*RI and *Xho*I sites. The solid line represents part of the vector. The open boxes represent non-coding regions. The closed box represents the coding region. The restriction enzymes were used to subclone *asgp*. Arrows indicate the extent and direction of the sequenced strands. bp, base pairs.

A

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                                                                                                                TTCTGTTAGTAAACCTA   17
AGGAAGCTGTATTGAAATCTGAACCTGCTAAGGCAAACGTTAAGTCCAACGGTTCGAACGGTGTATCACGCACGAAGTGAAAGGGAGCTCACACCAAAC  116

ATG ACG TCC AAA CCT GTA ACA GAT CAA GAG AAA CGC AAG CAA ATC TCT GTG CGT GGA ATA GCA TCT CTT GAA GGA  191
 M   T   S   K   P   V   T   D   Q   E   K   R   K   Q   I   S   V   R   G   I   A   S   L   E   G   25
GTT GCT GAC ATT AAA AAG TCG TTC AAT CGT CAC TTG CAT TTC ACT CTG GTG AAA GAC CGA AAT GTT GCA ACA CCA  266
 V   A   D   I   K   K   S   F   N   R   H   L   H   F   T   L   V   K   D   R   N   V   A   T   P   50
AGA GAT TAC TAT TTT GCT CTT GCC AAC ACT GTG AGA GAC CAA CTG GTT GGA AGA TGG ATT CGG ACA CAA CAA TAT  341
 R   D   Y   Y   F   A   L   A   N   T   V   R   D   Q   L   V   G   R   W   I   R   T   Q   Q   Y   75
TAT TAT GAG AAG GAC CCA AAG AGA GTG TAT TAC TTG TCA CTG GGA TTC TAC ATG GGA AGA GCT TTG CAA AAC ACG  416
 Y   Y   E   K   D   P   K   R   V   Y   Y   L   S   L   G   F   Y   M   G   R   A   L   Q   N   T  100
ATG CTC AAC CTT GGA ATT CAA AGT TCC TGT GAT GAG GCT ATG TAC CAG ATT GGA CTG GGT ATA GAA GAG TTA GAG  491
 M   L   N   L   G   I   Q   S   S   C   D   E   A   M   Y   Q   I   G   L   G   I   E   E   L   E  125
GAA ATG GAA GAA GAT GCC GGA TTG GGA AAT GGT GGT CTT GGT CGA TTG GCA GCC TGT TTC TTG GAC TCC ATG GCA  566
 E   M   E   E   D   A   G   L   G   N   G   G   L   G   R   L   A   A   C   F   L   D   S   M   A  150
ACT TTG GGC TTG GCT GCT TAT GGT TAT GGT ATT CGA TAT GAA TAT GGC ATT TTC AAT CAG AAG ATA AGG GAA GGT  641
 T   L   G   L   A   A   Y   G   Y   G   I   R   Y   E   Y   G   I   F   N   Q   K   I   R   E   V  175
TGG CAG GTT GAA GAA GCT GAT GAC TGG TTG AGA TAC GGA AAC GCT TGG GAC AAG GCC AGA CCA GAA TAC ATG ATT  716
 W   Q   V   E   E   A   D   D   W   L   R   Y   G   N   A   W   D   K   A   R   P   E   Y   M   I  200
CCA GTC CAT TTT TAT GGC CGT GTC GAC CAC GAG GAT GGA GAT TGG AGC AAG CCA AGC AAG TGG AGT GAC ACA AAT  791
 P   V   H   F   Y   G   R   V   D   H   E   D   G   D   W   S   K   P   S   K   W   S   D   T   N  225
GTT GTC TTT GCA ATG CCA TAC GAC ACA CCA ACC CCT GGT TAT GGC AAC AAC ACT GTC AAC ACA TTG AGG CTC TAC  866
 V   V   F   A   M   P   Y   D   T   P   T   P   G   Y   G   N   N   T   V   N   T   L   R   L   Y  250
