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Molecular and functional analysis of the (6-4) photolyase from the hexactinellid *Aphrocallistes vastus*☆

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Abstract

The hexactinellid sponges (phylum Porifera) represent the phylogenetically oldest metazoans that evolved 570–750 million years ago. At this period exposure to ultraviolet (UV) light exceeded that of today and it may be assumed that this old taxon has developed a specific protection system against UV-caused DNA damage. A cDNA was isolated from the hexactinellid *Aphrocallistes vastus* which comprises high sequence similarity to genes encoding the protostomian and deuterostomian (6-4) photolyases. Subsequently functional studies were performed. It could be shown that the sponge gene, after transfection into mutated *Escherichia coli*, causes resistance of the bacteria against UV light. Recombinant sponge photolyase was prepared to demonstrate that this protein binds to DNA treated with UV light (causing the formation of thymine dimers). Finally, it is shown that the photolyase gene is strongly expressed in the upper part of the animals and not in their middle part or their base. It is concluded that sponges not only have an excision DNA repair system, as has been described earlier by us, but also a photolyase-based photo-reactivating system.

Keywords: Sponges; Hexactinellida; *Aphrocallistes vastus*; (6-4) photolyase; Photolyase/blue-light photoreceptor family

1. Introduction

Marine sponges (phylum Porifera) are sessile filter feeders that evolved during the late Proterozoic [1], very probably between the two major glacial periods 750–700 and 610–570 million years ago [2]. Only a few species occupy the freshwater milieu [3]. The Porifera are subdivided into three classes: Hexactinellida, Demospongiae and Calcarea [4]. The earliest fossil sponge spicules are thought to belong to the class Hexactinellida from the Ediacarian age (late Proterozoic) [1], and recent protein sequence data have confirmed that the siliceous sponges, Hexactinellida and Demospongiae, are more closely related to the Urmetazoa, the common hypothetical ancestor of Metazoa [5], than the Calcarea, which share more similarities with the Cnidaria [6–9]. This conclusion has recently been confirmed using 18S rDNA sequence data [10].

The Neoproterozoic world in which the ancestors of modern sponges lived was characterized by lower oxygen and higher carbon dioxide levels than today [11]. The early sponges lived in an eutrophic environment on the outer shelf region of the supercontinent that existed at this time and have probably been exposed to high levels of ultraviolet light [1]. Hence, they must have developed mechanisms to repair DNA damaged by UV light. Molecules that result from DNA damage, including pyrimidine–pyrimidone photoproducts, and photolysases, are known from both deuterostome and protostome metazoans, but none are known from the Porifera, the most basal group of metazoans. The known functions of these molecules are varied; the photolyases as members of the Cryptochrome family are associated with blue light photoreceptors, which are essential light detectors for the development of plants [12]. One way to elucidate the function of the common ancestor of those genes is to clone related genes from the descendants of the phyloge-

☆ The sequence reported here is deposited in the EMBL/GenBank data base under the accession no. AJ437143 as *Aphrocallistes vastus* photolyase-related protein.

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netically most ancient group of metazoans, the Hexactinellida. In the past, this approach has been successful to determine the roles of other metazoan specific genes (e.g., Refs. [7,13,14]).

We have cloned a CRY [cryptochrome] sequence from the hexactinellid sponge *Aphrocallistes vastus* that has high sequence similarity to genes encoding (6-4) photolyases and related proteins. Functional studies show that this gene codes for a photolyase-related protein.

2. Materials and methods

2.1. Materials

Restriction endonucleases and modifying enzymes for recombinant DNA techniques and vectors were obtained as described earlier [15,16]. Monoclonal anti-thymidine dimer antibodies were purchased from Sigma (Deisenhofen, Germany). PCR-DIG (digoxigenin)-Probe-Synthesis Kit, anti-DIG AP Fab fragments and CDP [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] were from Roche Diagnostics (Mannheim, Germany).

2.2. Sponges

The specimens of *A. vastus* [Schulze] (Porifera, Hexactinellida, Hexasterophora, Hexactinosida, Aphrocallistidae) were collected from Saanich Inlet, British Columbia (Canada) by SCUBA diving from a depth of 35–40 m and transported in seawater to aquaria at the University of Victoria. Each sponge was higher than 1 m. Pieces were cut with scissors from three distinct parts of the sponge; (1) the uppermost region, which contained only newly formed tissue and no fused siliceous skeleton; (2) the middle region in which the glass skeleton was fused, and tissue plentiful; and (3) the most basal portion of the animal, which consisted of dense silica and little tissue. Pieces were broken into fragments by hand (using three pairs of vinyl gloves to prevent wounding by the glass shards) and the tissue was dissociated through 50-μm Nitex mesh into beakers of 0.45-μm filtered sea water (FSW). After the tissue was allowed to aggregate for 6 h, 1 or 2 days, it was washed once in FSW, frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Cloning of photolyase-related protein from A. vastus

