Vol. 136: 153–161, 1996 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published June 6

Cloning of a heat-inducible biomarker, the cDNA encoding the 70 kDa heat shock protein, from the marine sponge *Geodia cydonium*: response to natural stressors

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ABSTRACT: The biomarker concept involves the use of biochemical, cellular and physiological parameters as screening tools in environmental surveillance. Stress proteins, such as heat shock proteins (HSPs), fulfill many of the requirements for being ideal candidates in a biomarker strategy for environmental monitoring. Sponges (Porifera) are one of the major phyla found in the marine hard-substrate benthos, both with respect to the number of species and biomass. However, only recently have genes from sponges been cloned. Here we describe the isolation of the cDNA encoding a heat shock protein of M_r (relative molecular weight) 70 kDa (HSP70). The cDNA HSP70 from the marine sponge *Geodia cydonium* has a length of 2.3 kb and encodes an AA sequence of M_r 72 579. The sponge HSP70 displays characteristic features of the HSP70 family. The HSP70 protein is induced by natural stressors including changes in temperature as well as pH, as demonstrated by Western blot analysis. No response was observed after treating the samples with hypotonic or hypertonic conditions. Our results provide the first molecular evidence that HSP70 of sponges is a useful biomarker.

KEY WORDS: *Geodia cydonium* · Sponges · Heat shock protein · HSP70 · Environmental stress · Biomarker

INTRODUCTION

Recently phylogenetic analysis based on molecular DNA sequence data has shown that sponges (Porifera) have evolved from the same ancestor as other metazoa (Müller 1995). They have the basic structural elements and signal transduction pathways present in other multicellular organisms. Sponges are primarily filter-feeders and do not have effective structural defense systems. Hence, these animals have developed powerful metabolic strategies which enable them to resist unfavorable environmental conditions. Sponges react to environmental stress with the induction of a series of metabolic pathways, e.g. the heat shock system (Batel et al. 1993, Müller et al. 1995), the polyphosphate metabolism (Lorenz et al. 1995), the multixenobiotic resistance transport system (Müller et al. 1996) and programmed cell death (apoptosis) (Batel et al. 1993). For the quantification of the physiological adjustments to fluctuating environments, *in vitro* cultures (regenerating sponge cubes; single cell cultures) from both freshwater and marine sponges have been introduced (Müller & Müller 1996).

Stressors of both anthropogenic and natural origin have been shown to induce the synthesis of heat shock proteins (HSPs) or stress proteins in a number of organisms including bacteria, plants and mammals (Sanders 1990). The number of HSPs induced by stressors varies and their expression is both tissue and species specific. In general 5 families of stress proteins are found in eukaryotes; 4 of them are grouped according to their molecular weights as HSP90, HSP70,

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HSP58–60 and HSP20–30 whereas the fifth HSP is termed 'ubiquitin' (Schlesinger et al. 1982). The HSPs are essential cell components, most of them being involved in the formation of transient protein complexes (Nover 1991) They may also play key roles during cell cycle and development. For example, the members of the HSP60 and HSP70 families are ATP-binding proteins involved in the folding of nascent and denatured proteins or protein complexes (molecular chaperons) (Rothman 1989).

Here we present the first report of an HSP70 cDNA from a marine sponge, *Geodia cydonium*, and its potential for monitoring aquatic environmental stress. Findings obtained mainly from studies involving vertebrates indicate that HSP70s may protect cells against environmental stressors (Lindquist & Craig 1988). Previously, we identified the ubiquitin protein and cloned the corresponding gene from the marine sponge *G. cydonium* (Pfeifer 1993a). We found that ubiquitin expression in *G. cydonium* is influenced by environmental stress. Furthermore, we showed that the sponge species *Ephydatia fluviatilis* responds to temperature stress with the induction of another stress protein, HSP70 (Müller et al. 1995). In the present study we report the cloning of sponge HSP70 cDNA from *G. cydonium*. In addition we show that the expression of sponge HSP70 is regulated by natural stressors.

MATERIALS AND METHODS

Materials. Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained from Stratagene (Heidelberg, Germany) and USB (Cleveland, OH, USA); CSPD was from Tropix (Bedford, MA, USA).

The monoclonal antibody (McAb) anti-HSP70 Ab N27-F3-4 (human) was used; it recognizes an epitope in the N-terminus of HSP70 (Diehl & Schmidt 1993). This was a gift from H. W. Stürzbecher (Heinrich-Pette-Institut, Hamburg, Germany).

