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RESEARCH ARTICLE

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Innate immune defense of the sponge *Suberites domuncula* against gram-positive bacteria: induction of lysozyme and AdaPTin

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Abstract Sponges are filter feeders that are exposed to large amounts of bacteria present in their surrounding aqueous milieu. The characteristic cell wall component of gram-positive bacteria, peptidoglycan (PPG), was used as a model molecule to study the responsiveness of cells from the marine demosponge *Suberites domuncula* toward gram-positive bacteria. The sponge lysozyme, which hydrolyzes PPG, was isolated from the living sponge; in addition its gene was cloned (*SDLYS*) and expressed in *Escherichia coli*. Antibodies were raised against the recombinant protein to demonstrate that in the Western blot both molecules give the same signal. In situ hybridization with *SDLYS* as a probe showed that cells in the mesohyl, the gray cells, strongly react with *SDLYS*. Subsequent immunofluorescence studies with antibodies raised against lysozyme revealed that only bacteria react with anti-lysozyme and only those that are scattered within the mesohyl of the tissue. An *adaptor* gene (*AdaPTin-1*) was isolated from the same sponge species that encodes a putative protein involved in endosome formation. Based on its differential expression we conclude that sponge cells react to PPG with a rapid activation of endocytosis, followed by the release of lysozyme.

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The cDNA sequences from *Suberites domuncula* have been deposited (EMBL/GenBank): cDNA for the lysozyme (*SDLYS*) under the accession number AJ699166 and the AdaPTin-1 cDNA (*SDAP1*) under AJ699167.

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Introduction

Since 1922 (Fleming and Allison 1922) lysozymes [Enzyme Nomenclature (EC) no. 3.2.1.17] have been known to be present in different tissue fluids of mammalians where they act as bacteriolytic enzymes. After identification of their amino acid sequence (Jollès et al. 1963) and their functional role in mammalians (Jollès 1976), related enzymes were found also in plants (reviewed in: Jollès 1996). Sequence analyses revealed that the metazoan lysozymes form a distinct family of proteins (Bachali et al. 2002) that serve as innate or natural antimicrobial defense molecules against bacterial infection (Salton 1975). The metazoan lysozymes are grouped into the class of c-type (chicken) lysozymes, i-type (invertebrate) lysozymes, and lactalbumin and display considerable overall sequence homology (Bachali et al. 2002). They catalyze the hydrolysis of the β-1,4-glycosidic linkage between *N*-acetyl muramic acid and *N*-acetylglucosamine of peptidoglycans (PPGs). PPGs are an important constituent of bacterial cell walls (reviewed in Jollès 1996). Lysozymes have been proven to be effective against gram-positive bacteria since they digest their PPG, whereas they are quite ineffective against gram-negative bacteria (McKenzie and White 1991). When PPGs or whole bacteria are taken up by metazoan cells via endocytosis they cause inflammation (reviewed in Ginsburg 2002).

The animal groups that are exposed most to microorganisms in the marine milieu are sessile filter feeders, and in particular among them the sponges (phylum Porifera) are the most abundant multicellular animals found in the marine hard-substrate benthos. It has been calculated that some of these animals filter huge amounts of water, 0.002 to 0.84 cm^3 per second per cubic centimeter of sponge tissue, through their

aquiferous canal system, to extract edible material (see Osinga et al. 2003). To survive they must have developed very efficient defense strategies. Cloning studies have revealed that these phylogenetically oldest metazoans have, like the higher triploblastic animals, immune defense molecules, which are either cell-membrane-associated receptors, for example, molecules containing scavenger receptor cysteine-rich (SRCR) domains, or soluble factors, such as cytokines (reviewed in Müller et al. 1999). Besides having humoral and cellular defense/immune mechanisms, sponges eliminate microorganisms by engulfing them through phagocytosis (Metchnikoff 1892). Most of these molecular and cellular studies to assess the immune repertoire in sponges have been performed with the demosponge *Suberites domuncula* (Müller et al. 1999, 2004a). Recently, the first molecular data were presented that *S. domuncula* recognizes the lipopolysaccharide (LPS) molecule on the surface of the gram-negative bacteria, resulting in an activation of the mitogen-activated protein kinase (MAPK) pathway (Böhm et al. 2001). Sponges interact also with components of the cell wall of fungi, for example, $(1 \rightarrow 3)$-β-D-glucans, through the $(1 \rightarrow 3)$-β-D-glucan binding receptor by an increased expression of a series of defense molecules (Perović-Ottstadt et al. 2004).

In the present study, molecular data on the potential defense mechanism(s) of sponges against gram-positive bacteria are presented for the first time. It is shown that sponges possess a gene that codes for lysozyme. It is expressed after exposure to bacterial PPG from gram-positive cocci *Staphylococcus aureus*. Antibodies were raised against the recombinant sponge lysozyme to prove that also biologically active lysozyme exists in sponge extract. Simultaneously the adaptor-related protein complex 1 molecule (AdaPTin) is upregulated, which is indicative of the activation of the endocytic pathway.

Materials and methods

Chemicals and enzymes

The sources of chemicals and enzymes used were given previously (Kruse et al. 1997; Krasko et al. 2000). PPG isolated from *Staphylococcus aureus* was obtained from Fluka (Seelze, Germany), lyophilized cells from *Micrococcus lysodeikticus* (ATCC no. 4698), hematoxylin solution (Mayer's), eosine solution, DPX mounting medium, 4′,6-diamidino-2-phenylindole-dilactate (DAPI), and Cy3-conjugated sheep anti-rabbit IgG from Sigma-Aldrich (Taufkirchen, Germany). Anti-digoxigenin antibody conjugated with alkaline phosphatase, PCR-DIG Probe Synthesis Kit, and NBT/X-Phosphate were from Roche (Mannheim, Germany).

Sponges

Live specimens of *S. domuncula* (Porifera, Demospongiae, Hadromerida) were collected near Rovinj (Croatia) and kept in aquaria in Mainz (Germany) for more than 10 months prior to their use.

Isolation of the cDNA for lysozyme

The complementary DNA (cDNA) encoding the putative sponge lysozyme (*SDLYS*) was isolated from the *S. domuncula* cDNA library (Kruse et al. 1997) by polymerase chain reaction (PCR). The primers were designed against the highly conserved region within the metazoan lysozymes, with the mussel *Mytilus edulis* (Bachali et al. 2002) as an example. Two regions are highly conserved, the homologous domain and a stretch close to the C-terminus of the deduced proteins. Successful were the primers, designed against the terminal regions, between aa_{146} and aa_{153} in the *M. edulis* sequence. The degenerate primers were used in the PCR reaction together with the vector primer. The PCR was carried out at an initial denaturation at 95°C for 4 min, followed by 30 amplification cycles at 94°C for 30 s, 59°C for 45 s, 70°C for 1.5 min, and a final extension step at 70°C for 10 min. The reaction mixture was as described earlier (Wiens et al. 1998). The fragments obtained were used to isolate the cDNA from the library (Ausubel et al. 1995) and resulted in one clone with a 585-nucleotide (nt) insert [excluding the poly(A) tail].