The cDNA *APHVAPHR* coding for the photolyase-related protein [PHR_APHVA], was isolated from a library of *A. vastus*, which was prepared in lambda ZAP Express (Stratagene) and packaged in vitro with the MaxPlax™ Packaging Extract (Epicentre Technologies) as described [14]; the work was part of a systematic sequencing program of the expressed genome of *A. vastus* (http://spongebase.uni-mainz.de/; to be released). The fragment of 650 nt encoding the sponge photolyase-related protein was completed using vector-specific as well as internal *APHVAPHR*-specific primers. A 1822-nt long (excluding the poly(A) tail) and complete cDNA was obtained.

2.4. Detection of the (6-4) photolyase activity

The *Escherichia coli* strain SY2(*Δphr*::*Cm*^{r}, *ΔuvrA*::*Km*^{r}, *ΔrecA*::*Tc*^{r})/pGEX which is deficient in photoreversal activity for pyrimidine cyclobutane dimers (CPDs) that carry the plasmid *pGEX-4T-2* was used. The positive control was *E. coli* SY2/pMS969 which had the *phr* (6-4) photolyase gene from *E. coli* [17]. The *E. coli* SY2 was also used as a host for the *A. vastus* photolyase-related protein *APHVAPHR* gene (SY2/*APHVAPHR*). The cDNA was cloned into *pGEX-4T-2* vector (Pharmacia), *pGEX-4T-2/APHVAPHR*, and used for transformation as described [17,18]. Expression of the photolyase-related protein was induced by 1 mM isopropyl β-D-galactopyranoside (IPTG) [18].

The procedure described by Kim and Sundin [17] was applied. After growing for 6 h at 26 °C, the *E. coli* cells were harvested, suspended in 20-ml saline (0.9%) and transferred into a petri dish. Irradiation was performed with a UV-B lamp (peak at 312 nm; L+F-Labortechnik, Wasserburg). To eliminate UV-C (<280 nm) the light was filtered through cellulose diacetate (Kodacel; Eastman Kodak, Rochester). The energy output of the UV-B lamp was monitored with a UV-radiometer (UVM-CP with a B sensor; Peschel, Mainz). During irradiation the cells were gently mixed. Following ultraviolet irradiation, the *E. coli* cells were illuminated for 1 h with a lamp emitting white fluorescent light at 25 °C. Subsequently, the cells were incubated overnight at 26 °C. The survival rate, which is given in percent, was calculated on the basis of the number of colonies formed. Each point represents the mean (± standard error of the mean) from five replicate experiments.

2.5. Protein expression

Expression of the *APHVAPHR* gene was performed in *E. coli* BL21 using the GST (*S*-transferase) Fusion system (Amersham) as described [19] and following the instructions of the manufacturer. The *APHVAPHR* clone was introduced into the *pGEX-4T-2* plasmid containing the *Schistosoma japonicum* glutathione-*S*-transferase gene and expressed with IPTG. The fusion protein was purified by affinity chromatography on glutathione Sepharose 4B [20]. If not mentioned otherwise, this recombinant fusion protein, r-PHR_APHVA, was used for the experiments. In one series of experiments, the fusion protein was cleaved with thrombin (10 units/mg) to separate glutathione-*S*-transferase from the recombinant sponge photolyase as outlined [21].

2.6. Antibodies against photolyase

Polyclonal antibodies (PoAb) were raised against the recombinant photolyase, r-PHR_APHVA, in female rabbits (White New Zealand) as described [21]. In 4-week intervals, three boosts, 10 μg of protein each, were injected. The serum was collected. The PoAb were termed PoAb-PHR. In control experiments 100 μl of the PoAb-PHR was adsorbed to 20 μg of recombinant photolyase (30 min; 4 °C) prior to its use.

2.7. Western blotting

Gel electrophoresis in the presence of $NaDodSO_4$ ($NaDodSO_4$/PAGE) of the protein extracts was performed in 10% polyacrylamide gels containing 0.1% $NaDodSO_4$ according to Laemmli [22]. Protein samples were subjected to gel electrophoresis in the presence of 2-mercaptoethanol and stained with Coomassie brilliant blue. Semi-dry electrotransfer was performed according to Kyhse-Andersen [23] onto PVDF-Immobilon. Membranes were processed [24] and incubated with PoAb-PHR (diluted 1:500) for 90 min at room temperature. After blocking the membranes with 5% bovine serum albumin, the immune complexes were visualized by incubation with anti-mouse IgG (alkaline phosphatase conjugated), followed by staining with 4-chloro-1-naphthol.

