Evolutionary relationships of the metazoan $\beta\gamma$ -crystallins, including that from the marine sponge *Geodia cydonium*†

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SUMMARY

$\beta\gamma$ -crystallins are one major component of vertebrate lenses. Here the isolation and characterization of a cDNA, coding for the first $\beta\gamma$ -crystallin molecule from an invertebrate species, the marine sponge *Geodia cydonium*, is described. The size of the transcript as determined by Northern blotting was 0.7 kb in length. The deduced amino acid sequence consists of 163 aa residues and comprises four repeated motifs which compose the two domains of the $\beta\gamma$ -crystallin. Motif 3 contains the characteristic $\beta\gamma$ -crystallin 'Greek key' motif signature, while in each of the three other repeats, one aa residue is replaced by an aa with the same physico-chemical property. The sponge peptide shows striking similarities to vertebrate $\beta\gamma$ -crystallins. Analysis by neighbour joining of the sponge motifs with the two motifs present in spherulin 3a of *Physarum polycephalum* shows that motif 4 of the sponge $\beta\gamma$ -crystallin was added as the last single sequence to the tree. The data support the view that the $\beta\gamma$ -crystallin superfamily, present in eukaryotes, evolved from a common ancestor including also the sponge $\beta\gamma$ -crystallin.

1. INTRODUCTION

Recent phylogenetic analyses applying molecular biological techniques revealed that the lowest metazoan phylum, the sponges (Porifera), possess basic structural elements common to higher invertebrates and vertebrates. The cDNAs coding for those proteins have been cloned from the marine sponge *Geodia cydonium*, a member of the taxon Geodiidae, which is the oldest known group within the Demospongiae and can be traced back to the early Cambrian (ca. 550 million years) (Mehl *et al.* 1997). Among these proteins are cDNAs, coding for adhesion molecules (lectins (Pfeifer *et al.* 1993)), adhesion receptors (receptor tyrosine kinase (Schäcke *et al.* 1994a,b), integrin receptor (Pancer *et al.* 1997)), signal transduction molecules (protein kinases C (Kruse *et al.* 1996)) and an transcription factor (serum response factor (Scheffer *et al.* 1997)). Their deduced amino acid (aa) sequences have shown high homology in their signatures and domains when compared with respective metazoan sequences. Based on these findings the monophyletic origin of Metazoa has been established (Müller 1995).

Sponges are animals which are devoid of muscles and nerve cells (reviewed in Simpson 1984). However, cell biological and molecular data indicate that sponges react to certain transmitters (reviewed in

Müller & Müller 1997) and contract Ca^{2+} dependently using myosin type II as filaments (Lorenz *et al.* 1997). Sponges have primitive organs (Weissenfels cited in Mehlhorn (1989)) but light-sensitive organs have not yet been detected. Several experiments demonstrate, however, that sponges react to light, e.g. in *Tethya crypta* as a stimulus to open osculae (Reiswig 1971), or in *Ephydatia fluviatilis* as a modulator for hatching from gemmules (Rasmont 1970). It has been proposed that eyes have evolved in Metazoa independently approximately 40 times; this suggestion based on the clear differences in morphologies and modes of development of the compound eye in insects and the single-lens eyes of vertebrates and molluscs (summarized in Clayton 1978; Halder *et al.* 1995). However, recent cDNA sequence and expression data support the hypothesis of a monophyletic origin for photoreceptor cells (reviewed by Halder *et al.* 1995). It was reported that expression of the *Pax6* gene is involved in the lens induction process (Li *et al.* 1994) not only in vertebrates but also in insects and perhaps in molluscs (Halder *et al.* 1995). In addition, all visual systems studied until now, from insects, cephalopods to vertebrates, contain the visual pigment proteins opsins which trigger together with the vitamin A-derived chromophore a G-protein-mediated signalling cascade (Yarfitz & Hurley 1994). A further prominent protein group in ocular tissues are the crystallins which are highly expressed in the lens (Wistow & Piatigorsky 1988).

