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Origin of the interferon-inducible (2′-5′)oligoadenylate synthetases: cloning of the (2′-5′)oligoadenylate synthetase from the marine sponge *Geodia cydonium*[1]

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Abstract In vertebrates cytokines mediate innate (natural) immunity and protect them against viral infections. The cytokine interferon causes the induction of the (2′-5′)oligoadenylate synthetase [(2-5)A synthetase], whose product, (2′-5′)oligoadenylate, activates the endoribonuclease L which in turn degrades (viral) RNA. Three isoforms of (2-5)A synthetases exist, form I (40–46 kDa), form II (69 kDa), and form III (100 kDa). Until now (2-5)A synthetases have only been cloned from birds and mammals. Here we describe the cloning of the first putative invertebrate (2-5)A synthetase from the marine sponge *Geodia cydonium*. The deduced amino acid sequence shows signatures characteristic for (2-5)A synthetases of form I. Phylogenetic analysis of the putative sponge (2-5)A synthetase indicates that it diverged first from a common ancestor of the hitherto known members of (vertebrate) (2-5)A synthetases I, (2-5)A synthetases II and III. Moreover, it is suggested that the (2-5)A synthetases II and III evolved from this common ancestor (very likely) by gene duplication. Together with earlier results on the existence of the (2′-5′)oligoadenylates in *G. cydonium*, the data presented here demonstrate that also invertebrates, here sponges, are provided with the (2-5)A system. At present, it is assumed that this system might be involved in growth control, including control of apoptosis, and acquired its additional function in innate immune response in evolutionarily younger animals, in vertebrates.

Key words: (2′-5′)Oligoadenylate synthetase; Animal; Monophyly; Immunology; Interferon; Cytokine; *Geodia cydonium*

1. Introduction

The evolution of immunology has been widely neglected until today [1] especially in the taxon of invertebrates, even though more than 95% of all known species belong to this group [2]. Two mechanisms of immunity have been studied extensively in vertebrates, the adaptive immune response and the innate (natural) immune response (reviewed in [3]). Since there is no evidence for the presence of a major histocompatibility complex or high concentrations of circulating immunoglobulins (Ig) in invertebrates, it is generally assumed that the adaptive immune system is a vertebrate invention (reviewed in [4]). However, recently advances have been made especially by molecular biological approaches to detect at least some traits of the latter system in invertebrates, e.g. the existence of molecules revealing similarity to vertebrate antigen receptors in tunicates [5], or the high polymorphism of the gene encoding Ig-like domains of a receptor tyrosine kinase in sponges [6].

With the transition from unicellular to multicellular animals, defence mechanisms (i) against infection posed by foreign invaders and (ii) to distinguish self from non-self (reviewed in [7]) had to be developed. The biological model of choice to study the origin of the metazoan immune system is the sponges (Porifera), which represent the only extant metazoan phylum that already existed prior to the 'Cambrian explosion' [8]. It was Metchnikoff [9] who demonstrated that echinoderm macrophage-like cells participate in host defence by phagocytosis of foreign material as an early immune response. It is now established that macrophages and monocytes in mammals and hemocytes of invertebrates mediate many innate immune responses to microbial infection (reviewed in [10]). An innate immune system is activated when pathogens bind to non-clonally distributed pattern recognition receptors on immune cells [11]. Among those are the scavenger receptor(s), which have been implicated both in vertebrate and in invertebrate (insect) non-self recognition [12].

Recently, we reported that also in Porifera (the marine sponge *Geodia cydonium*) an amazingly polymorphic system of scavenger receptors, composed both of scavenger receptor cysteine-rich domains of group A [13,14] and group B [15], and of short consensus repeats [15], is present. These receptors are located at the cell surface [14]. Biochemical evidence suggests that invertebrate cytokine-related molecules, e.g. interleukin in echinoderms [16], or tumor necrosis factor in sponges [17], exist and may be involved in non-self recognition.