The following cDNA clones have been used to screen the cDNA library: 2 human HSP70 cDNAs, M78053 (Nagle et al. 1992) and M11717 (Wu et al. 1985); in addition HSP70 cDNA clones from rat (L16764) (Longo et al. 1993), *Hydra vulgaris* (T. C. G. Bosch unpubl.), and *Petunia hybrida* (X06932) (Winter et al. 1988). In one series of experiments the sponge *Geodia cydonium* receptor tyrosine kinase cDNA was used (X72622) (Schäcke et al. 1994a).

Sponge. Live specimens of *Geodia cydonium* (Porifera, Demospongiae, Geodiidae) were collected near Rovinj (Croatia). Immediately after being collected from a depth of 25 m at 16°C they were used for experimental analysis.

Incubation conditions. ***Thermal stress:*** Incubation was performed in filtered, oxygenated seawater. One piece each of the specimens remained untreated at 16°C for the entire period; a second piece was treated for 2 h at 26°C (heat shock) or 2 h at 6°C (cold shock) and subsequently for 18 h at 16°C. Then the material was immediately frozen in liquid nitrogen until use for RNA or protein isolation.

Ionic and pH stress: For this series of experiments the specimens were treated under the following conditions at 16°C for 2 h, and subsequently incubated in seawater at 16°C for 18 h. Hypotonic treatment: samples were incubated with diluted seawater (diluted 1:1 with distilled water); hypertonic treatment: incubation in seawater supplemented with 0.25 M NaCl; pH treatment: incubation in seawater at a pH of 10.0 (adjusted with NaOH) or in seawater of pH 6 (adjusted with conc. HCl).

Isolation of *Geodia cydonium* HSP70 cDNA. Screening of the cDNA library from *G. cydonium* (Pfeifer et al. 1993b) was performed under low stringency hybridization conditions of plaque lifts from 3×10^5 pfu (plaque-forming units) on nitrocellulose using the human HSP70 cDNA (Ausubel et al. 1995). Filters were hybridized overnight at 42°C in 35% formamide, 5 × sodium chloride/sodium citrate buffer (SSC), 0.02% $NaDodSO_4$, 0.1% N-laurylsarcosine and 1% blocking reagent (Fathallah et al. 1993). The dsDNA restriction fragment was labelled with digoxygenin-11-dUTP using the random primed labelling kit (Boehringer, Mannheim, Germany). Filters were washed twice in 2 × SSC, 1% $NaDodSO_4$ at room temperature, followed by 2 additional washes in 0.1 × SSC, 0.1% $NaDodSO_4$ (42°C). Positive clones were detected with an alkaline phosphatase conjugated anti-digoxygenin antibody using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) as substrate (Blake et al. 1984). Single phage plaques were obtained by 3 additional screening cycles. Following an *in vivo* excision procedure described by Stratagene (Heidelberg), phagemids (pBluescript SK^-) were excised from lambda phages using the filamentous helper phage R408 and the *Escherichia coli* strain XL-1-blue. The positive sponge HSP70 clones were named GCHSP70.

DNA sequencing: The DNA for sequencing was isolated by alkaline lysis according to Sambrook et al. (1989). DsDNA was sequenced by the dideoxy chain termination method (Sanger et al. 1977) using the 'Sequenase Sequencing Kit'.

Sequence analysis: Identification of sites and signatures was performed with programs available in PC/GENE (1995). Homology searches were performed via the E-mail servers at the European Bioinformatics Institute, Hinxton Hall, UK (blitz@ebi.ac.uk and fasta@ebi.ac.uk) and the National Center for Biotech-

nology Information, National Institutes of Health, MD, USA (blast@ncbi.nlm.nih.gov).

Northern blot. Total sponge RNA was isolated as described previously and subjected to denaturing agarose gel electrophoresis (Pfeifer et al. 1993a). Hybridization with heterologous cDNA probes was performed as follows: after denaturation of RNA at 68°C for 10 min in electrophoresis buffer (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA; pH 7.0) containing 50% formamide and 18% formaldehyde, the samples were separated on 1.2% agarose gels containing 660 mM formaldehyde and blot-transferred to Nylon 66 membranes (Ausubel et al. 1995). The detection of the digoxygenin-labelled probe was performed using CSPD [disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1]decan-4-yl)phenyl phospate] as substrate for alkaline phosphatase (Beck & Koestner 1990). Hybridization to sponge cDNA GCHSP70 was performed at high stringency (Ausubel et al. 1995).