2.8. Binding studies

DNA from herring sperm was modified by UV-irradiation using the UV-B lamp (peak at 312 nm) at an energy of 10 J/cm^2 as described [25]. After this procedure thymine dimers were introduced into the DNA. DNA was depurinated under acidic conditions prior to the spotting onto the membrane; after neutralization DNA (0.1 or 1 nmol) was spotted on a positively charged nylon filter (LI-COR) by a homemade vacuum dot-blot device and heated for 60 min at 80 °C. Control assays were performed with nontreated DNA. After washing in 2 × SSC the filters were air-dried.

Filters, loaded either with control nontreated DNA or with DNA after modification by UV irradiation, were incubated with anti-thymidine dimer specific antibodies (1:1000 dilution) and the immune complexes were visualized by incubation with anti-mouse IgG (alkaline phosphatase conjugated). In a separate experiment, filters with nontreated or UV-treated DNA were incubated with the fusion protein r-PHR_APHVA (1 μg/ml) after blocking. After incubation for 60 min (37 °C) the samples were further incubated with monoclonal antibodies (from mouse) raised against anti-glutathione-*S*-transferase (Sigma; 1:2000). After washing the immunocomplexes were visualized with anti-mouse IgG, labeled with alkaline phosphatase (Sigma; 1:5000), and finally developed by chemiluminescence technique using CDP, the chemiluminescence substrate of alkaline phosphatase, according to the instructions of the manufacturer (Roche).

2.9. Northern blot analysis

RNA was extracted from liquid-nitrogen pulverized sponge tissue as described [26]. RNA (total) samples (5 μg) were electrophoresed in a formaldehyde/1% agarose gel and blotted onto Hybond N^+ membrane following the manufacturer's instructions (Amersham; Little Chalfont, Buckinghamshire, UK). Hybridization was performed with a ≈ 550-nt-long *APHVAPHR* fragment in High-$NaDodSO_4$ hybridization buffer [7% $NaDodSO_4$, 50% formamide, 5 × SSC, 0.1% Na-laurylsulfate, 50 mM Na_3PO_4 (pH 7.0) and 2% of blocking reagent (Roche)] at 42 °C. The probe had been labeled using the PCR-DIG-Probe-Synthesis kit (Roche). The signals of the Northern blot were visualized by the chemiluminescence procedure [27]; CDP-Star was used as substrate.

3. Results and discussion

3.1. Photolyase-related protein from A. vastus

The cDNA, encoding a photolyase-related protein from *A. vastus*, was isolated from a cDNA library as described under Materials and methods. The nucleotide sequence *APHVAPHR* comprises 1822 residues. One open reading frame is found from nt_4 to $nt_{1693-1695(stop)}$; accession number AJ437143. The deduced aa sequence, PHR_APHVA, with 563 residues, represents a calculated 63,921-Da polypeptide (Fig. 1). It shares highest similarity to DNA photolyases with an "Expect value" [degree of similarity between each pair of proteins after alignment (*E*)] of $5.9e^{-11}$ [28]. In accordance to Kanai et al. [29] the photolyase sequences can be dissected into two segments, the N-terminal α/β domain (in the *A. vastus* protein spanning aa_1 to aa_{172}) and the C-terminal helical domain (aa_{267} to aa_{563}) which are separated by a inter-domain loop (aa_{173} to aa_{266}). The *A. vastus* protein shows highest similarity [30] to the protostome sequence from *Drosophila melanogaster*, the (6-4) photolyase (NP_477188 [31]; *E* of $7e^{-46}$), and photolyases from *Danio rerio* (AB042254 [32]; *E* of $4e^{-45}$) and from *Xenopus laevis* (BAA97126 [33]; *E* of $5e^{-45}$); Fig. 1. The instability index, as a calculated measure for the half-life of a protein, has been computed to be 44, suggesting that the sponge photolyase is an unstable protein [34].

3.2. Phylogenetic analysis

According to the classification of Kanai et al. [29] the photolyase-related proteins have been divided into class I and class II photolyases. The class I proteins are further subdivided into animal and plant cryptochromes (CRY) as well as 8-HDF and MTHF-type photolyases. Representative members from these groups were chosen from the databases for the phylogenetic analysis. The focus on animal CRYs/photolyases follows from recent studies, which have demonstrated

