International Journal of New Technology and Research (IJNTR)
ISSN:2454-4116, Volume-3, Issue-7, July 2017 Pages 01-07

Major Vault Protein of the Protozoan *Raphidiophrys contractilis* Has a Binding Property to β-1,3-Glucan and is Involved in Food Capturing

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***Abstract*— A centrohelid heliozoan *Raphidiophrys contractilis* was found to recognize curdlan, an insoluble β-1,3-glucan, as food. When a suspension of curdlan gel was added to the heliozoans, the gel particles were ingested into food vacuoles. By affinity purification with curdlan gel, a protein of 100 kDa was isolated as the only β-1,3-glucan-binding protein from the detergent-extracted cell homogenate of *R. contractilis*. The protein was subjected to mass spectrometry with a reference contig dataset obtained by transcriptome analysis. From the obtained partial nucleotide sequences and additional PCR-based sequence determination, the protein was identified as major vault protein (MVP), the main component of Vault complex. The *R. contractilis* MVP reacted with an antibody against human MVP, and specific binding to β-1,3-glucan was verified by a competition assay with laminarin, a soluble-type β-1,3-glucan. The heliozoans were mixed with prey flagellate *Chlorogonium capillatum* and extracellular fluid was collected during food-uptake. The fluid was then subjected to pull-down assay with curdlan gel, by which multiple protein species were detected including MVP as one of the major proteins, suggesting that MVP is secreted from the heliozoans during food uptake as a component of a large protein complex. The involvement of the heliozoan MVP in prey recognition suggests a possible novel function of this protein in self/nonself discrimination.**

***Index Terms*—β-1,3-glucan binding protein, Food capturing, Major vault protein, *Raphidiophrys contractilis*, Self/nonself discrimination.**

I. Introduction

In heterotrophic protists, self and nonself discrimination is essential for correct targeting of predatory organisms to the desired prey. Heliozoans are unicellular protists that usually feed on algae and protozoa [1]. In an attempt to search for the most primitive self/nonself recognition mechanism in eukaryotic organisms, identification of protozoan proteins that recognize the prey cell surface was carried out in the present study. Previously, we reported that food recognition in a heliozoan *Actinophrys sol* was mediated by β-1,3-glucan molecules on the surface of prey organisms [2], and another heliozoan species *Raphidiophrys contractilis* showed similar feeding behavior and preferences to microorganisms as food [3]. Here, isolation and characterization of β-1,3-glucan binding protein was carried out using *R. contractilis*, and we found that the protein has a strong homology to the major vault protein (MVP), the main component of the vault complex, that is widely conserved in eukaryotes but its function is still not fully understood.

Vault is a barrel-shaped ribonucleoprotein complex that is highly conserved across eukaryotic species [4] with some exceptions such as fungi, insects, and plants [5]. The biological functions of MVP are incompletely understood, while it has been suggested to be involved in a variety of cellular functions including cell signaling, innate immunity, drug resistance, and nuclear-cytoplasmic transport [6]. Recent research indicated that MVP may be a protein of ancient origin. Its original function seems to have been lost or modified in the history of evolution, and now it may have come to play a variety of different functions [5]. Regarding protozoa, although the gene sequence of MVP has been reported in many supergroups such as Stramenopiles, Alveolata, Rhizaria andExcavata, there is no report on its function at all [5]. Therefore, detailed studies of protozoan MVPs may contribute to the elucidation of the evolutionary origin of MVP.

II. Materials and Methods

A. Organism and culture medium

The centrohelid heliozoan *Raphidiophrys contractilis* was originally collected in Shukkeien Garden, Hiroshima, Japan. Cells were cultured monoxenically at 20±1°C in a culture medium which was based on 10% artificial sea water [7]. The medium consisted of 46.2 mM NaCl, 0.9 mM KCl, 0.8 mM $CaCl_2$, 2.3 mM $MgCl_2$, 0.6 mM $NaHCO_3$, 0.74 mM sodium acetate, 0.01% polypepton (Wako, Tokyo, Japan), 0.02% tryptone, and 0.02% yeast extract. Small flagellates, *Chlorogonium capillatum*, were added to the medium as food source, and the heliozoans were co-cultured with the food flagellates for about 2 weeks under constant illumination.

B. Light microscopy

Light microscopy was performed under Nikon Eclipse Ni microscope with differential interference contrast optics. Heliozoan cells were mixed with food materials (living *Chlorogonium capillatum* and *Saccharomyces cerevisae*, and substances recognized by heliozoans as food such as zymosan and curdlan) that were washed with 10% seawater. *Saccharomyces cerevisae* possesses cell wall with β-1,3-glucan as one of the main components [8]. Zymosan A (Sigma, St Louis. Mo., USA) is prepared from *Saccharomyces cerevi-*

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siae and composed mainly of insoluble β-1,3-glucan. Curdlan (Wako Pure Chemical Industries, Japan) is a linear β-1,3-glucan polymer.