SDS-PAGE and Western blot. Gel electrophoresis of the extracts was performed in 10% polyacrylamide gels containing 0.1% $NaDodSO_4$ (SDS-PAGE) according to Laemmli (1970). Total cellular extracts of the sponge were obtained as follows: frozen sponge was homogenized in liquid nitrogen and transferred to phosphate buffered saline, supplemented with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation (10 000 × *g*; 4°C; 30 min) the supernatant was subjected to gel electrophoresis.

The gels were either stained for proteins with Coomassie brilliant blue or were electrotransferred as described by Kyhse-Andersen (1984) onto polyvinylidenedifluoride-Immobilon P. Filters were processed (Towbin et al. 1979) and incubated with anti-HSP70 Ab after blocking the membranes with 3% bovine serum albumin. The immune complexes were visualized by incubation with anti-mouse IgG (alkaline phosphatase-conjugated), followed by staining with bromochloroindolyl phosphate/nitro blue tetrazolium substrate. For semiquantitative analysis, the blots were scanned with an integrating densitometer (Shimadzu CS-910/C-R1A).

Further analytical procedures. For determination of protein the Fluoram method was used (Weigele et al. 1973); the standard was bovine serum albumin.

RESULTS

Synthesis of a 70 kDa protein in *Geodia cydonium* after heat stress

In a preliminary approach to determine if the sponge *Geodia cydonium* reacts to thermal stress with expression of the HSP70 protein, the protein patterns of non-stressed and temperature-stressed (heat) specimens of *G. cydonium* were analyzed by SDS-PAGE (Fig. 1A). The proteins were then examined by Western blotting (Fig. 1B). As shown, one polypeptide present in the extract from stressed animals of M_r 70 000 immunologically crossreacted with McAb anti-HSP70 Ab (Fig. 1B, lane b); this protein is absent in the controls (lane a). These data suggest that the 70 kDa protein represents the HSP70.

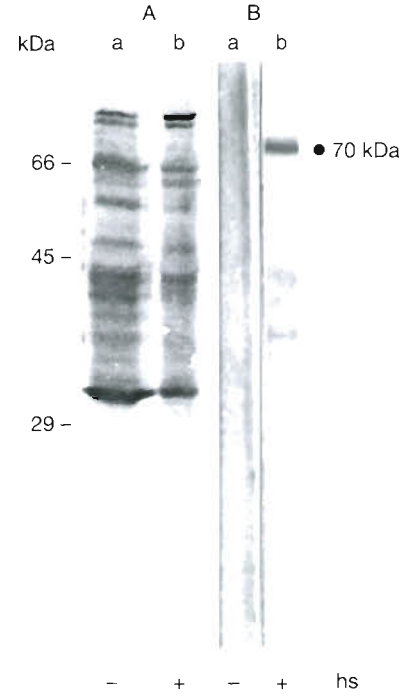

Fig. 1. Identification of a 70 kDa protein in a protein extract from temperature stressed *Geodia cydonium*. Animals either remained for the entire incubation period at the ambient temperature of 16°C (hs: –) or were heat-stressed at 26°C (hs: +) as described in 'Materials and methods' (A) Proteins (5 µg of protein per lane) from non-stressed (lane a) or stressed sponges (lane b) were size-separated by PAGE, and stained with Coomasie brilliant blue. (B) As in A, proteins extracted from a control animal (lane a) or from a heat-treated one (lane b) were transferred to Immobilon sheets after electrophoretic separation, and incubated with McAb anti-HSP70 Ab. The immunocomplex is visualized by a secondary antibody as described under 'Materials and methods'

Identification of HSP70 transcript with heterologous cDNA probes

Northern blot studies were performed with the following cDNA probes coding for HSP70: from human (2 clones), rat, *Hydra vulgaris* and *Petunia hybrida*. The results revealed that the 2 human HSP70 cDNAs showed the strongest positive signals with RNA iso-

