Caspase-mediated apoptosis in sponges: cloning and function of the phylogenetic oldest apoptotic proteases from Metazoa

Matthias Wiens, Anatoli Krasko, Sanja Perovic, Werner E.G. Müller*

Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität Mainz, Duesbergweg 6, 55099 Mainz, Germany

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Abstract

Sponges (phylum Porifera) represent the phylogenetically oldest metazoan phylum. These animals have complex cell adhesion and powerful immune systems which allow the formation of a distinct body plan. Consequently, an apoptotic machinery has to be predicted that allows sponges to eliminate unwanted cells accumulating during development. With the marine sponge Geodia cydonium, it is shown that allografts of these animals undergo apoptosis as demonstrated by apoptotic DNA fragmentation. Extracts from allografts contain an enzymic activity characteristic for caspases; as substrate to determine the cleavage activity, Ac-DEVD-AMC was applied. cDNAs encoding predicted caspase-3-related proteins were isolated; they comprise the characteristic structure known from caspases of other metazoan phyla. The two cDNAs are assumed to originate from one gene by alternative splicing; the longer form comprises a caspase recruitment domain (CARD), whereas the shorter one is missing CARD. The expression of sponge caspase genes is up-regulated during allograft rejection. In vivo incubation experiments with Ac-DEVD-CHO (a caspase-3 inhibitor) showed a reduction of apoptotic DNA fragmentation, whereas Ac-LEHD-CHO (an inhibitor of caspase-9) caused no effect. It is concluded, that for the establishment of the metazoan body plan, both the adhesion molecules and the apoptotic molecules (described here) were essential prerequisites.

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1. Introduction

Until 10 years ago, sponges (phylum Porifera) have been considered as multicellular animals merely composed of unspecialized flagellates that are held together by an extracellular matrix (ECM) [1,2]. This view implied that sponge cells are (almost) devoid of any differentiation capacity and are associated within an organism only in a very loose organization pattern. However, by molecular cloning, it became overt that sponges have signal transduction pathways induced by ECM molecules, for example, collagen [3,4] and lectins [5]; these ECM molecules are recognized by cell surface receptors with characteristic metazoan domains and modules, like the immunoglobulins (Ig).

Furthermore, sponges possess transcription factors restricted to Metazoa, for example, the Tcf-like transcription factor (in preparation). The findings that sponges have an immune system which is very related to that found in Deuterostomia, like in mammals, and only distantly related to the systems acting in Protostomia, like in Caenorhabditis elegans or in Drosophila melanogaster (reviewed in Ref. [6]) were very surprising. For example, sponges have (i) Ig-like domains present on cell surface receptors which comprise the features of mammalian variable domains [7] and (ii) immune receptors composed of tyrosine-based inhibitory/activation (ITIM/ITAM) motifs [8].

The new findings that sponges have complex gene regulatory systems that allow the formation of a distinct body plan led us to suggest that these metazoans are provided also with the regulated mechanism of a genetically programmed cell death, apoptosis. Because all sponge species have a defined final size and shape, it seems to be a prerequisite for the survival of these multicellular organisms, once the development is completed, to be provided with a controlled maintenance and renewal of the diverse...
sets of cell types. As can be deduced from the findings with metazoan animals, from Cnidaria [9] to mammals [10], also the processes of apoptosis in sponges might be prone to physiological, self-destructing signals executed by the neighboring cells and the ECM, and/or by pathological, toxic insults. Experimental evidence has been presented that marine sponges, for example Geodia cydonium and Suberites domuncula, undergo apoptosis during formation of the asexual reproduction bodies, the gemmules, [11] and also in response to environmental stress [12]. First molecular studies concentrated on the elucidation of molecules which are involved in the initiation of the apoptotic pathway, the death receptors, and the anti-apoptotic, cell survival proteins. As one molecule shown to be involved in the death receptor signaling complex, the proapoptotic molecule DD2 comprising two death domains has been cloned [13]; it is involved in cell death seen in response to allograft rejection [14]. Major molecules known to regulate cell survival are the Bcl-2 and related cytoplasmic proteins [15]. Also these have been isolated from G. cydonium and S. domuncula [13]; the G. cydonium Bcl-2-related gene was even found to confer resistance against environmental stress to human cells [16].