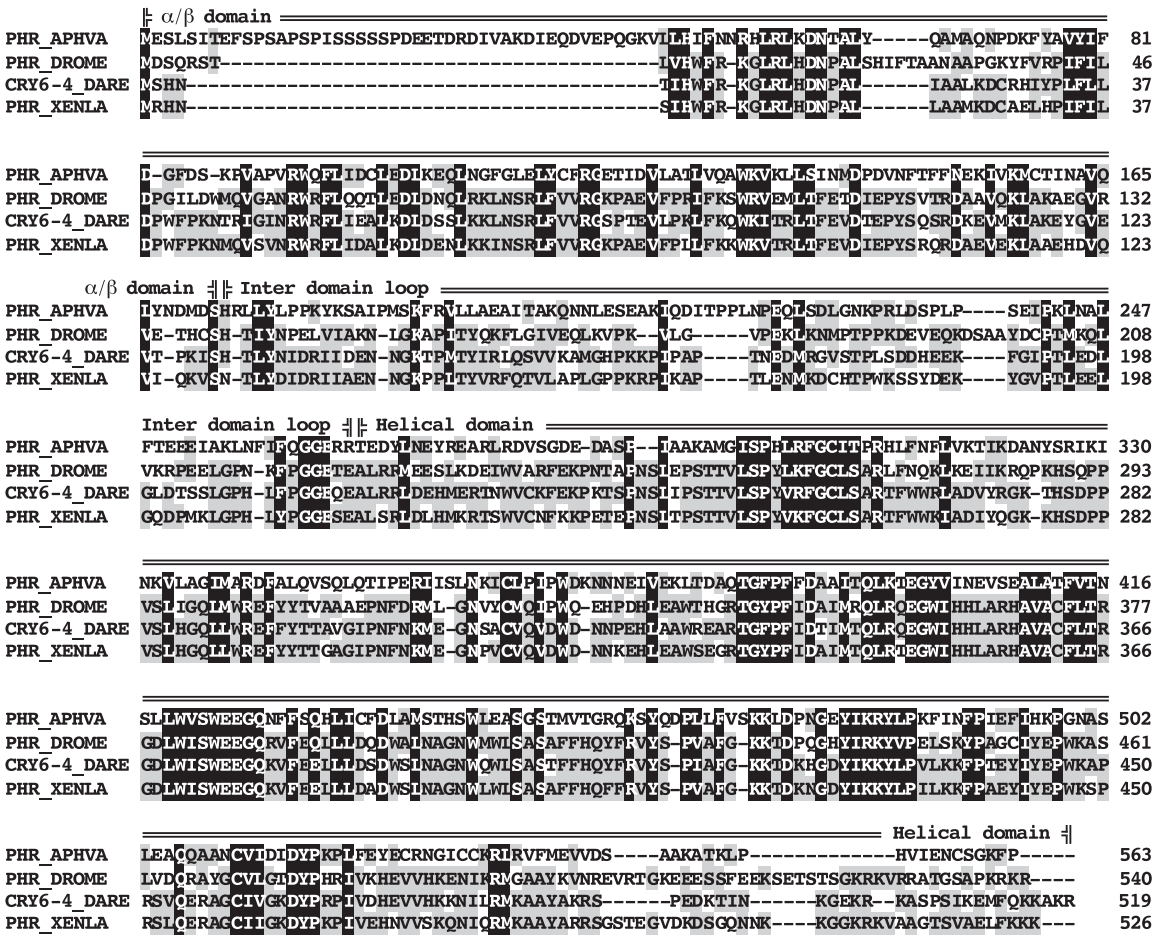

Fig. 1. The predicted sponge (6-4) photolyase-related molecule PHR_APHVA is aligned with the related (6-4) photolyases from *Drosophila melanogaster* (PHR_DROME; NP_477188), *Danio rerio* (CRY6-4_DARE; AB042254) and from *Xenopus laevis* (PHR_XENLA; BAA97126). Residues conserved (similar or related with respect to their similar physico-chemical properties) in all sequences are shown in white on black and those in at least two sequences in black on gray. The borders of the domains are in accordance with those given by Kanai et al. [29].

that the deuterostome photolyases/cryptochromes have DNA photolyase activity and are inhibitors for transcriptional activators [32]. The latter have been implicated in the regulation of the circadian rhythm (see Refs. [32,35]). Using the zebra fish (*D. rerio*) as a model, it has been shown that proteins belonging to the photolyases/cryptochromes, and having very high sequence similarity, comprise either (6-4) photolyase activity or have transcriptional activity [32].

In the rooted tree the bacterial DNA photolyase from *Methanothermobacter thermautotrophicus* was used as an outgroup; Fig. 2. The tree shows two main branches, one which comprises the class II photolyases, which have in addition to the cofactor $FADH_2$ either 8-HDF (8-hydroxy-5-deazaflavin) or MTHF (5,10-methyl-tetrahydrofolate), and the class I photolyases. With high significance (>98.9%) the class I photolyases split into the plant cryptochromes, the photolyase of the 8-HDF type, which include the bacterial (*Halobacterium salinarum*, *Synechocystis* sp. and *Streptomyces griseus*) photolyases and the photolyases of the MTHF type, which comprise the DNA photolyases from yeast, *Neurospora crassa*, *Salmonella typhimurium* and *Trichoderma harzianum*.