C. Glucan binding assay

Curdlan (an insoluble β-1,3 glucan) was used as a matrix for affinity purification of β-1,3 glucan binding proteins. Curdlan powder was suspended in a lysis buffer (0.5% Nonidet P-40, 200 mM NaCl, 50mM Tris-HCl (PH 7.4), 1 µg/ml leupeptin, 1 mM EDTA, and 1mM PMSF) and heated at 90°C for 10 min. After gelation by cooling, the curdlan gel was mechanically crushed into small fragments, and washed 3 times with the lysis buffer. *R. contractilis* cells (approximately 4×10^6 cells) were mixed with the lysis buffer and vortexed for several seconds at 4°C. After 10 min, it was centrifuged at 15,000 rpm at 4°C for 5 min. The supernatant was mixed with approximately 1/100 vol. of curdlan gel and incubated at 4°C for 3 h. The suspension was then centrifuged at 4,400 rpm for 5 min to separate into curdlan-bound (pellet) and unbound (supernatant) portions. The obtained pellet was washed with the lysis buffer for five times before subjected to SDS-polyacrylamide gel (12%) electrophoresis (SDS-PAGE).

D. Mass spectrometry and identification of gene

The curdlan-bound protein band was cut out from the silver-stained gel and processed for MASS spectrometry (LC-MS/MS, Thermo Fischer, San Jose, CA, USA) as described by Kawamura et al [9] and Mascot software to identify tryptic peptide sequences matched to the nucleotide sequence database constructed from *de novo* transcriptome analysis using the same heliozoan species (details of the analysis will be described elsewhere).

E. Cloning, cDNA sequencing and sequence analysis

Molecular cloning of the full-length of cDNA encoding the curdlan-binding protein was carried out by PCR reactions to fill in sequence gaps between identified contigs, and by 3'- and 5'- RACE PCR to identify terminal sequences of the gene. The gene-specific primers user for PCR are listed in Table I, and positions of primers are schematically shown in

Table I. Nucleotide sequences of gene-specific primers used in this study

Primer	Oligonucleotide
86138_Fw	5'-ACCCCGGCGAGAAGCTCGGAAG-3'
68024_Rv	5'- AGCGCGGGGGAGATCCTTTTG -3'
68024_Fw	5'-TGAGGTGGAGATCATCGACCGC-3'
85282_Rv	5'-CTTGAGCTGGGCTTGTGTGAC T -3'
85282_Fw	5'-AGGCCGAGAAGTCCAGGGCTGATT-3'

Fw and Rv denote forward and reverse primers, respectively. The DNA fragments were amplified by PCR with the primer pairs (86138_Fw/68024_Rv and 68024_Fw/85282_Rv) to fill in the gaps between detected contigs. The nucleotide sequence of the full-length transcript was determined by 3'- and 5'- RACE reactions using gene specific primers (85282_Fw primer at 3'-end and 68024_Rv primer at 5'-end) and Universal Primer A Mix (UPM) supplied in SMARTer RACE 5'/3' Kit (Takara Bio, USA).

Fig. 3A. A template cDNA library was prepared from the TRIzol-isolated total RNA of *R. contractilis* by using SMARTer RACE 5'/3' Kit (Takara Bio USA, USA). PCR-amplified fragments were cloned with TOPO TA Cloning Kit with PCR 2.1-TOPO vector (Applied Biosystems, USA). Sequencing was conducted using the Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems) and analyzedusing ABI 3100, ABI 3130xl, and ABI 3130 Genetic Analyzers (Applied Biosystems).

The sequence reported in this paper has been deposited in the DDBJ database (accession no. LC310985).

F. Sequence alignment and phylogenetic tree building

The deduced amino acid sequences of *R. contractilis* MVP and *Rattus norvegicus* MVP (accession no. Q62667) were multiple-aligned using Clustal W program (version 2.1, http://clustalw.ddbj.nig.ac.jp/). Amino acid sequences of MVP from various organisms were compared and a neighbor joining phylogenetic tree was constructed using MEGA software version 6 [10].Multiple alignment was generated by Clustal W. A bootstrap test was done with 1,000 replicates [11]. The evolutionary distances was computed by p-distance method [12].