In previous studies, it was shown that the key molecules, the proapoptotic molecule DD2 as well as the cell survival proteins, Bcl-2-related molecules, have no obvious homologues in the Saccharomyces cerevisiae genome even though regulatory apoptotic mechanisms have been described in those multicellular organisms (see Ref. [17]). Therefore, it was pressing to analyze if the executor molecules, the caspases, have evolved together with the appearance of the earliest Metazoa, the Urmetazoa [18]. In the present study, we demonstrate that in sponges, caspases exist, which comprise the features of the related enzymes known from higher metazoan phyla; sequence comparison revealed that the caspases in Porifera, here the marine sponge G. cydonium was studied, have — like the above-mentioned DD2/Bcl-2 proteins — no sequence relationship to yeast molecules. The data add further support to the assumption that sponges are model organisms to understand basic pathways which are characteristic for Metazoa.

2. Materials and methods

2.1. Materials

Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained as described earlier [19,20]. Ac-DEVD-AMC, Ac-DEVD-CHO and Ac-LEHD-CHO were from the Peptide Institute (Osaka, Japan). In addition, digoxigenin (DIG) DNA labeling kit, DIG-11-dUTP, anti-DIG AP Fab fragments, disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.1 3,7]decan}-4-yl)phenyl phosphate (CDP), “Cell Death Detection ELISA” and positively charged nylon membrane (no. 1209272) were from Roche (Mannheim, Germany).

2.2. Sponges

Specimens of the marine sponge G. cydonium (Porifera, Demospongiae, Geodiidae) were collected in the northern Adriatic near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) at a temperature of 17 °C.

2.3. Allo- and autografting procedure

The “insertion technique” was used for the grafting experiments [7,21]. Tissue pieces from the same (autograft) or from a different specimen (allograft) were removed with a cork drill (diameter of 1 cm; approximate length of 4 cm) and inserted into holes of the host, which had a slightly narrower diameter (0.9 cm). The sponges were kept for up to 5 days before being subsequently analyzed. Tissue samples were removed from the grafts and used for extraction of DNA (analysis of fragmented DNA), protein (caspase-3 assay) or RNA (Northern blotting). Control tissue samples were taken from an animal not involved in grafting.

2.4. In vivo effect of caspase inhibitors in grafts

To determine if also in vivo a caspase is involved in the process of apoptosis in allografts, the effects of two caspase inhibitors were determined. Ac-DEVD-CHO is known to inhibit specifically caspase-3, whereas the compound Ac-LEHD-CHO causes a relative inhibitory specificity for caspase-9 [22]. The compounds were added immediately after grafting of allogeneic tissue in a concentration of 10 μM. The specimens (graft with host) were subsequently incubated in the presence of the inhibitor for up to 5 days. Tissue samples of 5 g were removed at time zero (immediately after transfer into Ac-DEVD-CHO or Ac-LEHD-CHO containing seawater) from the grafts, or after 1, 3, or 5 days. Then apoptotic fragmentation was determined using the photometric immunoassay “Cell Death Detection ELISA” as described below.

2.5. Extract preparation

Tissue was extracted with lysis buffer (pH 7.4; 10 mM N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES)/KOH, 5 mM DTT, 4.2 mM EDTA, 1 mM PMSF and 0.1% [w/v] CHAPS) for 20 min at 4 °C. After centrifugation (14,000 x g; 10 min), cleared extract was collected and analyzed in the “Ac-DEVD-AMC cleavage assay”.

2.6. Ac-DEVD-AMC cleavage assay

The Ac-DEVD-AMC cleavage activity was determined as described [23]. The assay is based on the spectrophoto-
metric detection of the chromophore AMC (7-amino-4-methylcoumarin) after cleavage from the labeled substrate Ac-DEVD-AMC by caspase-3 (or caspase-3-like) protease. Released AMC was monitored continuously in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm [24]. The activity is given fluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm [24].

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caspase-3 (long form) from aa 273 to aa 277. This primer, 5‘-AAA/GCCIAAA/GC/GTIC/GTIA/TTA/C/TGTICAA/GGCITGC/T-3’ (I = inosine) was used in conjunction with a vector-specific primer. The PCR was carried out using a GeneAmp 9600 thermal cycler (Perkin Elmer), with an initial denaturation at 95 °C for 3 min, then 35 amplification cycles each at 95 °C for 30 s, 51 °C — 45 s, 74 °C — 15 min, and a final extension step at 74 °C for 10 min as described [29]. The amplified product ( ≈ 600 bp) was purified and used for screening of the library [30]. The plasmid DNAs were sequenced with an automatic DNA sequenator [Li-Cor 4200]. Two sets of longest inserts, GEOCYCAS3s and GEOCYCAS3l, have been obtained that had sizes of 1170 nt (GEOCYCAS3s) and 1412 nt (GEOCYCAS3l; excluding the poly(A) tail), respectively.