The sponge sequence from *A. vastus* is at the base of the branch that includes the animal CRY (cryptochromes/photolyases). The branch point of insect and vertebrate sequences is well supported (>90%) (Fig. 2). In spite of a high degree of similarity to the photolyases, the human polypeptide [36], the zebra fish and insect cryptochromes [37] are lacking DNA photolyase activity. However, another protein (z5-4phr) has high sequence similarity to the other cryptochromes/photolyases from zebra fish and has (6-4) photolyase activity [32]. A similar situation has been described for *X. laevis*; in this animal, genes coding for a photo-reactivating activity as well as cryptochromes not involved in DNA repair have been described [36,38].

3.3. (6-4) Photolyase activity of the A. vastus cDNA

To determine if the sponge photolyase-related protein confers resistance against ultraviolet light to the repair-defective cells of the *E. coli* SY2 strain, the bacteria were

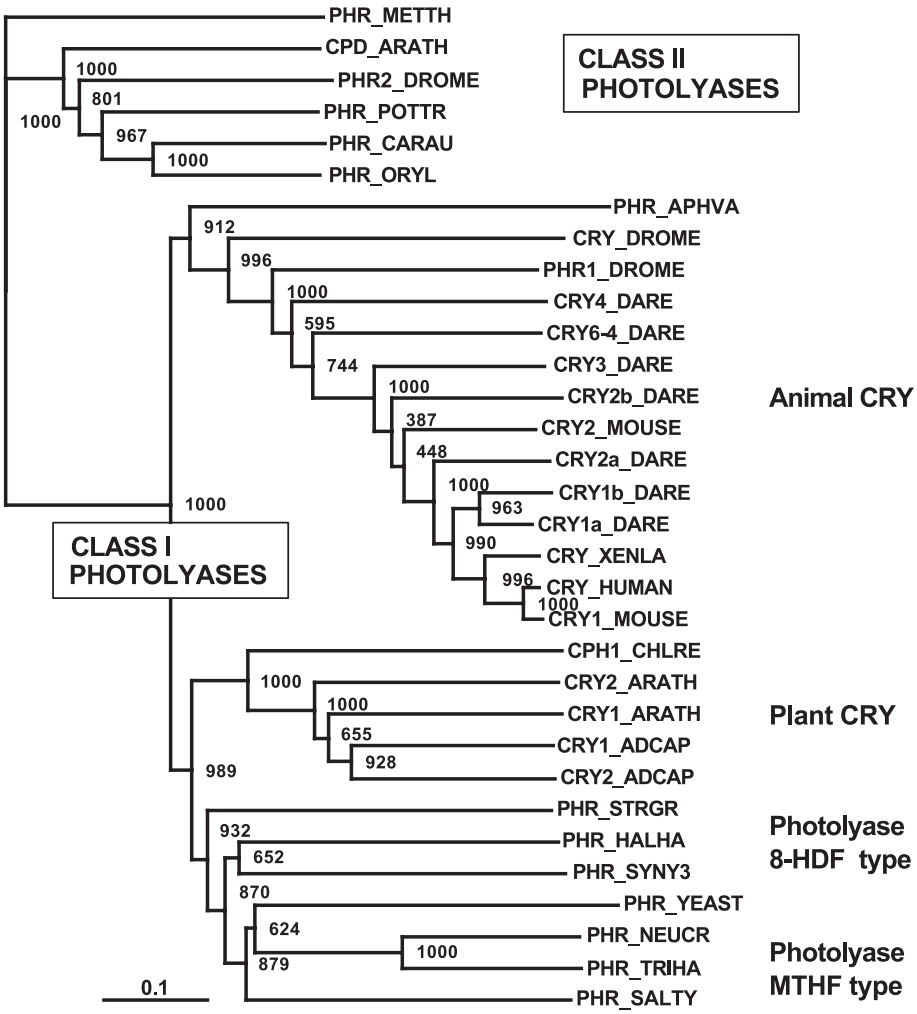

Fig. 2. The phylogenetic relationship of the photolyase/cryptochrome polypeptides. The sponge photolyase-related molecule (PHR_APHVA) has been aligned with related sequences; finally a rooted tree has been computed. The numbers at the nodes are an indication of the level of confidence for the branches 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. The following sequences (grouped according to Refs. [29,32]) have been included. *Class I photolyases*: (i) Group of animal CRY (cryptochromes/photolyases); the cryptochrome sequences from *Danio rerio* zcry1a (CRY1a_DARE; AB042248/AB042248), zcry1b (CRY1b_DARE; AB042249/AB042249.1), zcry2a (CRY2a_DARE; AB042250/AB042250.1), zcry2b (CRY2b_DARE; AB042251/AB042251.1), zcry3 (CRY3_DARE; AB042252/AB042252.1), zcry4 (CRY4_DARE; AB042253/AB042253.1) and (6-4) photolyase (CRY6-4_DARE; AB042254), the human photolyase (CRY_HUMAN; D83702), mouse photolyase/blue-light receptor homolog 1 (CRY1_MOUSE; AB000777) and homolog 2 (CRY2_MOUSE; AB003433), frog cryptochrome 1 (CRY_XENLA; AY049033), the photolyase from *D. melanogaster* (PHR1_DROME; BAA12067.1) and the cry gene product from *D. melanogaster* (CRY_DROME; AAF55649.1). (ii) Plant cryptochromes; the *Arabidopsis thaliana* cryptochrome 1 (CRY1_ARATH; Q43125) and cryptochrome 2 (CRY2_ARATH; Q96524), the *Adiantum capillus-veneris* blue-light photoreceptor 1 (CRY1_ADCAP; BAA32810.1) and photoreceptor 2 (CRY2_ADCAP; BAA32812.1) and the photolyase/blue light photoreceptor 1 from *Chlamydomonas reinhardtii* (CPH1_CHLRE; AAC37438). (iii) Photolyase of the 8-HDF type; the deoxyribodipyridine photolyases from *Halobacterium salinarum* (PHR_HALHA; P20377), from *Synechocystis* sp. (PHR_SYNY3; Q55081) and from *Streptomyces griseus* (PHR_STRGR; P12768). (iv) Photolyase of the MTHF type; the DNA photolyases from yeast (PHR_YEAST; P05066), *Neurospora crassa* (PHR_NEUCR; P27526), *Salmonella typhimurium* (PHR_SALTY; P25078) and *Trichoderma harzianum* (PHR_TRIHA; CAA08916.1). *Class II photolyases*: The corresponding enzymes from *Methanothermobacter thermautotrophicus* [DNA photolyase] (PHR_METTH; P12769), from *A. thaliana* [CPD photolyase] (CPD_ARATH; CAA67683.1), from *D. melanogaster* [photolyase] (PHR2_DROME; BAA05042.1), from *Potorous tridactylus* [DNA photolyase] (PHR_POTTR; Q28811), from *Carassius auratus* [DNA photolyase] (PHR_CARAU; P34205) and the enzyme from *Oryzias latipes* (PHR_ORYL; BAA05043.1). The tree was rooted with the *M. thermautotrophicus* DNA photolyase.

transformed with the sponge *APHVAPHR* cDNA, inserted into *pGEX-4T-2*.

The *E. coli* SY2 strain was very sensitive to ultraviolet light if the cells were exposed to UV-B at doses between 0.5 and 2 J/m^2, as shown in Fig. 3. However, if *E. coli* SY2 cells were transfected with *A. vastus* photolyase-related protein *APHVAPHR* gene (*E. coli* SY2/*APHVAPHR*), the UV-induced damages in these bacteria were almost completely repaired during the light-repair phase; at a dose of 2 J/m^2 only a small reduction of the survival rate was seen (Fig. 3). The same resistance is seen if *E. coli* SY2 was complemented with the *phr* (6-4) photolyase gene from *E. coli*. In contrast,

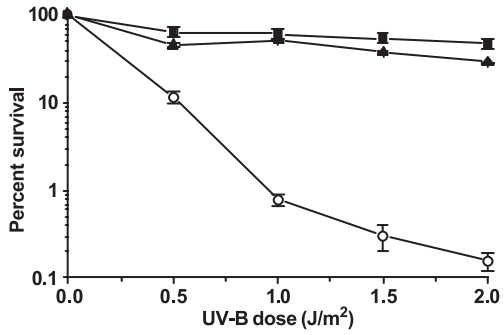

Fig. 3. Survival of *Escherichia coli* strain SY2/pGEX (which does not have photolyase activity; ○), *E. coli* SY2/pMS969 (complemented with the *phr* (6-4) photolyase gene from *E. coli*; ●) and *E. coli* SY2 carrying the *A. vastus APHVAPHR* gene (▲) after UV-B irradiation. After UV-B irradiation the cells were exposed to visible light and subsequently incubated over night. The experimental procedure is given under Materials and methods; each point represents the mean (± standard error of the mean) from five replicate experiments.