ACT GCC AAA TCA CCT AAT TCA TTT AAT CTT GGT GTT TTC AAC ACT GGA GAT TAC ATT CAA GCT GTT TGC GAC CGA  941
 T   A   K   S   P   N   S   F   N   L   G   V   F   N   T   G   D   Y   I   Q   A   V   C   D   R  275
AAC CTG GCT GAA AAT ATA TCA AGG GTC TTG TAT CCG AAT GAC AAC TTT TTT GAA GGC AAG GAA CTG AGA TTG AAG 1016
 N   L   A   E   N   I   S   R   V   L   Y   P   N   D   N   F   F   E   G   K   E   L   R   L   K  300
CAG GAG TAT TTT GTG GTG TGT GCG ACT GTC CAG GAT ATC ATA CGT CGA TTC AAG TCC TCA ATA TTT GGA TGT CGT 1091
 Q   E   Y   F   V   V   C   A   T   V   Q   D   I   I   R   R   F   K   S   S   I   F   G   C   R  325
GAC CCT GTC AGA ACA TCG CTT GAT GCT TTT CCT GAT AAG GTC GCC ATA CAG CTA AAC GAC ACC CAT CCG GCC TTG 1166
 D   P   V   R   T   S   L   D   A   F   P   D   K   V   A   I   Q   L   N   D   T   H   P   A   L  350
GCC ATC CCT GAG CTC ATG CGA CTC TTT GTC GAT GTT GAG AAA ATG CCT TGG GAA AGA GCA TGG AAC ATA GTG AGA 1241
 A   I   P   E   L   M   R   L   F   V   D   V   E   K   M   P   W   E   R   A   W   N   I   V   R  375
AAG ACA TGC GCC TAC ACA AAC CAC ACA GTC TTG CCT GAA GCG TTG GAA CGT TGG CCT GTG CAC TTG TTG GAA AGA 1316
 K   T   C   A   Y   T   N   H   T   V   L   P   E   A   L   E   R   W   P   V   H   L   L   E   R  400
ATG CTT CCA AGA CAT CTT GAG ATT ATT TAC ATC ATC ACC CAA AGC ACT TGG AAA ATG TGT CCA AAA ATG TTT CCA 1391
 M   L   P   R   H   L   E   I   I   Y   I   I   T   Q   S   T   W   K   M   C   P   K   M   F   P  425
GAT GAT CCC GAC CGA CTT CGT AGA ATG TCT CTC GTC GAG GAA GAA GGA GAG AAA CGA ATC AAC ATG GCA CAT CTT 1466
 D   D   P   D   R   L   R   R   M   S   L   V   E   E   E   G   E   K   R   I   N   M   A   H   L  450
TGT ATT GTG GGG TCG CAT GTT GTC AAT GGT GTG GCG GCA ATA CAT TCT GAA ATC ATC AGA ACA TCT GTT TTC AAG 1541
 C   I   V   G   S   H   V   V   N   G   V   A   A   I   H   S   E   I   I   R   T   S   V   F   K  475
GAC TTT GTT GAA CTT GCT GAA AAA ATG GGG GAG AAA AAT AAG TTC CAG AAT AAA ACT AAT GGC ATC ACT CCA AGG 1616
 D   F   V   E   L   A   E   K   M   G   E   K   N   K   F   Q   N   K   T   N   G   I   T   P   R  500
AGG TGG CTC CTG CTT TGT AAC CCT GGC CTG GCC GAT CTT ATT GCT GAG AAA ATA GGG GAA GAT TGG CCA AAG AAC 1691
 R   W   L   L   L   C   N   P   G   L   A   D   L   I   A   E   K   I   G   E   D   W   P   K   N  525
CTG GAC CAA CTT CGT GAG CTT GAA AGC TTC AAA GAC GAT GCA GCC TTC ATT CGA AGA GTT AGC CAA ATC AAA CAG 1766
 L   D   Q   L   R   E   L   E   S   F   K   D   D   A   A   F   I   R   R   V   S   Q   I   K   Q  550
GAA AAT AAG ATG AAG TTG GCA CAG TTC ATA AAC AAG CAG TGG GGA GTA AAA GTA GAC CCC TCA TCC ATG TTC GAT 1841
