Isolation and Characterization of Okadaic Acid Binding Proteins from the Marine Sponge *Halichondria okadai*†

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ABSTRACT: Okadaic acid, first isolated from the marine sponge *Halichondria okadai*, is a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A, respectively). Photoaffinity labeling experiments previously performed with biotinylated photoreactive okadaic acid revealed the presence of okadaic acid binding protein (OABP) in the crude extract of *H. okadai*. In this article, OABP1 and OABP2 were purified from *H. okadai* as guided by the binding affinity of [27-^{3}H]okadaic acid. OABP1 has an approximate molecular mass of 37 kDa in SDS−PAGE analysis. Edman degradation followed by molecular cloning and sequencing identified OABP1 as being 88% identical to the rabbit PP2Aβ catalytic subunit. On the other hand, HPLC analysis revealed that OABP2 consists of three 22 kDa proteins (OABP2.1, OABP2.2, and OABP2.3). Electrospray ionization mass spectrometry indicated that OABP2.1 and OABP2.2 form a complex with okadaic acid. The complete amino acid sequence of OABP2, determined by Edman degradation and molecular cloning, showed that OABP2.1 is 96% identical to OABP2.2 and 66% identical to OABP2.3, while being very slightly homologous to any protein phosphatases known to date. OABP2 did not exhibit phosphatase activity, though it bound to okadaic acid with a K_d of 0.97 nM. Furthermore, OABP2 was not detected in the sponge *Halichondria japonica* or the dinoflagellate *Prorocentrum lima*. We thus speculated that OABP2 might be involved in detoxifying okadaic acid.

Marine natural products have attracted chemists and biologists with their complicated structures and unique biological activities (*1*, *2*). Due to the recent development in analytical instruments such as NMR,[1] MS, and HPLC, numerous natural products have been identified, and their chemical structures have been determined in the past two decades. Many of them exhibit potent bioactivities, often providing useful molecular tools for scientists and potential new chemotypes leading to drug discovery. Unfortunately, it is still extremely difficult to elucidate their mode of action since biological function is frequently regulated by a complex of signaling cascades. Recent achievement in chemical biology has clearly demonstrated that binding of natural products often results in alteration of phenotype; their binding can be transmitted in the signal transduction cascade, which eventually activates physiologically important events controlling phenotype. To gain a better understanding of the biological activity of natural products, it is of great importance to identify their target proteins as the first step.

Okadaic acid (OA, **1**) was first isolated from the marine sponge *Halichondria okadai* (*3*) (Figure 1) and later discovered as a toxic constituent from the cultured dinoflagellate *Prorocentrum lima* (*4*). Because marine sponges are a filter feeder, which allows symbiosis of bacteria, OA presumably originates from symbiotic microorganisms (*5*). OA and its homologues tend to accumulate in shellfish, frequently causing human illness called diarrheic shellfish poisoning (*6*). OA exhibits strong cytotoxicity with an EC_{50} value of 1.7 nM against P388 cell line and exhibits tumor-promoting activity. All of these biological activities are attributed to

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[1] Abbreviations: Ac, acetyl; BSA, bovine serum albumin; cDNA, complementary DNA; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; DEAE, diethylaminoethyl; DEPC, diethyl pyrocarbonate; dNTP, deoxynucleotide triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; ESI-MS, electrospray ionization mass spectrometry; GSP, gene specific primer; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; K_d, equilibrium dissociation constant; LB, Luria-Bertani; LC, liquid chromatography; mRNA, messenger RNA; MS, mass spectrometry; NMR, nuclear magnetic resonance; OA, okadaic acid; OABP, okadaic acid binding protein; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PEEK, polyether ether ketone; PMSF, phenylmethanesulfonyl fluoride; *p*NPP, *p*-nitrophenyl phosphate; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; RACE, rapid amplification of cDNA ends; SCBP, sarcoplasmic calcium binding protein; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TOF, time of flight; Tris, tris(hydroxymethyl)aminomethane; UAP, universal amplification primer.

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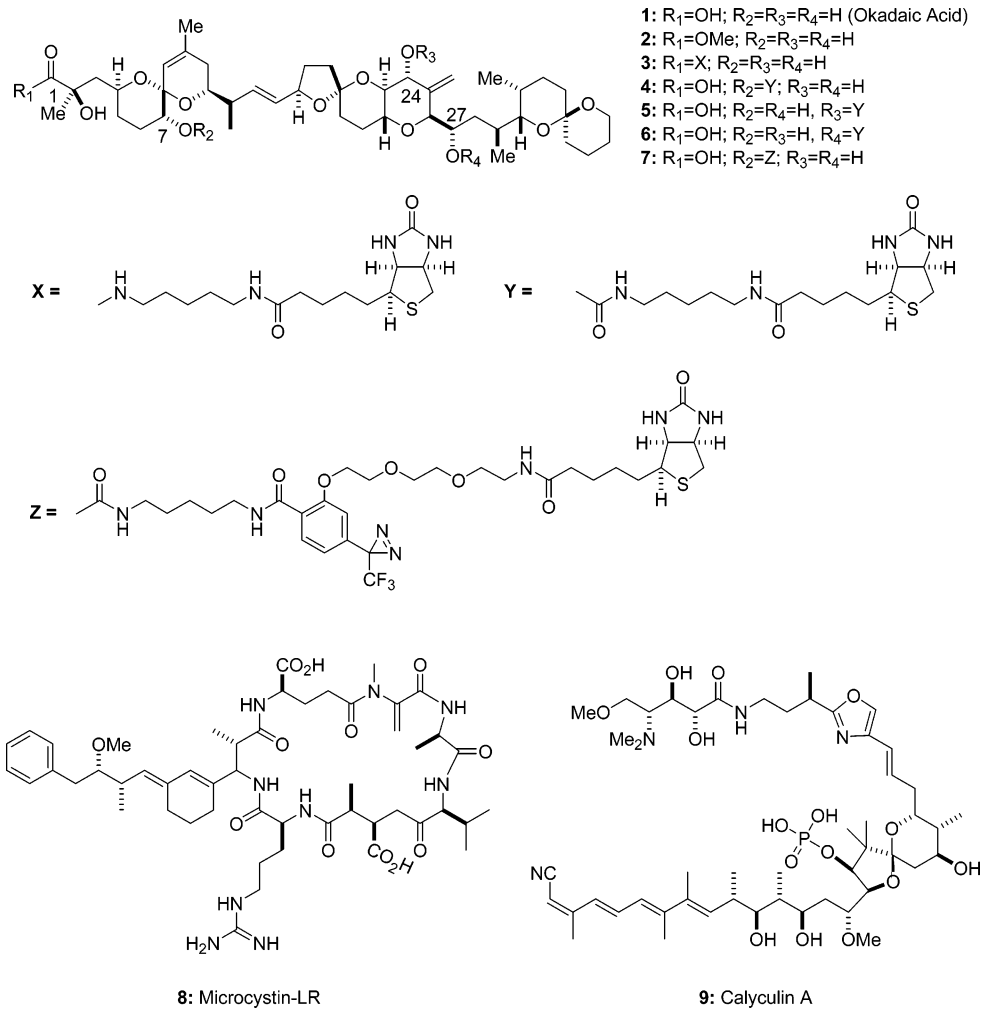


FIGURE 1: Structures of okadaic acid **1**, methyl okadaate **2**, biotinylated okadaic acid (**3**−**6**), the photoreactive biotinylated okadaic acid **7** (*12*), microcystin-LR (*18*), and calyculin A (*19*).

the strong inhibition of serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) (*7*).