Cloning of the AdaPTin-1 cDNA

AdaPTin proteins associate with clathrin via the conserved clathrin adaptor complex small chain signature peptide, which is located in the human adaptor-related protein complex 1 sigma 2 subunit (NP_003907.3) between aa_{56} and aa_{66}. Degenerate primers against this segment [nt_{176} to nt_{208} (in the sponge final sequence); accession no. AJ699167] together with the vector primers were used to isolated the complete cDNA by PCR procedure as described above. The complete clone, *SDAP1*, is 670 nts long.

Sequence analysis

The sequences were analyzed using the computer programs Blast (2003) and FASTA (2003). Multiple alignments were performed with CLUSTAL W version 1.6 (Thompson et al. 1994). Phylogenetic trees were constructed on the basis of aa sequence alignments by neighbor joining, as implemented in the "Neighbor" program from the PHYLIP package (Felsenstein 1993). The distance matrices were calculated using the Dayhoff PAM matrix model as described in Dayhoff et al. (1978). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein 1993). The graphic presentations were prepared with GeneDoc (Nicholas and Nicholas 1997).

Recombinant sponge lysozyme and raising of antibodies

The sponge *SDLYS* sequence was expressed in *E. coli* (TOP10). The complete open reading frame (ORF; nt_{19} to nt_{401}), was isolated by PCR using one forward primer [5′-ccatggcaaccaaaccctctggtccta-3′ (the *Nco*I restriction site is underlined)] and one reverse primer (5′-aagcttagatacttcttgactttcacc-3′; *Hin*dIII). The 397-bp-long part was cloned into the expression vector pBAD/gIII A, which contained at the 3′-terminus the *myc* epitope and the *polyhistidine* region. The insert was expressed overnight at 30°C in the presence of 0.0002% of L-arabinose. The fusion protein was extracted and purified with the His Tag Purification Kit (Novagen, Madison Wis., USA). The purity of the material was checked by 10% polyacrylamide gels containing 0.1% $NaDodSO_4$ (polyacrylamide gel electrophoresis, or PAGE) according to Laemmli (1970). The recombinant lysozyme was dialyzed against 25 m*M* Tris-HCl buffer (pH 7.2), supplemented with 10 m*M* of DL-dithiothreitol.

Polyclonal antibodies (PoAb) were raised against the recombinant lysozyme in female rabbits (white New Zealand) as described in Schütze et al. (2001); the PoAb were termed PoAb-LYS. In one control experiment 100 µl of the PoAb-LYS were adsorbed to 20 µg of recombinant lysozyme (30 min; 4°C) prior to use.

Staining procedure

The specimens were fixed in 4% (w/v) paraformaldehyde for 10 min; cryosections (8 µm) were prepared and—after fixation—washed with phosphate-buffered saline (PBS). The slides were transferred into distilled water (5 min) and then incubated with hematoxylin solution for 2 min. After washing in PBS, the sections were counterstained with 0.1% (w/v) eosine solution for 1 min. The slices were dehydrated with ethanol in xylene and then mounted with DPX mounting medium and inspected microscopically.

In situ localization studies

The method used was based on the procedure described by Polak and McGee (1998) with modifications described recently (Perović et al. 2003). Frozen sections (8 µm) were obtained, fixed with paraformaldehyde (4%), treated with Proteinase K, and subsequently fixed again with paraformaldehyde. To remove the sponge color, the sections were washed with increasing concentrations of ethanol. Hybridization [with the antisense and sense (control) probe] was performed in 2×SSC (sodium chloride/sodium citrate), supplemented with 50% formamide. The activation of the digoxigenin (DIG)-labeled probe (10 pmol/ml) was performed for 1 min at 95°C in 4×SSC buffer. Hybridization proceeded overnight in a glass chamber at 45°C. Subsequently the sections were washed at 55°C, 1×2 min in 2×SSC and 3×20 min in 0.2×SSC. Final washes (2×5 min) were with PBT [PBS that contained 0.1% (v/v) of Triton X-100 and 2 mg/ml of bovine serum albumin (BSA)] at room temperature. After blocking [1% blocking reagent for nucleic acid (Roche) in 1×PBS, containing 0.1% (v/v) Tween 20] for 15 min at room temperature, the slices were reacted with anti-DIG Fab fragments conjugated to alkaline phosphatase (dilution 1:100) for 1 h at 37°C in a humid chamber. After two washes (5 min each) with PBT at room temperature and one wash (5 min) with the Tris buffer (100 m*M* Tris/HCl, pH 9.5; 100 m*M* NaCl and 50 m*M* $MgCl_2$), the sections were incubated with Tris buffer and supplemented with the dye reagents NBT (4-nitro blue terazolium chloride) and X-Phosphate (Roche) for 45 min at 37°C in the dark. After washing the sections for 5 min in 1×PBS, they were covered in Glycergel (DAKO, Hamburg, Germany) and analyzed under the microscope. Hybridization was performed with a labeled lysozyme probe from *S. domuncula* (*SDLYS*; nt_{30} to nt_{480}). Sense probes were used in parallel as negative controls in the experiments.

Immunohistochemistry

Tissue was fixed in paraformaldehyde, embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany) and sectioned, essentially as described in Böhm et al. (2001). To remove the siliceous spicules the tissue samples were incubated with HF/NH_4F (1.8 ml of 20% HF plus 8.0 ml of 10 *M* NH_4F) for 60 min on ice. The 4-µm-thick slices were incubated with PoAb-LYS (1:300 dilution) overnight. Then the slides were incubated with Cy3-conjugated sheep anti-rabbit IgG for 2 h. The sections were inspected by immunofluorescence with an Olympus AHBT3 light microscope. Pre-immune serum was used as a control.

In parallel slices were stained with 5 µg/ml of DAPI for 30 min to identify DNA, especially in bacteria. In *S. domuncula* no autofluorescence is seen in this histological section.

Extraction of lysozyme-like protein from sponge tissue

Ten grams of frozen sponge tissue was homogenized in 30 ml of Ca^{2+}-free and Mg^{2+}-free artificial seawater (CMFSW; Rottmann et al. 1987). The supernatant was collected after centrifugation (20,000x *g*, 30 min) and dialyzed against CMFSW for 12 h (step 1: crude extract). The crude extract was acidified to 5% acetic acid; after standing overnight at 4°C the precipitate formed was collected by centrifugation. The sediment was dissolved in 2 ml of CMFSW (step 2: acid precipitation). This fraction was applied onto a DEAE-cellulose column (1×5 cm), which had been equilibrated with a 10 m*M* Tris-HCl buffer (containing 2 m*M* EDTA, 100 m*M* NaCl; pH 8.2). After washing with this buffer the fractions containing lysozyme activity were eluted

with 1 *M* NaCl. They were pooled and dialyzed against CMFSW (step 3: DEAE-cellulose). During the fractionation procedure both the protein content and the lysozyme activity were determined.

PAGE was performed in 15% polyacrylamide gels containing 0.1% $NaDodSO_4$ according to Laemmli (1970). Protein samples were subjected to the gels either in the native state, or after denaturation (presence of 2-mercaptoethanol and under heating). Staining of proteins was performed with Coomassie brilliant blue.