 E   N   K   M   K   L   A   Q   F   I   N   K   Q   W   G   V   K   V   D   P   S   S   M   F   D  575
GTC CAG GTG AAA CGA ATC CAC GAG TAC AAG CGT CAG CTC ATG AAC GCC CTG CAC ATT GTT GTA ATG TAC AAC CGA 1916
 V   Q   V   K   R   I   H   E   Y   K   R   Q   L   M   N   A   L   H   I   V   V   M   Y   N   R  600
ATC AAA ACT GAC CCG AAC AAA GAT TTT GTG CCC AGA ACA GTG ATG GTC GGT GGA AAG GCC GCA CCT GGA TAC CAC 1991
 I   K   T   D   P   N   K   D   F   V   P   R   T   V   M   V   G   G   K   A   A   P   G   Y   H  625
ACT GCC AAG ATG ATC ATC AAA CTG ATC AAC AAT ATC GCA CAT GTT GTC AAT AAT GAT CCT ATT GTT GGT GAC AGG 2061
 T   A   K   M   I   I   K   L   I   N   N   I   A   H   V   V   N   N   D   P   I   V   G   D   R  650
TTG AAG GTT GTT TAT CTG GAA AAC TAC AGA GTC TCT CTA GCC GAG AAA GTA ATT CCG GCC GCC GAT CTG TCA GAA 2141
 L   K   V   V   Y   L   E   N   Y   R   V   S   L   A   E   K   V   I   P   A   A   D   L   S   E  675
CAG ATT TCC ACG GCA GGA ACC GAA GCG TCA GGA ACA GGA AAC ATG AAA TTC ATG CTC AAC GGT GCT CTC ACC ATC 2216
 Q   I   S   T   A   G   T   E   A   S   G   T   G   N   M   K   F   M   L   N   G   A   L   T   I  700
GGG ACA CTA GAT GGG GCT AAC GTG GAG ATG GCT GAG GAA ATG AAT GGA GAA AAT ATT TTC ATA TTT GGG TTG AAG 2291
 G   T   L   D   G   A   N   V   E   M   A   E   E   M   N   G   E   N   I   F   I   F   G   L   K  725
GTT GAC GAA GTT GAG CAA TTG GAC AAG GAC GGT TAC AAT GCC CGA TCT TTC TAC GAG AAT GTT CCG GAA CTT CGA 2366
 V   D   E   V   E   Q   L   D   K   D   G   Y   N   A   R   S   F   Y   E   N   V   P   E   L   R  750
ACA GCC CTA GAC CAA ATT TCA TCC GGC TAC TTC AAC CCC AAC GAA CCG GAC CAG TTT GCC CAT TTC GTG GAA AAC 2441
 T   A   L   D   Q   I   S   S   G   Y   F   N   P   N   E   P   D   Q   F   A   H   F   V   E   N  775
CTC ATT AAA TTC GAC AGG TTT AAG CTT CTG GCG GAT TTC CAA TCT TAC GTT GAG TGT CAG GAT AAA GTC AGT GCT 2516
 L   I   K   F   D   R   F   K   L   L   A   D   F   Q   S   Y   V   E   C   Q   D   K   V   S   A  800
GCC TAC AAG GAC ACT TAC AAG TGG ACC CAG ATG TGC ATA GCC AAC ATT GCT GCA TCT GGA AAG TTC TCA AGT GAC 2591
 A   Y   K   D   T   Y   K   W   T   Q   M   C   I   A   N   I   A   A   S   G   K   F   S   S   D  825
CGA ACC ATC GCC GAG TAC GCG AGA CAA ATC TGG GGA GTC GAG CCA CAA CCA AAT CTC AAG ATA CCT GCG CCC AAC 2666
 R   T   I   A   E   Y   A   R   Q   I   W   G   V   E   P   Q   P   N   L   K   I   P   A   P   N  850
GAA CCT CTT GAA AGA GCG GAG AAC ACC GAA GGA TAC AAG GAT TTC TGA                                      2714
 E   P   L   E   R   A   E   N   T   E   G   Y   K   D   F   *                                      866