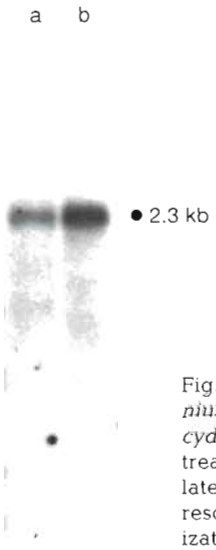

Fig. 2. Identification of *Geodia cydonium* HSP70 by Northern blotting. *G. cydonium* was temperature (heat)-treated; subsequently RNA was isolated and 5 µg of RNA per lane was resolved by electrophoresis. Hybridization was performed with the 2 human HSP70 cDNAs. M78053 (lane a) and M11717 (lane b)

lated from specimens of temperature-stressed *Geodia cydonium* (Fig. 2). The size of the hybridizing band was 2.3 kb (lanes a and b), which is compatible with other reported eukaryotic HSP70 genes (Gellner et al. 1992). A weaker signal of similar size was seen with the probe from rat and *P. hybrida* while no hybridization signal could be detected using the *H. vulgaris* gene probe (not shown). The human probe M11717 was used for the subsequent screening of the sponge cDNA library.

Cloning of the HSP70 cDNA and its deduced AA sequence from *Geodia cydonium*

A human HSP70 cDNA was used to identify and isolate the corresponding cDNA clone from the marine sponge *Geodia cydonium*. Seven independent clones, each with the same sequence (also at the 5'-termini) were analyzed; they are termed GCHSP70. All clones contained the 2.1 kb long cDNA insert. The open reading frame for GCHSP70 with the ATG-codon for Methionine [at nucleotide (nt) 19] is 1989 base pairs long (Fig. 3). The typical signal polyadenylation site AATAAA (Zarkower et al. 1986) is not present as in most cDNAs from *G. cydonium* (Pfeifer et al. 1993b).

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GCHSP70 GAGAGAAAGAAACGGACGATGGCTAAGAAGGCACCAGCCGTAGGGATCGATCTGGGCACG   60
GCHSP70 ACGTACTCGTGTGTCGGCGTTTTCCAGCACGGCAAGGTGGAGATTATCGCCAACGACCAG  120
GCHSP70 GGGAACAGAACCACTCCGAGCTACGTCGCCTTTACGGATTCGGAGCGACTCATCGGCGAT  180
GCHSP70 GCGGCCAAGAATCAGGTCGCTATGAACCCCACAAACACGGTCTTCGACGCCAAGCGGTTC  240
GCHSP70 ATCGGGCGCCGGAGCAACGACCCCGTCGTGAGCAGCGACAAGAAGCACTGGTCGTTTGAG  300
GCHSP70 GTCATCGACGAAGCCGGGCGACCAAGGGTCCGTGTCGAGTACAAGGGCGAGAAGAAGTCG  360
GCHSP70 TTCTTTGCTGAGGAAATCTCGTCCATGGTTCTGACCAAGATGAAGGAGACGGCCGAGGCT  420
GCHSP70 TACTTGGGCAAGACGATCACCGACGCTGTCGTAACGGTCCCGGCGTACTTCAACGACTCT  480
GCHSP70 CAGCGACAGGCCACAAAAGACGCAGGTATCATCTCAGGGCTCAACATCCTCCGAATCATC  540
