

## Okadaic Acid, an Apoptogenic Toxin for Symbiotic/Parasitic Annelids in the Demosponge *Suberites domuncula*†

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**The role of okadaic acid (OA) in the defense system of the marine demosponge *Suberites domuncula* against symbiotic/parasitic annelids was examined. Bacteria within the mesohyl produced okadaic acid at concentrations between 32 ng/g and 58 ng/g of tissue (wet weight). By immunocytochemical methods and by use of antibodies against OA, we showed that the toxin was intracellularly stored in vesicles. Western blotting experiments demonstrated that OA also existed bound to a protein with a molecular weight of 35,000 which was tentatively identified as a galectin (by application of anti-galectin antibodies). Annelids that are found in *S. domuncula* undergo apoptotic cell death. OA is one candidate inducer molecule of this process, since this toxin accumulated in these symbionts/parasites. Furthermore, we identified the cDNA encoding the multifunctional prosurvival molecule BAG-1 in *S. domuncula*; it undergoes strong expression in the presence of the annelid. Our data suggest that sponges use toxins (here, OA) produced from bacteria to eliminate metazoan symbionts/parasites by apoptosis.**

Sponges, the evolutionarily oldest metazoan phylum (*Porifera*), share one common ancestor with the other metazoan phylum, the *Urmetazoa* (31, 35). Most sponges are marine sessile filter feeders; some species daily filter tons of water containing millions of bacteria or viruses through their bodies (48; reviewed in references 18 and 26). Due to their efficient chemical and biological defense systems, sponges have survived not only adverse climatic periods (24) but also biotic and anthropogenic threats (34).

Two strategies are used by sponges to eliminate attacking (infectious) bacteria: first, chemical strategies via the production of secondary metabolites (see references 45 and 51) and, second, humoral and cellular defense mechanisms (33). The hitherto studied molecular biological or cell biological defense systems used by sponges against microorganisms have been elucidated primarily in the demosponge *Suberites domuncula*. This species is able to recognize and defeat gram-positive as well as gram-negative bacteria by the antimicrobial lectin tachylectin (52). In addition, *S. domuncula* has developed a specific system to recognize gram-positive bacteria via the peptidoglycan coat; subsequently bacteria are endocytosed and/or extracellularly digested by lysozyme (59). The lipopolysaccharide (LPS) outer membrane of gram-negative bacteria induces in *S. domuncula* an adaptive antibacterial response by increas-

ing the release of bioactive and antimicrobial lyso-platelet-activating factors (36). We have shown that in the aqueous environment also, fungi are recognized by sponges via a receptor, the (1→3)- $\beta$ -D-glucan binding protein; after interaction of fungal (1→3)- $\beta$ -D-glucans with the corresponding receptor, *S. domuncula* causes the expression of a series of defense molecules, e.g., a fibrinogen-like protein and an epidermal growth factor precursor (43).

Finally, the more general defense system against microorganisms should be highlighted; LPS activates the mitogen-activated protein kinase pathway which modulates the sponge immune system (8, 66). In conclusion, sponges are provided with a very efficient immune system (32) that involves an array of proteinaceous as well as nonproteinaceous defense molecules. In addition, sponges not only contain symbiotic/parasitic bacteria but also live in a symbiotic (mutualistic) relationship with prokaryotic microorganisms (reviewed in references 1, 8, 17, and 45).

Earlier it was shown that *S. domuncula* harbors bacteria that produce the secondary metabolite okadaic acid (OA) (66). OA was first isolated from the Caribbean marine sponge *Halichondria melanodocia* by Tachibana et al. (58); later, related OA polyethers were also isolated from other sponges (67). The polyether fatty acid derivative OA is rather toxic to mammals (55). The free-living microalgae *Prorocentrum lima* (39) is the prime producer of OA.

The biological activity of OA is linked with an inhibition of protein phosphatases (5), resulting in an induction of apoptosis (8, 41, 49). In *S. domuncula* OA was shown to augment the immune response against LPS and bacteria via increased phosphorylation of p38, a central kinase of the mitogen-activated protein kinase pathway, resulting in a dif-

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† This article is dedicated to R. A. Lewin (Scripps Institution of Oceanography, La Jolla, CA), who 30 years ago discovered *Prochloron*, an algal species that stimulated our present-day view on endosymbiosis between blue-green algae and tunicates/sponges.

ferential phosphorylation of proapoptotic and antiapoptotic molecules (66).

*S. domuncula* lives together with the hermit crab *Pagurites oculatus* (Decapoda: Paguridea), which resides predominantly in shells of the mollusk *Trunculariopsis trunculus* (Gastropoda: Muricidae) (4, 23) on which the sponge grows. In the natural environment the sponge body harbors surprisingly few symbiotic/parasitic metazoans, which might indicate that effective defense systems eliminate such organisms. Only some annelids, e.g., *Syllis* sp., *Haplosyllis* sp., or *Nereis* sp., have been identified (3). The elucidation of the molecular and cellular mechanism(s) by which sponges protect themselves against parasitic metazoans has begun only recently. It could be demonstrated that sponge-associated bacteria produce 2-methylthio-1,4-naphthoquinone, a compound that suppresses the CD36/LIMPII signal transduction pathway that modulates angiogenesis (in vertebrates) and canal formation (in *S. domuncula*) (37).

In the present study we show that in *S. domuncula* OA is accumulated in tissue, especially in those areas where annelids live in the sponge. The experiments were performed with antibody probes that were raised against the toxin and that had been successfully applied previously for an enzyme-linked immunosorbent assay (66). Our results indicate that the annelids die of apoptosis while—in parallel—the cells of the surrounding sponge tissue strongly express the multifunctional pro-survival molecule BAG-1 (see reference 63). We selected this gene for the studies because it exists only in metazoa (62). BAG-1 interacts with a series of cellular targets, e.g., the Bcl-2 apoptosis regulator, heat shock proteins, or components of the proteasome machinery (63). We assume that in *S. domuncula* OA causes an apoptogenic effect in response to invading symbionts/parasites.