TTCCTTCCTGAACCCAAAGTTCCAATTATCTAGAATTTATTGCTAAGTTGCAGTATTTTTTGTTTTCGCCTTAGAAACCAAAGAAACCTAAATTGTTCA 2813
CGTTATTGGTCAAATCCCACAGACTTCGTAGGCCTGATTTGCTATCCTGATGTGTATGCTTCCACTGTTAGGAATGATTTCAAGGACCTTTCACCTTCT 2912
CACTCACTGCGCATTGTGTGTCATTTGGTTCACGTTTGACTATTATTATTATTATTATTTTTGCGTTTATTTAGTTATAAAGGTTCAGAATATAAAGCA 3011
TGCATTACCTAAAAAAAAAAAAAAAAAAA                                                                       3041
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B

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RLGP   --MAKPLTDQEK-------------------------RRQISIRGIVGVENVAELKKGFNRHLHFTLVKDRNVATPRDYYFALAHTVRDHLVGRWIRTQ  72
RBGP   --MAKPLTDSER-------------------------QKQISVRGIAGLGDVAEVRKSFNRHLHFTLVKDRNVATPRDYFFALAHTVRDHLVGRWIRTQ  72
RMGP   ---SRPLSDQDK-------------------------RKQISVRGLAGVENVSDLKKNFNRHLHFTLVKDRNVATPRDYYFALAHTVRDHLVDRWIRTQ  71
AsGP   -MTSKPVTDQEK-------------------------RKQISVRGIASLEGVADIKKSFNRHLHFTLVKDRNVATPRDYYFALANTVRDQLVGRWIRTQ  73
YGP    MITEEPTSPHQIPRLTRRLTGFLPQEIKSIDTMIPLKSRALWNKHQVKKFNKAEDFQDRFIDHVETTLARSLYNCDDMAAYEAASMSIRDNLVIDWNKTQ 100
           * .                                .     . .       . .  *  *.  ** .    .     . * . ..** **  * .**

RLGP   QHYYDKCPKRVYYLSLEFYMGRTLQNTMINLGLQNACDEAIY-------------QLGLDMEELEEIEEDAGLGNGGLGRLAACFLDSMATLGLAAYGYG 159
RBGP   QHYYERDPKRIYYLSLEFYMGRTLQNTMVNLGLQTACDEATY-------------QLGLDLEELEEIEEDAGLGNGGLGRLAACFLDSMATLGLAAYGYG 159
RMGP   QHYYAKDPKRIYYLSLELYMGRTLQNTMVNLALENACDEATY-------------QLGLDMEELEEIEEDAGLGNGGLGRLAACFLDSMATLGLAAYGYG 158
AsGP   QYYYEKDPKRVYYLSLGFYMGRALQNTMLNLGIQSSCDEAMY-------------QIGLGIEELEEMEEDAGLGNGGLGRLAACFLDSMATLGLAAYGYG 160
YGP    QKFTTRDPKRVYYLSLEFLMGRALDNALINMKIEDPEDPAASKGKPREMIKGALDEGGFKLEDVLDQEPDAGLGNGGLGRLAACFVDSMATEGIPAWGYG 200
       * .  . ***.*****   ***.* *...*. ..   * *               . *  .*.. . * ****************.***** *. * ***

RLGP   IRYEYGIFNQKIREGWQVEEADDWLRHGNPWEKARPEFMLPVHFYGRVEHT------QAGTKWVDTQVVLALPYDTPVPGYMNNTVNTMRLWSARAPNDF 253
RBGP   IRYEFGIFNQKIVNGWQVEEADDWLRYGNPWEKARPEYMLPVHFYGRVEHT------PNGVLWLDTQVVLAMPYDTPVPGYKNNTVNTMRLWSAKAPNDF 253
RMGP   IRYEFGIFNQKICGGWQMEEADDWLRYGNPWEKARPEFTLPVHFYGRVEHT------SQGAKWVDTQVVLAMPYDTPVPGYRNNVVNTMRLWSAKAPPYF 252
AsGP   IRYEYGIFNQKIREVWQVEEADDWLRYGNAWDKARPEYMIPVHFYGRVDHEDGD--WSKPSKWSDTNVVFAMPYDTPTPGYGNNTVNTLRLYTAKSPNSF 258
YGP    LRYEYGIFAQKIIDGYQVETPDYWLNSGNPWEIERNEVQIPVTFYGYVDRPEGGKTTLSASQWIGGERVLAVAYDFPVPGFKTSNVNNLRLWQARPTTEF 300
       .***.*** ***    *.*  * **  ** *.  * *  .** *** *..           *     * *. ** * **. .  **..**  *.    *

