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Finding of the Same Antigens in the Polychaete, *Pseudopotamilla occelata*, as Those in the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea*

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ABSTRACT—The polychaete *Pseudopotamilla occelata* is the first animal revealed to contain high levels of vanadium besides ascidians. The present experiment disclosed that *P. occelata* has the same antigens with those in the ascidian *Ascidia sydneiensis samea*, which were recognized by two types of antibodies, a polyclonal antibody against vanadium-associated proteins extracted from blood cells and a monoclonal antibody against vanadocytes in the vanadium-rich ascidian *A. sydneiensis samea*. There is, therefore, a possibility that similar mechanism works on the accumulation of vanadium between the Polychaeta and the Ascidiidae.

INTRODUCTION

Recently, the polychaete, *Pseudopotamilla occelata*, was reported to be a new accumulator of high levels of vanadium (Ishii *et al.*, 1993). The level of vanadium contained in the polychaete was calculated to be 25.5 mg vanadium/g dry weight, which was 10^6 times higher than that in seawater (Cole *et al.*, 1983; Collier, 1984). After Henze's first finding of high levels of vanadium in ascidian blood cells (Henze, 1911), many analytical chemists joined in attempts to search animals having such high levels of vanadium. Although several species of animal, such as those belonging holothurian and nudibranch, were hitherto reported to accumulate high levels of vanadium (Phillips, 1918; Webb, 1937; Webb and Fearon, 1937; Noddack and Noddack, 1940), they were not necessarily reconfirmed to having such ability by the other investigators (Bertrand, 1950; Webb, 1939; Ciereszko *et al.*, 1963; Carlisle, 1968).

On the other hand, the highest concentration of vanadium, 350 mM, in the blood cells of the ascidian, *Ascidia gemmata*, was reported (Michibata *et al.*, 1986, 1991a) and, among the approximately ten types of blood cells in ascidians, signet ring cells were identified as the so-called vanadocytes (Michibata *et al.*, 1987, 1991b). Thereafter, we produced a monoclonal antibody specific to the signet ring cells, designated S4D5, which recognized an antigen of a single peptide of about 45 kDa (Uyama *et al.*, 1991). More recently, we extracted a vanadium-associated protein (VAP) from the homogenate of the blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea*, and prepared a polyclonal antibody, designated anti-VAP, against the protein composed of 12.5 kDa and 15 kDa peptides with a minor peptide of 16 kDa (Kanda *et al.*, 1997).

In the present experiment, we examined whether or not the polychaete, *Pseudopotamilla occelata*, has the same antigens as those in the vanadium-rich ascidian, *A. sydneiensis samea*, which were recognized by two types of antibodies: a polyclonal antibody anti-VAP and a monoclonal antibody S4D5 specific to ascidian vanadocytes.

MATERIALS AND METHODS

Leupeptin, pepstatin A and chymostatin were purchased from Peptide Institute, Inc. Osaka, Japan. Other chemicals used were analytical grade, commercial products. Specimens of the vanadium-rich fan worm, *Pseudopotamilla occelata*, and 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 of the Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo, Otsuchi, Iwate Prefecture, Japan. Both species of animals were maintained in each aquarium at our laboratory at 18°C until use.

One g of branchial crowns in wet weight composed of many bipinnate radioles was collected by cutting off from the trunk bodies of the fan worm *P. occelata*. The sample was washed with 0.2 M Tris (2-amino-2-methyl-1,3-propanediol)-HCl buffer (pH 8.5) containing 0.4 M sucrose and suspended in the solution containing protease inhibitors [leupeptin, pepstatin A, chymostatin and phenylmethylsulfonyl fluoride (PMSF)] at 10 μg/ml and was homogenized in 10 ml of the same buffer solution using a Potter-Elvehjem homogenizer. The homogenate

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obtained was centrifuged at 100,000 × g for 30 min at 4°C. Contents of protein and vanadium in the sample at each experimental step were determined by Bradford method (Bradford, 1976) and by an atomic absorption spectrometry (Michibata *et al.*, 1991a), respectively.