GCHSP70 AACGAGCCGACGGCCGCCGCCATTGCGTACGGACTGGACAAGAAACACGACTCCTCGGAG  600
GCHSP70 CAAAACATTCTCATTTTCGATCTTGGCGGAGGTACTTTTGATGTTTCCATCCTCACCATC  660
GCHSP70 GAGGAGGGGATATTTGAGGTGAAATCCACTGCTGGAGATACCCATTTGGGTGGCGAGGAC  720
GCHSP70 TTTGACAACCGGATGGTGAACCACTTCATATCCGAGTTCAAACGAAAGTTCAAAAAAGAC  780
GCHSP70 ATGTCGGGAAATAAGAGAGCTGTGCGTCGGTTGAGAACTGCCTGCGAACGTGCCAAGCGT  840
GCHSP70 ACCCTGTCCTCCATCACAGAGGCAAGTATCGAAATCGACTCGCTCTTTGAGGGAATCGAC  900
GCHSP70 TATTACACCAAGATCACTCGGGCCAGGTTCGAGGAGCTGTGCGGAGACTTATTCCGCGGT  960
GCHSP70 ACATTGGAGCCTGTCGAGAAAGCTCTGCGAGACTCCAAATTTGACAAGGGTCAGATCCAC 1020
GCHSP70 GAGATTGTCCTGGTCGGAGGATCCACTCGAATCCCTCGTATTCAGAAACTTCTCCAGGAC 1080
GCHSP70 TTCTTCAATGGGAAGACGCTGAACAAATCCATCAACCCTGACGAGGCTGTGGCGTACGGC 1140
GCHSP70 GCTGCCATCCAAGCCGATATCCTCACTGGAGACACCAGCGAGGAAGTTCAAGACCTCCTC 1200
GCHSP70 CTCCTCGACGTCACTCCACTGTCCCTCGGAATTGAGACAGCTGGTGGAGTCATGACTGCC 1260
GCHSP70 CTCATCAAGAGAAACTCTACGATCCCTAAAAAGGAAACAGAGACCTTTACGACATACTCT 1320
GCHSP70 GACAACCAGCCTGGTGTCCTCATCCAGGTGTACGAGGGAGAGAGAGCCATGACAAAAGAC 1380
GCHSP70 AACAACCTCCTGGGTAAATTTGAACTCACGGGGATCCCCCCTGCCCCAAGGGAAGTCCCC 1440
GCHSP70 CAGATTCAGGTGACCTTTGACATTGACAGAAACGGGATCCTGAATGTATCGGCCGCTGAC 1500
GCHSP70 AAGAGCACGGGGAAGGAGAACAAGATCACCATCACCAACGACAAAGGTCGATTGAGTGCA 1560
GCHSP70 GAAGAGATTGACCGAATGGTACGTGAAGCGGAGCAATACAAGGCGGCAGACGACGCCCAG 1620
GCHSP70 AGAGAGCGAGTATCGGCCAAAAACCAACTCGAGAGCTACGCATTCCAAATGAAGAGCACC 1680
GCHSP70 TTTGAGGAGGACAAGGTAAAGGAGAAGGTCCCGGAGGAAGACCGTGAGAAAGTCATCAGC 1740
GCHSP70 AAGTGCAAGGAGGTCATCGATTGGCTGGACAAGAACCAGTCGGCGGAGAAGGAAGAATTT 1800
GCHSP70 GAGCACCAGCAGAAGGAGTTGGAGGGTATCTGCACGCCCATTGTGACCAAGCTCTACCAG 1860
GCHSP70 GCCGGCGGTGCACCGGGTGGTGGGATGCCGGGAGGAATGCCAGGAGGTATGCCAGGCGGG 1920
GCHSP70 ATGCCTGGAGGGTTCCCTGGAGGTGCTGGCCCTACGTCTGGGGGGTCAACTGGAGGTGGG 1980
GCHSP70 AGTGGGCCAACCATCGAGGAGGTTGACTAGACAGTCACTGCTGCAGAGACAGAAAAACTG 2040
GCHSP70 CCTATTTATGTGTAGTGTATTATTGTGTGTGTGATCTCTGTATTCTCTTCAATACTCTGA 2100
GCHSP70 ACAAACACA20                                                   2109
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Fig. 3. Nucleotide sequence of the sponge HSP70 gene. The start ATG and the stop TAG triplets are double-underlined