RLGP   NLQDFNVGDYIQAVLDRNLAENISRVLYPNDNFFEGKELRLKQEYFVVAATLQDVIRRFKASKFGSKDGVGTVFDAFPDQVAIQLNDTHPALAIPELMRI 353
RBGP   KLKDFNVGDYIEAVLDRNLAENISRVLYPNDNFFEGKELRLKQEYFVVAATLQDIIRRFKSSKFGCRDPVRTCFETFPDKVAIQLNDTHPALSIPELMRI 353
RMGP   NLKDFNVGGYIQAVLDRNLAENISRVLYPNDKFFEGKELRLKQEYFVVAATLQDIIRRFKSSKFGCRDPVRTNFDAFPDKVAIQLNDTHPSLAIPELIRI 352
AsGP   NLGVFNTGDYIQAVCDRNLAENISRVLYPNDNFFEGKELRLKQEYFVVCATVQDIIRRFKSSIFGCRDPVRTSLDAFPDKVAIQLNDTHPALAIPELMRL 358
YGP    DFAKFNNGDYKNSVPQQQRAESITAVLYPNDNFAQGKELRLKQQYFWCAASLHDILRRFKKS--------KRPWTEFPDQVAIQLNDTHPTLAIVELQRV 392
           ** * *  .*  .. ** *. ****** * .********.**  .*...*..**** *              ***.**********.*.* ** *.

RLGP   FVDIEKLPWSKAWEITKKTFAYTNHTVLPEALERWPVDLVEKLLPRHLQIIYEINQKHLDRIVALFPKDIDRMRRMSLIEEEGGKR-INMAHLCIVGCHA 452
RBGP   LVDVEKVDWDKAWEITKKTCAYTNHTVLPEALERWPVSMFEKLLPRHLEIIYAINQRHLDHVAALFPGDVDRLRRMSVIEEGDCKR-INMAHLCVIGSHA 452
RMGP   LVDLERLDWDKAWDVTVKTCAYTNHTVLPEALERWPVHLMETLLPRHLQIIYEINQRFLNRVAAAFPGDVDRLRRMSLVEEGAVKR-INMAHLCIAGSHA 451
AsGP   FVDVEKMPWERAWNIVRKTCAYTNHTVLPEALERWPVHLLERMLPRHLEIIYIITQSTWKMCPKMFPDDPDRLRRMSLVEEEGEKR-INMAHLCIVGSHV 457
YGP    LVDLEKLDWHEAWDIVTKTFAYTNHTVMQEALEKWPRRLFGHLLPRHLEIIYDINWFFLQDVAKKFPKDVDLLSRISIIEENSPERQIRMAFLAIVGSHK 492
       **.*.. *  ** .  ** *******. ****.**  .   .*****.*** *.          ** * * . *.*..**    * * ** *.. *.*

RLGP   VNGVAKIHSDIVKTQVFKDFSELEPDK-----FQNKTNGITPRRWLLLCNPGLADLIAEKIG---EDYVKDLSQLTKLHSFVGDDIFLRELAKVKQENKL 544
RBGP   VNGVARIHSEIVKQSVFKDFYELEPEK-----FQNKTNGITPRRWLLLCNPGLAEIIVERIG---EGFLTDLSQLKKLLSLVDDEAFIRDVAKVKQENKL 544
RMGP   VNGVARIHSEILKKTIFKDFYELEPHK-----FQNKTNGITPRRWLVLCNPGLAEVIAERIG---EEYISDLDQLRKLLSYLDDQAFIRDVAKVKQENKL 543
AsGP   VNGVAAIHSEIIRTSVFKDFVELAEKMGEKNKFQNKTNGITPRRWLLLCNPGLADLIAEKIG---EDWPKNLDQLRELESFKDDAAFIRRVSQIKQENKM 554
YGP    VNGVVELHSELIKTTIFKDFIKFYGPS----KFVNVTNGITPRRWLKQANPSLAKLISETLNDPTEEYLLDMAKLTQLEKYVEDKEFLKKWNQVKLNNKI 588
       ****  .**....  .****            * * **********  .** ** .* * ..   *     . .*  *     *  *..   ..*  **.