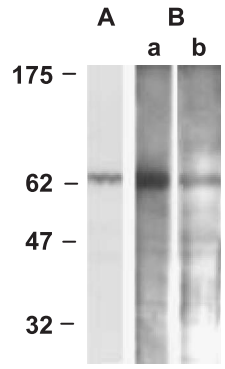

Fig. 4. Identification of the recombinant photolyase. (A) The recombinant photolyase (10 μg of protein) was size-separated by $NaDodSO_4$/PAGE using a 10% polyacrylamide gel and stained with Coomassie brilliant blue. (B) For the Western blot analysis the PoAb-PHR antiserum, raised against the recombinant *A. vastus* protein, was used. After transfer the filter with the recombinant protein was either incubated with untreated PoAb-PHR antiserum (B; lane a) or with the antiserum pre-adsorbed with the recombinant photolyase (B; lane b), as described under Materials and methods.

if the bacteria transformed with *pGEX-4T-2/APHVAPHR* remained in the dark the viability dropped almost to zero; not shown. A similarly strong UV-B sensitivity is observed if the cells were transformed with the empty vector, irrespective of a post treatment with light (not shown).

3.4. Preparation of recombinant PHR_APHVA and raising of antibodies

In order to prepare recombinant photolyase protein, the sponge gene *APHVAPHR* was inserted into the pGEX vector, comprising also the gene for glutathione-*S*-transferase as described under Materials and methods. The photolyase-related protein was expressed as a fusion protein. After induction with IPTG, the fusion protein was purified and subsequently cleaved with thrombin. The size of the recombinant sponge photolyase-related protein, r-PHR_APHVA, was determined to be 65 kDa by $NaDodSO_4$/PAGE, matching well with the deduced molecular weight (Fig. 4A).

In the second step, polyclonal antibodies were prepared against the *A. vastus* recombinant photolyase (r-PHR_APHVA), PoAb-PHR. They were used for the identification of the recombinant protein in the Western blot experiment (Fig. 4B). The nontreated antibody reacted strongly with the protein band of a size of 65 kDa (lane a); in contrast, only a weak signal is seen if PoAb-PHR had been pretreated with recombinant protein prior to the application in the Western blot experiment (lane b).

3.5. Binding of recombinant PHR_APHVA to modified DNA

Herring sperm DNA was modified by UV light to introduce thymine dimers. Treated DNA or nontreated control DNA, at concentrations of either 0.1 or 1 nmol per spot, was spotted onto Nylon filters and incubated with anti-thymidine dimer specific antibodies, as described under Materials and methods. After washing the immunocomplexes were detected using a secondary, labeled antibody. As shown in Fig. 5 (upper row), only the spots with thymine dimer-containing DNA were stained with anti-thymidine dimer specific antibodies (and the labeled secondary antibody). Both amounts of spotted UV-light treated DNA applied (0.1 and 1 nmol/spot) were recognized by the antibodies.

In a second series of experiments the DNA, immobilized onto Nylon filters, was incubated first with the r-PHR_APHVA (fusion protein) and subsequently with the anti-glutathione-*S*-transferase antibodies. It is shown that the recombinant sponge photolyase recognized only the UV-light modified DNA, while the recombinant protein did not bind to the nontreated DNA (Fig. 5; lower row).

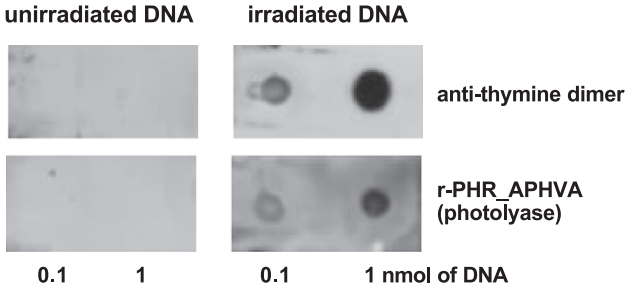

Fig. 5. Binding studies of anti-thymidine dimer specific antibodies or of recombinant photolyase from the sponge either to nontreated DNA (left panel) or to UV-light treated DNA (right panel). The two DNA preparations were spotted onto Nylon filters (either at 0.1 or 1 nmol of DNA) and incubated with antibodies as indicated. In the upper row the anti-thymidine dimer-specific antibodies were applied (detection of the immune complexes with labeled anti-mouse IgG), while in the lower row the DNA was reacted with the recombinant fusion protein r-PHR_APHVA and detection was performed with anti-glutathione-*S*-transferase antibodies. Further details are given under Materials and methods.

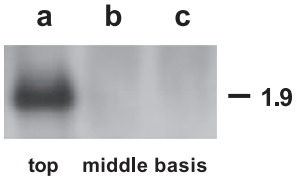

Fig. 6. Northern blot analysis to determine the relative steady-state level of expression of *APHVAPHR* gene in *A. vastus* tissue. Samples from the top (lane a), middle part (lane b) and the bottom part of the sponge (lane c) were taken and total RNA was extracted. Total RNA samples (5 μg each) were size-separated in an agarose gel and blotted onto Hybond N^+; the size (1.9 kb) is given on the right margin. Hybridization was performed with a labeled *APHVAPHR* fragment as described under Materials and methods. Finally the RNA:DNA signals were identified with applying the chemiluminescence procedure.