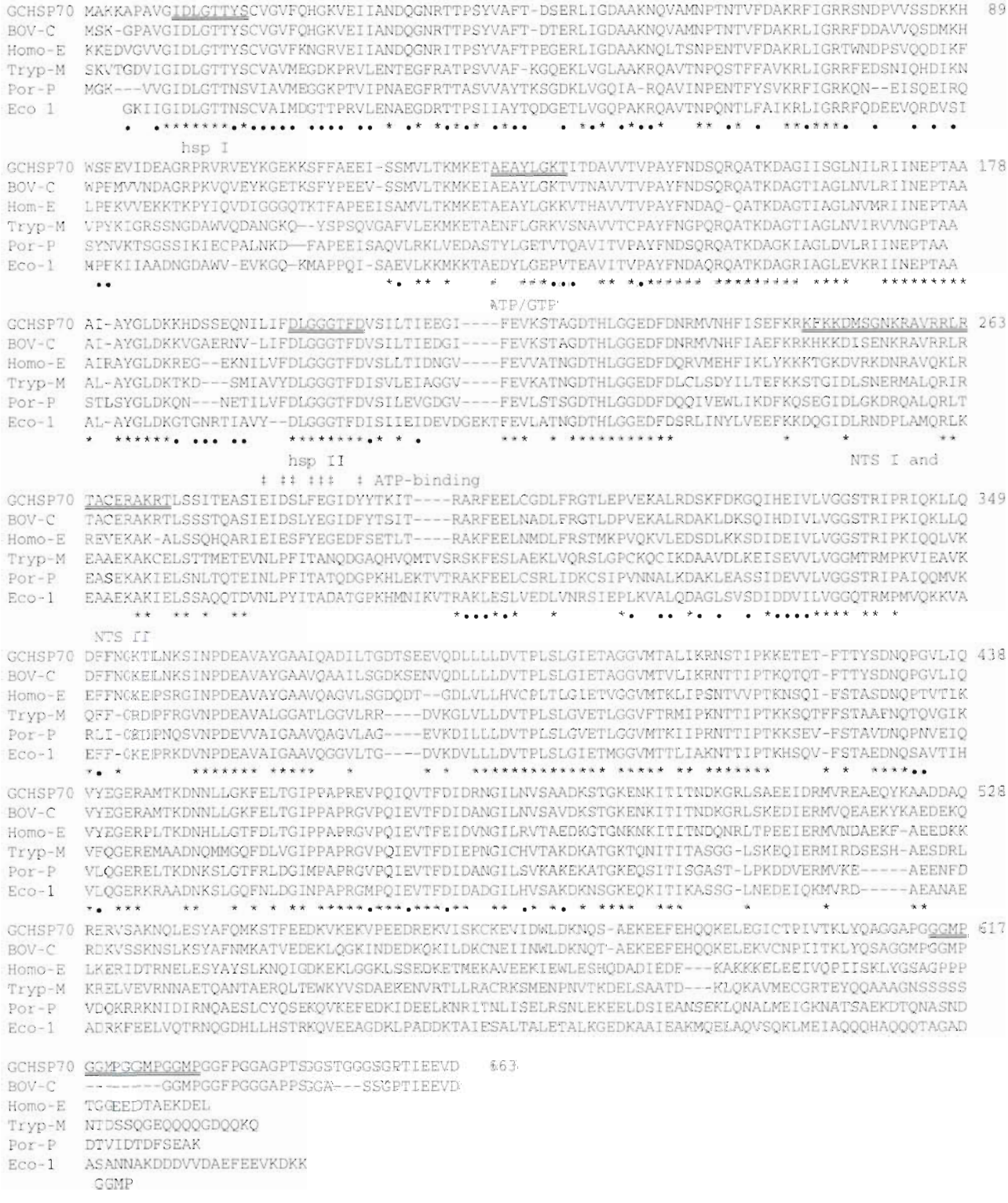

Fig. 4. Alignment of the AA sequence for HSP70, deduced from the cDNA isolated from the marine sponge *Geodia cydonium* (GCHSP70) with selected HSP70s from other organisms or from organelles. Alignment was performed with HSP70s from cow (BOV-C; accession number P19120), human (Homo-E; P11021), *Trypanosoma cruzi* (Tryp-M; M73627), chloroplast from *Porphyra umbilicalis* (Por-P; X62240) and *Escherichia coli* (Eco-1; P04475). Identities are marked with an asterisk (*); homologies (>50%) (•). The following signatures, characteristic for HSP70, are double underlined: heat shock HSP70 proteins family signature 1 (abbreviated hsp I), heat shock HSP70 proteins family signature 2 (hsp II), bipartite nuclear targeting sequences (NTS), ATP/GTP-binding site motif A (P-loop) (ATP/GTP) and the GGMP motif (GGMP). The putative ATP-binding site is also marked (‡)

The deduced AA sequence of the open reading frame for the sponge HSP70 is shown in Fig. 4. The 663 AA long sequence has a deduced M_r of 72 579, and isoelectric point of 5.04; the instability index has been computed to be 41.01, indicating an unstable protein (PC/GENE 1995).

Analysis of the deduced AA sequence from *Geodia cydonium* revealed that it has the typical characteristics for an HSP70; it comprises (1) the heat shock HSP70 proteins family signature 1, IDLGTTYS (AA 10 to 17); (2) the heat shock HSP70 proteins family signature 2, DLGGGTFD (AA 201 to 208) (Craig & Gross 1991); and (3) the ATP-binding site with the sequence LxFDxGGGxxD (AA 196, 198–199, 201–203, 206); the latter segment binds the Mg^{2+} ion and the β-phosphate of MgATP (Fletterick et al. 1975). In addition, the *G. cydonium* GCHSP70 displays the following characteristic sites: (1) one ATP/GTP-binding site motif A (P-loop) (AA 132 to 139) and (2) 2 bipartite nuclear targeting sequences at AA 248 to 264 and 259 to 275 (PC/GENE 1995). The ATP/GTP-binding site motif A (P-loop) interacts with one of the phosphate groups of the nucleotides (Walker et al. 1982).