Crystallins are categorized into two classes: the

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ubiquitous crystallins and the taxon-specific crystallins (Slingsby 1985; Wistow 1985; Slingsby & Bateman 1990; and reviewed in Wistow & Piatigorsky 1988; Jaenicke 1994; Kodama & Eguchi 1994; De Jong *et al.* 1994). No structural or functional characteristics are common to all crystallins. The α -, β - and γ -crystallins are classed as ubiquitous crystallins and are found in almost all vertebrate species. The second class, the taxon-specific crystallins, includes a series of 'enzyme crystallins', among them δ , ζ and ρ -crystallin, which display catalytic functions. One member of the ubiquitous crystallins, α -crystallin, is structurally related to the ubiquitous small heat-shock proteins (Ingolia & Craig 1982; Merck *et al.* 1993), whereas the $\beta\gamma$ -crystallins form a separate class of homologous proteins (Wistow 1993).

To further substantiate the assumption that light sensory organs might be of monophyletic origin (Halder *et al.* 1995) it was the task of the present study to elucidate if sponges contain genes homologous to the ubiquitous crystallin genes. The $\beta\gamma$ -crystallin genes were selected because they are, in vertebrates, expressed abundantly only in the eye lens.

2. MATERIALS AND METHODS

(a) Materials

Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained from Stratagene (Heidelberg) and USB (Cleveland, OH).

(b) Sponge

Live specimens of *Geodia cydonium* (Porifera, Demospongiae, Geodiidae) were collected near Rovinj (Croatia). Immediately after collection from a depth of 25 m at 16 °C they were frozen in liquid nitrogen.

(c) Isolation of *G. cydonium* GCCRBG cDNA

The 5'-end of the sponge crystallin cDNA was cloned by polymerase chain reaction (PCR) from a *G. cydonium* cDNA library (Pfeifer *et al.* 1993) in lambda ZAPII, using the degenerate reverse primer 5'-CTCRTANAGNCTCCAYTCNCC-3' (where N = A/C/G/T, R = A/G, Y = C/T) in conjunction with the ZAPII 5'-end vector-specific primer T3. The PCR reaction mixtures of 50 μ l included 10 pmoles of the degenerate primer and 5 pmoles of the primer T3 (Stratagene), 200 μ M of each nucleotide, 1 μ l of the cDNA library (approximately 10^9 pfu), buffer and 2.5 units of Taq DNA polymerase (Boehringer Mannheim). PCR amplifications were run on a GeneAmp 9600 thermal cycler (Perkin Elmer) with the following cycling parameters: an initial denaturation at 95 °C for 3 min, then 35 cycles of 95 °C for 45 s, 56 °C for 1 min, 72 °C for 1.5 min and a final extension step at 74 °C for 10 min. After electrophoresis, several bands were observed in the ethidium bromide-stained gel, ranging between 0.6 and 0.9 kb in length. Amplification products were purified through a QIAquick Spin column (QIAGEN), cloned in bulk into pGEM-T (Promega) and six independent clones were sequenced (Sequenase version 2.0, USB). The sponge crystallin was termed GCCRBG. Based upon the 5'-end sequence of

GCCRBG, we designed specific forward primers for PCR cloning of the C-terminus of GCCRBG from the cDNA library, in conjunction with the ZAPII 3'-end vector-specific primer T7. PCR reactions were as above except that 5 pmoles each of the internal forward primers and T7 (Stratagene) primers were used, and the cycling parameters were: 3 min at 95 °C, then 35 cycles of 95 °C for 45 s, 59 °C for 45 s, 74 °C for 90 s. The amplification product (approximately 0.7 kb in length) was cloned and sequenced.

DNA sequencing was performed with an automatic DNA sequencer (Li-Cor 4000S).