#### MATERIALS AND METHODS

**Chemicals, materials, and enzymes.** Restriction enzymes, a Total RNA Isolation kit, and reagents for the rapid amplification of cDNA ends (RACE) procedure were purchased from Invitrogen (Carlsbad, CA); *TripEx2* vector was from BD (Palo Alto, CA); TRIzol reagent was from GibcoBRL (Grand Island, N.Y.); Hybond-N<sup>+</sup> nylon membrane was from Amersham (Little Chalfont, Buckinghamshire, United Kingdom); a PCR-DIG-Probe-Synthesis kit, cell death detection kit (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL] reaction; fluorescein isothiocyanate [FITC] based), and a BM Chemoluminescence Blotting Substrate kit were from Roche (Mannheim, Germany); aprotinin, sodium orthovanadate, Nonidet-P40, and 4',6-diamidino-2-phenylindole-dilactate (DAPI) were from Sigma (St. Louis, MO).

**Sponges and extracts.** Specimens of the marine sponge *S. domuncula* (phylum Porifera, class Demospongiae, order Hadromerida) were collected in the northern Adriatic Sea near Rovinj (Croatia) and then kept in aquaria in Mainz (Germany) at a temperature of 17°C. Extracts were prepared from tissue samples; they were homogenized in lysis buffer (Tris-buffered saline, pH 7.5, 1 mM EDTA, 1% Nonidet-P40, 10 mM NaF, 0.1 μM aprotinin, 1 mM sodium orthovanadate).

**Preparation of antibodies against OA.** Polyclonal antibodies against OA (PcAb-OA) were raised in female rabbits (White New Zealand) as previously described (66). OA conjugate was injected at 4-week intervals into the animals; after three boosts serum was collected, and the antibodies were prepared (21). The titer of the antibodies was determined to be 1:2,000.

**Histological analysis.** Tissue was fixed in paraformaldehyde, embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany), and sectioned, essentially as previously described (7, 65). To remove the siliceous spicules, the tissue samples were incubated with HF/NH<sub>4</sub>F. The 4-μm-thick slices were incubated with PcAb-OA (1:500 dilution) overnight. Then the slides were treated with Cy3-conjugated goat anti-rabbit immunoglobulin G (IgG) for 2 h. The sections were inspected by immunofluorescence with an Olympus AHB3 microscope. In parallel, slices were stained with 5 μg/ml of DAPI for 30 min to identify DNA, especially in bacteria. In the tissue of the sponge *S. domuncula*, the bacteria are

almost exclusively found in clusters, which can easily be identified as such (8). Where indicated, the slices were stained with the trichrome stain ASTRIN (28) and inspected with an Olympus AHB3 microscope. In addition, the specimens were inspected directly using Nomarsky interference contrast optics. For in situ detection of apoptosis, tissue samples were permeabilized and subjected to a TUNEL reaction (in situ cell death detection kit) according to the instructions of the manufacturer. The DNA was end labeled with FITC-dUTP, which was followed by direct analysis of fluorescent cells.

As outlined previously (66) tissue sections were reacted with PcAb-OA (1:300 dilution) overnight to also identify cellular structures that form immunocomplexes with the antibodies. Then the slides were incubated with FITC-conjugated goat anti-rabbit IgG for 2 h, and subsequently the sections were inspected for immunofluorescence (Olympus AHB3 microscope).

**Electron microscopy.** Transmission electron microscopy was performed as previously described (38). Briefly, sponge samples were cut into pieces, incubated in phosphate buffer, treated with OsO<sub>4</sub>, and finally dehydrated with ethanol. The dried samples were incubated with propylene oxide, fixed in propylene oxide-araldite, and covered with araldite before being cut into 60-nm ultrathin slices (Ultracut S; Leica, Wetzlar, Germany). The samples were transferred onto coated copper grids and analyzed with a Tecnai 12 microscope (FEI Electron Optics, Eindhoven, The Netherlands).

Immunogold labeling with detection by electron microscopy was performed with tissue samples treated in glutaraldehyde or paraformaldehyde (38). After 2 h the material was dehydrated in ethanol and embedded in LR White resin (London Rein Company, Berkshire, England); the samples were blocked with 5% bovine serum albumin in phosphate-buffered saline (PBS) and then incubated with the primary antibody PcAb-OA (1:500) for 12 h at 4°C. Preimmune serum was used as a control. After three washes with PBS-1% bovine serum albumin, sections were incubated with a 1:100 dilution of the secondary antibody (1.4-nm nanogold anti-rabbit IgG, diluted 1:200; Nanoprobes, Yaphank, NY) for 2 h. Sections were rinsed in PBS, treated with 1% glutaraldehyde-PBS for 5 min, washed, and dried. Subsequently, enhancing of the immunocomplexes was performed with silver, as previously described (12). The samples were examined with a Tecnai 12 microscope.

**Western blotting.** Total cell extracts (10 μg/lane) were subjected to 0.1% sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (7). Western blotting experiments were performed using PcAb-OA (1:300 dilution). After incubation for 3 h, the blots were incubated with goat anti-rabbit IgG, peroxidase coupled (1:5,000 dilution; New England Biolabs). Detection of the immunocomplex was carried out using BM chemoluminescence blotting substrate. For one series of experiments a polyclonal antibody against the recombinant *S. domuncula* galectin (PcAb-rGAL) was used (H. C. Schröder et al., submitted for publication); the titer of this antibody preparation was 1:3,000.