For protein determination, the Fluoram method was used; the standard was bovine serum albumin [25].

2.7. Determination of apoptosis

To determine apoptotic fragmentation in G. cydonium tissue, the photometric immunoassay “Cell Death Detection ELISA” was applied. Tissue samples were removed and treated with Ca2+- and Mg2+-free artificial seawater containing EDTA [26,27] to allow dissociation into single cells. As described in the Manual provided by Roche, the cells were centrifuged (500 × g; 5 min) and the pellets were treated with lysis buffer (30 min; room temperature) after transfer to microtiter plates. After collecting the nuclei by centrifugation (2000 × g; 5 min), the released nucleosomes were assayed using the one-step sandwich immunoassay (using the biotin-labeled anti-histone and peroxidase-conjugated anti-DNA antibodies). The nucleosomes in the lysate were immobilized onto streptavidin-coated wells. After washing with PBS, the amount of nucleosomes was determined with the peroxidase substrate ABTS. The colored product, representing the immobilized nucleosomes, was determined at an absorbance at 405 nm and corrected for the absorbance at 480 nm. The values found were correlated with the amount of DNA present in the samples before lysis. DNA was determined according to a standard assay [28]. Five independent assays had been performed.

2.8. Polymerase chain reaction cloning of the two putative G. cydonium caspase-3 molecules

The two complete sponge cDNAs, encoding the short form, GEOCYCAS3s, as well as the long form, GEOCYCAS3l, of caspase-3 proteins (CAS3s_GEOCY and CAS3l_GEOCY), were cloned by polymerase chain reaction (PCR) from the G. cydonium cDNA libraries [5]. The degenerate forward primer included the conserved QACxG pentapeptide, containing the catalytic Cys. This stretch is located in the large subunit of caspases and found in the G. cydonium caspase-3 (short form) from aa 163 to aa 167 and in caspase-3 (long form) from aa 273 to aa 277. This primer, 5‘-AAA/GCCIAAA/GC/GTIC/GTIA/TTA/C/TGTICAA/GGCITGC/T-3’ (I = inosine) was used in conjunction with a vector-specific primer. The PCR was carried out using a GeneAmp 9600 thermal cycler (Perkin Elmer), with an initial denaturation at 95 °C for 3 min, then 35 amplification cycles each at 95 °C for 30 s, 51 °C — 45 s, 74 °C — 15 min, and a final extension step at 74 °C for 10 min as described [29]. The amplified product ( ≈ 600 bp) was purified and used for screening of the library [30]. The plasmid DNAs were sequenced with an automatic DNA sequenator [Li-Cor 4200]. Two sets of longest inserts, GEOCYCAS3s and GEOCYCAS3l, have been obtained that had sizes of 1170 nt (GEOCYCAS3s) and 1412 nt (GEOCYCAS3l; excluding the poly(A) tail), respectively.

2.9. Sequence comparisons

The sequences were analyzed using computer programs BLAST [31] and FASTA [32]. Multiple alignments were performed with CLUSTAL W Ver. 1.6 [33]. Phylogenetic trees were constructed on the basis of aa sequence alignments by neighbour joining, as implemented in the “Neighbour” program from the PHYLIP package [34]. The distance matrices were calculated using the Dayhoff PAM matrix model as described [35]. The degree of support for internal branches was further assessed by bootstrapping [34]. The graphic presentations were prepared with GeneDoc [36].

2.10. Northern blot

RNA was extracted from liquid-nitrogen pulverized sponge tissue with TRIzol Reagent (GibcoBRL, Grand Island, NY). Poly(A)+-RNA was purified from sponge tissue with Oligotex mRNA kit (QIAGEN) and analyzed. Then an amount of 1 µg of total RNA was electrophoresed through 1% formaldehyde/agarose gel and blotted onto Hybond N+ membranes following the manufacturer’s instructions (Amerham, Little Chalfont, Buckinghamshire, UK) [37]. Hybridization was performed with a 0.5 kb part of GEOCYCAS3l. The probe was labeled with the PCR-DIG-Probe-Synthesis Kit according to the “Instruction Manual” (Roche). For the quantification of the Northern blot signals, the chemiluminescence procedure was applied [38]; CDP-Star was used as substrate. The screen was scanned with the GS-525 Molecular Imagcr (Bio-Rad). In a parallel blot and using the vector-specific primer. The PCR was carried out using a GeneAmp 9600 thermal cycler (Perkin Elmer), with an initial denaturation at 95 °C for 3 min, then 35 amplification cycles each at 95 °C for 30 s, 51 °C — 45 s, 74 °C — 15 min, and a final extension step at 74 °C for 10 min as described [29]. The amplified product ( ≈ 600 bp) was purified and used for screening of the library [30]. The plasmid DNAs were sequenced with an automatic DNA sequenator [Li-Cor 4200]. Two sets of longest inserts, GEOCYCAS3s and GEOCYCAS3l, have been obtained that had sizes of 1170 nt (GEOCYCAS3s) and 1412 nt (GEOCYCAS3l; excluding the poly(A) tail), respectively.