3.6. Expression of photolyase-related protein in the animal

In order to obtain a (relative) measure for the steady-state expression of the gene encoding the photolyase-related protein, samples from different regions of the sponge were taken. Northern blot analysis revealed that RNA isolated from the top of the animals contained large amounts of photolyase transcripts (Fig. 6; lane a), while in the RNA from the middle part (lane b) or the bases of the animals (lane c) no signal could be detected. The transcript size was 1.9 kb.

4. Conclusion

We have previously demonstrated that demosponges have an efficient enzymatic DNA excision repair system that is inducible by ultraviolet light [39]. We now show that a representative of the most ancient group of sponges, the hexactinellid *A. vastus*, possesses a gene encoding the protein (6-4) photolyase, which displays DNA repair activity. The finding that this gene is expressed in those regions of the sponge which may be most exposed to UV light in the environment that these animals inhabit today suggests that this gene is also inducible by UV light.

The hexactinellid sponge *A. vastus* typically inhabits depths of up to 500 m on the coast of British Columbia, Canada, but specimens may be found at 35–40 m and even as shallow as 5 m [43]. Despite the oligotrophic waters of this coast, ultraviolet light (UV-A and UV-B) penetrates to a depth of over 20 m. Under those conditions the light intensity can reach the organisms to approximately 10% of that measured at the surface [40]. The Hexactinellida are the phylogenetically oldest metazoan taxon within the phylum Porifera. These sponges are estimated to have evolved from the common ancestor of the Metazoa, the Urmetazoa, approximately 600 million years ago [5,9]. The evidence that this period was characterized by a high oxygen content of the atmosphere [41] suggests that living organisms were exposed to higher levels of ultraviolet light than at present.

The major damage caused by ultraviolet light is the formation of pyrimidine cyclobutane dimers (CPD) and (6-4) pyrimidine–pyrimidine photodimers. Two types of DNA photolyases have been found that cleave CPD dimers: CPD photolyase, or (6-4) photolyase [42]. Based on sequence similarities, the DNA photolyases have been classified together with the chryptochromes, which include blue-light receptors, into a single DNA photolyase/chryptochrome protein family [29,32].

In the present study the polypeptide deduced from *A. vastus*, PHR_APHVA, was found to display high sequence similarity to the photolyase/blue-light photoreceptor family [29]. Although it has been established that among the class I photolyases, proteins that either mediate the repair of UV-B-induced pyrimidine dimers or are involved in circadian rhythms [32] have a high sequence similarity [29], the role of these proteins in the ancestral metazoans has remained unclear. The described *A. vastus* sequence, PHR_APHVA, is unequivocally assigned to the class I photolyases based on its high sequence similarity, especially within the N-terminal α/β-domain and C-terminal helical domain. The overall domain structure (N-terminal α/β-domain–inter-domain loop–C-terminal helical domain) is also found in the sponge photolyase.

The demonstration that *E. coli* deficient in DNA repair becomes resistant to UV irradiation after transformation with a vector containing the *A. vastus* protein PRH_APHVA strongly indicates that the *A. vastus* protein displays DNA repair activity. Furthermore, the sponge recombinant PHR_APHVA bound to UV-modified DNA that contained thymine dimers, while it failed to bind to nontreated DNA. As other studies have shown that recombinant fish [32] or frog photolyase [44] will bind with high affinity to thymidine dimer-modified DNA, our results suggest that the gene encoding the sponge photolyase-related protein displays a thymidine dimer repairing enzyme activity.

Finally, Northern blot analysis of gene expression in different regions of the sponge showed that there is a high level of expression at the top of the animals, a region which is most directly exposed to light; RNA extracted from tissue taken from the middle and the base of the sponge contained no transcripts of the *A. vastus* gene *APHVAPHR*. Although it is tempting to infer from this result that the *A. vastus* photolyase gene is inducible upon light exposure, as only the upper-most region of the sponge would be directly exposed to light, unfortunately we know too little about the light environment of this sponge. However, the hypothesis that the expression of the photolyase gene is inducible is also supported by the calculated instability index for this protein, which predicted an unstable protein.

Taken together, the data demonstrate that sponges not only have an DNA excision repair system described previously in the demosponge *G. cydonium* [39], but they also have photolyase-based photo-reactivating activity, as shown here for the hexactinellid *A. vastus*. These data lend support to the view that during the Precambrian, the

period in which sponges evolved from a common ancestor of all Metazoa, these animals were exposed to intense ultraviolet light. Furthermore, these data also suggest that proteins within the photolyase/chryptochrome family, which are involved in the maintenance of the circadian rhythm, may have evolved from an ancestral protein that had photolyase activity.

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