Previously, we demonstrated that OA exhibited specific binding to PP2A using surface plasmon resonance (SPR) (*8*). When the crude lysate of *H. okadai* was loaded into the SPR biosensor, the sensorgram showed the presence of substances that bind to the immobilized okadaic acid **4** (*9*). To identify these substances, a 7-OH biotinylated diazirine probe **7** (Figure 1) was synthesized for photoaffinity labeling experiments. Two proteins, ca. 25 and ca. 37 kDa, were found to cross-link to **7**, and thus, we were motivated to purify and characterize these proteins. This report presents the isolation of two okadaic acid binding proteins (OABP1 and OABP2) guided by [27-^{3}H]okadaic acid binding and determination of their primary sequences by molecular cloning and LC−MS/MS.

MATERIALS AND METHODS

Materials. The sponge *H. okadai* was collected off the coast of Aburatsubo Bay in Kanagawa Prefecture and stored at −30 °C. Edman degradation analysis was performed by APRO Life Science Institute, Inc. (Tokushima, Japan). Oligonucleotides for PCR were purchased from Sawady Technology (Tokyo, Japan). Lys-C endopeptidase was purchased from Wako Pure Chemicals (Osaka, Japan), and the enzyme solution dissolved in 10 mM Tris-HCl (pH 9.0) was stored at −20 °C. Microcystin-LR was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Calyculin A was a generous gift from N. Fusetani and S. Matsunaga of The University of Tokyo. Biotinylated okadaic acids (*8*, *9*), methyl okadaate, and [27-^{3}H]okadaic acid (specific radioactivity, 5.0 Ci/mmol) were prepared as described previously (*10*). The Nick Column, DEAE-Sephadex A-50, the Source 15 PHE 4.6/100 column, the MonoQ 5/5 column, the Hiload 16/60 Superdex 75 column, and the Quick Prep Micro mRNA Purification Kit were purchased from Amersham Bioscience (Uppsala, Sweden). The Superscript Preamplification System for First Strand cDNA Synthesis, the 5′RACE and 3′RACE System for Rapid Amplification of cDNA Ends, and the Glassmax Spin Cartridge were purchased from Invitrogen Corp. (Carlsbad, CA). T4 DNA ligase and the pGEM-T vector were from Promega (Madison, WI). The Wizard Plus Minipreps DNA purification system (Promega) and the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) were kindly provided by M. Sato and Y. Umezawa of The University of Tokyo. The TSK-gel Super ODS column (1.0 mm × 50 mm) was purchased from Tosoh Corp. (Tokyo, Japan). The Develosil 300 C4 HG-5 column (4.6 mm × 150 mm) was purchased from Nomura Kagaku Co., Ltd. (Aichi, Japan). The Mightysil RP-18 GP column (4.6 mm × 250 mm) was purchased from Kanto Chemical Co., Inc. ImmunoPure

Table 1: Isolation of OABP1 and OABP2 from *H. okadai*[a]

	purification step	protein (mg)	total binding (nmol)	specific activity (nmol/mg)	yield (%)	phosphatase activity (pmol min^{-1} μg^{-1})
	soluble extract	6500	6.5	0.001	−	150
OABP1	$(NH_4)_2SO_4$ fractionation	3730	1.12	0.0003	100	21
	DEAE-Sephadex A-50	174	0.7	0.004	63	39
	Source 15 PHE	11	0.55	0.05	49	58
	MonoQ	2.3	0.345	0.15	31	82
	Superdex 75	0.234	0.152	0.65	14	710
OABP2	$(NH_4)_2SO_4$ fractionation	1670	310	0.17	100	110
	DEAE-Sephadex A-50	323	154	0.47	49	2.4
	Source 15 PHE	15.9	150	9.4	48	<0.2
	Superdex 75	13.2	190	14.4	62	1

[a] Purification of the two proteins was monitored via binding of [27-^{3}H]okadaic acid.

Immobilized Monomeric Avidin was purchased from Pierce (Rockford, IL). Other chemicals were purchased from Wako Pure Chemicals.

Binding Assays. All operations were carried out at 4 °C. A protein fraction was diluted to the desired concentration with 20 mM Hepes-NaOH (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 0.1 mg/mL BSA. To a 20 μL aliquot was added 1 mL of ice-cold acetone (*11*). After incubation at 4 °C for 30 min, the precipitate was recovered with centrifugation at 10000*g* for 10 min. The pellet was suspended in 1 mL of ice-cold acetone again and centrifuged at 10000*g* for 10 min. Then the pellet was dissolved in 100 μL of 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 0.1 mg/mL BSA, and 0.01% Tween 20 and incubated with 6.2 nM [27-^{3}H]okadaic acid (*10*) for 2 h. The mixture was loaded onto the NICK column and eluted with 800 μL of 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1.0 mM EDTA, and 0.01% Tween 20 (*12*). The recovered sample was mixed with scintillation cocktail, and then its radioactivity was quantified with a liquid scintillation counter. Nonspecific binding was assessed in the presence of an ~100-fold excess of unlabeled OA.

Protein Phosphatase Assays. A protein solution diluted with 50 mM Tris-HCl (pH 7.0) containing 0.12 mg/mL BSA, 1 mM $NiCl_2$, 32.5 μM $CaCl_2$, and 0.9 mg/mL *p*NPP was incubated at 37 °C for 90 min (*13*). Phosphatase activity was calculated by measuring the absorbance of *p*-nitrophenol (extinction coefficient for *p*-nitrophenol, 1.78×10^4 M^{-1} cm^{-1}) at 405 nm.

Isolation of OABP1. The sponge stored at −40 °C was cut into small pieces with a razor blade and homogenized with 20 mM Tris-HCl (pH 8.2) containing 540 mM NaCl, 7.0 mM Na_2SO_4, 0.2 mM $NaHCO_3$, 10 mM KCl, 20 mM EDTA, 0.5 mM DTT, and a series of protease inhibitors (*11*). After centrifugation at 75000*g* for 60 min, $(NH_4)_2SO_4$ powder was added to the supernatant to bring its level of saturation to 20%. The mixture was centrifuged at 75000*g* for 30 min. This procedure was repeated twice with use of 30 and 40% saturation of $(NH_4)_2SO_4$. The final supernatant was saved as fraction I for the purification of OABP2. The pellet was dissolved in buffer A, which consisted of 30 mM Tris-HCl (pH 7.5) containing 1.0 mM EDTA, 0.1% CHAPS, 0.5 mM DTT, and protease inhibitors, and dialyzed against the same buffer. The dialyzed solution was mixed with DEAE-Sephadex A-50 gel for 30 min. The gel was washed with buffer A containing 100 mM NaCl, and then bound proteins were eluted with 300 mM NaCl in buffer A. The eluate was concentrated and diluted with 50 mM sodium phosphate (pH 7.0) containing 1 mM EDTA, 0.5 mM DTT, and 1.0 M $(NH_4)_2SO_4$. The solution was loaded onto a SOURCE 15 PHE 4.6/100 column and eluted with 34 mL of a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ at a flow rate of 0.5 mL/min. The positive fractions were concentrated and diluted with 30 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 0.05% CHAPS, 0.5 mM DTT, and protease inhibitors. The solution was loaded onto a Mono Q HR 5/5 column and eluted with 20 mL of a linear gradient from 0 to 300 mM NaCl at a flow rate of 1.0 mL/min. The positive fractions were concentrated and subjected to a Hiload 16/60 Superdex 75 gel filtration column with 10 mM Hepes-NaOH (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 0.05% CHAPS, 0.5 mM DTT, and protease inhibitors at a flow rate of 0.7 mL/min. The purified protein (234 μg of protein; specific binding activity, 0.65 nmol/mg of protein) was concentrated and stored at −40 °C in 50% glycerol (Table 1).