G. Western blotting

Table II. List of contigs detected by mass spectrometry for the 100-kDa β-1,3-glucan binding protein isolated by curdlan-gel pull-down assay

Contig number	Mascot score*	Nucleotide sequence
86138	177	GTGTGCCTTGCCCACTCCGACAACGAGATCCGCTTCGCACAGGACCCGTTCCCCTTGTACCCCGGCGAGAAGCT CGGAAGCAAAGTCACCCCCTTGCAAGTGTTGCCACCAAACAAGGCCTTGCGTCTGCGTGCTCTTCGTGATTTCAC GGACGAGGCAGGAAAGGAGATCCGTGCTGGTGACGAATGGTTGTTCCACGGCCCAGGAACCTACACTCCTCGG GTAGATGTCGAAGAGGTGGACACGGTCAATGCCATTATCATCAAGCCCAATGAGGCCCTCAAGCTGCGCGCCCG CCAAACCTTTGTCGCCCGCAGCGGCGAGACCCGCAGGGCCGG
68024	331	GAGCATCGTCAAGGGGTACGTCCTGACCGAGAAGACCGGACTTCACGTGCGCTCCACAAGAACCTTCAAGGATG TCTTTGGAAAGGAGAGAAAGGCTGGTTCCGAGTGGTTGGTGACCATCAAGGATGCCGAGACTTACATTCCAGGA GTGTACGAGGAGGTTGTGGGTGAGGTTGCCGCTGTGACCTTGACCACTCGCCAGTACTGTGTCGTCTTGGACCC AATTGACACCACAGGAACACCCAAGTATGGCCAACGCGAGCTTCGCAAGGGAGAGATGACATTCTTCCTGTACC CCGGCGAGAGCTTGGAGTCTGGCGTTCAGAACGTGTTCGTTTTGGGCGAGGAGGATGGTTTGTTGGTGCGTGCA CGCCAAACCTTTGATGACCACAAGCCAGGCGATCGCTGGATGATTCTCGGTCCATGCGAGTACGTCCCATCTGTT GAGGTGGAGATCATCGACCGCCGCAAGTCCATCCCCTTGGACGAGAACGAGGGTGTGTACGTGCGCGACACAA GAACCGGTAAGGTTCGCCCAGTGACCGGCCAATCGTACATGCTTGAGCCCTTTGAGGAGCTGTGGCAAAAGGAT CTCCCCCGCGCTGTTGAGGACCTGCTCACCTCGGACCTCGACCCACTCGCCGACAGAACATCTGGTGCCCCGCC GCACGCACTGGACCTCGTGACAAGACAAAGGTGGTGACCTACCGTGTGCCCCACAAT
85282	266	CCTCGGCGTCTTGTCGGAGCTTGGTTTGGGTCAACTCGGCGTCGGACTCGATCTTCATGGCCTGTGCCTTGAGCT GGGCTTGTGTGACTGCGGCGCGACCCTCAATCTCCGCGGCCTCTGCGCGGGCCTGTGCCTCGGCCTTGGCTTGAC CGGTGGACTCGACGGCAGCCGAGGCTGCTTGCAAAAGGAGCAAATCAGCCCTGGACTTCTCGGCCTCGGCCTCA TCCTTGATCTTTTGGCGCTCAAGCTTACCGCGGGC

*Scores > 65.6 indicate identity or extensive homology ($p < 0.05$)

For immunoblotting, the gels were blotted onto polyvinylidene difluoride (PVDF) membranes and washed 3 times with 0.1% Tween-20 in Dulbecco's phosphate-buffered saline (DPBS). Membranes were treated with blocking solution (5% milk powder and 0.1% Tween-20 in DPBS) for 1 h, and incubated for 2 h in the primary antibody (rabbit anti-human major vault protein polyclonal antibody, Flarebio Biotech LLC., USA) at 1:2,000 dilution. Membranes were then washed and incubated for 2h with the secondary antibody (peroxidase-labelled anti-rabbit antibody, GE Healthcare, USA). After washing with 0.1% Tween-20 in DPBS, membranes were treated with Amersham ECL Western Blotting Detection Reagent (GE Healthcare) and visualized with Amersham Hyperfilm MP (GE Healthcare).