In the last 3 years our group has discovered that in tissue from the sponge *G. cydonium* high levels of (2′-5′)oligoadenylates [(2-5)A_n] are synthesized [18]. In mammalian cells (2-5)A_n is found after viral infections (reviewed in [19]). The oligomer (2-5)A_n with a chain length of $2 \leq n \leq 9$ is synthesized from ATP by the enzyme (2′-5′)oligoadenylate synthetase [(2-5)A synthetase; EC 2.7.7.-] [20–22]. (2-5)A_n activates the endoribonuclease L [23,24] that consequently degrades RNA (reviewed in [25]). In vertebrate systems the expression of (2-5)A synthetase is under control of interferons. These initiate a signal transduction pathway, via the interferon receptors-JAK/STAT pathway (reviewed in [26]); ultimately the transcription factor ISGF is bound to the ISRE elements, which are located upstream of interferon-responsive genes, thus activating their transcription [27]. Among the interferon-responsive genes is the one which encodes (2-5)A synthe-

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[1] The sequence reported here is deposited in the EMBL/GenBank data base (accession number Y18497).

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tase [28]. Three isoforms have been described in mammalian systems [20,29,30]: (2-5)A synthetase I corresponding to 40–46 kDa, (2-5)A synthetase II with 69 kDa, and (2-5)A synthetase III with 100 kDa. Forms I and II are present in the nucleus and the mitochondria and are associated with the microsomes [20]. The (2-5)A synthetases are known to be activated by binding to double-stranded RNA (dsRNA) (reviewed in [31,32]). (2-5)A synthetase I has been cloned from mouse [33,34], rat [22,35], human [36] and chicken [37]. (2-5)A synthetase II has been isolated from human [38]; the 683 aa long open reading frame (ORF) of the cDNA encoding (2-5)A synthetase II is composed of two very similar parts [38]. (2-5)A synthetase III has been recently cloned also from human [39]; the p100 synthetase ORF is composed of three adjacent synthetase domains and is presumably involved in splicing of pre-messenger RNA.

Until now, (2-5)A synthetases have been identified enzymatically or by molecular cloning in vertebrates. In non-vertebrate systems the product of (2-5)A synthetase, (2-5)A_n, has only been unequivocally characterized in the sponge *G. cydonium* by both biochemical and immunological methods [18] and recently also by matrix-assisted laser desorption-ionization time-of-flight and nuclear magnetic resonance analyses as well [40]. The activity of the enzyme (2-5)A synthetase was also identified in *G. cydonium* [18].

Here we describe the cloning of a first putative invertebrate (2-5)A synthetase, identified in the sponge *G. cydonium*, and provide evidence that the deduced amino acid sequence is the predecessor of the present-day (2-5)A synthetases I, as well as of the two segments of (2-5)A synthetase II or the three domains in the (2-5)A synthetase III.

2. Materials and methods

2.1. Materials

Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained from Stratagene (La Jolla, CA, USA), Qiagen (Hilden, Germany), Boehringer (Mannheim, Germany), USB (Cleveland, OH, USA), Amersham (Buckinghamshire, UK) and Promega (Madison, WI, USA).

2.2. Sponge

Specimens of *G. cydonium* [Jameson] (Metazoa; Porifera, Demospongiae, Tetractinomorpha, Astrophorida, Geodiidae) were collected near Rovinj (Croatia) from a depth of 25 m at 17°C from their habitat, the muddy sand bottoms. They were kept in aquaria in Mainz (Germany) for up to 8 months at a temperature of 16°C.