3. Results

3.1. Allograft rejection in G. cydonium

Grafting experiments were performed as described previously [7,21]. In Fig. 1, it is shown that G. cydonium (Fig.
1A) can distinguish between specimens of the same species. If grafts consisted of tissue from the same specimen, they fused completely (Fig. 1B); the host/graft zone became invisible 5 days after transplantation (Fig. 1C). Allografts rejected each other and the graft underwent tissue resorption (Fig. 1D).

3.2. Induction of apoptosis in sponges during allograft rejection

As described earlier, sponges undergo apoptotic removal of unwanted tissue, developed during exogenous and endogenous apoptotic processes [39]. Previously, the TUNEL procedure was used to demonstrate that during allograft rejection, DNA in those tissues undergoes fragmentation which is characteristic for apoptosis [14]. In addition, DNA fragmentation was analyzed by gel electrophoresis. In control tissue used for transplantation, no degradation of DNA is seen; however, if DNA is analyzed from allografts, DNA fragmentation to \( \leq 200 \text{bp} \) can be detected. In contrast, in autografts, no sign of DNA fragmentation is seen (Ref. [14]; and not shown).

3.3. Identification of caspase-3 activity in allografts

The first evidence that during the process of DNA fragmentation in allografts caspases are activated, came from studies using one substrate for caspase-3. Extracts were prepared from tissue samples taken from auto- and allografts and protein was extracted. The Ac-DEVD-AMC cleavage assay was applied. The protease activity in the extracts from both controls or autografts (5 days after transplantation) is low and amounts to 80 units/mg (3 h of reaction time) of protein (Fig. 2A). However, in extracts prepared from 5-day-old allografts, the activity is high and reaches 580 units/mg (Fig. 2A). In one series of experiments, the supernatant from allografts, taken 5 days after transplantation, was preincubated (30 min, 4 °C) with 4 \( \mu \text{M} \) of Ac-DEVD-CHO, an inhibitor for caspase-3 activity. This preparation was subsequently used in the enzymic assay using Ac-DEVD-AMC as substrate. After this pretreatment, the caspase activity was found to be reduced by over 82% (data not shown).

The caspase-3-like activity increases steadily during the 5 days incubation period (Fig. 2B). At time zero, a level of 80 units/mg is found in both auto- and allografts. This amount increases significantly already 1 day after grafting to 230 units/mg while the maximum is measured after 5 days with 620 units/mg (Fig. 2B). Based on these results, we had strong evidence for the presence of a caspase-3-like enzyme in the sponge G. cydonium.
3.4. Cloning of two forms of caspase-3-like proteins from *G. cydonium*

The cDNAs encoding the caspase-3-related polypeptides were isolated from the sponge *G. cydonium* as described under “Materials and methods”. Two forms have been obtained. The long form whose nt sequence, GEOCYCAS3l is 1413 nt long and has an ORF of 1278 nt (accession number AJ344144) encodes a 426-aa-long deduced protein sequence (Fig. 3). The short form, GEOCYCAS3s, with a length of 1178 nt has an ORF from nt107 to nt1054 encoding a 316-aa-long protein (accession number AJ417903). It is interesting that the nt sequences of the two cDNAs are identical within the ORFs starting from nt370 (long form) and from nt40 (short form), suggesting that two cDNAs derived from one gene by alternative splicing. Northern blot analyses performed with both sponge clones as a probe yielded one band of 1.6 kb (GEOCYCAS3l) and 1.3 kb (GEOCYCAS3s), respectively. These sizes confirm that full-length cDNAs had been isolated (see below).