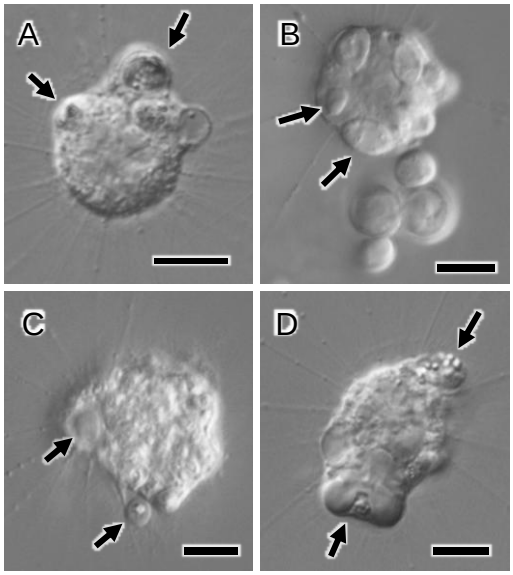

Fig. 1. Phagocytotic uptake of food materials by *Raphidiophrys contractilis*. A: Uptake of food flagellates (*Chlorogonium capillatum*). Food vacuoles (arrows) were formed on the periphery of the cell body. B: Uptake of living *Saccharomyces cerevisiae* cells (arrows). C: Uptake of zymosan-A (insoluble β-1,3-glucan prepared from the cell wall of *S. cerevisiae*) as shown by arrows. D: Uptake of curdlan gel (high molecular weight insoluble polymer of β-1,3-glucan) as shown by arrows. Bars, 10 µm.

H. Competitive glucan binding assays

To verify β-1,3-glucan-mediated specific binding of curdlan and MVP, laminarin (a soluble β-1,3-glucan; Nacalai Tesque, Japan) was used for competitive binding to MVP. After cell extract was prepared as described above, curdlan gel was added to the extract with or without surplus amount of laminarin (50 times as much as curdlan in dry weight). Following incubation for 3 h on ice, curdlan gel was collected by centrifugation as mentioned above, and examined by SDS-PAGE.

I. Induction of protein release during food capturing

To examine if MVP is released from *R. contractilis* cells during food capturing, an experiment was carried out using *Chlorogonium* as prey. *Chlorogonium* cells were first washed three times with 10% seawater and mixed with a cell suspension of *R. contractilis*. Cell mixture was left still for 10 min, and extracellular fluid was collected by centrifugation at 4,400 rpm for 10 min. The extracellular fluid was mixed with curdlan gel, and proteins trapped by curdlan was examined by SDS-PAGE and further characterized by mass spectrometry as described above.

III. Results

A. Uptake of β-1,3-glucan by R. contractilis as food

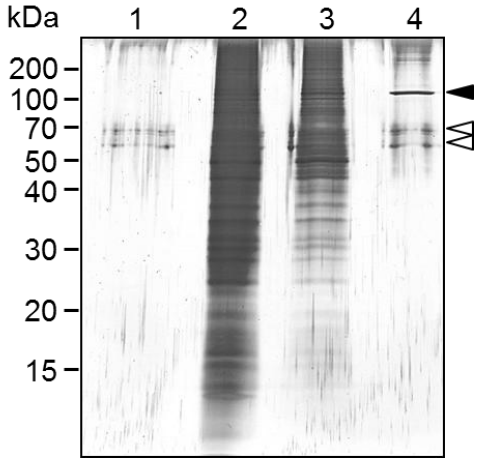

Fig. 2. Isolation of curdlan-binding protein as analyzed by 12% SDS-PAGE. Lane 1: Negative control (curdlan only). Lane 2: cell lysate of *R. contractilis*. Lane 3: curdlan-unbound fraction of the cell lysate. Lane 4: curdlan-bound fraction of the cell lysate. A prominent curdlan-binding protein band appeared at around 100 kDa (solid triangle), while open triangles show non-specific silver-stained bands derived from curdlan gel.