(d) Sequence comparisons

Sequences were analysed using computer programs PCGene (1995), BLITZ (blitz@ebi.ac.uk) and FASTA (fasta@ebi.ac.uk). Phylogenetic trees were constructed on the basis of aa sequence alignment by neighbour-joining, applying 'Neighbor' program from the PHYLIP package (Felsenstein 1993). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein 1993). The distance matrix was calculated as described (Dayhoff *et al.* 1978). The graphical output of the bootstrap figure was produced by program 'Treeview' (Roderic D. M. Page, University of Glasgow, UK (available at <http://http://taxonomy.zoology.gla.ac.uk/software.html#treeviewing>). Multiple alignment (the default options were used) was performed with CLUSTAL W version 1.6 (Thompson *et al.* 1994) and the graphic presentation was composed with GeneDoc (Nicholas 1996).

(e) Northern blot

RNA was extracted from liquid nitrogen pulverized sponge samples with TRIzol Reagent (GibcoBRL) as recommended by the manufacturer, with the additional isolation step for samples with high content of polysaccharides. Poly(A) RNA was purified with Oligotex mRNA kit (QIAGEN). One microgram of mRNA was electrophoresed through 1% formaldehyde/agarose gel and blotted onto Hybond N⁺ membrane following the instructions of the manufacturer (Amersham). The RNA molecular weight marker I (Boehringer Mannheim) was used for size estimates. Hybridization to the ³²P-labelled GCCRBG probe was at 65 °C overnight in: 0.25 M NaH₂PO₄ (pH 7.2), 1 mM EDTA and 0.5% blocking reagent (DUPONT). Washes were done at 65 °C as follows: twice in 0.125 M NaH₂PO₄ (pH 7.2), 2.5% SDS, 0.05 mM EDTA and then twice in 0.025 M NaH₂PO₄ (pH 7.2), 0.5% SDS, 0.01 mM EDTA. The film was exposed for two weeks at -80 °C.

3. RESULTS

(a) Cloning of the GCCRBG cDNA from *G. cydonium*

Employing degenerate PCR cloning, a sponge cDNA clone was isolated whose deduced protein revealed striking similarity to vertebrate $\beta\gamma$ -crystallins. The sequence, which is termed GCCRBG, is 682 bp long and contains an open reading frame extending from nt 72 with the start codon for Met to the stop codon TAA at nt 561 (figure 1). The typical signal polyadenylation site AATAAA (Zarkower *et al.* 1986) is not present as in most cD-

Figure 1. The nucleotide sequence and deduced aa sequence of the sponge *GCCRBG* cDNA. The typical 'Greek key' motifs are underlined; the conserved 'fold-over' segments are in superscript and marked. The small letters a₁–a₄ to d₁–d₄ refer to the predicted β -strands (performed with the program of Chou & Fasman (1978)) present in motif 1 to 4.

NAs from *G. cydonium* (Pfeifer *et al.* 1993).

Northern blot analysis using the original sponge *GCCRBG* clone as a probe (figure 2) revealed one band of a size of 0.7 kb, confirming the presence of the complete transcript.

(b) The deduced aa sequence of sponge *CRBG_GEOCY*

Six clones were analysed and were found to contain an identical insert. The deduced aa sequence of 163 residues (which is designated *CRBG_GEOCY*) has an *M_r* (molecular weight) of 17912 and an isoelectric point of 9.01; the instability index has been computed to be 39.86, indicating a stable protein (PC/GENE 1995).