Sequence comparison of sponge HSP70 with HSP70s from other organisms

The AA sequence of the *Geodia cydonium* HSP70 cDNA, GCHSP70, was aligned (Fig. 4) with HSP70 sequences of the group of cytoplasmic [cow (BOV-C)], endoplasmic reticulum [human (Homo-E)], mitochondrial [*Trypanosoma cruzi* (Tryp-M)], chloroplast [*Porphyra umbilicalis* (Por-P)] and eubacterial HSP70s [*Escherichia coli* (Eco)] as summarized by Boorstein et al. (1994). The analysis of the pairwise similarity scores revealed (PC/GENE 1995) that GCHSP70 shows highest homology to cow (BOV-C) HSP70 with 70% similarity and to a lower degree to human (Homo-E) (26%), *P. umbilicalis* (Por-P) (15%), *E. coli* (Eco) (14%) and *T cruzi* (Tryp-M) (12%).

These data indicate that the GCHSP70 belongs to the subfamily of cytoplasmic HSP70s. This conclusion is also supported by the existence of 4 GGMP motifs within AA 614 to 629 (McKay et al. 1994); this tetrapeptide repeat sequence is thought to be involved in the folding/unfolding function (Gellner et al. 1992).

RNA analysis

Sponge HSP70 mRNA was identified by Northern blots using GCHSP70 as a probe. As shown in Fig. 5A the RNA isolated from animals after thermal stress contained a 2.3 kb long transcript (lane b), which was absent in control animals (lane a). This size matches those found to be typical of HSP70 mRNAs from other sources (Gellner et al. 1992).

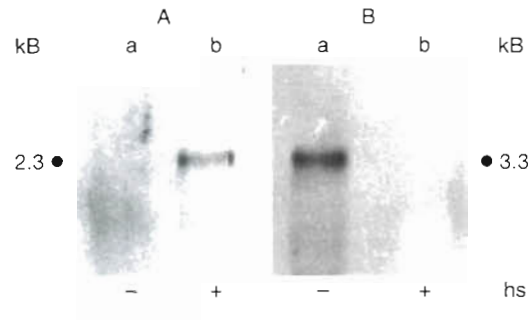

Fig. 5. Northern blot analysis using HSP70 and RTK gene probes. From animals that remained either untreated (lanes a) or have been temperature-stressed (lanes b) RNA was isolated and 5 µg each were analyzed. Separate blots were either hybridized with the *Geodia cydonium* GCHSP70 clone (A) or with the RTK cDNA from the same species (B). Further details are given in 'Materials and methods'

In one series of experiments it was determined that the steady state level of RTK transcript changes after heat stress. It was found (Fig. 5B) that the level of RTK mRNA is strongly down-regulated in animals which had been treated by heat (lane b); in the controls the 3.3 kb transcript is present (lane a).

Expression of sponge HSP70 under different ionic and pH conditions

In these studies the animals were kept at a constant ambient temperature but were treated for 2 h under the following conditions: in hypotonic or hypertonic environment or at pH 6 or 10; the latter extreme pH condition was chosen to estimate the degree of resistance potential of the sponge species. After incubation the animals were transferred to fresh seawater at ambient temperature. Protein extracts were prepared and analyzed by Western blotting for the presence of HSP70.

The experiments show (Fig. 6) a low expression of HSP70 in the controls (lane k). If the animals were treated by heat stress, either at 10°C above (lanes g and i) or below the ambient temperature (lane h) an approximately 15-fold increase in the amount of immunologically cross-reacting protein, HSP70, in *Geodia cydonium* is seen. Treatment of the samples in an hypotonic (lanes d and f) or hypertonic environment (lanes c and e) did not change the expression significantly. However, if the sponge was treated in an pH 6 (lane a) or pH 10 environment (lane b) expression of HSP70 is increased by 8- and 4-fold, respectively.