RLGP   KFSQFLEKEYK------VKINPSSMFDVHVKRIHEYKRQLLNCLHVITMYNRIK---------KDPKKFFVPRTVIIGGKAAPGYHMAKMIIKLVTSVAE 629
RBGP   KFSAQLEKEYK------VKINPCSMFDVHVKRIHEYKRQLLNCLHIITLYNRIK---------KDPTKTFVPRTVMIGGKAAPGYHMAKMIIKLVTSIGD 629
RMGP   KFSAYLETEYK------VHINPNSLFDVQVKRIHEYKRQLLNCLHIITLYNRIK---------REPNRFMVPRTIMIGGKAAPGYHMAKMIIKLITAIGD 628
AsGP   KLAQFINKQWG------VKVDPSSMFDVQVKRIHEYKRQLMNALHIVVMYNRIK---------TDPNKDFVPRTVMVGGKAAPGYHTAKMIIKLINNIAH 639
YGP    RLVDLIKKENDGVDIINREYLDDTLFDMQVKRIHEYKRQQLNVFGIIYRYLAMKNMLKNGASIEEVAKKYPRKVSIFGGKSAPGYYMAKLIIKLINCVAD 688
       .    .  .               ..**..********** .*   ..  *  .*          .  .    .  . ***.****  **.****.. .

RLGP   VVNNDPMVGSKLKVIFLENYRVSLAEKVIPATDLSEQISTAGTEASGTGNMKFMLNGALTIGTMDGANVEMAEEAGEENLFIFGMRVDDVAALDKKGYEA 729
RBGP   VVNHDPVVGDRLRVIFLENYRVSLAEKVIPAADLSQQISTAGTEASGTGNMKFMLNGALTIGTMDGANVEMAEEAGEENLFIFGMRVEDVEALDQKGYNA 729
RMGP   VVNHDPAVGDRFRVIFLENYRVSLAEKVIPAADLSEQISTAGTEASGTGNMKFMLNGALTIGTMDGANVEMAEEAGEDNFFIFGMRVEDVERLDQRGYNA 728
AsGP   VVNNDPIVGDRLKVVYLENYRVSLAEKVIPAADLSEQISTAGTEASGTGNMKFMLNGALTIGTLDGANVEMAEEMNGENIFIFGLKVDEVEQLDKDGYNA 739
YGP    IVNNDESIEHLLKVVFVADYNVSKAEIIIPASDLSEHISTAGTEASGTSNMKFVMNGGLIIGTVDGANVEITREIGEDNVFLFGNLSENVEELR---YNH 785
       .**.*.      .*...  * ** ** .***.***..*********** ****..** * ***.******.. * . .* *.**   . *  *    *

RLGP   KEYYEALP-ELKLVIDQIDNGFFSPNQPDLFKDIINMLFYH-DRFKVFADYEAYVKCQEKVSQLYMNQK-AWNTMVLRNIAASGKFSSDRTIREYAKDIW 826
RBGP   QEFYERLP-ELRQAVDQISSGFFSPKDPDCFKDVVNMLMYH-DRFKVFADYEAYIQCQAQVDHLYRNPK-DWTKKVIRNIACSGKFSSDRTITEYAREIW 826
RMGP   QEYYDRIP-ELRQIIEQLSSGFFSPKQPDLFKDIVNMVMHH-DRFKVFADYEEYIKCQDKVSELYKNPR-EWTRMVIRNIATSGKFSSDRTIAQYAREIW 825
AsGP   RSFYENVP-ELRTALDQISSGYFNPNEPDQFAHFVENLIKF-DRFKLLADFQSYVECQDKVSAAYKDTY-KWTQMCIANIAASGKFSSDRTIAEYARQIW 836
YGP    QYHPQDLPSSLDSVLSYIESGQFSPENPNEFKPLVDSIKYHGDYYLVSDDFESYLATHELVDQEFHNQRSEWLKKSVLSLANVGFFSSDRCIEEYSDTIW 885
       .    .*  *   .  .  * * *  *  *   .  .   * . .  *.. *. ..  *   .      *    .  .*  * *****.* .*.  **