2.3. Isolation of a cDNA encoding the putative G. cydonium (2-5)A synthetase

The complete sponge *GC2-5AS* cDNA was cloned by polymerase chain reaction (PCR) from the *G. cydonium* cDNA library in λZAP Express [41]. The degenerate reverse primer 5′-YGY HGG RTY IGC IGG RTC-3′ (where I = inosine, Y = T/C, H = T/A/C, R = A/G) in conjunction with the ZAPII 5′-end vector-specific forward primer T3 was used. The degenerate primer was designed against the conserved amino acid (aa) segment in the mouse (2-5)A synthetase I ([33]; accession number P11928) of signature 2, between aa_{302} and aa_{307} (DPADPT). The PCR reaction was carried out using a GeneAmp 9600 thermal cycler with initial denaturation at 95°C for 3 min, then 35 amplification cycles of 95°C for 30 s, 60°C for 45 s, 74°C for 1.5 min, and a final extension step at 74°C for 10 min. The reaction mixture was as described earlier [42]. A fragment of ~900 bp was used to isolate the cDNA [43]. The longest insert obtained had a size of 1141 nt (excluding the poly(A) tail). The clone was termed *GC2-5AS* (Y18497) and was sequenced using an automatic DNA sequenator (Li-Cor 4200).

2.4. Sequence comparisons

Analyses of the sequence composition, structure and features were performed by the computer programs PC/GENE [44]. Homology searches and sequence retrieval were done via the E-mail servers at the European Bioinformatics Institute, Hinxton Hall, UK (BLITZ@ebi.ac.uk) and the National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA (BLAST@ncbi.nlm.nih.gov). A phylogenetic tree was constructed on the basis of aa sequence alignment by neighbor joining [45] applying the PHYLIP ver. 3.5c program [46]; the distance matrix was calculated as described [47]. The degree of support for internal branches was further assessed by bootstrapping [46]. Graphical output of the bootstrap figure was produced by the program 'Treeview' (Roderic D.M. Page, University of Glasgow, UK; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Multiple alignment – with the default options (gap opening: 10.00; gap extension: 0.05; delay divergent sequence: 40%; Blossum series; gap separation distance: 8) – was performed with CLUSTAL W version 1.6 [48], and the graphic presentation was composed with GeneDoc [49]. The automatic alignment was optimized manually.

Hydropathy analysis, based on the method of Kyte and Doolittle [50], was performed using the PC/GENE Soap [44].

2.5. Northern blot

RNA was extracted from liquid nitrogen-pulverized sponge tissue with TRIzol Reagent (Gibco BRL) and the procedure for Northern blot analysis on Hybond N^+ membrane was performed as described [42]. Hybridization experiments were done with the complete cDNA for (2-5)A synthetase from *G. cydonium*, *GC2-5AS*, as a probe. The probe was labeled with DIG-11-dUTP by the DIG DNA labeling kit. After hybridization the DIG-labeled nucleic acid was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase) and visualized by the chemiluminescence technique using CDP, the chemiluminescence substrate alkaline phosphatase, according to the instructions of the manufacturer (Boehringer).

3. Results and discussion

3.1. Cloning of a cDNA encoding the putative (2-5)A synthetase from G. cydonium

Applying a degenerate reverse primer, designed against one conserved region of the (2-5)A synthetase I (see Section 2) a fragment of ~900 bp was obtained. The complete cDNA, *GC2-5AS*, 1141 nt in length (EMBL accession number Y18497) encoding the putative sponge (2-5)A synthetase was isolated by primer walking. The size of the transcript was 1.3 kb (Fig. 1), indicating that the complete cDNA was obtained. The first ATG, position 79, starts the ORF of 981 bp. The ATG codon is surrounded by a medium strong Kozak consensus sequence which in *GC2-5AS* reads GAT**ATG**G (the start codon is in bold) [51].

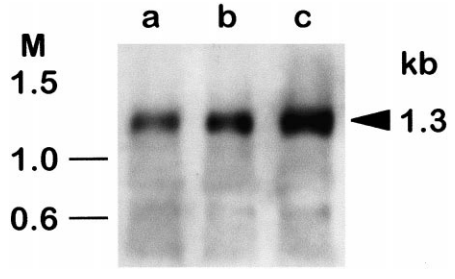

Fig. 1. Northern blot analysis of *GC2-5AS*. Two (lane a), four (lane b) and six μg (lane c) of *G. cydonium* total RNA were electrophoresed through 1% formaldehyde/agarose gel, blotted and hybridized to the DIG-labeled *GC2-5AS*. Left: Molecular weight markers (M; in kb).