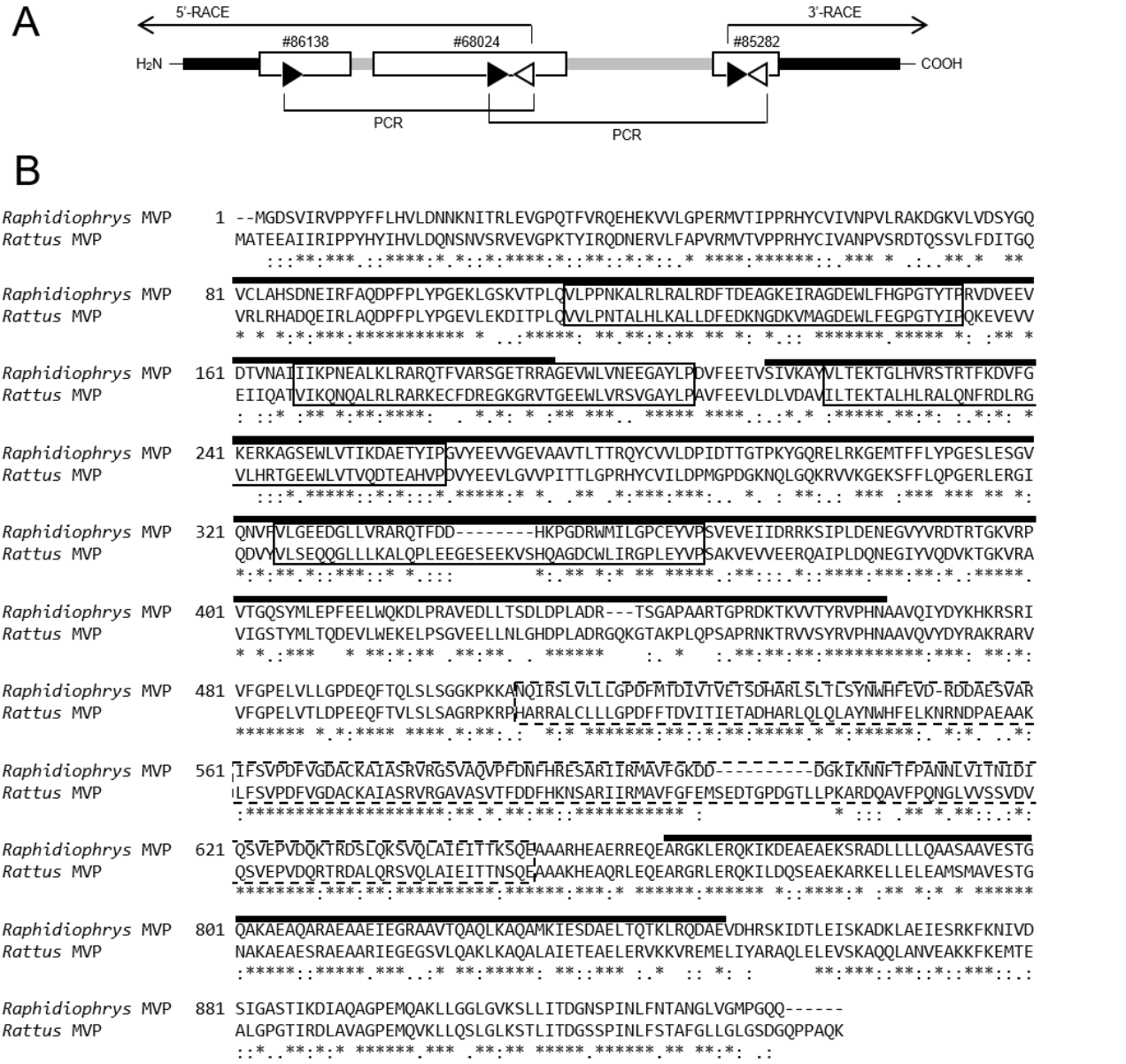

Fig. 3. Positions of the detected three polynucleotide fragments by mass spectrometry (#86138, #68024, #85282) in the full-length transcript of the β-1,3 glucan-binding protein of *R. contractilis* as determined by PCR and 3'- and 5'-RACE procedures. A: positions of the three fragments (open rectangles) and PCR primers (forward and reverse primers as indicated by open and closed triangles, respectively) are shown in the full-length transcript. B: Comparison of the amino acid sequences of *Raphidiophrys contractilis* curdlan-binding protein and *Rattus norvegicus* MVP (accession no. NP_073206.2) aligned using the ExPASy SIM alignment tool (web.expasy.org/sim). Sequences encircled in rectangles with solid lines show repeated "vault units", and a rectangle with broken line denote the "MVP shoulder domain". Sequences with thick lines on top shows positions of the three protein fragments initially identified by mass spectrometry.

Food uptake of *R. contractilis* is initiated when prey organisms such as protozoans and algal cells are attached at the surface of heliozoan axopodia that are radiating from the spherical cell body as a trap for catching prey. The prey is then engulfed by pseudopodia and ingested into the cell body for digestion. As shown in Fig. 1A, *Chlorogonium capillatum* cells are captured and enclosed in food vacuoles. *Saccharomyces cerevisiae* (Fig. 1B) and its cell wall derivative zymosan-A (Fig. 1C) were also ingested into food vacuoles. As the main component of the cell wall of *Saccharomyces cerevisiae* is β-1,3-glucan, curdlan (insoluble polymer of β-1,3-glucan) was applied to *R. contractilis*. As shown in Fig. 1D, fragments of curdlan gel were recognized as prey and ingested into food vacuoles just in the same manner as seen in normal food uptake by *R. contractilis*.