Lysozyme assays

The lysoplate assay was performed as described in Lie et al. (1986). Agarose (1%) plates in 50 m*M* phosphate buffer (pH 6.2) were prepared, containing 1 mg/ml of *M. lysodeikticus*. Tissue cubes (facial length of 3 mm) were challenged for 1–3 days with PPG from *S. aureus* (5 μg/ml) or remained without this glycan (controls) and then transferred onto the plates for 6 h at 17°C. Subsequently, the sponge samples were removed from the agarose and the plates were incubated at 30°C for 24 h. The lysed zones are caused by the lytic activity of lysozyme.

For a quantitative determination the turbidimetric assay was applied (Parry et al. 1965). A suspension of *M. lysodeikticus* (0.2 mg/ml) in a 50 m*M* phosphate buffer (pH 6.2) was mixed with the enzyme samples in a final volume of 1 ml. The reaction was allowed to stand at 25°C and the absorbance was measured at 530 nm after 30 s (start) and 4.5 min. One unit (U) was defined as that lysozyme activity that caused a decrease in absorbance of 0.001 per minute.

Western blotting

For the identification of lysozyme in extracts, the samples were subjected to 15% or 10% PAGE (Laemmli 1970). After protein transfer, the membranes were incubated with PoAb-LYS (1:500 dilution); the immune complexes were visualized by incubation with anti-rabbit IgG (alkaline phosphatase conjugated), followed by staining with 4-chloro-1-naphthol.

RNA preparation and Northern blot analysis

RNA was extracted from liquid-nitrogen pulverized tissue with TRIzol reagent (GibcoBRL, Grand Island, N.Y.) as described in Grebenjuk et al. (2002). Then 5 μg of total RNA was electrophoresed and blotted onto Hybond-N^+ nylon membrane (Amersham, Little Chalfont, Buckinghamshire, UK). Hybridization was performed with the following probes: the lysozyme cDNA (*SDLYS*; nt_{30} to nt_{380}), the adaptor *SDAP1* cDNA (nt_{53} to nt_{423}), or a part of β-tubulin, *SDTUB* (accession no. AJ550806; nt_{83} to nt_{483}). The probes were labeled with the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany). After washing, DIG-labeled nucleic acid was detected with anti-DIG Fab fragments and visualized by chemiluminescence technique using disodium chloro-(methoxyspiro dioxetane chloro) tricyclo decan phenyl phosphate (CDP; Roche).

Fig. 1A–C The *Suberites domuncula* lysozyme (LYS_SUBDO); the putative protein was deduced from the complete cDNA *SDLYS*. **A** The sponge enzyme is aligned with the next similar lysozyme, the one from the mussel *Mytilus edulis* (LYS_MYTEDU, AAN16207.1 (aa_{67} to aa_{122}; Bachali et al. 2002). Conserved residues (similar or related with respect to their similar physico-chemical properties) are shown in *white on black*. The homologous domain, according to Bachali et al. (2002), is marked. **B** This domain was aligned with the corresponding regions of the group of i-type lysozymes from the following animals: the clam (*Calyptogena* sp.) lysozyme-1 and lysozyme-2 [LYS1_CAL, AAN16211.1 (aa_6 to aa_{60}) and LYS2_CAL, AAN16212.1 (aa_{41} to aa_{92}); Bachali et al. 2002], the 14.9 kDa and the 18.0 kDa lysozymes from *Caenorhabditis elegans* [LYS2_CAEEL, NP_500206.1 (aa_{23} to aa_{85}) and LYS5_CAEEL, NP_501313.2 (aa_{34} to aa_{97})], the lysozyme from the mollusc *Tapes japonica* [LYS_TAJA, BAC15553.1 (aa_{24} to aa_{79})], the lysozyme from the mussel *Bathymodiolus thermophilus* [LYS_BATTHE, AAN16209.1 (aa_{55} to aa_{109}); Bachali et al. 2002], chlamysin from *Chlamys islandica* [CLAMY_CHLAI, CAB63451.1 (aa_{33} to aa_{87}); Nilsen et al. 1999] the "destabilase-2" from the medicinal leech *Hirudo medicinalis* [DEST2_HIRUDO, S78428 (aa_{29} to aa_{84}); Zavalova et al. 1996], the two putative lysozymes from *Drosophila melanogaster* [LYS-c_DROME, AAF47445.1 (aa_{46} to aa_{102}) and LYS-i_DROME, CAA21317.1 (aa_{38} to aa_{101})], c-type lysozymes from the chicken [LYS_CHICKEN, CAA23711.1 (aa_{48} to aa_{104}); Jung et al. 1980)] and from human [LYS-c_HUMAN, CAA32175.1 (aa_{30} to aa_{86}); Peters et al. 1989] and lactalbumin, with the human alpha-lactalbumin as an example [LACT-a_HUMAN, CAA28800.1 (aa_{28} to aa_{82}); Hall et al. 1987]. Residues conserved in all sequences are shown in *white on black* and those in at least seven sequences in *black on gray*. The consensus amino acids are given above the alignment (*capital letters* present in all sequences; *small letters* in more than seven; *5* aromatic aa; *6* aliphatic aa). **C** After alignment the unrooted tree was constructed; the sequences could be classified into the three mentioned lysozyme families. The *numbers at the nodes* are an indication of the level of confidence—given as percentage—for the branches as determined by bootstrap analysis [1,000 bootstrap replicates]. *Scale bar* indicates an evolutionary distance of .1 aa substitutions per position in the sequence