The predicted translation product of the long form GEOCYCAS3l, 426 aa in length (Fig. 3), has a size of *M*$_r$ 48,024 and a *pI* of 5.2. The calculated size of the small form deduced from GEOCYCAS3s is 35,319 kDa comprising 316 aa, with *pI* of 5.6. The polypeptide predicted from the short cDNA, termed CAS3s_GEOCY, shares highest sequence similarity to the human caspases-3 Yama [accession number U26943; the “Expect value” (*E*)] is 4e-17]. Therefore, the sequences were termed caspase-3-related proteins. The long form of the sponge caspase, CAS3l_GEOCY, shares highest sequence similarity with caspases class I based on the presence of a caspase recruitment domain (CARD) [41], which is missing in CAS3s_GEOCY (class II caspases).

The caspases occur as inactive zymogens that undergo activation by proteolysis (reviewed in Ref. [42]). The large and small subunits are formed from the proenzyme by cleavage at Asp(P1)-X(P1') sites. The borders of the large form of the caspase-3 from *G. cydonium* have been predicted following the rules for vertebrate caspases (reviewed in Ref. [43]); they are found within the segments aa$_{120}$–aa$_{281}$ for the large subunit and aa$_{324}$–aa$_{426}$ for the small subunit (Fig. 3); the numbering refers to the large form of the sponge caspase. The size of the large subunit is 161 aa and that of the small subunit is 104 aa (within the large form of caspase-3). The small form of the sponge caspase-3 does not have the Asp cleavage site at the N terminus, suggesting that this polypeptide is not or is unusually processed.

Within the large subunits of caspase, in general, two consensus stretches are present that frame the aa residues His and Cys which are involved in the catalytic mechanism of caspases [43]. Also the sponge caspases comprise in the deduced proteins these two sites, which are characteristic for caspase family active sites (ISREC, Ref. [44] and are found between aa$_{219}$–aa$_{233}$ (HSAYDCVVAILTHG) and aa$_{226}$–aa$_{277}$ (KPVFVQACRG); Fig. 3. Besides these two
residues, His and Cys, two Arg residues are present (at aa175 and aa352; long form) that are brought into structural arrangement for catalysis [43]; Fig. 3.

The caspases can be divided according to the N-terminal prodomain into (i) class I caspases that possess long N-terminal prodomains and (ii) class II caspases that have only short or no prodomains (reviewed in Ref. [41]). Class I caspases, like caspase-8 and -10, associate via protein/protein interactions with their prodomains to the specific death complexes, receptors, as well as soluble molecules that comprise the death domain [45]. In contrast, class II caspases, like caspase-3 and -6, are involved in downstream functions of the death cascade and are involved in cleavage of the target proteins [42], for example, gelsolin [46]. Based on the predicted polypeptide sequences, it must be concluded that the two forms of the sponge caspases should be functionally grouped to class I caspase with the long form of caspase-3 [CAS3l_GEOCY], and to class II caspase with the short form [CAS3s_GEOCY]. Sequence analysis of the prodomain of the sponge caspase-3 (long form) reveals that it comprises the characteristic amino acids found in CARDs [45]. CARD domain interacts with the death effector

Fig. 4. Comparison of the large subunit of the sponge caspase-3 protein with the related molecules. (A) The large subunit of CAS3_GEOCY [short form; CAS3-s_GEOCY] (CAS3l_GEOCY; aa120 –aa281) is aligned with the most related segment of caspase-9 of X. laevis (CAS9l_XENLA; BAA94750.1, aa101 –aa276). Similar aa in white on black. The borders of the subunits and the conserved aa are marked. (B) Unrooted phylogenetic tree constructed after the alignment of the deduced aa sequence of the large subunit of sponge CAS3_GEOCY (CAS3l_GEOCY) with the related sequences from caspase-1 from D. melanogaster (CAS1l_DROME; O02002, aa86 –aa209), human caspase-2 (ICE2l_HUMAN; P42575, aa140 –aa271), human caspase-3 (CAS3l_HUMAN; XP_003524.2, aa140 –aa271), mouse caspase-3 (CAS3l_MOUSE; NP_033940.1, aa97 –aa227), the caspase-3 from Ovis aries (CAS3l_OVIS; AAC25713.1, aa14 –aa123), the fish caspase-3 from Danio rerio (CAS3l_DIANIO; BAB32409 1, aa14 –aa123), caspase-1 [CED3] proteins from C. elegans (CED3l_CAEEL; P42573, aa251 –aa76), Hydra vulgaris caspase-3A (CAS3Al_HYDRA; AF15127_1, aa251 –aa321), H. vulgaris caspase-3B (CAS3Bl_HYDRA; AF15128_1, aa251 –aa321), the D. melanogaster caspase-6 (CAS6l_DROME; AF22007_1, aa199 –aa318), caspase-6 from Oncorhynchus mykiss (CAS6l_ONCOR; AF21221_1, aa199 –aa318), caspase-7 from rat (CAS7l_RAT; NP_071596.1, aa199 –aa318), caspase-7 from Mecocricetus auratus (ICE7l_MESAU; P55214, aa212 –aa316), caspase-7 from X. laevis (CAS7l_XENLA; BAA94748.1, aa212 –aa316), caspase-8 from X. laevis (CAS8l_XENLA; BAA94749.1, aa265 –aa394), mouse caspase-8 (CAS8l_MOUSE; CA07677.1, aa265 –aa394), caspase-9 from rat (CAS9l_RAT; NP_113820.1, aa201 –aa315), caspase-9 from X. laevis (CAS9l_XENLA, aa201 –aa315), human caspase-9 (CAS9l_HUMAN; NP_001220.1, aa165 –aa297) and the human caspase-10 (ICE10AI_HUMAN; Q92851, aa288 –aa501). The numbers at the nodes refer to the levels of confidence as determined by bootstrap analysis. The scale bar indicates an evolutionary distance of 0.1 aa substitution per position in the sequence.
domain [47]; a CARD domain is predicted in the long form of the sponge caspase within the segment spanning from aa8 to aa97 (Fig. 3).