B. Identification of β-1,3-glucan binding protein as MVP

To identify β-1,3-glucan binding proteins of *R. contractilis*, pull-down assay was carried out by mixing the cell extract with curdlan gel. Proteins were co-precipitated with curdlan gel and curdlan-binding proteins were examined by SDS-PAGE as shown in Fig. 2. A major protein band of 100

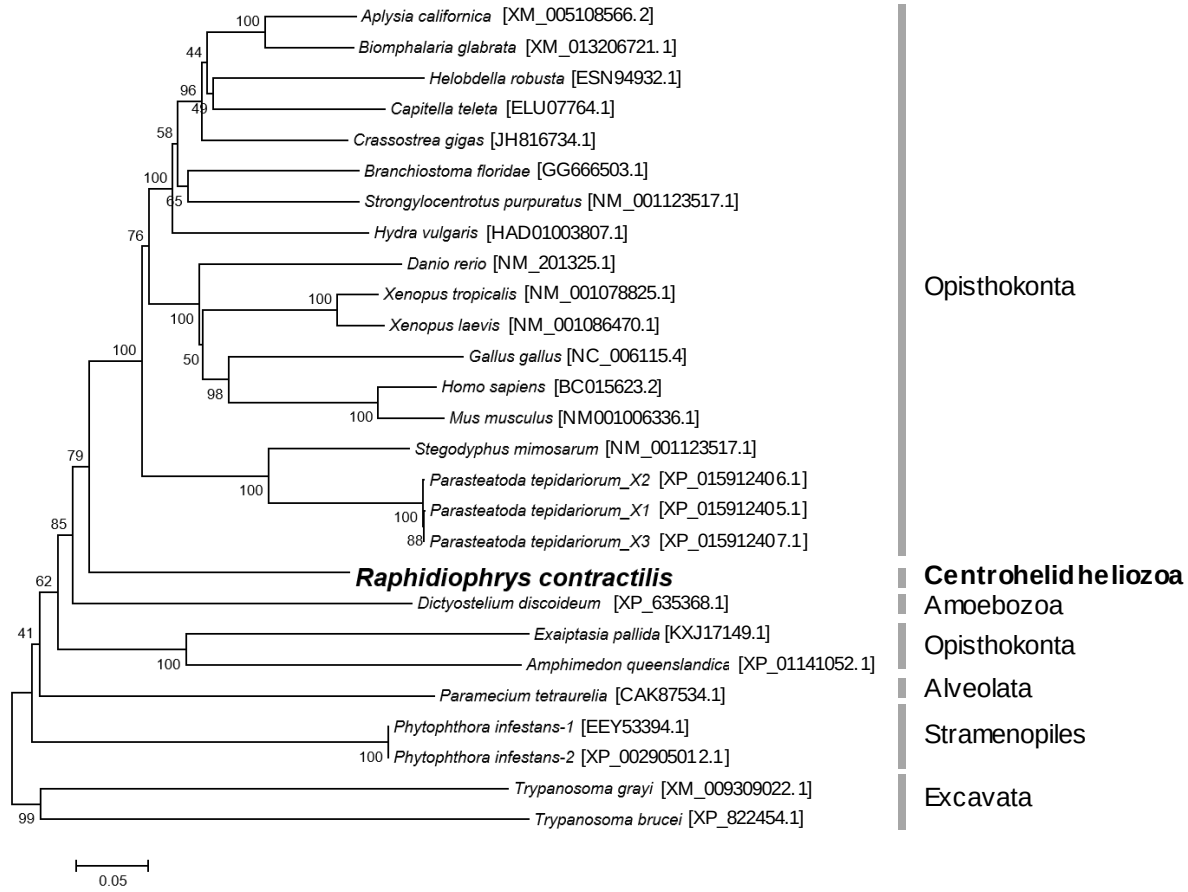

Fig. 4. Neighbor-joining phylogenetic tree of the amino acid sequences of MVPs from different species. Accession numbers used to construct the phylogenetic tree are shown in parentheses. The β-1,3 glucan-binding protein of *R. contractilis* is indicated in bold italic. Bar (0.05) indicates the genetic distance.

kDa (indicated by solid triangle in lane 4) was detected, and was further analyzed by mass spectrometry using a nucleotide database obtained from a transcriptome analysis of *R. contractilis*. As the result, three nucleotide fragments (contigs) were detected at high significance ($p<0.05$) by mass spectrometry, and all of the sequences were identified by NCBI BLAST as homologs of major vault proteins (MVP). The nucleotide sequences are listed in Table II. The sequence gaps between adjacent sequences were filled with the sequence data obtained from PCR reactions using gene-specific primers as schematically shown in Fig. 3A. The 5' and 3' ends of the protein coding sequences were determined by 5'- and 3'-RACE procedures, respectively. The deduced amino acid sequence shows a strong homology to *Rattus norvegicus* MVP (accession no. NP_073206.2). Identity between these sequences is 57.6% in 847 residues overlap (score: 2473.0; gap frequency: 2.6%), with characteristic MVP domains (four "vault units" and a "MVP shoulder domain") shared between these proteins. (Fig. 3B).