**Isolation of a cDNA for BAG-1 from *S. domuncula*.** The complete cDNA of the sponge BCL2-associated athanogene (BAG1\_SUBDO) was isolated from the cDNA library (29) by PCR. The degenerate primers were designed against the conserved ubiquitin domain signature, which in the human BAG-1 sequence (CAH72742.1) spans the segment from amino acid 50 to 57. The forward primer 5'-CARAARCTIATHTTYAARGGIAAR-3' (I, inosine) was successful together with the vector primer [5'-TAATACGACTCACTATAGGG-3']. PCR was carried out at an initial denaturation at 95°C for 5 min, followed by 35 amplification cycles of 95°C for 30 s, 58°C for 45 s, and 74°C for 1.5 min, with a final extension step at 74°C for 10 min. Fragments of the expected sizes were obtained and cloned into the TOPO TA (pCRII) vector in *Escherichia coli* TOP10 cells. Sequencing was performed with primers directed to the SP6 promoter and the T7 promoter. The sequence was completed with insert-specific primers in combination with 5' RACE primer or with 3' RACE primer using the CapFishing full-length cDNA premix kit. The final sequence (SDBAG1) was confirmed by an additional PCR using primers directed against the nontranslated region of the cDNA, followed by sequencing.

**Sequence comparison.** The BAG-1 deduced protein was analyzed using the computer programs BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi>) and FASTA (<http://www.ebi.ac.uk/fasta33/>). Multiple alignments were performed with CLUSTAL W, version 1.6 (61). Phylogenetic trees were constructed on the basis of amino acid sequence alignments by the neighbor-joining method, as implemented in the Neighbor program from the PHYLIP package (16). The distance matrices were calculated using the Dayhoff PAM matrix model as previously described (13). The degree of support for internal branches was further assessed by bootstrapping (16). The graphic presentations were prepared with GeneDoc (40).

**RNA preparation and Northern blot analysis.** Tissue samples (1 g each) from the mesohyl were taken from regions of the sponge which contained no annelids

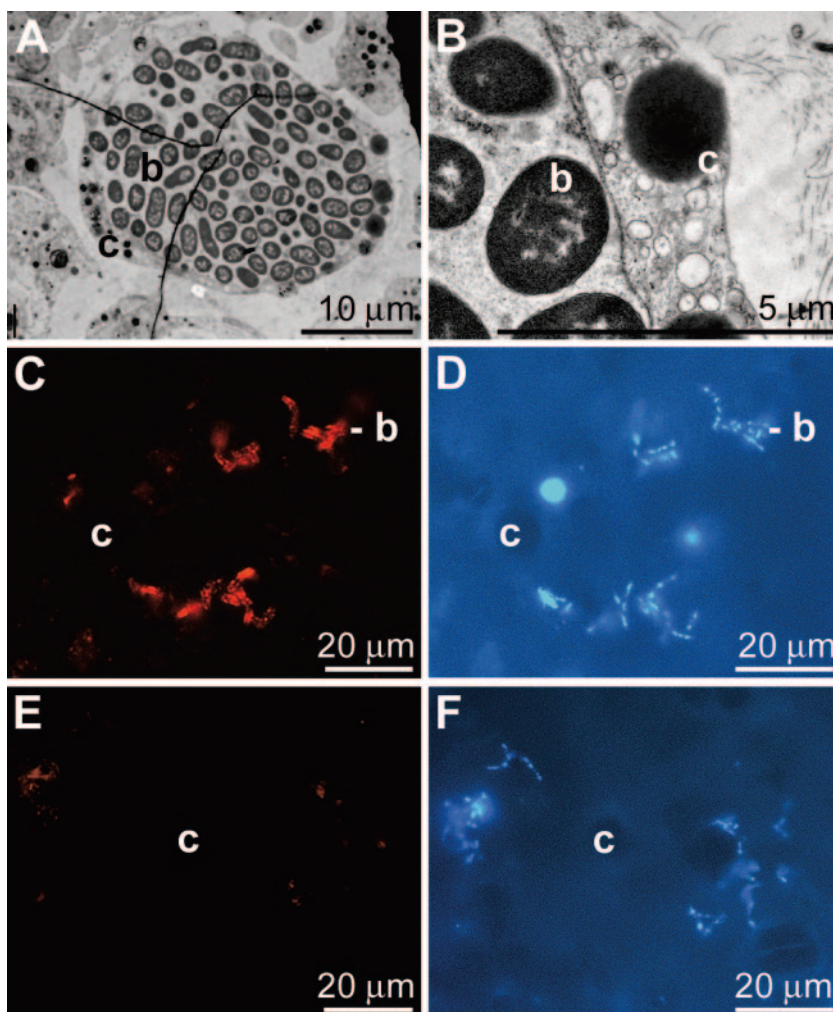


FIG. 1. Localization of bacteria in *S. domuncula*. (A) Transmission electron microscopy through *S. domuncula* tissue showing one bacteriocyte (c) which is filled with bacteria (b). (B) A higher magnification shows that the cell body (c) surrounds the bacteria clusters (b). Besides these compartmented bacteria, microorganisms are also scattered throughout the mesohyl of the tissue. In the sections they can be seen by light microscopy after staining with DAPI (D and F). By using PcAb-OA and then a Cy3-conjugated anti-rabbit secondary antibody the bacteria become brightly stained (C). As a control, preimmune serum was used, which gave no signal (E).

and from tissue around parts in which these organisms had been identified. RNA was extracted from liquid-nitrogen-pulverized tissue with TRIzol reagent as previously described (19). Then 5  $\mu\text{g}$  of total RNA was electrophoresed and blotted onto Hybond-N<sup>+</sup> nylon membrane. Hybridization was performed with a 420-nucleotide (nt) fragment of the SDBAG1 cDNA; this region was within the open reading frame. The housekeeping gene  $\beta$ -tubulin, *SDTUB* (accession number AJ550806), of *S. domuncula* was used as an internal standard. The probes were labeled with a PCR-DIG Probe Synthesis Kit (Roche, Mannheim; Germany). After the slices were washed, digoxigenin (DIG)-labeled nucleic acid was detected with anti-DIG Fab fragments and visualized by chemiluminescence using CDP (Roche).