3.5. Phylogenetic analyses of the putative sponge caspases

Based on the facts that (i) the two forms of the sponge caspases can be grouped to both classes of caspases with respect to the domain composition and that (ii) the overall amino acid sequences of the large and small subunits of the sponge enzymes are identical, a clear homology to known caspases from vertebrates was not expected. Therefore, the large and the small subunits of the sponge caspases (long form was chosen for this comparison) were aligned with the next closest related sequences found in the BLAST data-bank. Caspases were selected which had been classified to the group I caspases, caspase-1, group II caspases, caspase-2, -3 and -7, as well as group III caspases, caspase-6, -8, -9 and -10 [42]. With caspase-4 and -5, no high homology was found.

The unrooted tree built from the large subunit of the sponge caspases with the related subunits from metazoan caspases revealed a high similarity to the D. melanogaster caspase-6 (AF222007_1) and vertebrate caspase-9, for example, from Xenopus laevis (BAA94750.1); Fig. 4A and B. The sponge large subunit shares 65% similar and 39% identical amino acids with those related subunits; the relationships to the other classes of caspases is only slightly less [≈ 55% similarity and ≈ 33% identity]. No obvious sequence similarity exists to other multicellular organisms, for example, S. cerevisiae or Arabidopsis thaliana.

An equally high similarity with respect to caspases from other metazoan phyla was found for the small subunit of the sponge caspases (Fig. 5A). In the unrooted tree, the highest similarity/identity is found with the human caspase-6 (XP_003600.2), caspase-10 (e.g. human caspase-10;
NP_001221.1) and human caspase-8 (AAD24962.1) with \( \approx 60\% \) similarity and \( \approx 40\% \) identity; Fig. 5B.

### 3.6. Expression of caspase

The expression of the sponge gene, encoding the caspase(s), was studied in tissue from allografts as well as from autografts. It is seen that the expression level of GEOCYCAS3 in both autografts (5 days after transplantation) and in control tissue was almost identical (Fig. 6A, lanes b and c). However, if RNA from allografts (in 5-day-old grafts) was analyzed, then a strong up-regulation was seen (lane a). Setting the expression level of the gene in control tissue to 1-fold, a 4.2-fold expression of the caspase gene was monitored in tissue from allografts. Besides the 1.6-kb transcripts (corresponding to the long form of GEOCYCAS3l) which were clearly seen, also the transcripts of the 1.3 kb short form (GEOCYCAS3s) were visible. In a parallel experiment, it was shown that the same amount of RNA was loaded onto the gels as demonstrated by the strength of the signals which appear from the hybridization with the *G. cydonium* \( \beta \)-tubulin as a probe (Fig. 6B). From these data, we conclude that during grafts rejection in allogeneic tissue, the expression of caspases increases.

### 3.7. Involvement of caspase-3 in the process of apoptosis during allograft rejection

The effects of two inhibitors of caspases were determined during allograft reaction in vivo; Ac-DEVD-CHO, an inhibitor of caspase-3 and Ac-LEHD-CHO (caspase-9 inhibitor) as described under “Materials and methods”. Immediately after grafting (time 0) or 1, 2, or 3 days later, tissue specimens were taken and the integrity of the DNA was assayed for apoptotic fragmentation using a photometric immunoassay. The amount of colored product was measured spectrophotometrically. The absorbance values were correlated to 50 ng of DNA (present in the samples before lysis). The values came from five independent determinations each; the means are given (the standard deviations were less than 15%).