Further methods

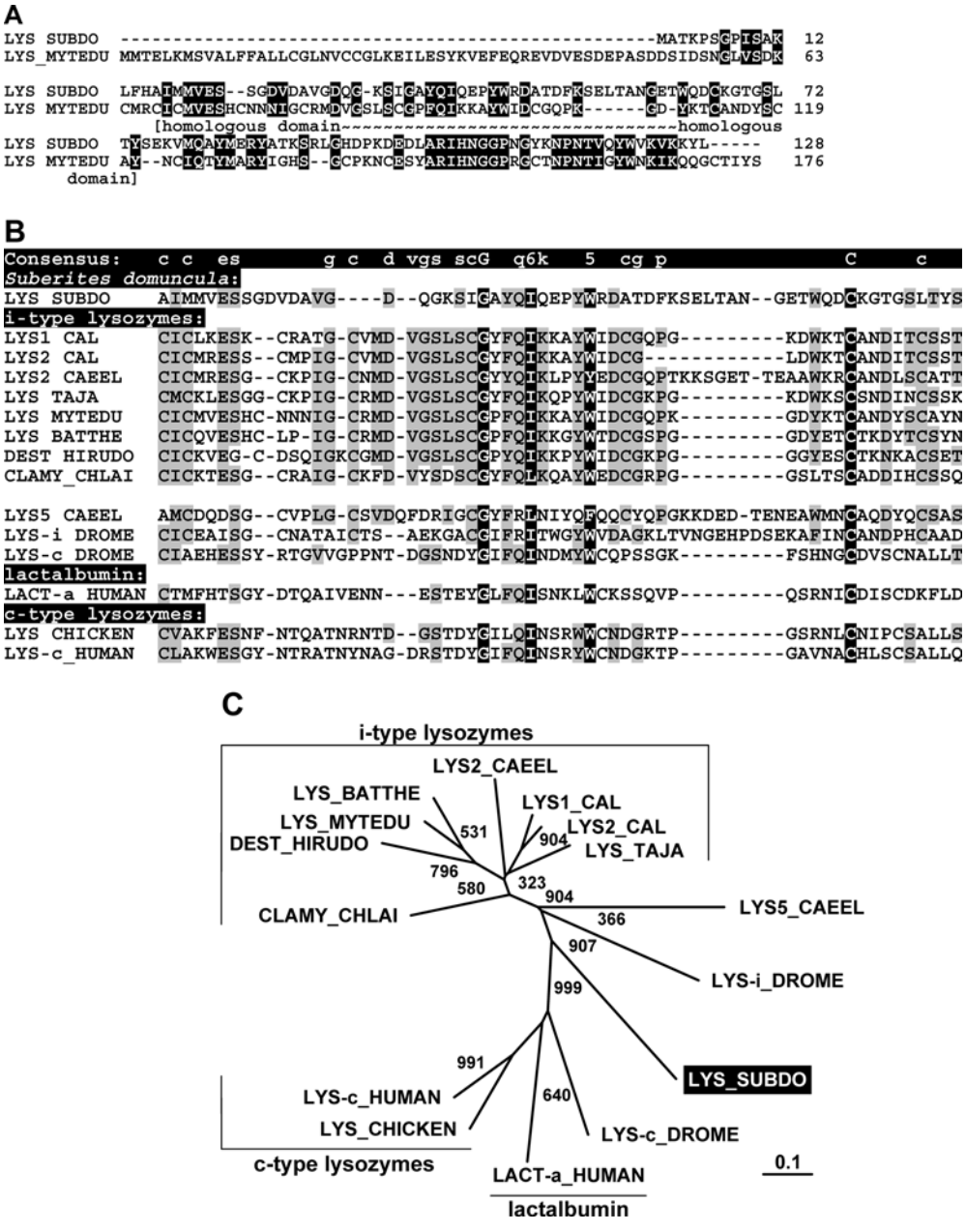
Transmission electron microscopy was performed with glutaraldehyde/PBS fixed sections that had been embedded in Epon resin as described in Böhm et al. (2001). Protein concentration was measured according to Lowry et al. (1951) using bovine serum albumin as standard.

Results

Cloning of the cDNA for lysozyme

Sequence

The cDNA encoding the putative sponge lysozyme (*SDLYS*) was obtained from the *S. domuncula* cDNA library. One ORF ranges from $nt_{19/21}$ to $nt_{403/405(stop)}$.



The cDNA is of full length as shown by Northern blot analysis (0.8 kb; see below). The 128-aa-long deduced protein, LYS_SUBDO, has a calculated size of 14,298 Da. The polypeptide shares the highest sequence similarity with the molluscan lysozymes, for example, the one from *Tapes japonica* (accession no. BAC15553.1) or from *Mytilus edulis* (AAN16207.1). The significance value (E-value) is E-12. The characteristic "homology domain" occurs within the *S. domuncula* sequence between aa_{16} and aa_{75} (Fig. 1A).

Phylogenetic analysis

The sponge lysozyme was aligned with the major enzymes of this family listed in Bachali et al. (2002). For the characteristic grouping the "homology domains" of the sequences were chosen (Fig. 1B). An unrooted phylogenetic tree was computed that revealed a distinct grouping of the "i-type lysozymes" (similar to the overall invertebrate lysozymes) and the "c-type lysozymes" (chicken-like lysozymes). More to the center of the tree the sponge lysozyme is found, which displays similarity to both the "i-type lysozymes" (25% identical/40% similar aa) and "c-type lysozymes" (10%/25%). Hence the sponge lysozyme, together with the two other invertebrate lysozymes, that from *Caenorhabditis elegans* (NP_501313.2) and the putative lysozyme from *Drosophila melanogaster* (AAF47445.1), form the basis of the metazoan enzymes of the lysozyme group.

Recombinant lysozyme and antibodies

To identify lysozyme in sponge extract, the recombinant lysozyme was prepared. The cDNA was cloned into the expression vector pBAD/gIII as described under Materials and methods. After induction with arabinose the protein could be identified in the bacterial lysate (Fig. 2B, lane a vs b). The recombinant protein was purified (Fig. 2B, lane c) and used to raise antibodies in rabbits. These PoAb-LYS were found to react with the purified recombinant fusion lysozyme (171 aa); the size of this protein was determined to be 20 kDa, compatible with the size of the recombinant protein (14 kDa) and the protein stretch covering the myc epitope and polyhistidine (Fig. 2B, lane d).

Sponge lysozyme

The activity of lysozyme in sponge tissue was determined quantitatively by applying the turbidimetric assay as described under Materials and methods. First, the lysozyme was enriched from sponge tissue extracts. Frozen sponge was homogenized and the cleared crude extract was obtained (step 1; Table 1). The specific activity for lysozyme was determined with 1.2 U/mg. Subsequently the enzyme was further enriched by an acid precipitation step (specific activity: 9.8 U/mg). Finally, a DEAE-cellulose chromatography (step 3) was added resulting in a 22.7-fold enrichment of the lysozyme with respect to the crude extract. The final fraction contained 0.15 mg/ml of protein (4.1 U lysozyme activity/ml); Table 1.

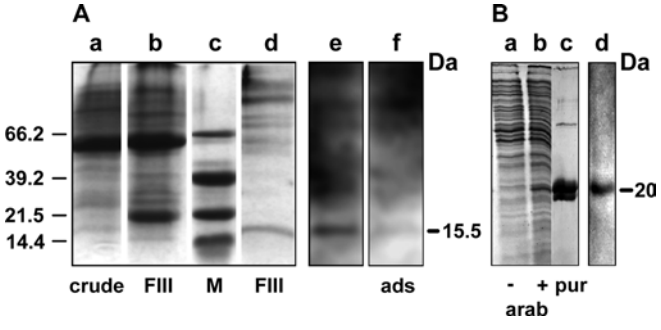


Fig. 2A, B Analysis of the sponge lysozyme. **A** Native lysozyme. Gel electrophoretic analysis (PAGE) of the crude extract (lane *a*) and fraction III (lane *b*) under native and under denatured conditions (lane *d*). The separation was performed in a 15% polyacrylamide gel (30 μg of protein were applied on the native gel and 5 μg of protein on the denaturing gel). In lane *c* the size markers were separated. Staining of proteins was performed with Coomassie brilliant blue. The enriched lysozyme fraction III was subjected to Western blot analysis using the rabbit PoAb-LYS antibodies (lane *e*). The PoAb-LYS antibodies, adsorbed with recombinant lysozyme fusion protein, almost failed to react with the 15.5 kDa protein in fraction III (lane *f*). **B** Recombinant lysozyme was obtained in *Escherichia coli*. Lane *a* PAGE analysis (10% gels) of bacterial lysate obtained from *E. coli* grown in the absence of arabinose (- arab). Lane *b* Lysate from bacteria that had been induced with arabinose (- arab). Lane *c* Affinity purified fusion protein (pur). Lane *d* Western blot analysis of purified fusion protein using PoAb-LYS antibodies

Electrophoresis of the crude extract and the fraction 3 was performed in 15% polyacrylamide gels either under native or denatured conditions (2-mercaptoethanol and heat). Under native conditions, one major band was detected in the crude extract that corresponds to a size of 60 kDa (Fig. 2A, lane a). After purification, in addition, a major protein with an apparent size of 20 kDa became visible (Fig. 2A, lane b). Analysis of fraction 3 under denaturing conditions (presence of 2-mercaptoethanol and heat) revealed in the low-molecular-weight range one major low-molecular-weight band with 15.5 kDa (Fig. 2A, lane d). Western blot analysis was performed with antibodies, raised against the recombinant lysozyme, PoAb-LYS. These antibodies recognized the 15.5 kDa protein from the natural source as well (Fig. 2A, lane e). In one control experiment the antibodies were pretreated with the recombinant lysozyme. This sample failed to react with this natural lysozyme (Fig. 2A, lane f).