Isolation of OABP2. Fraction I, described in Isolation of OABP1, was precipitated by adding $(NH_4)_2SO_4$ to bring its saturation to 50%. After centrifugation at 75000*g* for 30 min, the precipitate was dissolved in and dialyzed against buffer A. The solution was purified with DEAE-Sephadex A-50 at pH 7.5. The major binding resided in the unabsorbed fraction, which was concentrated, and diluted with 50 mM sodium phosphate (pH 7.0) containing 1 mM EDTA, 0.5 mM DTT, and 1.5 M $(NH_4)_2SO_4$. The solution was loaded onto a SOURCE 15 PHE 4.6/100 column and eluted with 34 mL of linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$ at a flow rate of 0.5 mL/min. The active fractions were concentrated and subjected to Hiload 16/60 Superdex 75 gel filtration column chromatography. The purified OABP2 (13.2 mg of protein; specific binding activity, 14.4 nmol/mg of protein) was stored at −40 °C in 50% glycerol (Table 1).

Preparation of cDNA from H. okadai. Extraction and purification from the homogenized sponge tissue were carried out using the Quick Prep Micro mRNA Purification Kit. *H. okadai* was collected off the coast of Aburatsubo Bay in Kanagawa Prefecture on January 12th, 2000, and was immediately stored in liquid N_2. The frozen sponge (200 mg) was powdered with a mortar and was homogenized in 0.4 mL of an extraction buffer containing guanidinium thioacetate and *N*-lauroylsarcosine with a Potter-Elvehjem homogenizer. The suspension was diluted with 0.8 mL of the elution buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. After centrifugation at 15000*g* for 1 min, the supernatant was mixed with 1 mL of oligo (dT)-cellulose.

The resin was washed with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.5 M NaCl and then washed with the same buffer containing 1 M NaCl. The mRNA was collected with 0.4 mL of the elution buffer prewarmed at 65 °C.

For additional purification and concentration, 10 μL of 10 mg/mL glycogen and 40 μL of 2.5 M potassium acetate (pH 5.0) were added to 400 μL of a mRNA solution. One milliliter of 95% EtOH was added to the mixture, and the mixture was kept at −40 °C overnight for precipitation. The precipitated mRNA was concentrated by centrifugation at 4 °C for 5 min at 13000*g* and dissolved in 20 μL of DEPC-treated water. Reverse transcription was performed using the Superscript Preamplification System for First Strand cDNA Synthesis.

Edman Degradation and PCR for Molecular Cloning of OABP2.1. On the basis of the partial amino acid sequences WVEQLK and YTFLQFDPAP for OABP1 determined by Edman degradation (*14*), the oligonucleotides were designed as follows. The forward primers are 5′-TGGGTNGARC-ARCTNAA-3′, 5′-TGGGTNGARCARTTRAA-3′, and 5′-CARTTYGAYCCNGCNCC-3′. The corresponding reverse primers are 5′-TTNAGYTGYTCNACCCA-3′, 5′-TTYAAY-TGYTCNACCCA-3′, and 5′-GGNGCNGGRTCRAAYTG-3′, respectively. The peptides LTESQYVQNA and FFFT-DQDNTT for OABP2 were determined by Edman degradation (*14*). The designed forward primers are 5′-CARTAYGT-NCARAAYGC-3′ and 5′-TTYTTYACNGAYCARGAYAA-3′. The reverse primers are 5′-GCRTTYTGNACRTAYTG-3′ and 5′-TTRTCYTGRTCNGTRAARAA-3′. For OABP2.3, the partial amino acid sequences were determined to be EASQIIWDGLDK and AQRFxFTDQNDESHPFN, where x could not be determined. The forward primers were designed as ATNATNTGGGAYGGNYT and the reverse primers as TCRTCRTTYTGRTCNGT. The letters Y, N, and R in the primer sequence stated in the above represent C/T, A/C/G/T, and A/G, respectively.

The following PCR is described for cloning OABP2.1. Two microliters of the cDNA solution was mixed with 2 μM degenerated primers, 0.2 mM dNTP in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$. PCR was initiated by addition of *Taq* polymerase (0.04 unit/μL). The amplification of cDNA involved 30 cycles of denaturation at 95 °C for 30 s, annealing at 44 °C for 30 s, and extension at 72 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 49 °C for 30 s, and extension at 72 °C for 2 min. After final extension at 72 °C for 7 min, the reaction was terminated when the mixture was cooled at 4 °C. The PCR fragment was separated by 2% agarose gel electrophoresis. The gel portion containing a DNA band was excised and was melted at 65 °C for 15 min. After the DNA was extracted with phenol and chloroform and precipitated with 70% ethanol, the pellet was dissolved in 10 μL of water. The PCR fragment was sublconed into the pGEM-T vector and transformed into *Escherichia coli* DH5α in an ampicillin/LB agar plate.

After colonies had been inoculated in ampicillin/LB overnight, the plasmid DNA was extracted using the Wizard Plus Minipreps DNA purification system. The DNA was digested with *Sac*I and *Apa*I and analyzed by 2% agarose gel electrophoresis. Sequencing of the DNA was performed by the dye terminator method using an ABI prism 310 Genetic Analyzer (Applied Biosystems) and the Big Dye Terminator Cycle Sequencing Kit. PCR primers T7 RNA polymerase primer (5′-TAATACGACTCACTATAGGG-3′) and SP6 RNA polymerase primer (5′-TATTTAGGTGA-CACTATAG-3′) were used for the PCR.

3′RACE for OABP2.1. The 3′RACE System for Rapid Amplification of cDNA Ends was used to extend cloning in the 3′-direction (*15*). cDNA was synthesized as described above, except that the oligo (dT)-containing adapter primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTT-TTTTTTT-3′) was used. After the digestion of mRNA, the first PCR was performed using 0.2 μM GSP1 (5′-GCTGCT-CATTCACTCGCCTGATTT-3′) and 0.2 μM UAP (5′-GGCCACGCGTCGACTAGTAC-3′). The condition involved 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min. Final extension was carried out at 72 °C for 7 min, followed by cooling at 4 °C. The PCR product was diluted 100-fold with water, which was used as the template DNA for nested PCR. The nested PCR was performed using GSP2 (5′-CCAGC-CAAACTTTTTGGGAAGGAA-3′) and UAP.

5′RACE for OABP2.1. The 5′RACE System for Rapid Amplification of cDNA Ends was used to extend the cloning in the 5′-direction (*15*). cDNA was synthesized in the manner described in 3′RACE for OABP2.1, except that GSP3 (5′-TCCTGGTCTGTGAAGAAG-3′) was used. To the solution of cDNA was added 120 μL of 6 M sodium iodide. The mixture was transferred to a GlassMax Spin Cartridge and centrifuged at 15000*g* for 20 s to remove unincorporated dNTP and GSP3. The cartridge was washed with ice-cold washing buffer supplied with the kit, and the cDNA was eluted with 50 μL of water preheated to 65 °C.