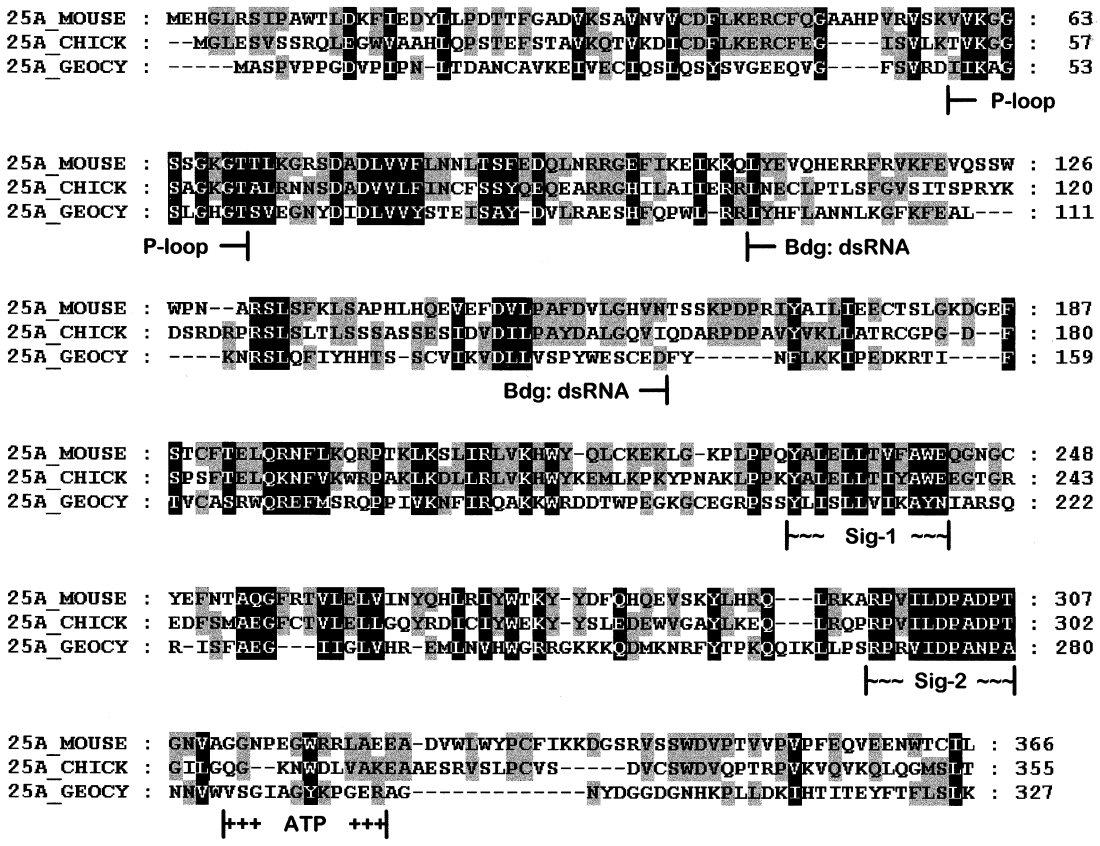

Fig. 2. Alignment of the deduced aa sequence of the *G. cydonium* putative (2-5)A synthetase, 25A_GEOCY (Y18497), with the corresponding proteins from mouse (25A_MOUSE, accession number P11928 [33]) as well as from chicken (25A_CHICK, AB002586 [37]). The sequences were truncated as follows: mouse at aa 366 and chicken at aa 355. The alignment was performed using the CLUSTAL W program. Amino acid residues similar among all three sequences are in inverted type and residues conserved in at least two sequences are shaded. The characteristic signatures of the (2-5)A synthetase are indicated: the two conserved signatures (Sig-1 and Sig-2), the P-loop motif (P-loop), the potential ATP binding region (ATP) and the dsRNA binding segment (Bdg: dsRNA).