All $\beta\gamma$ -crystallin sequences show a characteristic arrangement into four repeated motifs orga-

nized into two domains, as originally described by Blundell *et al.* (1981). Each motif is composed of four β -strands and one 'Greek key' signature. In the typical $\beta\gamma$ -crystallin 'Greek key' motif signature Ala-Thr-Leu-Tyr-Lys-His-Val-Asn-Phe-Gly-Gly-Lys-Glu-Leu-Leu (Wistow & Piatigorsky 1988), the underlined aa residues Tyr, Phe and Gly, as well as a Ser located approximately 20 aa closer to the C-terminal, are conserved. Inspection of the sponge sequence shows that it also can be arranged into four repeated motifs which span as follows: 1: aa₂ to aa₃₆; 2: aa₃₇ to aa₈₁; 3: aa₈₅ to aa₁₂₀; 4: aa₁₂₁ to aa₁₆₃ (figures 1 and 3) (compare Blundell *et al.* 1981; Chambers & Russell 1993). The motifs are each composed of four antiparallel β -strands (figure 1). Both N-terminal and C-terminal arms, seen in the human β - but not γ -sequences (Chambers & Russell 1993), are lacking; the connecting

Figure 2. Northern blot analysis of *GCCRBG*. One microgram of *G. cydonium* mRNA was electrophoresed through 1% formaldehyde/agarose gel, blotted and hybridized to the ^{32}P -labelled *GCCRBG* probe.

peptide is 3 aa long. Motifs 3 and 4 contain the $\beta\gamma$ -crystallin 'Greek key' signature as well as the conserved Ser residue. In motifs 1 and 2, two residues are replaced: Ser by Arg_{#17} in motif 1 and Gly by Asp_{#35} in motif 2. Although each is replaced by an aa with the same physico-chemical property (Taylor 1987), model building will have to show whether these residues indeed fit the $\beta\gamma$ -crystallin fold. It is interesting to note that the sponge sequence does not contain any Cys, while γ -crystallins of vertebrates have a high content of this aa (den Dunnen *et al.* 1986). Like in other crystallins, a signal peptide is missing in the sponge sequence, suggesting that it is an intracellular structural protein.

(c) *Sequence comparison of sponge CRBG_GEOCY with crystallins β/γ from other organisms*

Data bank searches (BLITZ and FASTA) revealed that CRBG_GEOCY showed significant similarity to the $\beta\gamma$ -crystallins (19% with respect to βB2 -crystallin from human (accession number P43320)). CRBG_GEOCY exhibited somewhat lower similarity to γ -crystallin (17% to γS -crystallin from human; P22914). For the alignment shown in figure 3, the sponge sequence was compared with aa sequences from vertebrates (human, chick, frog and fish) as well as with the crystallin-related molecules from the protozoan *Physarum polycephalum*—spherulin 3A—(Bernier *et al.* 1987) and the bacterial protein S from *Myxococcus xanthus* (Inouye *et al.* 1983). The comparison shows sequence identity and conservative

amino acid substitutions within the 'Greek key' motif signatures and the Ser containing triad, adjacent to the β -strands d (figure 1 and 3). Eye inspection already shows that the motifs from the bacterial sequence are different in length compared to the eukaryotic members.

For the phylogenetic analysis, the sponge $\beta\gamma$ -crystallin sequence as well as the vertebrate sequences were separated into the four motifs and aligned. The subsequent neighbour-joining analyses revealed that motif 4 of the *G. cydonium* sequence diverged first from a common ancestor of the sponge motifs. A comparison of the sponge motifs with the two motifs, present in spherulin 3A of *P. polycephalum*, revealed a tree with motif 4 in one branch, sponge motif 2 and motif 1 of *P. polycephalum* in a second one and the other motifs (*P. polycephalum* motif 2 and *G. cydonium* motif 1 and 3) in a third one (figure 4).

An unrooted neighbour-joining tree was constructed from the sponge motifs GEOCY-1 to -4, and the motifs of the vertebrate $\beta\gamma$ -crystallins (human, chick, frog and fish) (figure 5). The trichotomous tree shows that GEOCY-2 branches off first from the internal node 1, which includes also GEOCY-1 and -3; GEOCY-4 separated first from the two other motifs starting from node 2. The robustness of the inferred phylogenies was tested by bootstrap test and revealed that the branching of GEOCY-2 and GEOCY-4 from the respective nodes is of lower significance, with 48% and 24%, respectively, which is in contrast to all other relationships computed.