**In situ localization studies.** The method applied is based on a previously described procedure (42, 44). Frozen cross-sections (8  $\mu\text{m}$  thick) of tissue were prepared, fixed, treated with proteinase K, and subsequently fixed again with paraformaldehyde. After rehydration the sections were hybridized with the labeled probe, a 420-nt long part of the SDBAG1 cDNA portion. After blocking, the sections were incubated with an anti-DIG antibody conjugated with alkaline phosphatase. The dye reagent NBT/X-phosphate was used for visualization of the signals. Antisense and sense single-stranded DNA DIG-labeled probes were synthesized by PCR using a PCR DIG probe synthesis kit. Sense probes were used in parallel as negative controls in the experiments.

**Analytical procedures.** The OA concentration present in sponge tissue was assessed by coupled high-performance liquid chromatography-mass spectrometry

as described before (25, 66). The amount of OA detected was correlated with the weight of fresh tissue used for the analysis ( $n = 5$ ; the mean values  $\pm$  standard deviations [SD] are given). Protein concentrations were determined as previously described (30) using bovine serum albumin as a standard.

**Nucleotide accession number.** The sequence reported here was deposited in the EMBL/GenBank database under accession number AM183251 for the *S. domuncula* BCL2-associated athanogene (SDBAG1).

## RESULTS

**Production of OA in bacteria from *S. domuncula*.** The OA concentration was determined in tissue from *S. domuncula* either immediately after collection from the sea or after maintenance in the aquarium for 12 months. Tissue from freshly collected sponges had an OA content of  $58 \pm 7$  ng/g of wet weight (70 nM), while tissue from specimens which grew in the aquarium had a lower content of  $32 \pm 9$  ng/g (39 nM).

Tissue from *S. domuncula* contained bacteria either compartmented in cells, in bacteriocytes (Fig. 1A and B), or scattered within the mesohyl (Fig. 1C to F). The bacterio-

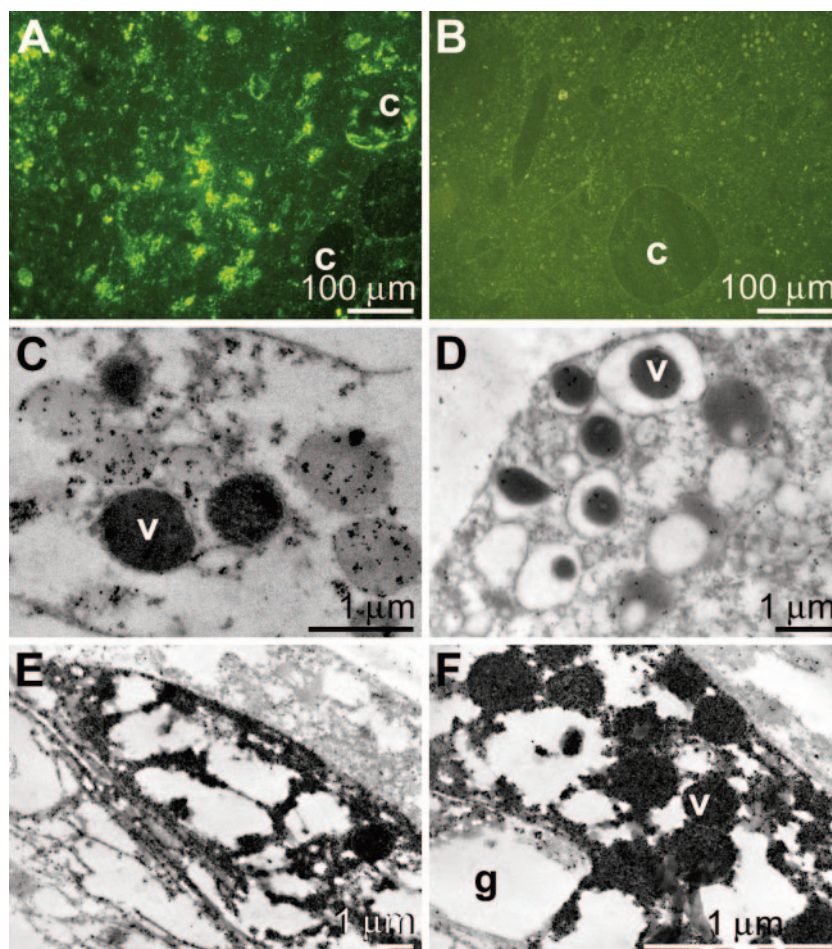


FIG. 2. Cellular structures reacting with PcAb-OA. (A) Tissue was sliced and reacted first with PcAb-OA and then with FITC-anti-rabbit IgG. The cells that surround the aquiferous canals (c) are especially well stained (light microscopy inspection). (B) Reaction of the tissue with preimmune serum. (C, E, and F) Application of the electron immunogold labeling technique (with detection by electron microscopy), using PcAb-OA, to identify OA in cells. The immunogold particles are accumulated in vesicles (v), which can be seen at a higher magnification (C and F). At lower magnification it becomes evident that the vesicles are arranged within cells around larger granules (g) containing no electron-dense material (E). (D) In a control the sections were treated with a preimmune serum.

cytes completely surrounded the bulky clusters of bacteria (Fig. 1A and B). The bacteria within the mesohyl could be visualized by DAPI (Fig. 1D and F) and were detected immunohistochemically by applying PcAb-OA. With this tool the bacteria became brightly stained (Fig. 1C). Preimmune serum was used as a control and produced no significant fluorescence (Fig. 1E).

**Storage of OA in tissue from *S. domuncula*.** In an earlier study we demonstrated that PcAb-OA reacted in *S. domuncula* with bacteria as well as with tissue structures (66). These antibodies were also applied in this study; the experiments revealed that especially the cells that surround aquiferous canals were brightly stained (Fig. 2A). Preimmune serum did not show clear reactions (Fig. 2B). Electron immunogold labeling was applied to identify the structures that were brightly stained by the antibodies (Fig. 2C to F). The results showed that PcAb-OA strongly accumulated as electron-dense grains in vacuoles which were localized within cells (Fig. 2C). In contrast, if preimmune serum was used, no significant reactions could be visualized (Fig. 2D). Lower magnification showed

that these granules were stored in distinct cells, which were heavily filled with 200- to 300-nm vacuoles, positively reacting with PcAb-OA (Fig. 2E). It was striking that these cells were composed of large granules containing no electron-dense material, suggesting that these cells were archaeocytes.