3.2. The deduced aa sequence

The deduced 327 long aa sequence from the ORF termed 25A_GEOCY has a calculated peptide mass of 37 400 Da and an isoelectric point of 9.6 ([44]; Physchem program). The putative sponge (2-5)A synthetase displays the two conserved motifs – of unknown function [52] –, signature 1 between aa_{206} and aa_{217} [Y(A/L)(L/I)xLL(T/V)(V/I)(Y/K) A(Y/W)(E/N)] and signature 2 between aa_{270} and aa_{280} [RPx(I/V)(L/I)DPA(D/N)P (T/A)] (Fig. 2). The P-loop motif [53] in the sponge sequence (aa_{49} to aa_{59}) differs in one position at aa_{52} where glycine is replaced by alanine. The other segment of (2-5)A synthetase essential for enzyme activity [34,54], residues aa_{285} to aa_{296}, which binds to ATP, is also present in the sponge sequence (Fig. 2). By progressive deletion mutation [34] the dsRNA binding region of (2-5)A synthetase has been detected between aa_{104} and aa_{158} of the murine enzyme or aa_{95} and aa_{143} in the sponge sequence. The sponge sequence has been aligned with the mouse and the chicken (2-5)A synthetases I; the conserved regions among all three are highlighted in Fig. 2.

Homology searches (BLITZ and BLAST) with the 25A_GEOCY deduced protein revealed highest similarity to (2-5)A synthetases I. The sponge sequence shares the following percentages of identical aa (similar, including identical, aa) with the two vertebrate sequences: (2-5)A synthetase from mouse (accession number P11928) 18% (39%), and (2-5)A synthetase from chicken (AB002586) 17% (37%) (Fig. 2).

In order to further support the high similarity between the putative sponge (2-5)A synthetase and the mammalian (2-5)A synthetases a hydropathy analysis was performed. The putative sponge (2-5)A synthetase shows hydrophobic peaks for the P-loop motif, the potential ATP binding region, the dsRNA binding segment, and the two conserved (2-5)A synthetase signatures at the positions that are also prominent in the murine sequence (data not shown).

3.3. Phylogenetic analysis and evolution of (2-5)A synthetases

Based on sequence comparisons, and on polypeptide lengths, it has been concluded that the (2-5)A synthetase II is composed of two segments which both are similar to (2-5)A synthetase I [38]. Therefore, the human 69 kDa (2-5)A synthetase II sequence (accession number M87284) was cut at position aa_{336} into two segments; both parts were aligned with the putative sponge (2-5)A synthetase, belonging to class I (Fig. 3A). The identity of the sponge sequence to the NH_2- or COOH-terminus of the human (2-5)A synthetase II is 16% or 17%, while the respective similarities (including the identical aa) were 29% or 33%.

An unrooted phylogenetic tree was constructed with the putative sponge (2-5)A synthetase as well as the two segments of the human (2-5)A synthetase II, the sequences from mouse,