4. DISCUSSION

The $\beta\gamma$ -crystallins, with the exception of the α -crystallins, are the major class of soluble proteins in lenses of mammals (Wistow & Piatigorsky 1988). The $\beta\gamma$ -crystallins of M_r 20 to 30 kDa form dimers or mixed aggregates, while γ -crystallins of M_r = 21 kDa are monomeric molecules (Blundell *et al.* 1981; Bax *et al.* 1990). It has been proposed (Wistow 1990) that the $\beta\gamma$ -crystallins of Metazoa are distantly related with the bacterial protein S, a development-specific protein from *M. xanthus* (Inouye *et al.* 1983), and spherulin 3a, an encystment-specific protein of *P. polycephalum* (Raub & Aldrich 1982). The aa sequences deduced from the respective cDNAs showed that protein S of *M. xanthus* consists of four crystallin motifs linked together by a connecting peptide (Inouye *et al.* 1983), while the spherulin sequence from *P. polycephalum* consists only of two motifs starting with an N-terminal arm (Bernier *et al.* 1987).

Until now, no molecular sequence data are available from $\beta\gamma$ -crystallins in invertebrates. From the squid, a water soluble S-crystallin has been isolated and designated SIII-crystallin. Although it shows in the N-terminal region of the sequence some analogy with the first 19 residues of the $\beta\gamma$ -crystallin folding unit, SIII-crystallin is most related with glutathione S-transferase (Siezen & Shaw 1982; Tomarev *et al.* 1992) and belongs to the class of enzyme-related

Figure 3. Alignment of the aa sequence for CRBG_GEOCY, deduced from the cDNA isolated from the marine sponge *G. cydonium* (*GCCR*) with selected crystallin sequences from other organisms. Alignment was performed with the $\beta\gamma$ -crystallins from vertebrates: human (CRB2_HUMAN; accession number P43320 (Chambers *et al.* 1993)), chick (CRB2_CHICK; Q05714 (Sawada *et al.* 1992; Duncan *et al.* 1996)), frog (CRBA_RANTE; P07317 (Luchin *et al.* 1987)), fish (CRG2_CYPCA; P10044 (Chang *et al.* 1984)), the slime mold: *P. polycephalum*—spherulin 3A—(SR3A_PHYPO; P09353 (Bernier *et al.* 1987)), the bacterium: *M. xanthus*—protein S—(DEST_MYXXA; P02967 (Inouye *et al.* 1983)), and the sponge *G. cydonium* sequence CRBG_GEOCY. Residues of the 'Greek key motifs' are shown in inverted type, while residues conserved in more than half of the sequences are shaded. The borders of the motifs are indicated (●), the N-terminal and C-terminal (N/C-term) arms as well as the connecting peptide (con pep) are indicated. The numbers below the sequences refer to the *G. cydonium* sequence and mark the aa flanking the motifs. The alignment was performed using CLUSTALW program.

crystallins. The aa sequence deduced from the *G. cydonium* $\beta\gamma$ -crystallin cDNA shows four γ -crystallin motifs; motif 1 (35 aa in length) and 2 linked (45 aa) with 3 (36 aa) and 4 (45 aa) via a connecting peptide which comprises three aa residues, conforming with the ABAB motif pattern of vertebrate crystallins (Wistow & Piatigorsky 1988), assuming that the aa substitutions seen in motif 1 and 2 allow the typical $\beta\gamma$ -fold. Future protein structural analyses is required to determine if the aa substitutions seen in motif 1 and 2 warrant the typical $\beta\gamma$ -fold. The N- and C-terminal arms, as well as the connecting peptide (linker), are required for the association of $\beta\gamma$ -crystallins homodimerization; the linker allows dimerization, while the arms prevent higher homooligomerization (Trinkl *et al.* 1994). The sponge sequence is devoid of these arms, indicating that it is a true γ -crystallin and suggesting that it does not form multimers in the native state (Berbers *et al.* 1984; reviewed in Wistow & Piatigorsky 1988). In the 'Greek key' motifs of the sponge sequence, the conserved Tyr-6, Glu-7, Gly-13 and Ser-34, or their equivalents, are present which are known to be important for the stabilization of the folded hairpin of $\beta\gamma$ -crystallins (Wistow *et al.* 1983). An interesting feature of the deduced sponge sequence is the lack of Cys residues, not allowing the formation of disulphide bonds as possible in some γ -crystallins of vertebrates (Blundell *et al.* 1981). In addition, the content of Met is low in the sponge sequence (2%) in contrast to some corresponding vertebrate sequences, which can comprise up to 14% of Met in fish (Chang *et al.* 1988).