The PcAb-OA showed a positive reaction in a protein extract from *S. domuncula* tissue. Total tissue extracts were prepared and subjected to SDS-PAGE (Fig. 3), as described in Materials and Methods. Western blotting experiments were performed using PcAb-OA. After incubation, the immunocomplexes on the blots were visualized by labeled secondary antibodies (Fig. 3, WB). One band with a molecular weight of 35,000 was prominently stained (lane a), strongly suggesting that in the sponge OA was (perhaps covalently) bound to a protein. In order to assess the nature of the postulated protein, a panel of antibodies that had been raised in our laboratory was screened. PcAb-rGAL raised earlier against *S. domuncula* galectin (53) was able to detect this band; it cross-reacted with this protein (molecular weight of 35,000), suggesting that OA was linked to galectin (lane b). Preimmune sera were used as

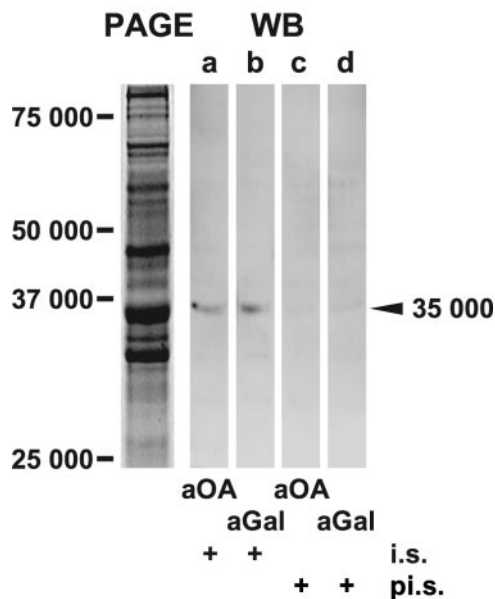


FIG. 3. Identification of a macromolecule, reacting with antibodies against OA. Extracts were prepared from tissue of *S. domuncula* and separated by 0.1% SDS-10% PAGE (PAGE). The crude extract was size separated and stained with Coomassie brilliant blue. In a parallel series of experiments, the proteins were blot transferred, and the Western blots (WB) were reacted with PcAb-OA (aOA; lane a) or PcAb-aGAL (aGal; lane b). In both blots a protein with a molecular weight of 35,000 was recognized. In addition to immune sera (i.s.), preimmune sera (pi.s.; lanes c and d) were also applied. Size markers are given.

controls after the blotting; they did not stain the protein species on the blot (lanes c and d).

**Annelid in *S. domuncula* tissue.** Cross-sections of the tissue occasionally disclosed annelids in the sponge (Fig. 4). The annelid, which could not at this point be identified or determined, was intensively stained with the trichrome stain ASTRIN (Fig. 4A and B). This staining also showed that the annelid was encapsulated by a coat that contained cells (Fig. 4B). In sections, the symbiont/parasite could also be identified by Nomarsky interference contrast optics (Fig. 4C). The annelid was characterized by a higher cell density than the sponge host, as was seen after DAPI staining (Fig. 4D). The size of the annelids in *S. domuncula* varied between 2 and 4 mm in length with diameters between 0.3 and 0.8 mm.

**Enrichment of OA in the annelid.** The high cell density of the annelid was used as a marker to identify this symbiont/parasite within sponge tissue (Fig. 5B and D). If slices were reacted with PcAb-OA, the cells of the annelid were also prominently and brightly stained (Fig. 5A). No immunocomplexes were seen if the slices were stained with preimmune serum (Fig. 5C).

**Induction apoptosis in the annelid.** A cell death detection kit was used to determine if the sponge (host) eliminated the symbiont/parasite by inducing it to programmed cell death (apoptosis). During the TUNEL assay procedure both single- and double-stranded DNA breaks were labeled with fluorescent nucleotides. Inspection by fluorescence microscopy showed that the annelids were stained green (Fig. 4E). In a parallel series

the sections were not treated with the labeled nucleotides, and these specimens did not show any significant staining (Fig. 4F).

**The *S. domuncula* BAG-1 molecule.** The above results indicate that OA is accumulated in the symbiont/parasite (Fig. 5), resulting in induction of apoptosis in the symbiotic/parasitic animal (Fig. 4E). To clarify whether the host (*S. domuncula*) responds to the annelid with a prosurvival reaction, the expression of the prosurvival molecule BAG-1 was studied.

The complete cDNA (SDBAG1) of the sponge BCL2-associated athanogene (BAG1\_SUBDO) was isolated. The 791-nt sequence has an open reading frame from nt 6 to 8 and nt 780 to 782 (stop). The deduced 258-amino-acid protein (BAG1\_SUBDO) (Fig. 6A) has a predicted molecular weight of 29,139. It displays (i) the ubiquitin domain signature ranging from amino acid 29 to amino acid 54 and (ii) the BAG domain from amino acid 101 to amino acid 181 (Motif Scan [http://myhits.isb-sib.ch/cgi-bin/motif\_scan]).

In the database, the highest sequence similarity of BAG1\_SUBDO was found to the human BAG-1 protein (accession number CAH72742.1) with a probability score  $E$  (11) of  $1e^{-23}$ . Only low similarity exists to predicted proteins from *Drosophila melanogaster* ( $1e^{-3}$ ), the Bag-1 (human) homolog protein *Caenorhabditis elegans* ( $1e^{-9}$ ), the predicted protein from *Saccharomyces cerevisiae* ( $2e^{-4}$ ), and the putative protein from *Arabidopsis thaliana* ( $1e^{-6}$ ). The phylogenetic tree, which was rooted with the plant sequence, reflects these relationships. One branch comprises the sponge protein and the human BAG-1 and shares a common ancestor together with the *C. elegans* Bag-1-like molecule (Fig. 6B).