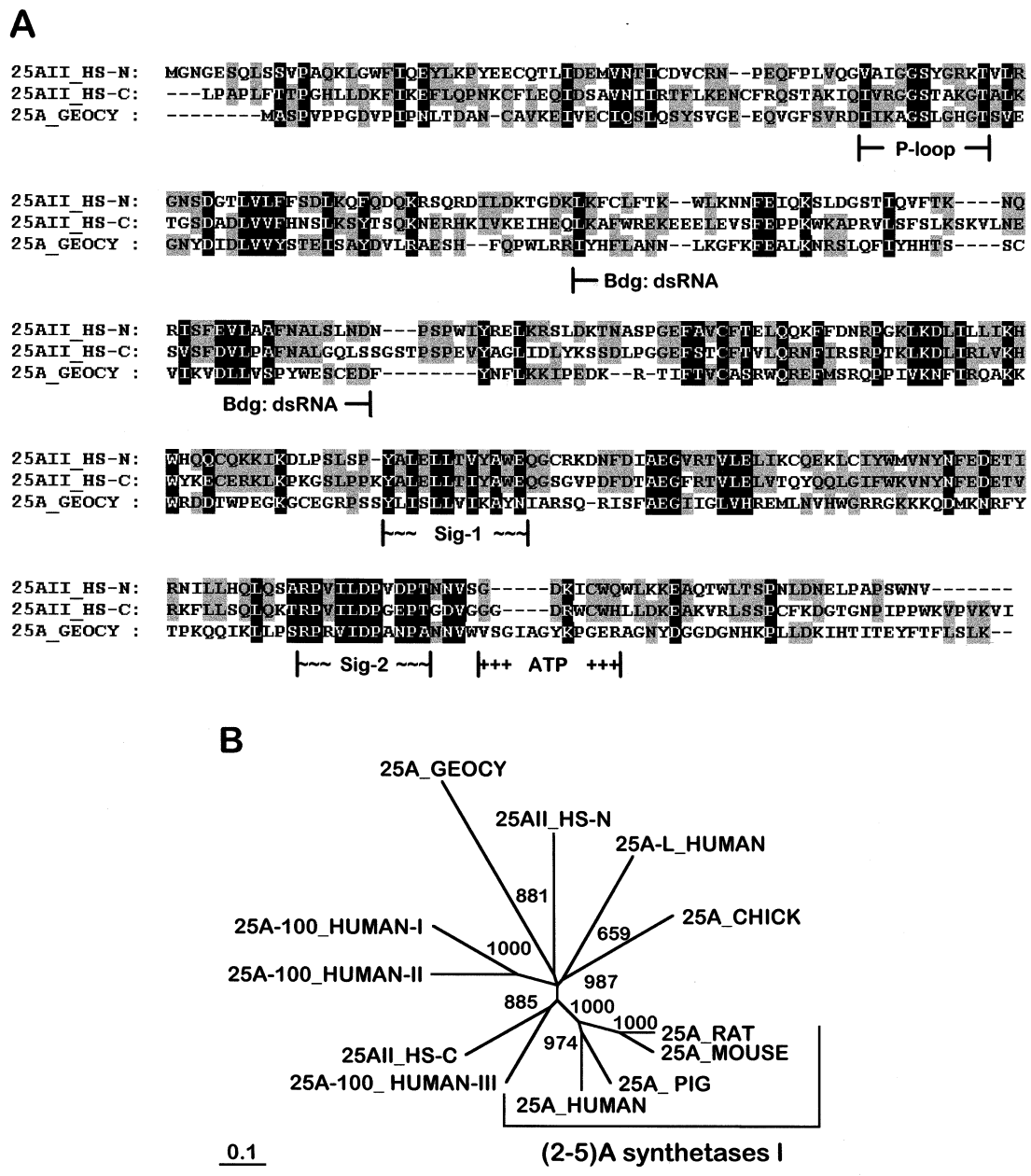

Fig. 3. Relationship between (2-5)A synthetases I, II and III to the sponge putative (2-5)A synthetase sequence (Y18497). A: Alignment of the putative sponge (2-5)A synthetase, 25A_GEOCY, with the two segments of the human (2-5)A synthetase II (25AII_HS, M87284 [38]); the human sequence was cut at position aa_{336}, and the NH_2-terminal part (25AII_HS-N) and the COOH-terminus (25AII_HS-C) were aligned with the sponge sequence. B: Unrooted phylogenetic tree, constructed from the putative sponge (2-5)A synthetase sequence (25A_GEOCY) and (i) with the group of mammalian (2-5)A synthetases I: mouse (25A_MOUSE; P11928), pig (25A_PIG; AJ225090), rat (25A_RAT; 461431) and human (25A_HUMAN; P00973) as well as the chicken (2-5)A synthetase (25A_CHICK; AB002586), (ii) the second group, the (2-5)A synthetase II and the related molecules: the two parts of the human (2-5)A synthetase II (25AII_HS-N and 25AII_HS-C; accession number M87284) as well as the human (2-5)A synthetase-like protein (25A-L_HUMAN; AJ225089) and (iii) the three domains of the human (2-5)A synthetase III (25A-100_HUMAN; AAD28543). The three domains of synthetase III are delimited as described [39] and have been termed 25A-100_HUMAN-I to -III. The analysis was performed by neighbor joining as described in Section 2. The numbers at the nodes are an indication of the level of confidence for the branches as determined by bootstrap analysis (1000 bootstrap replicates). The scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence.