A poly(dC) tail was added to the cDNA using terminal deoxynucleotidyl transferase and 2′-deoxycytidine 5′-triphosphate (200 μM) in 10 mM Tris-HCl (pH 8.4) containing 25 mM KCl and 1.5 mM $MgCl_2$. The mixture was incubated at 37 °C for 10 min and 65 °C for 10 min. The first PCR was run using 0.4 μM GSP4 (5′-GCCTTGAGCGTATCCT-CAAACGTG-3′) and 0.4 μM deoxyinosine-containing anchor primer (5′-GGCCACGCGTCGACTAGTACGGGI IGGGIIGGGIIG-3′, where I represents deoxyinosine). The process consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min. The final extension was at 72 °C for 7 min, and then the reaction mixture was chilled at 4 °C.

The PCR product was diluted 100-fold with water and used as the template for nested PCR. The nested PCR was performed using GSP5 (5′-CCATAGCCTCAAAGATGCCAGT-GC-3′) and UAP (5′-GGCCACGCGTCGACTAGTAC-3′).

ESI-MS Analysis of OABP2. The stored OABP2 solution was diluted with 100 μL of 0.1% aqueous TFA and purified with a Develosil 300 C4 HG-5 column. The column was equilibrated with 0.1% TFA, and the concentration of 0.05% TFA in CH_3CN was increased from 0 to 30% in the initial 10 min and from 30 to 45% over the next 110 min. The flow rate was 1 mL/min and was controlled by two PU-980 pumps (Jasco Corp., Tokyo, Japan). The absorbance wavelength was set at 280 nm (UV-970, Jasco Corp.).

The protein fraction was diluted with a 10-fold volume of 0.1% formic acid and 50% CH_3CN and was analyzed with a Q-TOF2 ESI mass spectrometer (Waters Corp., Milford, MA). A syringe pump was used to maintain a flow rate of 5 μL/min. The spectrometer was operated at a capillary

voltage of 3000 V, a source block temperature of 80 °C, and a desolvation temperature of 150 °C.

LC−MS Analysis of OABP2. A 100 μL solution of OABP2 (120 μg) in 10 mM Tris-HCl (pH 9.0) containing 6 M urea and 1 mg of DTT was incubated at room temperature under a N_2 atmosphere. After 2 h, 2 μL of 4-vinylpyridine was added, and then the mixture was incubated for 30 min. Pyridylethylated OABP2 was purified by HPLC. Each fraction was lyophilized and dissolved in 50 μL of 10 mM Tris-HCl (pH 9.0). It was digested with Lys-C endopeptidase at 37 °C for 24 h and was added with 0.1% TFA for termination. The peptide mixture was purified with HPLC on a TSK-gel Super ODS column (1.0 mm × 50 mm) using 0.1% TFA as solvent A and 0.05% TFA/MeOH as solvent B. The column was equilibrated and run with 100% solvent A. After 5 min, the concentration of solvent B was linearly increased from 0 to 80% over the next 80 min. The flow rate was maintained at 50 μL/min. The absorbance wavelength was set at 210 nm. The Q-TOF2 ESI mass spectrometer was connected online to a semimicro HPLC interface, which was consisted of two PU-1585 pumps (Jasco Corp.), a UV-1575 UV detector (Jasco Corp.), an HG-1580-32 dynamic mixer (Jasco Corp.), and a G1332A degasser (Agilent Technologies, Inc., Palo Alto, CA). Those were connected with PEEK tubings [80 cm × 0.13 mm (inside diameter)].

MS/MS analysis of the N-terminal fragment of OABP2.1 and OABP2.2 was carried out as follows. The Lys-C endopeptidase digest was subjected to LC−MS. A single charged parent ion (*m*/*z* 487.3) was selected with a quadrupole detector for collision-induced decomposition with argon gas. The collision energy was set to 30 eV. Daughter ion spectra were recorded with a scanning TOF detector.

*Photoaffinity Labeling of OABP2 Using Okadaic Acid Photoaffinity Probe **7***. The purified OABP2 was treated with acetone as described in Binding Assays, and then 26 μg of the protein was dissolved in 200 μL of 100 mM phosphate buffer. To the solution were added 5 μL of MeOH and 5 μL of **7** in MeOH (1 μg/μL). The mixture was incubated at room temperature for 60 min and irradiated at 360 nm with an XX-15L lamp (UVP, Inc., Upland, CA) for 60 min. The protein solution was precipitated with acetone, and the pellet was dissolved in 100 μL of 10 mM Tris-HCl (pH 9.0). After separation by SDS−PAGE, the proteins were transferred to a nitrocellulose membrane and incubated with HRP-conjugated streptavidin. The membrane was exposed to X-ray film for visualization.

When a peptide sequencing analysis was conducted on demand after photolabeling, the protein mixture was digested with 0.1 AU/mL of Lys-C endopeptidase at 37 °C for 12 h. The reaction was terminated via addition of 1 μM PMSF. The solution of the digest was loaded onto the column equipped with 500 μL of ImmunoPure Immobilized Monomeric Avidin. The column was washed with 5 mL of 0.1 M sodium phosphate (pH 7.2) containing 150 mM sodium chloride, and the biotinylated substances that remained on the column were subsequently eluted with 0.1 M glycine (pH 2.8) for LC−MS analysis.

RESULTS

To identify and characterize OA binding proteins from the sponge *H. okadai* (*9*), we monitored the purification process using binding assays with [27-^{3}H]okadaic acid (*12*). From 1.0 kg of *H. okadai*, collected from the coast of Aburatsubo Bay in Kanagawa Prefecture, 6.5 mg of extract was obtained (Table 1). The precipitate obtained in 40% $(NH_4)_2SO_4$ showed binding to [27-^{3}H]okadaic acid. The subsequent purification using DEAE Sephadex A-50 increased the specific activity by 13-fold. This fraction was subjected to hydrophobic interaction chromatography followed by negative ion exchange chromatography. The final step of purification was carried out by gel filtration chromatography to yield 234 μg of OABP1. The phosphatase activity of OABP1 measured by hydrolysis of *p*NPP was 710 pmol min^{-1} μg^{-1} (*13*).

Elucidation of the partial amino acid sequences and molecular cloning of OABP1 were carried out as follows. The apparent molecular mass of OABP1 was 37 kDa as determined via SDS−PAGE (Figure 2A, lane 1). OABP1 was digested within the gel by Lys-C endopeptidase and separated by reverse-phase HPLC (Figure S1A of the Supporting Information). Two peptides thus obtained were sequenced to be WVEQLK and YTFLQFDPAP by Edman degradation (*14*). Random primers were designed to amplify the DNA fragment of OABP1. mRNA was extracted from *H. okadai*, and reverse transcription of the mRNA provided the first strand cDNA sequence. The PCR yielded two distinct products (see Figure S2 of the Supporting Information). The amino acid sequence for one of the PCR products with 850 bp was 88% identical with that of a rabbit PP2Aβ catalytic subunit (*16*) (Figure S3 of the Supporting Information).