C. Characterization of R. contractilis MVP

MVP cDNA of *R. contractilis* has an open reading frame of 2,496 nucleotides corresponding to a deduced amino acid sequence of 832 amino residues. No signal peptide is predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP). According to the prediction by SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/), it is a soluble protein. The Expasy PI/MW server estimated pI of 5.70 and 92.50 kDa molecular mass, which is smaller than the estimated size by SDS-PAGE, suggesting possible protein modification. The ScanProsite analysis (http://ca.expasy.org/tools/scanprosite/) strongly stated that this protein has two N-linked glycosylation sites at Asn^{22} and Asn^{601}.

D. Phylogenetic analysis of MVP

A neighbor-joining phylogenetic tree was constructed using deduced amino acid sequences of MVPs from different groups of organisms, and shown in Fig. 4. The constructed phylogenetic tree indicates that MVP of *R. contractilis* is included in the family of MVP, closely related to Amoebozoa and Opisthokonta.

E. Confirmation of MVP by Western blotting

As an additional confirmation of MVP, Western blotting was performed with an antibody against human MVP. As shown in lane B2 in Fig. 5, the MVP antibody reacted with the curdlan-bound 100 kDa protein. Also in the whole cell extract, the 100 kDa component reacted with the antibody, but in addition, some minor protein bands were also found to be reacted with the antibody in the lower molecular weight region (indicated with asterisks in lane B1).

F. Competitive glucan binding of R. contractilis MVP with laminarin

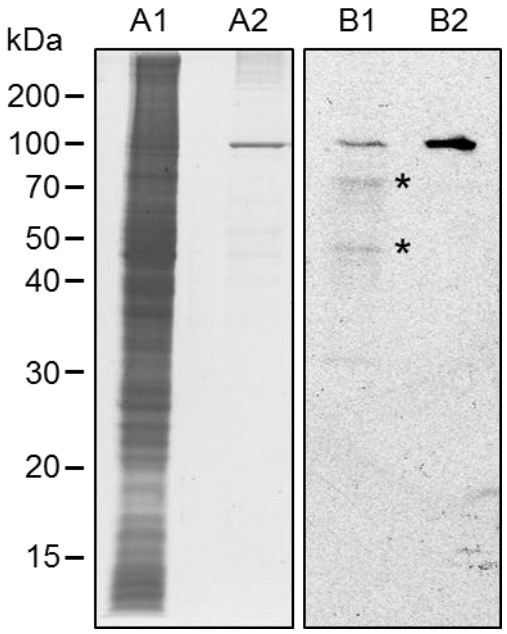

Fig. 5. Western blotting of whole-cell extract and curdlan-isolated fraction of *Raphidiophrys contractilis*, using anti-MVP antibody against human MVP. Lanes A1 and A2 are silver-stained SDS-PAGE profiles, and lanes B1 and B2 are the corresponding Western blots. Lanes A1 and B1 show the whole-cell extract, and A2 and B2 show the curdlan-bound fraction, containing MVP at approximately 100 kDa. Asterisks in lane B1 show minor protein bands reacted with the antibody

In order to confirm that the binding between MVP and curdlan is a β-1,3-glucan-dependent phenomenon, we investigated whether competitive inhibition with other type of β-1,3-glucan occurs. When the cell lysate was pre-treated with a surplus amount of laminarin, a soluble-type polymer of β-1,3-glucan, the amount of MVP bound to curdlan gel was greatly reduced (Fig. 6). This result clearly shows that MVP of R. contractilis has a binding property to β-1,3-glucan.

G. MVP is secreted extracellularly during predation

An experiment was conducted to ascertain whether MVP is secreted extracellularly during predation and is used for prey recognition. *Chlorogonium* cells were added as food to a R. contractilis cell suspension, and the extracellular fluid was collected and examined by curdlan pull-down assay. By SDS-PAGE and corresponding Western blotting with MVP antibody (lanes 1 and 2 in Fig. 7, respectively), we found that MVP appeared in the extracellular medium. In addition to MVP, the curdlan gel also trapped many other proteins as shown in lane 1, suggesting that MVP is present in the extracellular medium as a large complex with other proteins.