For phylogenetic analyses, crystallin or crystallin-like sequences from the sponge and spherulin of *P. polycephalum* have been cut into the respective mo-

Figure 4. Unrooted phylogenetic tree computed from the four motifs of the *G. cydonium* $\beta\gamma$ -crystallin, termed GEOCY-1 to -4, and the two motifs, present in spherulin 3A of *P. polycephalum* (PHYPO-1 and -2). The analysis was performed by neighbour joining, as described in §2. The numbers at the nodes are an indication of the level of confidence—given in percentage—for the branches as determined by bootstrap analysis (100 bootstrap replicates). Scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence.

tifs and aligned. The unrooted tree showed that the sponge motif 4, GEOCY-4, branches off first from the other sponge motifs and from the two spherulin motifs. The sponge motifs GEOCY-1 to -4 were subsequently compared with the vertebrate $\beta\gamma$ -crystallin motifs 1 to 4. GEOCY-2 and GEOCY-4 were found to branch off first at the nodes, while the sponge motifs 1 and 3, as well as those from the vertebrates,

Figure 5. Phylogenetic tree (unrooted) of metazoan $\beta\gamma$ -crystallin motifs. The comparison is based on the aa sequence of the four motifs, present in the sequences from human (CRB2.HUMAN; HUMAN-1 to -4), chick (CRB2.CHICK; CHICK-1 to -4), frog (CRBA.RANTE; RANTE-1 to -4) and fish (CRG2.CYPCA; CYPCA-1 to -4), as well as the motif 4 from the *G. cydonium* sequence GEOCY-1 to -4. Calculation was performed by neighbour joining. The numbers at the nodes refer to the respective level of confidence (per 100 bootstrap replicates); the internal nodes are marked (#1 to #3). Scale bar indicates the evolutionary distances.

diverged later. As outlined earlier (Wistow & Piatigorsky 1988; Piatigorsky & Wistow 1989), the four crystallin motifs present in protein S from *M. xanthus* are arranged in a different order than in eukaryotes, suggesting different lines of evolution from the gene encoding a single motif.

Recently, Wistow *et al.* (1995) constructed a phylogenetic tree using the motifs in a β B-crystallin, protein S and the EDSP protein from the amphibian *Cynops pyrrhogaster* (Takabatake *et al.* 1992). They reported that the EDSP conforms more closely with the vertebrate crystallins. This analysis using the sponge crystallin motifs confirms this view in the sense that the sponge motif 4 separated earlier in the tree than the four *Cynops* motifs (not shown).

Only recently, with the elucidation of cDNAs coding for adhesion molecules, adhesion receptors and signal transduction molecules from the lowest metazoan phylum, the Porifera (sponges) (see § 1), has it been possible to establish the monophyly of Metazoa (Müller 1995). The present study substantiates and extends this view also to molecules from light-sensory organs, in this case crystallins. However, it should be stressed that recent results show that crystallins are synthesized both in lens and non-lens tissues (Head

et al. 1991; Smolich *et al.* 1994), suggesting a specific role during embryogenesis (Duncan *et al.* 1996). Localization studies with antibodies raised against recombinant sponge crystallin will help to answer the question about the function of this molecule in *G. cydonium*.

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