Purification of OABP2 at first proved to be problematic because it could not be detected in binding assays. It is possible that protein−protein interactions preclude binding of [27-^{3}H]OA to OABP2 under these purification conditions, or because the sponge contains high concentrations of OA, OABP2 may form a complex with endogenous OA, thus preventing radioligand binding. Rinsing the fraction precipitated with 50% $(NH_4)_2SO_4$ in cold acetone (*17*) resulted in a drastic enhancement of the specific binding, confirming the presence of a binding protein in addition to OABP1 in the sponge. This protein named OABP2 was further purified in a manner similar to that for OABP1, except without the MonoQ separation, to yield 13.2 mg of OABP2, which corresponds to 0.79% (w/w) of the initial $(NH_4)_2SO_4$ extract (Table 1). It did not exhibit phosphatase activity when examined with *p*NPP as the substrate (*13*).

Characterization of OABP2 was first carried out by binding assays using [27-^{3}H]okadaic acid (Figure 3). The equilibrium dissociation constant (K_d) was calculated to be 0.97 nM by nonlinear fitting of the saturation binding curve (Figure 3A). Microcystin-LR (**8** in Figure 1) (*18*) and calyculin A (**9** in Figure 1) (*19*), which are known to compete for binding with OA to PP2A (*20*), did not replace [27-^{3}H]-okadaic acid even at 10 μM (Figure 3B). Methyl okadaate **2** exhibited competitive binding to OABP2 with a 1 order of magnitude weaker affinity (IC_{50} = 26 nM) than OA. Biotinylated okadaic acid (*9*) at C7-OH (**4** in Figure 1), C24-OH (**5** in Figure 1), and C27-OH (**6** in Figure 1) inhibited binding of OA to OABP2 with IC_{50} values of 6.8, 2.76, and 224 nM, respectively.

Analysis of OABP2 by SDS−PAGE under reducing conditions revealed that OABP2 was composed of two

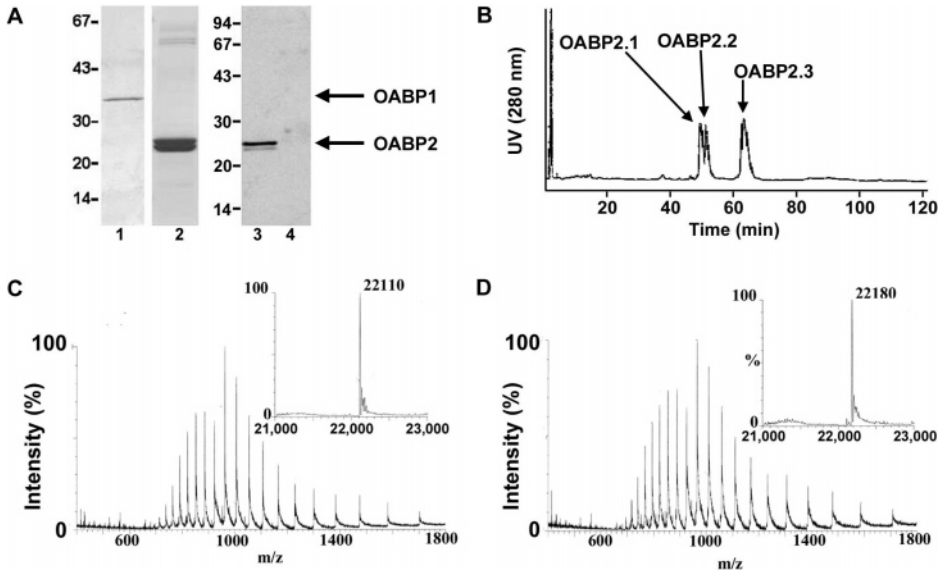


FIGURE 2: OABP2 consists of three proteins. (A) SDS−PAGE of OABP1 (lane 1) and OABP2 (lane 2) under reducing condition, stained with Coomassie Blue. Lane 2 shows two bands around 25 kDa. Photoaffinity labeling of OABP2 by compound **7** in the absence (lane 3) or presence (lane 4) of unlabeled OA. Molecular mass marker positions are indicated at the left. (B) HPLC analysis of OABP2. OABP2.1, OABP2.2, and OABP2.3 eluted at 48.4, 50.2, and 61.7 min, respectively. (C and D) LC−MS analysis of (C) OABP2.1 and (D) OABP2.2. The eluate from the HPLC analysis shown in panel B was analyzed with the Q-TOF2 ESI mass spectrometer. The insets show deconvoluted parent ions at (C) *m*/*z* 22 110 and (D) *m*/*z* 22 180 for OABP2.1 and OABP2.2, respectively.

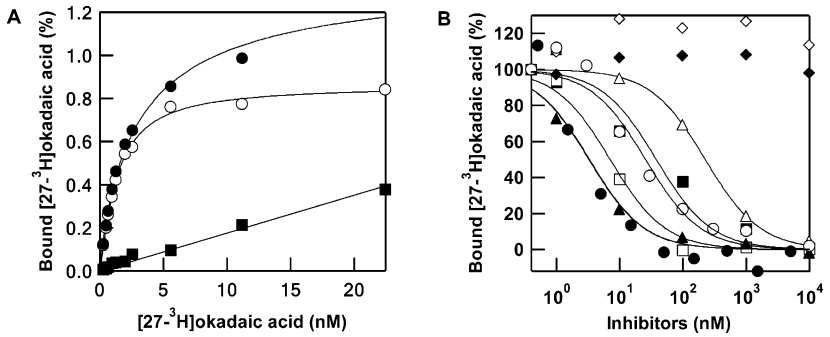


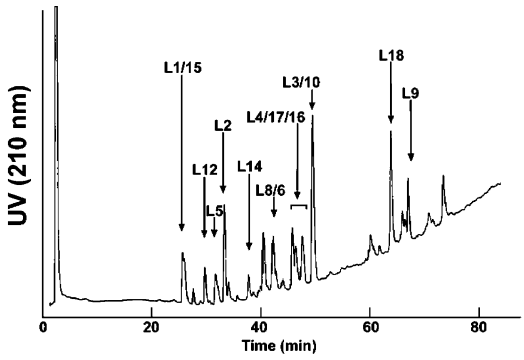
FIGURE 3: High-affinity binding of [27-^{3}H]okadaic acid to OABP2. (A) Saturation binding curve for binding of [27-^{3}H]okadaic acid to OABP2. Total (●) and nonspecific binding (■) was assessed in the absence and presence of excess unlabeled OA, respectively. The specific binding (○) was obtained by subtracting the nonspecific binding from the total binding. (B) Competitive inhibition of binding of [27-^{3}H]okadaic acid to OABP2 by OA [**1** (●)], methyl okadaate [**2** (○)], biotinylated derivatives at the carboxyl group at C-1 [**3** (■)], 7-OH [**4** (□)], 24-OH [**5** (▲)], and 27-OH [**6** (△)], microcystin-LR [**8** (◆)], and calyculin A [**9** (◇)] (*12*).

similar-sized proteins of ∼22 kDa (Figure 2A, lane 2). A photoaffinity labeling experiment was performed using OA derivative **7** (*8*, *9*). As a result, the upper band was labeled more intensely than the lower band (Figure 2A, lane 3), both of which disappeared in the presence of excess OA (Figure 2A, lane 4).