RLGP   NMEPS-DLKISLSKESSNGVNANGK---- 851
RBGP   GVEPS-DLQIPPPNLPKD------------ 843
RMGP   GLEPS-RQRLPAPDEKI------------ 841
AsGP   GVEPQPNLKIPAPNEPLERAENTEGYKDF 865
YGP    NVEPVT---------------------- 891
       ..**
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Fig. 4. Sequence alignments of the *asgp* gene encoding the 100-kDa antigen (**A**) and the deduced amino acids (**B**). The sequence of the functional cDNA isolated from clone *asgp* has an open reading frame (ORF) 2,598 nucleotides long-including the termination codon. The ORF extends from the first methionine codon at nucleotide 117 of the fragment to a TGA codon at nucleotide 2,714. The stop codon is indicated by an asterisk (**A**). Alignments between the amino acid sequence (AsGP) deduced from the nucleotide sequence of the *asgp* gene and those of GP derived from rat liver, brain, muscle, and the yeast *Saccharomyces cerevisiae* were compared. Amino acids that are identical in the four sequences are marked by an asterisk, while those that are similar are marked by a dot (**B**). The ORF encoded an 865 amino acid protein with 71.4% identity and 83.4% similarity to rat liver GP (Schiebel *et al*., 1992), while *asgp* and yeast GP only share 45.7% amino acid identity and 63.3% similarity (Hwang and Fletterick, 1986). RLGP, rat liver GP; RBGP, rat brain GP; RMGP, rat muscle GP; YGP, yeast GP.

correlated well with the expected molecular weight determined by Western blot analyses using S8E4 monoclonal antibody (Fig. 2). In addition, it was preliminarily revealed that S8E4 monoclonal antibody specifically recognized a recombinant 100-kDa asgp protein as well as the 100-kDa peptide from vanadocytes (data not shown).

DISCUSSION

In this study, an antigen specific to vanadocytes, recognized by the newly prepared S8E4 monoclonal antibody (Fig. 1), was revealed to be a 100-kDa peptide (Fig. 2). The predicted amino acid sequence of the cDNA clone *asgp* encoding the 100-kDa antigen shares approximately 70% identity with the amino acid sequence of GP derived from the rat (Fig. 4). These results demonstrate that the 100-kDa peptide from vanadocytes is GP, which is the third enzyme involved in carbohydrate metabolism to be found in vanadocytes. In addition to GP, massive amounts of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), which are both involved in the pentose phosphate pathway, are localized in vanadocytes (Uyama *et al.*, 1998a, b).

Since almost all of the vanadium ions dissolved in the +5 oxidation state (V^V) in seawater are reduced to V^{III} via V^{IV} in ascidian vanadocytes (Hirata and Michibata, 1991), reducing agents must participate in the accumulation process. We have already proposed that the NADPH produced in the pentose phosphate pathway in vanadocytes reduces V^V to V^{IV} in the cytoplasm of the vanadocytes, for two reasons. First, V^V is reduced to V^{IV} by the addition of NAD(P)H *in vitro* (Liochev and Fridovich, 1990; Shi and Dalal, 1993). Second, massive amounts of two enzymes in the pentose phosphate pathway, the major supplier of reducing agents in the form of NADPH, are localized in vanadocytes (Uyama *et al.*, 1998a, b). GP, newly identified to be localized in vanadocytes, is an enzyme that catalyzes the phosphorolysis of glycogen to produce glucose 1-phosphate. Glucose 1-phosphate is interconverted into glucose 6-phosphate, which is the initial substrate in both the pentose phosphate and Embden-Meyerhof pathways. Therefore, the discovery of GP provides strong additional evidence for the participation of the pentose phosphate pathway in the reduction of vanadium accompanying the accumulation of vanadium in vanadocytes. It may be safely said that the abundant expression of enzymes in the pentose phosphate pathway reflects a requirement for the NADPH produced in the pathway.