Methods for isolation of a vanadium-associated protein (VAP) from ascidian blood cells and for preparation of anti-VAP were described elsewhere in detail (Kanda *et al.*, 1997). In brief, ascidian blood cells were collected in 20 volumes of 50 mM Tris-HCl buffer solution containing 400 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM DTT (dithiothreitol), 1 mM PMSF and 10% (v/v) glycerol at pH 8.5 and were frozen at -80°C. Following the blood cell sample was thawed on ice and centrifuged at 100,000 × g for 60 min at 4°C, the supernatant obtained was dialyzed against 50 mM Tris-HCl buffer at pH 7.4 for 3 hrs at 10°C. The dialyzed solution was centrifuged at 100,000 × g for 60 min at 4°C and the supernatant was applied to a DEAE-Sephacel anion-exchange column which had been equilibrated with 50 mM Tris-HCl buffer at pH 7.4. Concentrations of protein and vanadium in each fraction were determined as described above. Consequently, VAP composed of peptides of 12.5 kDa and 15 kDa was obtained. Anti-VAP antibody was prepared by immunization of rabbits. Approximately 100 μg of the protein was lyophilized and dissolved in 700 μl of 0.9% NaCl. The solution was mixed with Freund's complete adjuvant and injected subcutaneously. The injection was repeated at intervals of two weeks. Three days after the last injection, the blood was used for the preparation of the anti-VAP serum. The serum was removed from the blood clot and any remaining insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4°C.

In order to obtain monospecific anti-VAP antibody, VAP was subjected to SDS-PAGE and electrophoretically blotted onto nitrocellulose paper. The paper was exposed to the anti-VAP serum which was diluted 20-folds with Tris-buffered saline (TBS), consisted of 140 mM NaCl, 10 mM Tris-HCl buffer at pH 7.2 and 1% BSA, for 2 hrs at room temperature. After washed three times with TBS buffer containing 0.01% (v/v) Tween 20 for 10 min, the nitrocellulose paper was incubated in 0.1M glycine-HCl buffer (pH 3.0) and agitated vigorously in order to release the antibodies from the antigen. Immediately, the solution was neutralized with 1M Tris-HCl (pH 9.0) containing 100 μg BSA. Then, the solution was dialyzed against 10 mM $(NH_4)HCO_3$ for 3 hr at 4°C and concentrated with Centricon 10 (Amicon, Inc., Massachusetts, USA) and used as purified anti-VAP antibody. Method for the preparation of monoclonal antibody, S4D5, which was used for a positive control, was described previously (Uyama *et al.*, 1991).

For immunoblot analysis, the homogenate of branchial crowns of *P. occelata*, the 100,000 × g pellet of branchial crowns, the 100,000 × g supernatant of branchial crowns, and the 100,000 × g supernatant of ascidian blood cells (each containing 20 μg protein) were dissolved, separately, in 62.5 mM Tris-HCl buffer solution at pH 6.8 which containing 5% 2-mercaptoethanol, 10% glycerol and 2.3% SDS, and were submitted to electrophoresis in a 12.5% polyacrylamide gel in the presence of 2 % SDS. The proteins separated on SDS-PAGE were electrophoretically blotted onto nitrocellulose paper. The paper was then soaked in TEN (Tris, EDTA and NaCl) buffer which consisted of 150 mM NaCl, 1 mM EDTA and 25 mM Tris-HCl buffer at pH 7.4, containing 1% BSA and was exposed to supernatant of the culture medium of hybridoma, S4D5, which was diluted at a ratio of 1 to 100 in TEN buffer or anti-VAP which was diluted at a ratio of 1 to 500 in TEN buffer. The nitrocellulose paper was washed with TEN buffer containing 0.05% Tween 20 for 30 min and incubated with anti-mouse IgG (H+L)-horseradish peroxidase (HRP) conjugated (Vector Laboratories, Inc., USA) or anti-rabbit IgG (H+L)-HRP conjugated (Organon Teknika Corporation, Philadelphia, USA) which was diluted at a ratio of 1 to 2000 with TEN buffer containing 0.05% Tween 20 for 30 min. After washed six times with TEN buffer containing 0.05% Tween 20 for 15 min, the nitrocellulose paper was incubated with ECL Western blotting detection reagents (Amersham International, plc, Buckinghamshire, England). Finally, the nitrocellulose blots were exposed to Hyperfilm-ECL (Amersham International, plc, Buckinghamshire, England).