Expression of lysozyme gene in sponge tissue

Sponge specimens were exposed to 5 μg/ml of PPG from *Staphylococcus aureus* for 3 days. Cryosections were prepared that were stained with hematoxylin/eosine as described under Materials and methods. Cross sections showed besides the predominant number of 7- to 10-μm cells clusters of 25-μm cells that stained intensively purple (Fig. 3A, a–d). The latter cells are scattered within the mesohyl, the intercellular compartment in sponges, which is surrounded by epithelial layers.

In parallel studies the cryosections were subjected to in situ hybridization using the *S. domuncula* lysozyme cDNA, *SDLYS*, as a probe (Fig. 3B, a–d). The antisense probe of lysozyme reacted primarily with cells present in the surface region of the sponge (Fig. 3B, a) and in the large 25-μm cells that are scattered within the mesohyl (Fig. 3B, b–d). Control studies were performed with the

Table 1 Enrichment of *Suberites domuncula*lysozyme. *U* unit

Purification step	Protein (mg)	Total activity (U)	Recovery (%)	Specific activity (U/mg)	Purification (fold)
Step 1: crude extract	85.5	102.0	100	1.2	1
Step 2: acid precipitation	2.55	25.0	25.5	9.8	8.2
Step 3: DEAE-cellulose	0.46	12.5	12.8	27.2	22.7

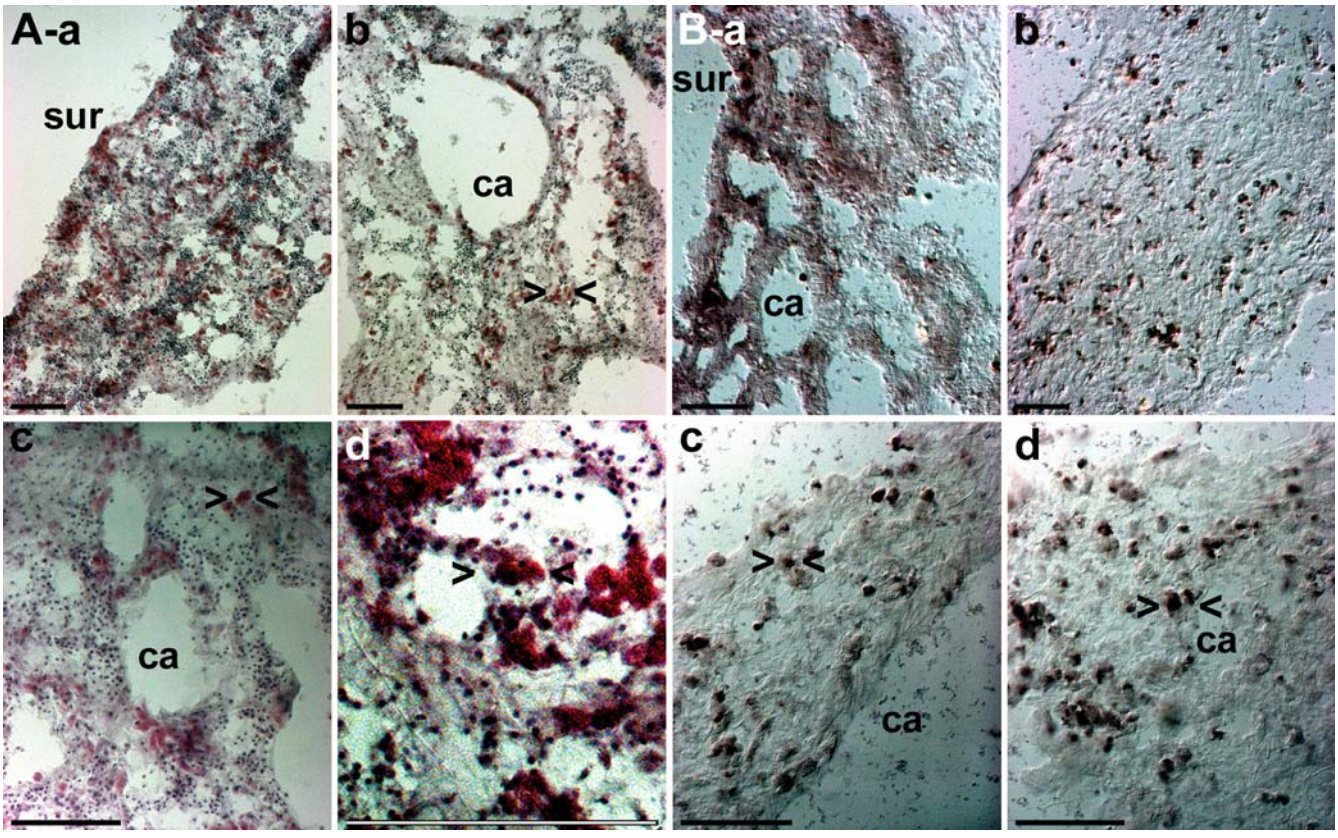


Fig. 3A, B Cells in *S. domuncula* expressing lysozyme. Cryosections were prepared from specimens exposed to 5 µg/ml of peptidoglycan (PPG) from *S. aureus* for 3 days. **A** The sections were stained with hematoxylin/eosine. The 25-µm large cells that stained intensive purple are marked (> <). **B** In situ hybridization was performed with parallel sections using the sponge lysozyme cDNA (antisense orientation). The following regions are seen: *sur* surface of the sponge; *ca* canal system. *Size bars* correspond to 500 µm

sense lysozyme probe, which gave no staining (not shown).

Increased lytic activity released by *S. aureus* treated tissue

The release of lysozyme from sponge tissue was studied by applying the lysoplate assay. Specimens were incubated in seawater in the absence or presence of PPG from *S. aureus* (5 µg/ml) for up to 3 days. Then the samples were placed (for 6 h) onto agarose plates that contained *M. lysodeikticus* as substrate for the lysozyme. Finally the tissue was removed and the plates were incubated at 30°C as described under Materials and methods. The experiments showed that the tissue samples that had not been challenged with *S. aureus* caused only a weak cleared zone reflecting lysozyme activity (Fig. 4, left). In contrast, the specimens that had been preincubated with PPG for 1 or 3 days caused a large cleared zone against *M. lysodeikticus* (Fig. 4, right), which indicated a strong lytic activity by lysozyme.