pig, rat, human and chicken (2-5)A synthetases I and the human (2-5)A synthetase-like protein. In addition, the three adjacent synthetase domains of the human (2-5)A synthetase III [39] were included (Fig. 3B). The result shows that the putative sponge (2-5)A synthetase originates from a node from which also the domains of the (2-5)A synthetases I–III and the human (2-5)A synthetase-like molecule derive. All mammalian (2-5)A synthetases I fall into one cluster, while the domains of the (2-5)A synthetases II and III and the chicken enzyme form separate groups (Fig. 3B).

Until now, in invertebrates only the *G. cydonium* putative (2-5)A synthetase cDNA, described here, could be identified. The sponge molecule shares a common ancestor with the members of the vertebrate (2-5)A synthetases of isoform I;

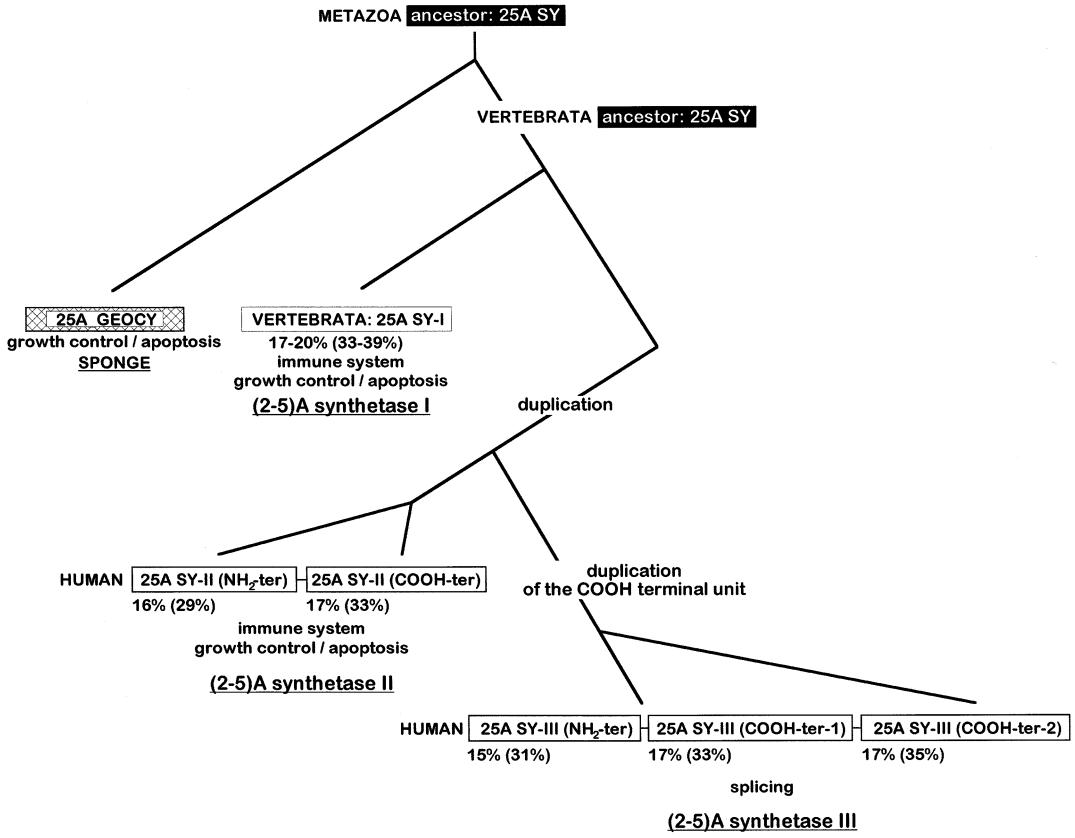

Fig. 4. Proposed branching order of the putative sponge (2-5)A synthetase (25A SY) from a common ancestor of metazoan (2-5)A synthetases (ancestor: 25A SY). The sponge synthetase, 25A_GEOCY, splits off first from this common ancestor. Later in evolution the vertebrate (2-5)A synthetases I branch off; they are more closely related to the sponge molecule than any of the single domains present in the synthetases II and III. Subsequently, the ancestral (2-5)A synthetase underwent gene duplication and gave rise to the evolution of (2-5)A synthetases of group II, with the two termini, 25A SY-II (NH_2-ter) and 2-5A SY-II (COOH-ter). From this level, a second process of domain addition, very likely duplication of the COOH-terminal unit, occurred, resulting in the formation of the synthetases III which comprise three synthetase domains. The percentages of aa identity and conservative aa homology (in parentheses) between the domains of the vertebrate synthetases I–III and the putative sponge synthetase are given.

17–20% identical aa (33–39% similar, including identical, aa) are recorded (Fig. 4). We suggest that in the course of evolution the ancestral molecule duplicated and gave rise to the ancestor of the (2-5)A synthetase II (Fig. 4). The sponge synthetase has 16% (17%) identical aa within the NH_2-terminus (COOH-terminus) and 29% (33%) similar aa to the human (2-5)A synthetase II. In a further stage an additional synthetase unit was formed; this molecule gave rise to the (2-5)A synthetase III. The similarity of the sponge sequence to the two COOH-terminal units of the human (2-5)A synthetase III is higher than to that of the NH_2-terminus. Since the similarity of the two COOH-terminal units of synthetase III to each other and also to the COOH-terminal unit of (2-5)A synthetase II is high, it is likely that a duplication occurred at the COOH-terminus of