IV. Discussion

In this study, we aimed to investigate the molecular mechanism of food recognition by the protozoan *Raphidiophrys contractilis*. Previously, we reported that a kind of extrusomes called kinetocyst discharges its content when *R. contractilis* attaches to the surface of prey organisms. Although the nature of the content of extrusomes was unknown, it was thought to be related to the recognition of food [3]. In *Actinophrys sol*, which is a relatively distantly related heliozoan, it has been shown that a 40 kDa β-1,3-glucan binding protein is involved in the food recognition system [2].

In this study, we found that a 100-kDa β-1,3-glucan

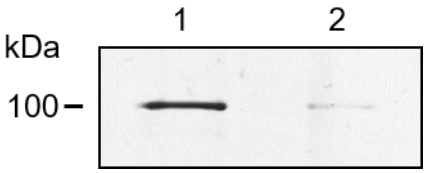

Fig. 6. A silver-stained SDS-PAGE gel, showing competitive inhibition of glucan binding of *Raphidiophryscontractilis* MVP by laminarin. Lane 1 shows a curdlan-binding MVP band, while it became reduced in lane 2 where the cell lysate was pretreated with laminarin (soluble β-1,3 glucan) prior to the addition of curdlan gel. Only the 100-kDa region of the gel is shown.

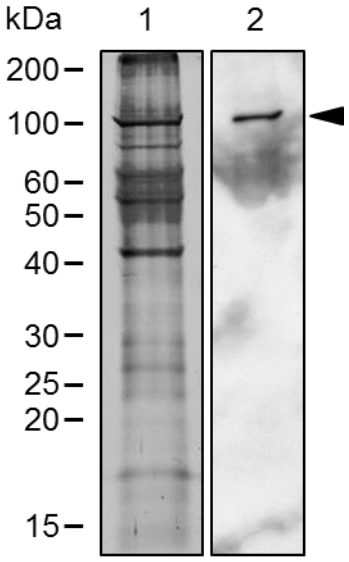

Fig. 7. Protein profile of the food-induced and curdlan-binding fraction and detection of MVP by Western blotting. Extracellular fluid was collected immediately after a cell suspension of *Raphidiophrys contractilis* was mixed with prey flagellate *Chlorogonium capillatum*, and curdlan-binding fraction was subjected to SDS-PAGE (lane 1) and Western blotting using anti-human MVP antibody (lane 2). A triangle shows the position of MVP. In addition to MVP, many other proteins were found to be secreted from the heliozoan, suggesting many proteins form a complex with MVP and co-precipitated by the curdlan gel.

binding protein is employed in food recognition by *R. contractilis*, which is released during food uptake. To our surprise, this protein has a strong homology to MVP of a variety of different organisms. The biological functions of MVP are not very clear, while in animals, MVP is suggested to be involved in a variety of functions including intracellular regulation of multiple signaling pathways, innate immunity, and drug resistance [6]. All of these are intracellular functions in the cytoplasm, and MVP is thought to be a protein functioning intracellularly. In this study, we presented evidence that MVP of *R. contractilis* is secreted outside the cell and plays a role in the recognition of food, a novel cellular function of MVP that has not been presented at all in other MVPs so far. Daly et al. [5] suggested that MVP is an ancient eukaryotic protein that was probably already present in the common ancestry of eukaryotes, and the original function of MVP might have been lost or completely different functions were assigned to MVP, as eukaryotic organisms experienced evolution of multicellularity.

Self-nonself discrimination is an essential cellular function for heterotrophic unicellular organisms, not only to distinguish food and non-food but also to avoid possible cannibalism [13]. It is also regarded as the basic and most primitive type of adaptive immunity, probably already possessed by the last eukaryotic common ancestor. The involvement of the heliozoan MVP homolog in prey recognition indicates a possible novel function of this protein in self/nonself discrimination, and also suggests that this function of MVP may have been lost in other eukaryotic species.

V. Conclusion

The centrohelid heliozoon *Raphidiophrys contractilis* was found to recognize β-1,3-glucan molecules to capture food organisms. A β-1,3-glucan-binding protein was purified by co-sedimentation with β-1,3-glucan gel, and identified as a major vault protein (MVP). The protein was found to be secreted from *R. contractilis* during feeding. The vault is a eukaryotic organelle found in most eukaryotic cells, but the function of this organelle is not fully understood. The discovery of MVP in β-1,3-glucan-mediated food recognition by unicellular protist may shed light on the evolution of self-nonself recognition and innate immunity systems of eukaryotes.

Acknowledgment

This study was supported by the Watanuki International Scholarship Foundation.

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