RESULTS

Contents of vanadium in the branchial crowns of *P. occelata* were determined as shown in Table 1. Homogenate of the branchial crowns contained 1.75 mg vanadium/g wet weight. Approximately 80% of the total amounts of vanadium in the branchial crowns was found in insoluble fraction obtained after centrifugation at 100,000 × g. Soluble fraction obtained after the centrifugation has only 20% of the total amounts of vanadium but the ratio of vanadium to protein was twice than that in the insoluble fraction.

SDS-PAGE analysis of the ascidian blood cells and of the branchial crowns of the fan worm (the left of Fig. 1) showed that polypeptides of various molecule masses were contained in both cases. Immunoblot analysis revealed that a monoclonal antibody, S4D5, recognized a single peptide of 45 kDa not only of ascidian blood cells (lane 4 of the right upper of Fig. 1) but also of the fan worm proteins (lane 1 of the right upper of Fig. 1). The antigenicity was detected in soluble protein fraction (lane 3 of the right upper of Fig. 1) but not in precipitated protein fraction (lane 2 of the right upper of Fig. 1). On the other hand, a polyclonal antibody, anti-VAP antibody, recognized mainly a peptide of 15 kDa with a minor peptide of 16 kDa of the fan worm (lane 1 of the right lower of Fig. 1). The antigenicity was found in precipitated fraction (lane 2 of the left of Fig. 1), but not in soluble fraction of the fan worm (lane 3 of the left of Fig. 1), although VAP was extracted from soluble fraction of ascidian blood cells (see Materials and Methods).

Table 1. Contents of vanadium and protein in the branchial crown of *Pseudopotamilla occelata*

Samples	Vanadium contents (μg)	Protein contents (mg)	Vanadium/Protein (μg/mg)
Homogenate	1,750	50	35
100,000 × g ppt	1,390	45	35
100,000 × g sup	350	5	70