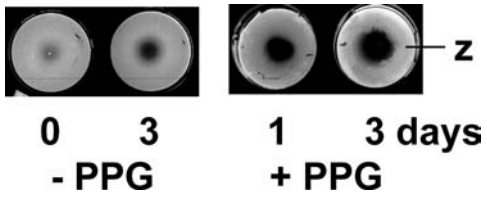


Fig. 4 Induction of lysozyme release from sponge tissue after challenging with PPG from *S. aureus*. The tissue cubes were incubated for 1–3 days in the absence or presence of the soluble PPG. Then the specimens were placed onto the agarose plates supplemented with *Micrococcus lysodeikticus* for 6 h at 17°C. Finally the plates were incubated at 30°C (24 h). the cleared zone in the center (*z*) is indicative of the digestion caused by lysozyme

Localization of lysozyme enzyme in the tissue

The results of the previous section suggest that the sponge lysozyme activity increases after treating the sponge with gram-positive bacteria, for example, *S. aureus*. Therefore, it was important to determine those regions within the sponge where the enzyme reaches its target. This was achieved by immunohistochemical experiments.

Earlier studies could be confirmed by transmission electron microscopy (Böhm et al. 2001), which revealed that in *S. domuncula* tissue bacteria occur primarily in clusters that are usually embedded in special cells, the bacteriocytes. One such cluster is shown in Fig. 5A. In addition, bacteria occur in less organized arrangements, which are more or less compact. For the

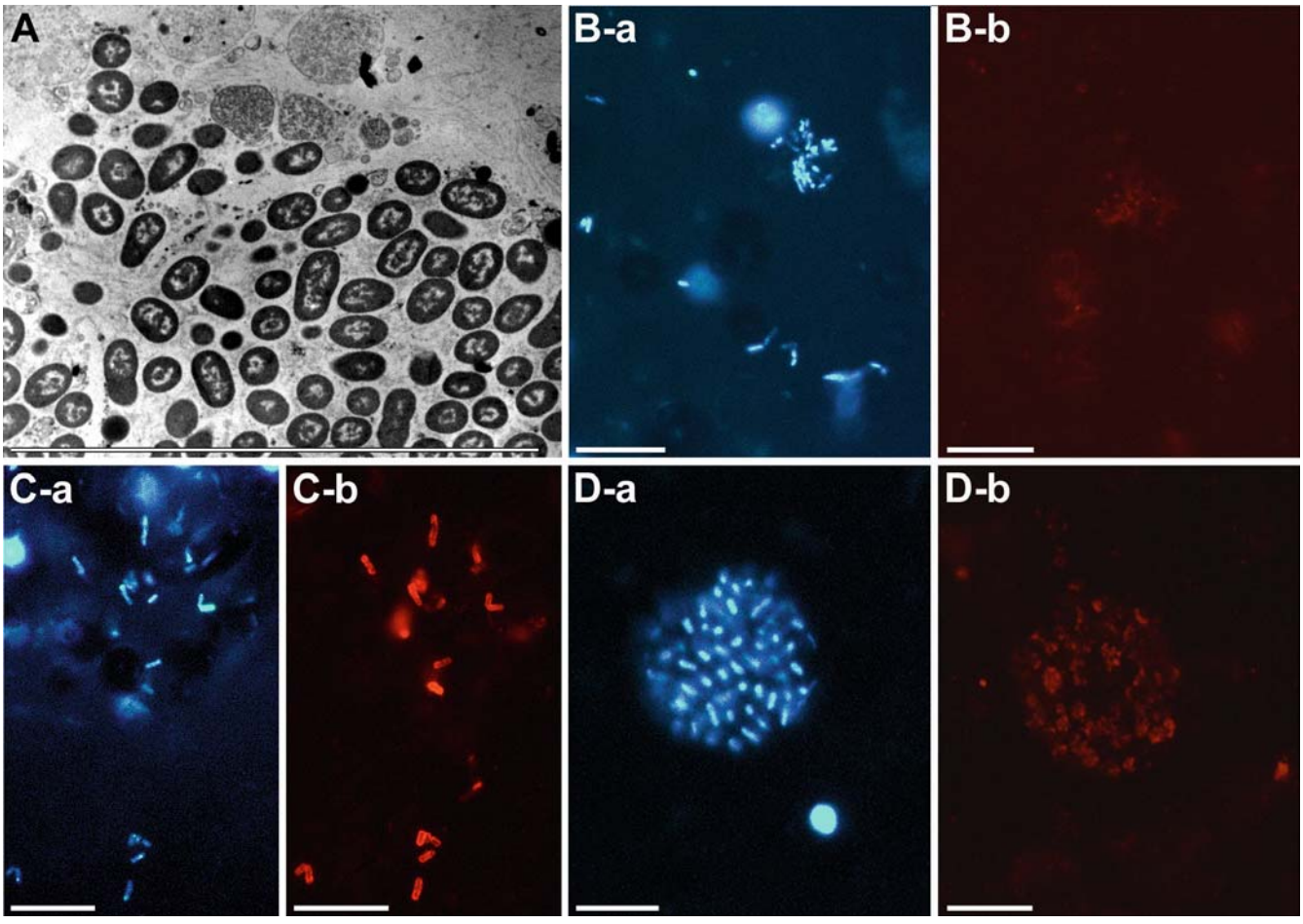


Fig. 5A–D Immunohistological identification of sponge lysozyme in the tissue. **A** Transmission electron microscopy through *S. domuncula* showing the bacteria clusters. **B–D** Light microscopical analysis of sections, stained with DAPI (**a**), or (**b**) which had been incubated with antiserum. In (**B**-*b*) the sections have been reacted with pre-immune serum and then with a Cy3-conjugated anti-rabbit secondary antibody. In **C**-*b* and **D**-*b*, the slices were incubated with the anti-lysozyme antiserum PoAb-LYS. It is evident that the bacteria that are interspersed in the tissue became brightly stained with PoAb-LYS (**C**-*b*), while those that reside in clusters were not (**D**-*b*). *Size bars* 20 μm

immunohistological identification of the bacteria in sponge tissue, slices were prepared and first incubated with pre-immune serum. This preparation did not react specifically with any structure in the sponge tissue, if Cy3-conjugated sheep anti-rabbit IgG had been added as secondary antibodies (Fig. 5B, b); in the parallel double staining (antibodies and DAPI) theDNA of bacteria appeared very brightly (Fig. 5B, a). Two staining patterns could be seen with the lysozyme antibodies PoAb-LYS if parallel staining was performed with PoAb-LYS, together with the Cy3-labeled secondary IgG and DAPI. First, those bacteria that are irregularly scattered within the sponge tissue and are readily stainable with DAPI (Fig. 5C, a) were strongly stained with PoAb-LYS (Fig. 5C, b). In contrast, if the compact nests of bacteria were stained with PoAb-LYS alone, the bacteria could hardly be visualized (Fig. 5D, b); simultaneously the DAPI staining was very bright (Fig. 5D, a).