The amino acid sequence of the *asgp* gene encoding the 100-kDa antigen is highly homologous with that of GP derived from the rat (Fig. 4). Although the amino acid sequence was highly conserved, the *asgp* gene exhibits a remarkable divergence in G + C content. For some unaccountable reason, only 48.4% of the nucleotides at the third codon position in the ORF of *asgp* are deoxyguanosine or deoxycytidine residues, while approximately 70% are deoxyguanosine or deoxycytidine residues in the rat GP sequence (Schiebel *et al.*, 1992; Hudson *et al.*, 1993).

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REFERENCES

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

Hudson JW, Herreon KL, Crerar MM (1993) Comparative analysis of species independent, isozyme-specific amino-acid substitutions in mammalian muscle, brain and liver glycogen phosphorylase. Biochim Biophys Acta 1164: 197–208

Hwang PK, Fletterick RJ (1986) Convergent and divergent evolution of regulatory sites in eukaryotic phosphorylase. Nature 324: 80–84

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

Kaneko A, Uyama T, Moriyama Y, Michibata H (1995) Localization, with monoclonal antibodies and by detection of autonomous fluorescence, of blood cells in the tissues of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 12: 733–739

Liochev SI, Fridovich I (1990) Vanadate-stimulated oxidation of NAD(P)H in the presence of biological membranes and other sources of O_2^-. Arch Biochem Biophys 279: 1–7

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

Michibata H, Iwata Y, Hirata J (1991) Isolation of highly acidic and vanadium containing blood cells from among several types of blood cell from Ascidiidae species by density gradient centrifugation. J Exp Zool 257: 306–313

Michibata H, Kanamori K (1998) Selective accumulation of vanadium by ascidians from seawater. In "Advances in Environmental Science and Technology" Ed by Nriagu J, John Wiley & Sons, Inc, pp 217–249

Michibata H, Uyama T, Kanamori K (1998) The accumulation mechanism of vanadium by ascidians –an interdisciplinary study between biology and chemistry on extraordinary high levels and reduced form of vanadium in vanadocytes. Am Chem Soc Symp Ser, in press

Schiebel K, Pekel E, Mayer D (1992) The nucleotide sequence of rat liver glycogen phosphorylase cDNA. Biochim Biophys Acta 1130: 349–351

Shi X, Dalal NS (1993) One-electron reduction of vanadium(V) by flavoenzymes/NADPH. Arch Biochem Biophys 302: 300–303

Uyama T, Nishikata T, Satoh N, Michibata H (1991) Monoclonal antibody specific to signet ring cells, the vanadocytes of the tunicate, *Ascidia sydneiensis samea*. J Exp Zool 259: 196–201

Uyama T, Moriyama Y, Futai M, Michibata H (1994) Immunological detection of a vacuolar-type H^+-ATPase in the vanadocytes of the ascidian *Ascidia sydneiensis samea*. J Exp Zool 270: 148–154

Uyama T, Nose Y, Wuchiyama J, Moriyama Y, Michibata H (1997) Finding of the same antigens in the polychaeta, *Pseudopotamilla occelata*, as those in the vanadium-rich ascidian, *Ascidia*

sydneiensis samea. Zool Sci 14: 43–47
Uyama T, Yamamoto K, Kanamori K, Michibata H (1998a) Glucose-6-phosphate dehydrogenase in the pentose phosphate pathway is localized in vanadocytes of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci, 15: 441–446
Uyama T, Kinoshita T, Takahashi H, Satoh N, Kanamori K, Michibata H (1998b) 6-Phosphogluconate dehydrogenase is a 45-kDa antigen recognized by S4D5, a monoclonal antibody specific to vanadocytes in the vanadium-rich ascidian, *Ascidia sydneiensis samea*. J Biochem, 124: 377–382
Wright RK (1981) Urochordata. In "Invertebrate Blood Cells Vol 2" Ed by Ratcliffe NA and Rowley AF, Academic Press, London, pp 565–626
Wuchiyama J, Michibata H (1995) Classification, based on autonomous fluorescence, of the blood cells of several ascidians that contain high levels of vanadium. Acta Zool (Stockholm) 76: 51–55

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