The ESI mass spectrum of OABP2 in aqueous 5% MeOH revealed the presence of three components named OABP2.1, OABP2.2, and OABP2.3 (Figures S4A and Figure S4B of the Supporting Information). The multivalent ion spectrum was deconvoluted to yield three parent ion peaks at *m*/*z* 22 916 (OABP2.1), 22 985 (OABP2.2), and 22 251 (OABP2.3). When OABP2 was dissolved in 50% CH_3CN and subjected to ESI-MS analysis, however, these ion peaks were observed at *m*/*z* 22 112, 22 181, and 22 252, respectively (Figure S4C and Figure S4D of the Supporting Information). The *m*/*z* values for OABP2.1 and OABP2.2 in 5% MeOH were shifted by 804 Da, indicating that the two components of OABP2 form a complex with OA. The LC−MS analysis of OABP2 successfully separated the three components (Figure 2B). The mass spectra for OABP2.1 and OABP2.2 are shown in panels C and D of Figure 2.

To determine their amino acid sequences, the proteins were digested by trypsin within the SDS−PAGE gels, and the peptide mixture was separated by HPLC. Then partial peptide sequences of the two peptides were determined to be FFFTDQDNTT and LTESQYVQNA by Edman degradation (Figure S1B of the Supporting Information) (*14*). On the basis of these sequences, oligonucleotide primers were designed and used for molecular cloning. The sense primer 5′-CARTAYGTNCARAAYGC-3′ and the antisense primer 5′-TTRTCYTGRTCNGTRAARAA-3′ yielded two products of 250 bp (Figure S2 of the Supporting Information). The two fragments later assigned to OABP2.1 and OABP2.2 are 92% identical to each other. RACE methods (*15*) enabled us to determine the entire sequence of OABP2.1 (Figure S5 of the Supporting Information). There is good agreement between the theoretical molecular mass, 22 199 Da, and the deconvoluted molecular mass, 22 110 Da (Figure 2C). The LC−MS analysis of the proteolytic digest of OABP2.1 with

A



B

Fragment	Residue	Sequence deduced from cDNA	Theoretical	Observed (*m/z*)			
			$[M+H]^+$	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+4H]^{4+}$
L11	120-120	K	147.11				
L7	56-57	DK	262.14				
L1	1-5	MANLK	576.31	487.36			
L13	126-130	EEATK	577.28				
L12	121-125	GYITK	581.33	581.44			
L15	138-142	DPNLK	586.32	586.42			
L14	131-137	LGIRVTK	786.52	786.65	393.83		
L6	48-55	FRELAPTK	902.50	902.68	451.82		
L4	30-38	GYVTEEVFK	1071.54	1071.72	536.35		
L5	39-47	DGVQRRLEK	1100.62	1100.83	550.91		
L2	6-14	EPSAHWC'RK	1218.58	1218.80	609.9		
L16	143-153	STGIFEAMDEK	1227.56	1227.79	614.38		
L17	154-165	NTGRITFEDTLK	1394.73	1394.97	698.00		
L10	106-119	ESSQTFWEGIDREK	1711.79		856.51		
L3	15-29	MRTVFRPWDVEGGGSK	1764.89		883.59	589.06	
L8	58-75	MYERSHRHWVN	2371.15		1186.83	791.50	
L18	166-190	HCNLGVKAQLFFFTDQDNTTHPFNYVRGALVD	2915.39		1458.96	972.98	
L9	76-105	MPEGYRLTESQYVQNAWLLIHSPDFEASLK	3524.98			1175.80	881.84

FIGURE 4: Peptide mapping of OABP2.1 by LC−MS. (A) HPLC analysis of the Lys-C digest of OABP2.1. The conditions are described in Materials and Methods. Peaks from L1 to L18 were assigned from the predicted proteolytic fragments of OABP2.1. (B) Comparison of the proteolytic peptides with those deduced from the cDNA sequence. The theoretical and observed fragments were identical except for the N-terminal fragment L1. The N-terminal fragment, which theoretically affords an ion at *m/z* 576.31 as $[M + H]^+$, was observed at *m/z* 487.36.

Lys-C endoproteinase accomplished the peptide mapping except for the N-terminal pentapeptide MANLK (Figure 4A). The calculated *m/z* value for the N-terminal peptide is 576.31, while it is absent from Figure 4B. Instead, fragment L1 was observed at *m/z* 487.36, which was unassigned at this stage. MS/MS analysis for the L1 peptide revealed that the protein lacks the N-terminal methionine and is acetylated at Ala^1 (data not shown). This posttranslational modification was confirmed by ESI-MS analysis of a synthetic tetrapeptide Ac-ANLK, which provided a fragmentation pattern identical to that observed (data not shown).

Elucidation of the primary structure by molecular cloning and protein sequencing for OABP2.2 and OABP2.3 were performed in the same manner. The peptide mass fingerprinting of OABP2.2 was identical with that of OABP2.1 in amino acid positions from Ala^1 to Lys^{104}. The same posttranslational modification also occurred at the N-terminus of OABP2.2 and OABP2.3. The protein sequences for the three components of OABP2 are aligned in Figure 5, indicating that they are highly homologous to each other.

The amino acid sequences of OABP2.1, -2.2, and -2.3 were referred to a database. A BLAST search did not hit any protein phosphatase homologous to OABP2 but revealed that OABP2 is approximately 24% identical to a sarcoplasmic calcium binding protein (SCBP2) originating from earthworm *Lumbricus terestris* (GenBank accession number CAD29318) (*21*) (Figure 5) and 20% identical to a putative calcium binding protein derived from *Streptomyces ambofaciens* (GenBank accession number CAI77998).

We then carried out a photoaffinity labeling experiment to investigate whether OABP2 is present in the sponge *Halichondria japonica* and the dinoflagellate *P. lima*. The sponges *H. japonica* and *H. okadai* were collected at the same spot within a 1 m radius off the coast of Aburatsubo Bay in Kanagawa Prefecture in Japan. They were simply homogenized and centrifuged to yield the crude extract. SDS−PAGE analysis shown in Figure 6 indicates that the photoaffinity probe of OA **7** appeared to bind to several proteins, including OABP1 and OABP2 in the *H. okadai* extract (lane 1), while the labeling did not occur in the presence of an excess of OA (lane 2). On the other hand, only OABP1 was detected in the *H. japonica* extract (lane 3), which disappeared in the presence of OA (lane 4). The same technique demonstrated that the dinoflagellate *P. lima*,

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OABP2.1     ANLKEPSAHWCRKMRTVFRPWDVEGGSKGYVTEEVFKDGV
OABP2.2     ANLKEPSAHWCRKMRTVFRPWDVEGGSKGYVTEEVFKDGV
OABP2.3     AVSGEPSKHWCRKIRSMFRQWDVEAGLKGYVTEEDFKNRV
CAD29318    -----MSAFYLRKLKTYFAATDTD--KDGVLTENDYHEMA
                  * .: **::: *   *.:   .* :**: ::: .