![Fig. 6. Expression of caspase gene (GEOCYCAS3) in tissue from G. cydonium during grafting (Northern blot). (A) Five days after transplantation, RNA both from allografts and autografts was isolated and poly(A)\(^+\)-RNA was purified. After size separation and blot transfer, the caspase transcripts are detected by the GEOCYCAS3 probe. In lane a, the steady-state level of this gene in allografts is shown; in parallel, the expression in control tissue (lane b) and in autografts (lane c) is shown. The bands corresponding to a size of 1.6 and 1.3 kb are marked. (B) To verify that equal amounts of RNA were loaded, the blots were hybridized with the *G. cydonium* \( \beta \)-tubulin probe.](image)

![Fig. 7. Caspase-3 involvement in allograft reaction. Allografting was performed and the specimens were immediately coincubated with either Ac-DEVD-CHO (an inhibitor of caspase-3) or Ac-LEHD-CHO (caspase-9 inhibitor) as described under “Materials and methods”. Immediately after grafting (time 0) or 1, 2, or 3 days later, tissue specimens were taken and the integrity of the DNA was assayed for apoptotic fragmentation using a photometric immunoassay. The amount of colored product was measured spectrophotometrically. The absorbance values were correlated to 50 ng of DNA (present in the samples before lysis). The values came from five independent determinations each; the means are given (the standard deviations were less than 15%).](image)

### 4. Discussion

Sponges are colonial organisms with a high degree of integrated complexity. An oscule joining several choanocyte chambers can be considered as a functional unit. After several steps of duplication, these units form a modular sponge [48] which reaches the stage of individuation. The
latter process became possible in sponges because the basic molecules of cell—cell and cell—matrix adhesion are present [49] which allow a tuned interplay between the cells. This communication within their own environment as well as with the extra-individual milieu is based on complex intracellular signaling networks which originate from ligand/receptor elements [20,50]. Like other metazoans, sponge species also have a defined body plan which is controlled by an effective immune system, that include the function of cytokines and their interacting receptors [6]. In addition, during the process of apoptosis (programmed cell death), unwanted cells are eliminated that accumulate during development and homeostasis [27,40].

In previous studies, we found in the sponges G. cydonium and S. domuncula polypeptides that trigger apoptosis, for example, extra-individual stressors [11] and (patho) physiological signals [51]. These signals ignite the intrinsic apoptosis machinery in which an adapter protein comprising two death domains [13] becomes up-regulated [14]. The apoptotic process in sponges is under the control of anti-apoptotic/cell survival proteins, encoded by a Bel-2-related gene [13,14,16]. In the present study, the next step in the process that kills the cells is summarized. The major genes that encode enzymes, involved in the executing of the death signal(s), are the caspases found in vertebrates (reviewed in Ref. [9]), which are homologues to CED-3, found in C. elegans (reviewed in Ref. [52]). Likewise complex is the apoptotic pathway in C. elegans (see Refs. [42,53]).

In the first part of this study, it is demonstrated that in sponges — like in vertebrates [54] — apoptosis is an essential element of the immune response. To obtain a first indication that caspases are necessary for apoptosis also in sponges, enzymic assays were performed using the synthetic peptide Ac-DEVD-AMC [24]. It is shown that extracts from allografts have caspase activity which reached 5 days after transplantation high levels. This in vitro cleavage of the substrate could be blocked by the competitive caspase inhibitor Ac-DEVD-CHO [24]. Because Ac-DEVD-AMC is a caspase-3 substrate, this result is a first evidence that such an enzyme exists also in sponges.