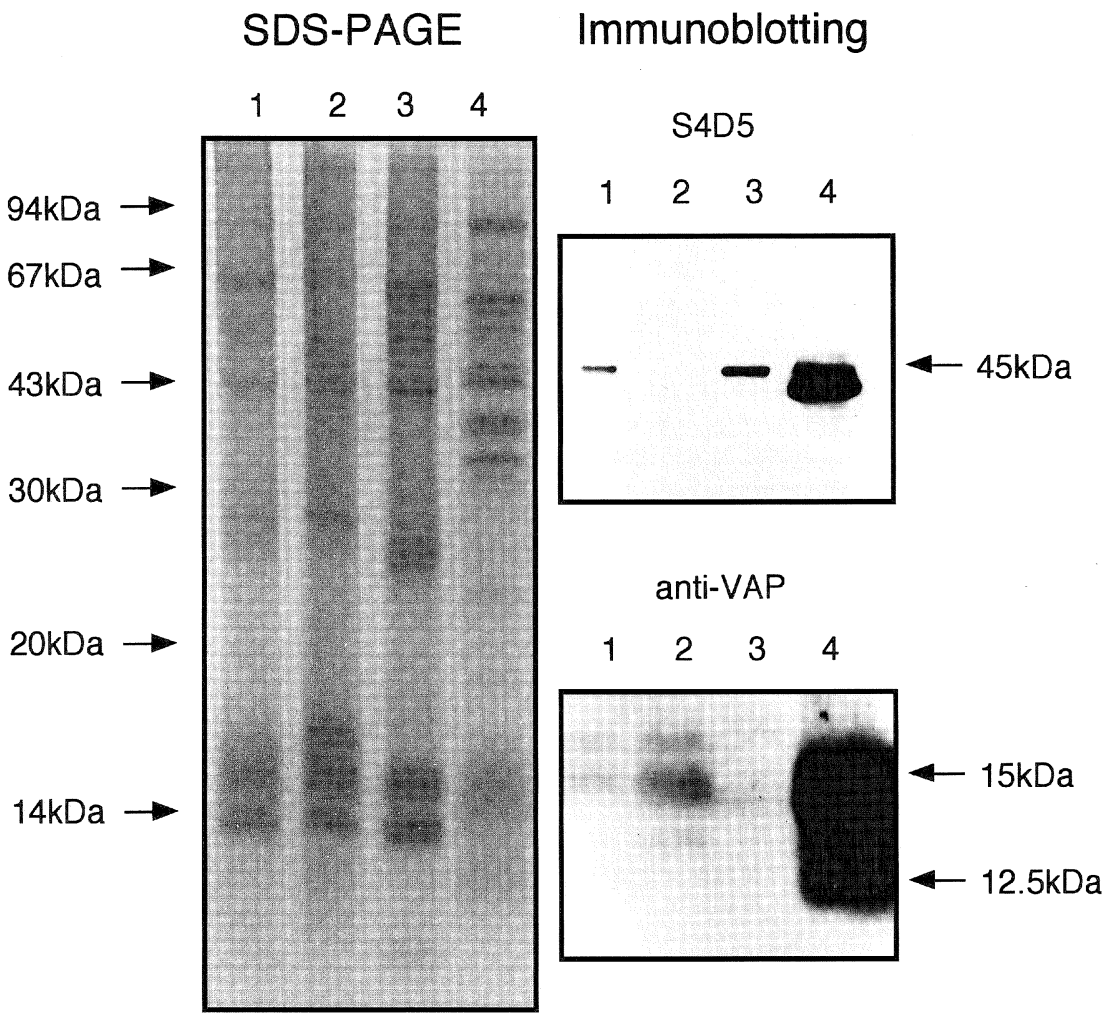

Fig. 1. Immunoblotting analysis of the S4D5 antigen and vanadium associated protein. Samples of branchial crowns of *P. occelata* and of blood cells of *A. sydneiensis samea* were subjected to SDS-PAGE. The separated proteins were blotted onto nitrocellulose paper and were reacted with a monoclonal antibody S4D5 and with a polyclonal antibody anti-VAP, respectively. Lane 1, the homogenate of branchial crowns of *P. occelata*; lane 2, the 100,000 × g pellet of branchial crowns; lane 3, the 100,000 × g supernatant of branchial crowns; lane 4, the 100,000 × g supernatant of ascidian blood cells. SDS-PAGE analysis for the blood cells of the ascidian and for the coelomic cells of the polychaete showed that polypeptides of various molecule masses were contained in both cases. Immunoblot analysis revealed that soluble 45 kDa proteins both of the fan worm and of the ascidian reacted with the monoclonal antibody S4D5 raised against the signet ring cells. Anti-VAP antibody recognized a peptide of 15 kDa with a minor peptide of 16 kDa of the fan worm (lanes 1 and 2 of anti-VAP). The antigenicity was found in the precipitated proteins but not in soluble proteins of the fan worm (lane 3 of anti-VAP) although it was found in peptides of 12.5 kDa and 15 kDa in soluble form in the ascidian.

DISCUSSION

The family Polychaeta is far from the family Ascidiidae phylogenetically. It is, therefore, of great interest that they are bound together by common characteristics of having high levels of vanadium and the same antigens that might be involved in the accumulation of vanadium. The level of vanadium found in the branchial crown of *P. occelata*, 1.75 mg vanadium/g in wet weight, is comparable with that in the blood cells of ascidians belonging the family Ascidiidae (Michibata *et al.*, 1986). In the case of the fan worm, high levels of vanadium were detected from the vacuoles of the epidermal cells covering the radioles of branchial crown but not from coelomic cells with electron probe X-ray microanalysis (Ishii *et al.*, 1993), in contrast to the case of ascidians that high levels of vanadium were reported to be localized in the vacuoles of signet ring cells (so-called vanadocytes) in ascidians (Uyama *et al.*, 1991; Botte *et al.*, 1979; Scippa *et al.*, 1982, 1985).