**Defense system of *S. domuncula*: expression of BAG-1.** Northern blotting experiments were performed to determine if the expression level of SDBAG1 changes in response to the symbiosis/parasitism of the annelid with the sponge. In the regions that contained annelids, a high expression level was seen, while in tissue that did not contain annelids, no expression could be detected by Northern blotting (Fig. 7). Four animals were analyzed in parallel. The housekeeping gene  $\beta$ -tubulin cDNA (*SDTUB*) was used as a control; its expression level did not vary among the four animals (Fig. 7).

**Expression and localization of BAG-1.** To support the assumption that BAG-1 is involved in the defense of *S. domuncula* against the metazoan symbiont/parasite, in situ hybridization was performed with the SDBAG1 cDNA probe. In tissue that did not harbor an annelid, no cells were positively stained with the antisense probe (Fig. 8A). However, if sections were reacted with the SDBAG1 probe, a layer of cells around the symbiont/parasite was brightly stained (Fig. 8B). No reaction could be observed if the sense SDBAG1 probe was used (not shown).

## DISCUSSION

The first illustrated description of a symbiotic relationship between metazoans and a demosponge (*Halichondria panicea*) was given by Ellis (15), who reported on the frequent occurrence of an annelid on the surface of this sponge. Sponges have developed several strategies to resist or eliminate such symbionts/parasites; some species secrete mucins on their surfaces as a protective shield (54). Another efficient defense against sponge-attacking metazoans was thoroughly studied by Cimino

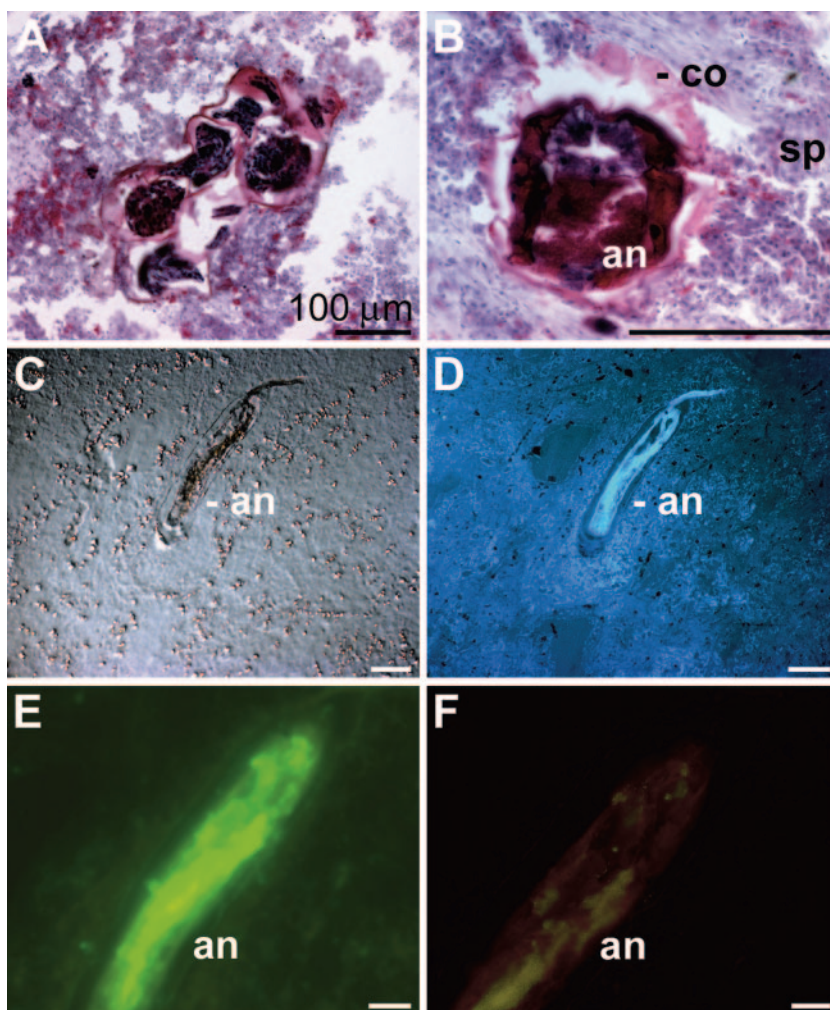


FIG. 4. Annelid in *S. domuncula*. (A and B) Cross-sections of *S. domuncula* were stained with the trichrome stain ASTRIN to distinguish between the sponge tissue (sp), which appears bluish, and the more reddish annelid (an). Around the annelid an encapsulating coat (co) can be identified. (C and D) Longitudinal sections through the symbiont/parasite by Nomarsky interference contrast optics (C) or by fluorescence after DAPI staining (D). (E) Apoptotic cell death of the annelid. The section was subjected to a TUNEL enzymatic labeling assay and subsequently analyzed by fluorescent light microscopy. (F) No fluorescence was seen when the specimens were subjected to the assay lacking the FITC-labeled nucleotides. Scale bars, 100  $\mu\text{m}$  (A, B, E, and F) and 25  $\mu\text{m}$  (C and D).