OABP2.1     QRRLEKFPELAPTKDKMYERSHRHWVNHCNLGVKMPEGYR
OABP2.2     QRRLEKFPELAPTKDKMYERSHRHWVNHCNLGVKMPEGYR
OABP2.3     GKTLERFPELASSKDKLIERAHHHWVEHCNLGVKMPPGYR
CAD29318    RRFIDIVKLDDAQGKKLHALAAKVWNDFFKG--WATDGKS
             : :: .    .  .*:   : : * :. :     . *

OABP2.1     LTESQYVQNAWLLIHSPDFEASLKESSQTFWEGIDREKKG
OABP2.2     LTESQYVQNAWLLIHSPNFEASLKESSQAFWEGIDREKKG
OABP2.3     LTEAQYIQNFWLFIHSPKFDQYLKEASQIIWDGLDKEKKG
CAD29318    LTQDQLIASFLKRRSDPKFLESLKELMTVEFHVVDINKDG
            **: * : .      .*.*   ***     :. :* :*.*

OABP2.1     YITKEEATKLGIRVTKDPNLKSTGIFEAMDEKNTGRITFE
OABP2.2     YITIEEATKLGIRVTKDPNLQSTGIFEAMDEKNTGRITFE
OABP2.3     FLTKEEAHAHGIRVTNDKDHASRGIFETMDEKKSGRITFE
CAD29318    SIQLDEFTIMFRFHGIDAAHAKA-SFEAIDSNSDGVISLD
             :  :*          *    .   **::*.:. * *:::