the ancestor of synthetase II. This conclusion is also supported by the calculation presented earlier [39]. The duplication process resulting in the evolution of the present-day (2-5)A synthetase II might have occurred $\sim$310 million years ago (based on the percentage of divergent aa and the estimated evolutionary rate deduced from the human and chicken enzymes [55–57]; 36% identical aa exist between the human synthetase I and the NH_2-terminus of the human (2-5)A synthetase II).

In an earlier study it was estimated that the *G. cydonium* galectin molecule diverged from a common metazoan ancestor 800 million years ago [40,58]; recently paleontological data demonstrated the existence of sponges already 580 million years ago [59]. The time of the first duplication, based on the calculation presented here, was 310 million years ago, hence later in evolution than the Cambrian explosion, which is dated back to 530 million years ago [8].

4. Conclusion

It is well established that multicellular animals are of monophyletic origin as shown by the examples of the receptor tyrosine kinase [60] and the integrin receptors [42]; these are autapomorphic characters restricted to all Metazoa including Porifera [61]. Until this study (2-5)A synthetases had only been cloned from vertebrates; now a putative (2-5)A synthetase was found also in a sponge. Future studies will show if the existence of this enzyme, or enzyme family, is restricted to Metazoa. In *Caenorhabditis elegans* no (2-5)A synthetase gene has been found during the genome project (www.The Sanger Centre Web Server.ac.uk).

All hitherto known (2-5)A synthetases are stimulated by interferon (reviewed in [62]). The (2-5)A system protects against virus infection [63] and plays a role in growth suppres-

sion [64]. Recently, experiments have been presented which indicate that the (2-5)A system is involved in apoptosis [65]. Based on the different roles of the (2-5)A system it can be proposed that in sponges in an early stage of evolution this mechanism was involved in growth control, including control of apoptosis. Later, in vertebrates, the (2-5)A system acquired a further function related to immunology (reviewed in [19]). It has been suggested [65] that growth suppression and apoptosis contribute to the antiviral activity displayed by the (2-5)A system; this (these) function(s) have been attributed also to the (2-5)A synthetase-like protein [52]. Future studies must show if an interferon-like antiviral system which also involves the (2-5)A synthetase exists in sponges as well. It should be stressed that experimental evidence is available for the exposure of sponges to virus loads [66].

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