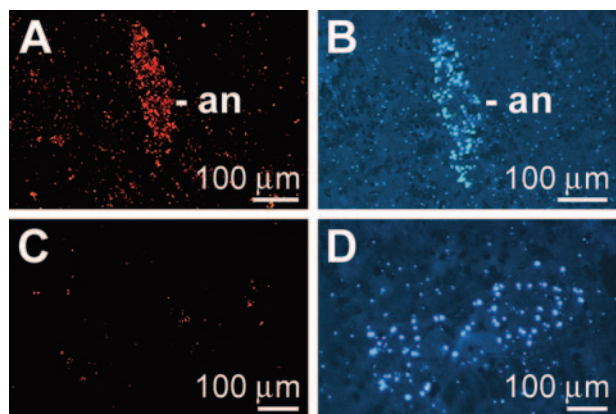


FIG. 5. Accumulation of OA in the annelid (an) living in *S. domuncula*. (A) The sections were reacted with PcAb-OA to show the highly fluorescent signals coming from the cells of the symbiont/parasite. (B) In parallel, the sections were stained with DAPI. (C) When the sections were treated with preimmune serum, no signals were seen. (D) Parallel image after DAPI staining. Scale bars, 100  $\mu\text{m}$ .

et al. and Proksch (9, 10, 45). They demonstrated that secondary metabolites, produced by the sponges or their associated microorganisms, are transferred from them to the predator(s). When taken up by the predators, the original secondary metabolites from sponges can undergo biotransformation (10). In particular, the sponge *Aplysina aerophoba*, which is rich in brominated tyrosine derivatives, was found to display an efficient protection system against metazoan predators (14, 46). These secondary metabolites are taken up by symbiotic/parasitic metazoans and then may also act as defense molecules (60).

The polyether OA studied here is toxic for vertebrates (20) due to its inhibitory effect on protein phosphatases 1 and 2A (22). This algal toxin is an inducer of apoptotic cell death in vertebrates (6) and also in sponges (66). It was first found in sponges of the genus *Halichondria* and later in dinoflagellates, especially in benthic species of the taxon *Prorocentrum*. Furthermore it was reported that OA is accumulated in mussels,

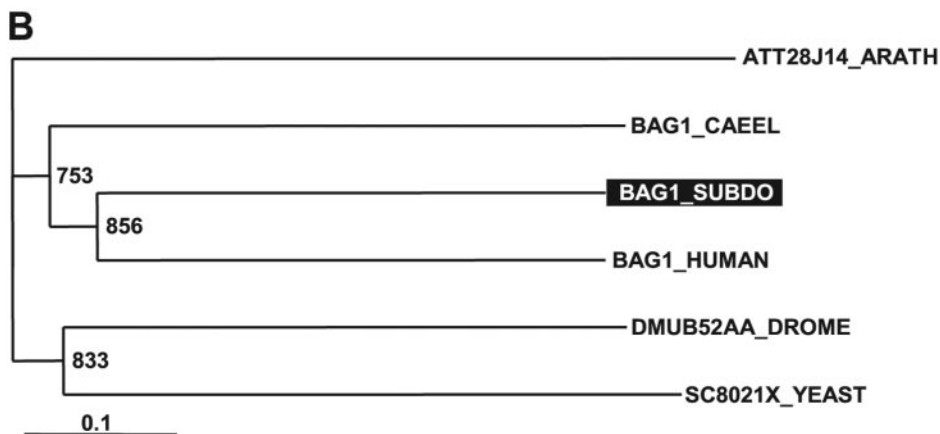
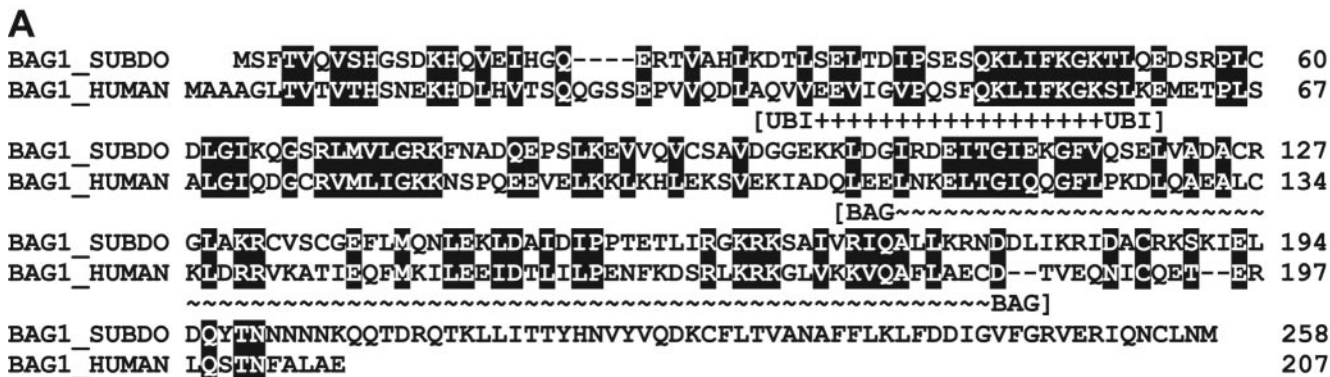


FIG. 6. (A) BAG-1 from *S. domuncula* (BAG1\_SUBDO) was aligned with the human BAG-1 (BAG1\_HUMAN; accession no. CAH72742.1). The two characteristic domains are marked: the ubiquitin domain (UBI) signature and the BAG domain. Amino acids that are similar in both sequences are shown boxed and in white type. (B) A rooted tree was constructed after alignment of these two sequences with the predicted protein from *D. melanogaster* (DMUB52AA\_DROME; CAA37227.1), the Bag-1 (human) homolog protein 1 from *C. elegans* (BAG1\_CAEL; AAB96728.1), the predicted protein from *S. cerevisiae* (SC8021X\_YEAST; CAA89774.1), and the putative protein from *A. thaliana* (ATT28J14\_ARATH; CAB87278.1). After alignment the tree was built (1,000 replicates). The plant sequence was used as an outgroup. The scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position in the sequence.

e.g., *Mytilus edulis*, to high concentrations (2 to 2,000 ng/g) (64). Hence, OA displays a potential threat to human health not only because it causes acute toxicity but also because it acts as a tumor promoter (56).