This set of experiments indicates that caspase-3 or caspase-3-related enzymes are present in sponges like in other metazoan phyla, phylogenetically younger than Porifera [9,10]. By PCR cloning with degenerate primers designed against the conserved stretch within the large subunit, two forms of cDNAs had been isolated. The deduced proteins revealed high similarity to mammalian caspases. Interestingly enough was the finding that the two forms of cDNA are identical at the ORF spanning the two putative subunits and differ considerably in the N-terminal part of the proteins. This finding strongly suggests that the two cDNAs derive from one gene by alternative splicing. Alternative splicing in the formation of caspase transcripts is not unusual, but the variants formed give rise to caspases from the same group, for example, for caspase-3 [55]. However, the two forms of predicted sponge caspase proteins are distinguished by the presence of the CARD domain [41]. This domain is found in the long form of the caspase-3-related sponge enzyme, whereas in the small sponge caspase, this characteristic feature of class I caspases is missing and in consequence, it should be grouped to the class II caspases [41]. The caspases are synthesized as inactivezymogens and are themselves activated by proteolysis [56] which gives rise to the large and small subunits. The processing sites, the aa residues His and Cys, which are involved in the catalytic mechanism of caspases and the cleavage sites, are present in the sponge enzyme(s) [43].

Until now, screening for further caspase cDNAs in G. cydonium was not successful; however, cDNAs encoding for caspases have recently also been identified in S. domuncula that showed a related sequence similarity. Therefore, we assume that the number of caspases in sponges is much lower than in mammalian systems; in consequence, one could predict that in sponges, the degree of redundancy is coupled with a lower level of regulation. Because the sponge extract cleaves Ac-DEVD-AMC, a substrate for caspase-3 and -related enzymes from this family [22], it is suggestive to assume that the common ancestor of the variety of caspases, which are present only in Metazoa, is a caspase-3-related enzyme. Perhaps the sponge sequence described here represents a prototype. This assumption is also supported by the finding (i) that the small sponge caspase shares highest similarity to caspase-3 enzymes on one hand and (ii) the fact that the long as well as the small subdomains of the sponge caspases share no distinct grouping to the homologous in the different caspases from metazoans, if unrooted trees are constructed, while the overall sponge sequence (short form) shares highest sequence similarity to the human caspases-3 (accession number U26943). The comparisons of the large subunit of the sponge enzyme showed highest similarity/identity to the D. melanogaster caspase-6 (AF222007.1) and the vertebrate caspases-9 (X. laevis; BAA94750.1), while the small subunit comprises highest similarity/identity to human caspase-6 (XP_003600.2), caspase-10 (NP_001221.1) and caspase-8 (AAD24962.1).

In this context, it might be stressed that the relationships between the large subunits of the different caspases and the small subunits is very close [43]. In line with these relationships, it appears to be reasonable to assume that a caspase-3-related enzyme from a sponge was the ancestor for the different groups of vertebrate caspases: class I caspases which comprise the recruitment domain CARD and the class II caspases, the executing caspases, which lack this domain [41]. While in sponges the long form (group I-related) and the short form (group II-related) of the caspase very likely derived from the same gene by alternative splicing, these enzymes are encoded in higher phyla by several genes. This fact implies that during the evolution which proceeded after the sponges, the different caspase subgroups evolved by gene duplication.
To support the documented results on the increased expression of the caspase gene during allograft reaction, functional experiments were performed. The allografts were incubated in seawater supplemented with either Ac-DEVD-CHO (caspase-3 inhibitor) or Ac-LEHD-CHO (caspase-9 inhibitor). After incubation, the extent of DNA fragmentation was determined. The results revealed that only Ac-DEVD-CHO suppressed apoptotic DNA fragmentation, whereas Ac-LEHD-CHO was without effect. This result strongly suggests that also in vivo a caspase-3 (like) enzyme is involved in the initiation of apoptosis during allograft reaction in the G. cydonium system. These data also imply that during allograft reaction, the sponge caspase-3 is proteolytically processed and thereby activated. Moreover, because the sponge caspase does not contain a death domain together with an Ig domain and also no zinc finger motif, it should not be classified to the paracaspases or the meta-caspases [57].

In higher bilaterian phyla, the caspases are activated by increase of zymogen concentration through zymogen clustering in the vicinity of the death receptor(s), under formation of the death-inducing signaling complex [58,59], rather than by increase of gene transcription. In the sponge apoptotic system using allografts, it is shown that in tissue which underwent apoptosis in response to the rejection process, an induced steady-state level of 1.6 kb caspase-3 transcripts is measured. This finding is taken as an indication that caspase-mediated apoptosis is also controlled on the transcriptional level.

Taken together, the data reported here provide evidence that with the appearance of sponges during evolution, the apoptotic machinery with the caspases as the executing enzymes was established. The similarity of the sponge caspase(s) to the caspases known from other metazoan molecules (reviewed in Ref. [8]) and the apoptotic molecules (described here) were essential prerequisites. In continuation of this line of research, investigations are now in progress which address the question for the sequence and potential function of the caspase-activated DNase (see Ref. [60]).

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References