AdaPTin-1 cDNA

The sponge cDNA (*SDAP1*) encoding AdaPTin-1 was isolated. The 670-nts-long cDNA comprises one ORF that ranges from $nt_{11–13}$ to $nt_{494–496(stop)}$. From the predicted 161 aa in this protein a relative molecular weight (M_r) of 19,081 Da was estimated (Fig. 6A). The sponge molecule (AP1_SUBDO) comprises the conserved clathrin adaptor complex small chain signature (Prosite no. PDOC00760), which reads VVYKryasLYF (the capital letters are conserved); this adaptor occurs also in other adaptor-related proteins, for example, AdaPTin-2.

The overall aa sequence of the AdaPTins is highly conserved among the multicellular organisms. The *S. domuncula* AdaPTin-1 has a high degree of similarity to the human adaptor-related protein complex 1 (sigma 2 subunit; accession no. NP_003907.3; 86% similar and 68% identical aa). In spite of this high similarity/identity between the human and sponge sequence and also down to the plant *Arabidopsis thaliana* clathrin adaptor complex (NP_195267.1; 74% similar and 59% identical with respect to AP1_SUBDO) a significant

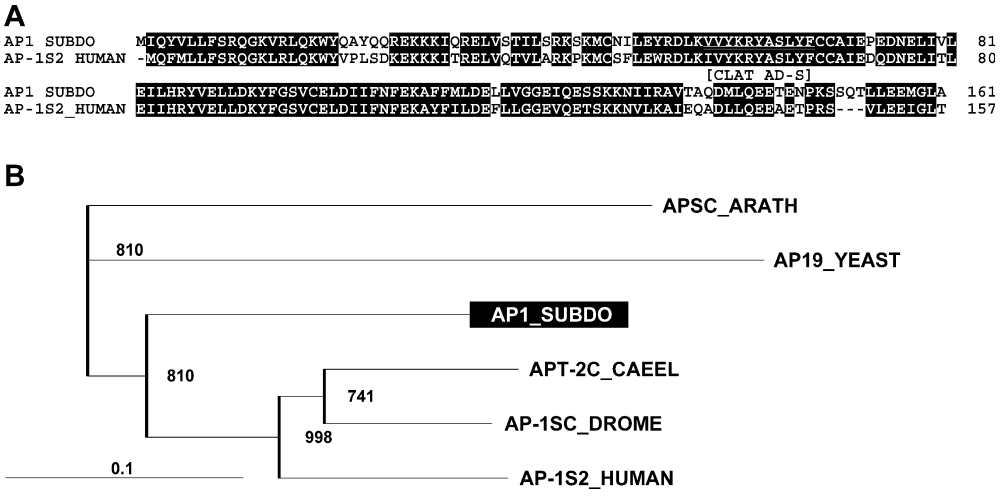


Fig. 6A, B Sponge AdaPTin-1. **A** Alignment of the *S. domuncula* deduced AdaPTin-1 protein (AP1_SUBDO) with the human adaptor-related protein complex 1 sigma 2 subunit (AP-1S2_HUMAN; NP_003907.3). The conserved clathrin adaptor complex small chain signature (CLAT AD-S) is marked. **B** The rooted tree was constructed with these two sequences and the *D. melanogaster* clathrin-associated adaptor complex AP-1 small chain sigma 1 (AP-1SC_DROME; AF228054_1), the *C. elegans* AdaPTin or adaptin-related protein (18.6 kDa; apt-2C; APT-2C_CAEEL;NP_504559.1), the clathrin coat assembly protein AP19 from *Saccharomyces cerevisiae* (AP19_YEAST; P35181) and the *Arabidopsis thaliana* clathrin adaptor complex (APSC_ARATH; NP_195267.1). The latter molecule was used to root the tree

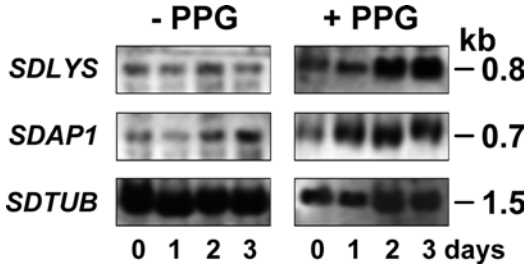


Fig. 7 Differential expression of the lysozyme (SDLYS) and the adaptor gene (AdaPTin-1) in response to exposure of sponge tissue to PPG. In one series of experiments the tissue specimens were incubated in the absence of PPG (−PPG) or were incubated with 5 μg/ml of PPG (+PPG). After the indicated period of time tissue samples were subjected to RNA extraction. The RNA was size separated and after blot transfer hybridized with the labeled probes as described under Materials and methods. Equal amounts of RNA were loaded onto the gel as can be deduced from the signals obtained with the probe encoding the house-keeping gene β-tubulin (*SDTUB*)

rooting could be calculated. The sponge sequence forms the basis of the branch comprising the other metazoan AdaPTins and is separated form the yeast clathrin coat assembly protein AP19 (P35181) and plant sequence (NP_504559.1).

Effect of PPG on lysozyme gene expression

Based on these results, experiments were designed to determine if after incubation of tissue with PPG, the expression of the *lysozyme* gene and also of the gene *AdaPTin-1*, whose products is (very likely) involved in endocytosis and/or sorting events in the Golgi network (Boehm and Bonifacino 2001), are modulated during the course of exposure to PPG.

Northern blot analyses performed with the labeled cDNA probe for lysozyme (*SDLYS*) and the adaptor protein AP-1 (*SDAP1*) revealed that the expression level of *SDLYS* and *SDAP1* did change during the course of a 3-day incubation in the absence of PPG (Fig. 7). However, if the tissue was incubated for this period of time with 5 μg/ml of PPG a strong upregulation of the *AdaPTin* gene is seen after an exposure of 1 day, whereas the strong *lysozyme* expression is apparent after a time lapse of 2 days (Fig. 7). Control blots were performed with the house-keeping gene β-tubulin (*SDTUB*) to demonstrate that the same amount of RNA was loaded onto the gels.

Discussion

Metchnikoff (1892) discovered that one powerful tool of sponges to eliminate microbes is intracellular digestion. This cellular defense mechanism against foreign invaders is well developed from Porifera to humans or insects. For this process, sponges possess specialized amoeboid cells, the archaeocytes, which have been considered as macrophages (Van de Vyver 1981). Until recently it remained unclear by which molecules sponges recognize the huge amount of microbes that they ingest, sort them in their aquiferous system into nutritional or infectious ones, and digest or eliminate them from their canals. The first molecular studies to understand the interaction between gram-negative bacteria (Böhm et al. 2001) and fungi (Perović-Ottstadt et al. 2004) demonstrated that the most thoroughly studied demosponge *S. domuncula* recognizes these groups of microbes through pathways (MAPK) or receptors [(1 → 3)-β-D-glucan binding receptor] that are known to act also in crown taxa against these invaders. It can be assumed that these

molecular recognition systems contribute to the amazing fact that sponges harbor sponge-specific bacterial communities (Hentschel et al. 2002).