The occurrence of OA is not restricted to *Halichondria*; in addition to the evidence of the present study, it has previously

been described in a second sponge, *S. domuncula*, at a level of 50 ng/g (66) as well as in *Geodia cydonium* (unpublished data). *S. domuncula* uses OA as a metabolite to modulate or augment its immune status against LPS-mediated bacterial effects (66). The data shown here demonstrate that *S. domuncula* harbors bacteria in special cells, bacteriocytes, and in cells scattered throughout the mesohyl. The bacteria are the producers of the toxin as demonstrated by immunohistological techniques. Now we show that in *S. domuncula* the protein which is presumptively galectin ( $M_r$  of 35,000) is linked to OA. It appears to be most likely that OA is transferred to the protein via an acetyl-transferase, with ATP and acetyl-coenzyme A as the coenzymes (2). Future studies must also show if OA is deposited in the protein-bound form and released, if required, by deacetylase (27). Several cDNAs of such enzymes exist in the *S. domuncula* expressed sequence tag database at a higher copy number (<http://spongebase.genoserv.de/>).

As shown by the studies presented here, OA exists in sponges (covalently) bound to protein. Therefore, it may be postulated that the toxin could also act as an antigen; this assumption is supported by our results showing that OA also exists in mussels in a protein-bound form (unpublished data).

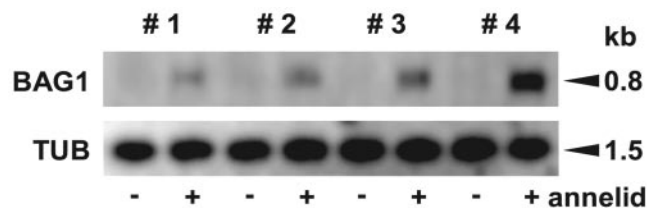


FIG. 7. Expression of BAG-1 in four sponge specimens (1 to 4); tissue samples from areas that did not contain symbionts/parasites (-) and from regions where the annelids were present (+) were analyzed. Following RNA isolation, the same amounts were loaded onto gels and subsequently size separated. After blot transfer the membranes were hybridized with the SDBAG1 probe (BAG1). The housekeeping gene  $\alpha$ -tubulin (TUB) was used as a control to show that the same amounts of RNA were loaded onto the gels.

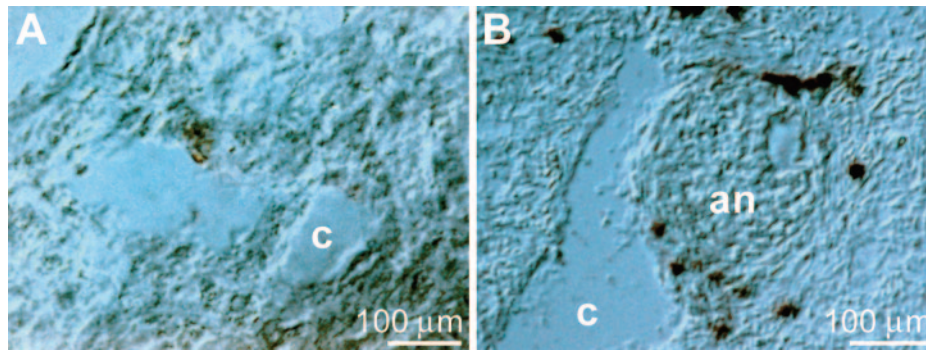


FIG. 8. In situ hybridization analysis applying the antisense probe for BAG-1 (SDBAG1). With this probe no signals are detected in regions where no annelid is seen (A), while in regions that contain the annelid several cells frame the symbiont/parasite (an) (B). Two aquiferous canals are marked (c).

The size of the free toxin ( $M_r$  of 804) is only large enough to act as a hapten; however, together with a macromolecule (here the protein with a molecular weight of 35,000), it has the potential to act as an immunogen. This finding might also have implications for human health since until now OA had not been detected bound to proteins; previously only extracts were prepared from potential toxic food by methanolic extraction, which allowed the isolation of only unbound OA (47). Future studies must show if humans produce, e.g., after eating toxin-containing mussels, antibodies against this toxin resulting in subchronic symptoms. It should be stressed here that OA occurs in the producer and/or the host in an ester form also, covalently linked to low-molecular-weight, nonproteinaceous organic moieties (57).

It had been demonstrated that OA may initiate a signal transduction pathway which differentially influences apoptotic cell death via a protein phosphatase (50). Based on this finding we investigated whether OA functions in *S. domuncula* as an inducer of apoptosis against foreign metazoan invaders. The antibodies against OA were used to demonstrate that this toxin accumulates in the annelid and settles in the sponge tissue. With the in situ cell death detection technique (TUNEL reaction) a high level of single- and/or double-stranded DNA breaks could be demonstrated in the cells of the symbiont/parasite, indicating the (early) stages of apoptosis. This strongly suggests that sponges, which harbor the bacteria producing OA, eliminate their metazoan symbionts/parasites by intoxication with this secondary metabolite.

To support this notion we studied whether the host, *S. domuncula*, is protected against the apoptogenic effect caused by the increased levels of OA in the cell layers surrounding the symbiont/parasite. BAG-1 has been selected as a molecular marker and as a molecule of choice. This prosurvival molecule is a multifunctional protein protecting cells against apoptotic cell death (see references 62 and 63). BAG-1 physically interacts particularly with heat shock proteins in response to stress factors during morphogenesis or to toxic effectors.

Two lines of evidence indicate that the host responds to a settling of annelids in *S. domuncula* with an upregulation of the expression of the prosurvival gene *BAG-1*. First, it had been demonstrated that only tissue that is adjacent to the symbiont/parasite expresses detectable amounts of BAG-1 protein. Secondly, it is strikingly shown, by application of the in situ hy-

bridization technique, that the cells that are in direct contact with the annelid strongly express *BAG-1*.

Taken together, our data indicate that in *S. domuncula* OA is a candidate apoptogenic molecule to eliminate invading symbionts/parasites. This study underscores the fact that sponges use secondary metabolites, including those that are synthesized by symbiotic microorganisms, as a chemical defense system against metazoan invaders.

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