The most striking finding in the present experiments is that *P. occelata* contains the same antigens as those of ascidians in addition to high levels of vanadium. In the ascidian, a vanadium-associated protein (VAP) has been recently isolated from the homogenate of the blood cells and a polyclonal antibody, anti-VAP antibody, has been prepared (Kanda *et al.*, 1997). Although characterization of VAP is under

progress, VAP is at least comprised of two main peptides of 12.5 kDa and 15 kDa with a minor peptide of 16 kDa and is estimated to associate with vanadium at an approximate ratio of 1 mol : 16 mols, indicating that VAP might be involved in the accumulation of vanadium (Kanda *et al.*, 1997). As shown in Fig. 1, the present experiments using a polyclonal antibody, anti-VAP antibody, and a monoclonal antibody, S4D5, against the vanadocytes in the vanadium-rich ascidian (Michibata *et al.*, 1988, 1991; Uyama *et al.*, 1991; Hirata and Michibata, 1992) disclosed that *P. occelata* contains the immunologically same proteins as those of the vanadium-rich ascidian, *A. sydneiensis samea*.

It is, however, noticeable that anti-VAP antibody recognized a peptide of 15 kDa with a minor peptide of 16 kDa in insoluble fraction of the fan worm (Fig. 1) while it was prepared against VAP extracted from soluble fraction of the ascidian blood cells. In fact, anti-VAP antibody reacted with two main peptides of 12.5 kDa and 15 kDa with a minor peptide of 16 kDa in soluble fraction of ascidian blood cells (Fig. 1). The reason is unexplained in present although pointing out the difference in vanadium distribution between the fan worm and the ascidians.

Vanadium is a multivalent transition metal and the ions under physiological conditions are known to be in the +3, +4 and +5 oxidation states (Chasteen, 1981, 1983; Kustin *et al.*, 1983; Boas and Pessoa, 1987). K-edge absorption spectrometry indicated that the chemical form of vanadium ions contained in living *P. occelata* is the +3 oxidation state in a highly symmetrical octahedral coordination environment with a lack of any significant quantity of the VO^{2+} component (Ishii *et al.*, 1993). On the other hand, wide spread agreement that the chemical form of vanadium ions in vanadocytes of ascidians is predominantly the +3 oxidation state has been established (Hirata and Michibata, 1991). However, a few species of the genus Amanita in the Kingdom Fungi, *Amanita muscaria, A. regalis* and *A. velatripes* are known to contain a high concentration of vanadium in the +4 oxidation state. Amavadine isolated from *A. muscaria* is a pale blue compound composed of N-hydroxyimino-α,α'-dipropionic acid and vanadium in a 2:1 ratio (Bayer and Kneifel, 1972). Several kinds of algae in the Kingdom Plant are known to contain vanadium in the +5 oxidation state in an active site of vanadium-dependent enzyme, peroxidases (De Boer *et al.*, 1986a, b). It is, therefore, unusual for living organisms to contain high levels of vanadium in the +3 oxidation state, as shown both in the polychaete and the ascidians. A problem awaiting solution is to isolate a reductant to reduce vanadium in the +3 oxidation state.

On the basis of the data obtained in the present experiments, there is a possibility that similar mechanism works on the accumulation of vanadium between the Polychaeta and the Ascidiidae. Characterization of the proteins recognized by the antibodies would lead us to solve the problems why several species of the Polychaeta and the Ascidiidae accumulate vanadium at extremely high levels and how they accumulate selectively vanadium ions from the seawater where many kinds of metal ions are dissolved.

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