In the present study we show that one major constituent from the cell wall of gram-positive bacteria, the PPGs, can modulate gene expression in *S. domuncula* resulting in the increased expression of *lysozyme* and the AdaPTin gene *SDAP1*. PPGs are released during bacteriolysis. Whole bacteria or their PPGs are known to be endocytozed (Ginsburg 2002), a process that is followed by an activation of an overall cell metabolism, including induction of inflammation and the synthesis of a series of hydrolytic enzymes. Among those are the lysozymes that degrade PPG (reviewed in Jollès 1996). Here the molecular tools for the investigation of the functional response of sponges toward PPG have been elaborated. *S. domuncula* lysozyme protein comprises the characteristic lysozyme domain that classifies this molecule as an ancestor for the other metazoan lysozyme and lysozyme-related enzymes, the i-type and c-type lysozymes and the lactalbumins. This cDNA (*SDLYS*) and antibodies (PoAb-LYS) prepared against the expressed, recombinant protein (r-LYS_SUBDO) were used to demonstrate that the cloned enzyme is identical with the 15.5 kDa naturally occurring lysozyme in this species.

The naturally occurring lysozyme is induced in tissue after exposure to PPG. This observation provides the first clue that bacterial cell-wall proteins elicit in *S. domuncula* the same response, increase of the lytic (lysozyme) activity, as in invertebrates (e.g. insects: Morishima et al. 1995) or vertebrates (e.g. human: Cole et al. 1999). This finding corroborates recent findings that the innate immune repertoire in sponges occurs with the same/similar diversity as in other metazoans and, even more surprising, comprises elements that are inducible. This property has been described recently also for the tachylectin lectin (Schröder et al. 2003), or the (1 → 3)-*β*-D-glucan binding receptor (Perović-Ottstadt et al. 2004). In the insects lysozyme is produced by their immune cells, the coelomocytes (Goven et al. 1994), and in mammals in the cells of the epithelial layers (e.g. Cole et al. 1999). In *S. domuncula* the *lysozyme* gene is expressed especially in cells that are embedded in the mesohyl compartment, which is lined by a surface layer of pinacoderm cells. The mesohyl in sponges is very bulky and composed of lectins, collagen, and glycoproteins (Müller 1982). There, the 25-µm large cells, intensively stained with hematoxylin/eosine, were identified as those that strongly express the *lysozyme* gene.

Based on earlier cytological and functional observations these 25-µm large cells are the gray cells (Boury-Esnault and Doumenc 1979); they have been described as eosinophiles displaying phagocytic activity (Sarà 1955). In the mesohyl compartment the symbiotic as well as the "infectious" bacteria are found (reviewed in Simpson 1984). Especially the symbiotic bacteria occur in clusters in specialized cells, the bacteriocytes (Lévi and Porte 1962); this localization has also been described for *S. domuncula* (Böhm et al. 2001). Besides the gray cells also the archaeocytes have been demonstrated to phagocytose bacteria and digest them in the mesohyl (Weissenfels 1983, and reviewed in Simpson 1984), from which the released bacterial proteins/particles are transported to the surface cells, the pinacocytes.

Striking and impressive was the finding that antibodies against the sponge lysozyme stain bacteria very brightly and (almost) none of the other cells that are scattered within the mesohyl. The antibodies failed to recognize those bacteria that are present in clusters within bacteriocytes. This result suggests that only the extracellularly present bacteria are digested by lysozyme while the intracellular microorganisms are protected against the lytic enzyme. From a previous study it can be assumed that the bacteria in the bacteriocytes are symbiotic (Böhm et al. 2001; Müller et al. 2004b). In consequence, the elimination process of the gram-positive bacteria, present in a scattered arrangement in the extracellular space of the mesohyl, can be divided into an activation phase of gray cells and an effector phase. During the activation phase PPG causes an increased expression of the genes encoding AdaPTin and also lysozyme; during the effector phase the bacteria are lysed extracellularly in the mesohyl (Fig. 8).

Even though it has been suggested since Metchnikoff (1892) that sponges phagocytose/endocytose bacteria this process has not been demonstrated on the molecular level. In eukaryotic cells the clathrin-coated vesicles mediate the endocytosis of surface receptors and also

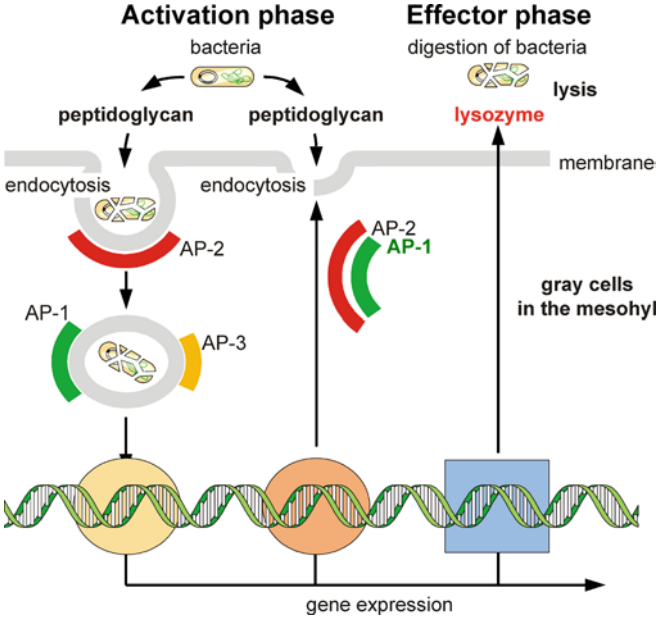


Fig. 8 Scheme describing the immune response of *S. domuncula* toward exposure to gram-positive bacteria. The microorganisms are taken up by endocytosis and PPG is released. This glycan causes during the activation phase an induction of gene expression for *AdaPTin* and *lysozyme* in the gray cells. During the effector phase lysozyme lyses the bacteria and their PPG

extracellular bacteria (Le Borgne and Hoflack 1998). The formation of these vesicles is initiated by the assembly of the adaptor proteins (AdaPTins) that form complexes on the target membranes. The AdaPTins are highly conserved proteins in the metazoans (Boehm and Bonifacino 2001). For the studies here the AdaPTin protein AP-1, the small sigma 2 subunit protein, had been selected for the isolation of the corresponding gene. As expected, this sponge molecule AP1_SUBDO was found again as the ancestral molecule of this group of adaptor proteins for Metazoa. AP-1 molecules function together with clathrin and are involved in the arrangement of the *trans*-Golgi network and/or the endosomes (Boehm and Bonifacino 2001). Northern blot studies, to estimate the steady-state expression of this *SDAP1* gene, revealed that it undergoes strong upregulation after the exposure of sponge tissue to PPG. This increase was seen as early as after a 1-day exposure. The simultaneous determination of the expression level of the *lysozyme* gene reveals that the expression of the enzyme was delayed by approximately 1 day. These expression studies show that a coordinated expression proceeds in which first those molecules appear after exposure to PPG that are involved in the endocytic process, while the extracellular digestion of the bacteria occurs after a lapse of an additional day (Fig. 8).

In conclusion, these studies indicate that gram-positive bacteria elicit in sponges an immune response, which is triggered by the cell-wall PPG. As known also from the $(1 \rightarrow 3)$-β-D-glucan responsive pathway in *S. domuncula* (Perović-Ottstadt et al. 2004), the activation phase of the cell is paralleled by the upregulation of the expression membrane-associated molecule(s), here the AdaPTin, which proceeds prior to the synthesis of the effector molecules, the lysozyme. Studies are now in progress that describe the enzymic generation of reactive oxygen in response to bacterial infection.

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