OABP2.1     DTLKAQLFFFTDQDNTTHPFNYVRGALVD
OABP2.2     DTVRAQRFFFTDQDNTTHPFNYVRGKLVD
OABP2.3     DTIKAQRFFFTDQNDESHPFNHIRGPLVD
CAD29318    EFLTAVVDFFTGEDEKSSSR-LFWGPLV-
            : : *   ***.::: : .   . * **
```

FIGURE 5: Alignment of deduced amino acid sequences of OABP2.1, OABP2.2, OABP2.3, and SCBP2 (*21*). The identical amino acid residues are denoted with asterisks, the conserved residues with colons, and the weakly conserved residues with dots. The highlighted residues (gray) are identical among OABP2.1, OABP2.2, and OABP2.3. Acetylation in the N-terminus of OABP2.1, OABP2.2, and OABP2.3 was confirmed by MS/MS analysis for the Lys-C endopeptidase digest (data not shown). The bold sequences were determined by Edman degradation. The underlined sequence was determined to be the binding site for the photoaffinity probe **7**. The GenBank accession numbers are AB078740 for OABP2.1, AB078741 for OABP2.2, and CAD29318 for SCBP2 (*21*).

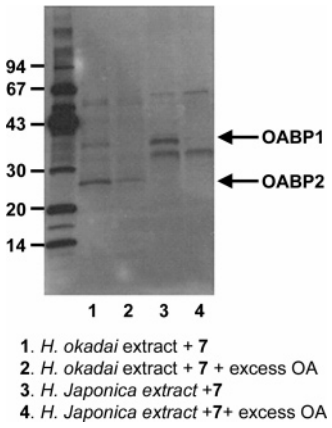

FIGURE 6: Photoaffinity labeling experiments identifying okadaic acid binding proteins in different sponges. The soluble fractions from the sponges *H. okadai* (lanes 1 and 2) and *H. japonica* (lanes 3 and 4) were photolabeled by **7**. OABP1 was detected in both of the sponge extracts (lanes 1 and 3), while OABP2 was detected only in *H. okadai* (lane 1). Labeling on OABP1 and OABP2 was abolished in the presence of excess OA (lanes 2 and 4).

which is a species known to produce OA, possesses no detectable amount of okadaic acid binding proteins (data not shown).

DISCUSSION

Okadaic acid (OA) and its derivatives are isolated from the sponge *H. okadai*, *Suberites domuncula* (*5*), and *Halichondria malanodocia* (*3*). Numerous studies have demonstrated that OA inhibits protein phosphatases 1 (PP1) and 2A (PP2A) and consequently exhibits cancer-promoting activity. Dinophysistoxin (*6*), one of the OA derivatives that bivalves accumulate through diet where toxic algal blooms occur, is thought to be responsible for serious human-threatening food poisoning called diarrhetic shellfish poisoning. Thus, OA is one of the well-known marine natural products used in many biochemical and pharmacological studies. The dinoflagellate *P. lima* is the most primitive species that produces OA, while its presence in the sponge *H. okadai* has not been identified. Sponges are a filter feeder, which takes up bacteria and plankton contained in seawater, and harbor bacteria for symbiosis. PP2A is one of the inevitable proteins conserved in many species playing roles in signal transduction (*22*, *23*). Considering the OA toxicity and the possible presence of protein phosphatases, we raised the simple question of how the sponge itself is tolerant of OA.

We searched for okadaic acid binding proteins and initially identified OABP1. OABP1 turned out to be 88% homologous to a rabbit PP2Aβ catalytic subunit (Figure S3) and retained the phosphatase activity. We thus speculated that OABP1 may be PP2A. Extensive washing of the 50% $(NH_4)_2SO_4$ fraction with acetone at 0 °C for 30 min made us identify OABP2 as a protein complex consisting of OABP2.1, OABP2.2, and OABP2.3 (Figure 5). OABP2.1 was 96% identical to OABP2.2 and 66% identical to OABP2.3. A database search revealed that OABP2.1 is 24 and 20% homologous to a sarcoplasmic calcium binding protein (SCBP2) originating from the earthworm *L. terestris* (GenBank accession number CAD29318) (*21*) (Figure 5) and a putative calcium binding protein derived from *S. ambofaciens* (GenBank accession number CAI77998), respectively. In spite of the fact that these calcium binding proteins possess the EF-hand motif, which accommodates calcium ion, no characteristic pattern for the EF-hand motif was found in OABP2. In addition, calcium ion did not affect the binding of OA to OABP2 (data not shown). Since physiological roles for the sarcoplasmic calcium binding protein SCBP2 (GenBank accession number CAD29318) and the putative calcium

binding protein (GenBank accession number CAI77998) have not been investigated in detail, there were no clues for evaluating the function of OABP2.

Two okadaic acid proteins, which are not homologous to PP1 and PP2A, were identified from crude extracts of other sponges. The first example is galectin (*5*). Western blotting analysis for the crude extract of *S. domuncula* demonstrated that both the anti-OA antibody and anti-galectin antibody detected the same 35 kDa protein band, suggesting that galectin covalently binds to OA. In the second example, the same approach was applied to the sponge *Lubomirskia baicalensis*, where Müller et al. (*24*) identified a 14 kDa protein, implying the presence of a covalent bond between this protein and OA. Since the crude extract prepared from either of the sponges would have contained numerous numbers of proteins with a size of ~35 or ~14 kDa, however, it seems difficult to address the possibility that they are OA binding proteins. In contrast, this study reveals that endogenous OA was dissociated upon cold acetone treatment and that the maximum number of binding sites for [27-^{3}H]-okadaic acid in the *H. okadai* extract was significantly increased, unveiling the presence of OABP2. Since acetone does not cleave covalent bonds, the interaction between OA and OABP2 was determined to be noncovalent, suggesting that OA binds to OABP2 in a manner different from that for galectin (*5*) or the 14 kDa protein (*24*).

Next, we investigated the structure−activity relationship for binding of OA to OABP2 in reference to binding of OA to PP2A. The X-ray crystal image of OA displays an intramolecular hydrogen bond between the carboxyl group and 24-OH (*3*). Since modification at either functional group causes a significant reduction in the binding affinity for PP2A (*9*, *25*, *26*), the cyclic structure of OA is important for the biological activity. Recently, Xing et al. (*27*) reported an X-ray crystal structure of the OA−PP2A complex at 2.6 Å resolution, revealing that the characteristic three-dimensional structure remains when binding to PP2A. The X-ray crystal structure shows that the hydrophobic end of OA is accommodated in the hydrophobic cage created at the OA binding site in PP2A and that microcystin-LR occupies the same surfaces sharing a similar set of amino acids that affect binding of both of the toxins. This study using [27-^{3}H]-okadaic acid determined the equilibrium dissociation constant of OA against OABP2 to be 0.96 nM (Figure 3A). Since the K_d values of OA for PP1 and PP2A are 145 nM and 30 pM in the literature, respectively (*25*), binding of the radioligand to OABP2 is 150 times stronger than binding to PP1 and 32 times weaker than binding to PP2A. Then various OA derivatives and other potent inhibitors to PP2A were tested to evaluate the molecular determinant for this high-affinity binding of OA to OABP2. Surprisingly, methyl okadaate (compound **2**) and the 24-OH biotinylated OA (compound **5**) exhibited moderate binding affinity for OABP2 (Figure 3B), implying that disruption of the intramolecular hydrogen bond does not significantly affect binding of OA to OABP2. Palmitoylation at C7-OH in OA decreases the binding affinity to PP2A by more than 3000-fold (*25*), while biotinylation at the same position (compound **4**) decreased binding affinity to OABP2 by only 7-fold (Figure 3B). In addition, since biotinylation at 27-OH reduced the binding affinity for OABP2 by 230-fold, the hydrophobic tail (FG rings) may be the recognition site for OABP2. Furthermore, neither microcystin-LR (*18*) nor calyculin A (*19*) inhibited binding of OA to OABP2. We thus concluded that the molecular determinant for the high-affinity binding of OA to OABP2 is different from that to PP2A.

The binding of OA to OABP2 was also characterized by photoaffinity labeling experiments followed by LC−MS measurements. As a result, the probe **7** cross-linked with a D^{139}PNLK peptide (Figure S7 of the Supporting Information). This peptide sequence exists in only OABP2.1 and OABP2.2, but not in OABP2.3. This finding may account for the two experimental results. First, the photoaffinity labeling experiments (Figure 2A) showed that the upper band corresponding to a mixture of OABP2.1 and OABP2.2 was detected more intensely than the lower band for OABP2.3. Second, ESI-MS results indicated that OABP2.1 and OABP2.2 form a complex with OA (Figure S4 of the Supporting Information). In spite of the fact that it is not clear whether OA is endogenously bound to OABP2 or OA becomes accessible to OABP2 during the course of purification, we speculated that the pentapeptide region is important for formation of a complex with OA.

When a defensive mechanism in *H. okadai* to prevent self-intoxication by OA is discussed, the stoichiometry between OA and OABP2 in the sponge has to be taken into consideration. The amount of OA in *H. okadai* was quantified to be 0.96 ± 0.04 μg/g of sponge tissue (see Figure S6 of the Supporting Information) (*28*). The molar ratio of OA to OABP2 (13.2 μg/g of sponge tissue) isolated from the sponge did not appear stoichiometric. Thus, the presence of OABP2 may not be the only mechanism against the endogenous OA. The first hypothesis to compensate for the apparent difference in quantity between OA and OABP2 is that OA may be present as a nontoxic form in the sponge. In the biosynthesis of monensin (*29*), a polyketide metabolite like OA, the carboxyl group is thought to hang onto the polyketide synthase module (*29*). Before the final step of hydrolysis proceeds, OA would remain esterified on the module, being inactive to PP2A. Alternatively, OA would be simply esterified, which abolishes the toxicity to the sponge (*30*, *31*). The second hypothesis explaining the apparent difference in quantity between OA and OABP2 is that OA molecules would be compartmentalized in the sponge (*24*). To examine compartmentalization of OA, we tried to separate the symbiotic bacteria from the sponge (*32*). Due to species-specific reasons observed in only *H. okadai*, we failed to do so. The physiological significance of OABP2 to *H. okadai* would be addressed via investigation of other species whether they possess OABP2. Quantification of OA by the fluorometric method (*28*) and detection of OABP2 by photoaffinity labeling using the probe **7** were carried out. We confirmed that the sponge *H. japonica*, found in the same area where *H. okadai* was collected, accumulated neither OA (Figure S6) nor OABP2 (Figure 6) and that the dinoflagellate *P. lima*, one of the primitive producers of OA, did not possess OABP2 but is known to express OA-insensitive protein phosphatases (*33*). Thus, all of these results suggested that OABP2 is uniquely present in the particular sponge *H. okadai* and that it may play a certain role in avoiding self-intoxication by OA.

Recent studies may provide a clue for ecological roles of OA in the sponge *H. okadai*. The sponge *L. baicalensis* harbors the OA-producing dinoflagellate *Gymnodinium san-*

guineum in Lake Baikal (*24*). OA appears to facilitate expression of heat shock protein *hsp70* in winter, which is necessary for the sponge to tolerate the cold temperature under the ice. According to the Japan Meteorological Agency, however, the surface temperature of seawater at a latitude of approximately 35° north, where Aburatsubo Bay is located, ranges between 10 and 25 °C during a year, which does not seem fatal to the sponge as observed between 0 and 17 °C in Lake Baikal. We thus ruled out the possibility that OA in *H. okadai* functions as in the case of *L. baicalensis*. In contrast, Schröder et al. (*5*) demonstrated that OA is localized either in symbiotic bacteria dwelling in the mesophyl of the marine demosponge *S. domuncula* or in vacuoles of the sponge cells. OA appears to play a role in triggering apoptosis against bacterial cells in the sponge *S. domuncula*, while OA does not attack the sponge cells due to the presence of prosurvival protein BAG-1. Besides OA, *H. okadai* has been known to accumulate other neurotoxins such as halichlorines (*34*) and alteramide A (*35*), and symbiotic species would be considered the source of these compounds. To maintain symbiosis between the sponge and symbiotic microbes or between the symbiotic species, it is thought that OA permits the producer to be predominant over other symbiotic species by exhibiting a similar function as in the case of *S. domuncula*.

OA is one of the most toxic secondary metabolites that has been widely used in biochemical and pharmacological research studies, since it specifically inhibits PP1 and PP2A by exhibiting high-affinity binding. Besides the dinoflagellate *P. lima*, OA has been thought to be produced by symbiotic species living in sponges. Considering the toxicity and concentration of okadaic acid in the sponge, we hypothesized the presence of a self-defense mechanism. This study unambiguously discloses a new finding; the sponge *H. okadai* contains OA binding proteins OABP2, a mixture of OABP2.1, OABP2.2, and OABP2.3, which turned out to be unknown proteins with no similarity to protein phosphatases. OA binds with high affinity to OABP2 ($K_d = 0.96$ nM), recognizing a specific region found in OABP2.1 and OABP2.2. Furthermore, this protein complex is detected only in the sponge *H. okadai*. All these results suggest that OABP2 may play a certain role in preventing self-intoxication in the sponge.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

HPLC chromatograms for Edman degradation, agarose gel electrophoresis for PCR, amino acid sequence of OABP1, ESI-MS spectra of OABP2, complete cDNA sequence of OABP2.1, chromatograms for quantification of OA in the sponges, and ESI-MS spectra from the photoaffinity labeling experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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