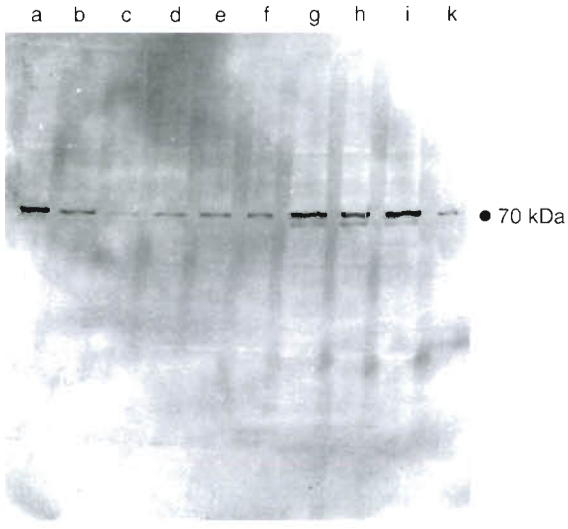

Fig. 6. Level of expression of HSP70 in *Geodia cydonium* after treatment of the animals under different stress conditions. Samples were treated as follows: at different temperature stress conditions of 6°C (lane h) or of 26°C (lanes g and i), at hypertonic (lanes c and e) or hypotonic (lanes d and f) environmental conditions or at pH 6 (lane a) or pH 10 (lane b); the control (non-treated sample) is shown at lane k. Extracts were prepared, size-separated and analyzed by Western blotting using the McAb anti-HSP70 Ab

DISCUSSION

Heat shock proteins, the 'classic' stress proteins, have the potential to act as biomarkers for general stress (Sanders 1990). In earlier reports and in the present study it was shown that this heat shock response is also a fundamental aspect of cellular physiology in freshwater and marine sponges. Exposure to temperature stress (Müller et al. 1995 and references therein) or xenobiotics (Batel et al. 1993) results in a dramatic reaction including induction of HSPs (especially HSP70) and suppression of other cellular proteins. With the isolation of the cDNA, encoding for HSP70 from the marine sponge *Geodia cydonium*, a tool is now available to analyze the reaction of a sponge to stressors at the molecular level.

HSP70s are the most prominent proteins inducible by heat (summarized in Becker & Craig 1994). They allow adaptation of cells to high temperatures by chaperoning proteins damaged during thermal stress. *In vivo* the role of the inducible form of HSP70s has been studied both in prokaryotic and in eukaryotic organisms (summarized in Parsell & Lindquist 1994). Unlike in *Escherichia coli* and in *Saccharomyces cerevisiae*, the HSP70s in insects and in vertebrates are the major proteins involved in thermotolerance. In mammalian cells, the HSP70 family comprises at least the following major members: the glucose-regulated protein (GRP) 78 (localized in the endoplasmic reticulum), GRP75 (mitochondria), the constitutive HSP (HSC) (cytoplasm) and the inducible HSP70 (Welch 1990). Based on data from sequence comparison and the experimental finding that the respective gene in *Geodia cydonium* is expressed during thermal stress, this sponge protein is likely to be a member of the group of inducible HSP70.

Besides their function in chaperoning unfolded protein during heat stress, the HSP70s are also involved in the elimination/correct folding of proteins after exposure of cells to metals. Previously, it has been described that in response to exposure to organotin and methyl mercury the *Geodia cydonium* HSP70 is induced prior to apoptosis (Batel et al. 1993). Similar findings have been described for rat hepatocytes (Bauman et al. 1993).

HSP70 in *Geodia cydonium* is expressed not only during heat and cold shock, but also if the animals are exposed to pH stress. The latter natural stressor is of ecological importance, as it has been established that a large fluctuation in pH results in periodic fish kill especially in freshwater and brackish water zones (Mason 1991). There is little present information about HSP70 expression in response to these natural stressors, although it has been shown that promastigotes (a stage of the parasite) of *Leishmania donovani* respond with synthesis of HSPs after both heat and pH shock (Salostra et al. 1994). In the present study we demonstrate experimentally that the sponge under investigation adapts to this unfavourable change in the environment with the expression of HSP70. A further outcome of this study is the finding that *G. cydonium* does not react to changes in the osmolarity of the seawater. This finding might be due to the fact that this species lives in a depth below 20 m where changes in the osmolarity are unlikely to occur. The animals used for the determinations of the expression of the HSP70 gene in response to stress were collected from 3 to 16 August 1995. The results reported here were qualitatively identical with those obtained with sponges collected during 5 to 20 March 1995. It should be noted here that we only found a positive or negative expression of HSP70 gene under heat shock in a few cases (2 out of 10 experiments), as shown in Fig. 1B, lanes a and b. In most samples, a small amount of HSP70 protein could be detected by Western blotting, which reflects the fact that HSP70 is also—to some extent—physiologically expressed (Sanders 1990).

HSP70 has been described to be associated with the hepatic glucocorticoid receptor (Diehl & Schmidt 1993). In addition, it has recently been shown that tyrosine kinases, e.g. MEKK1, are activated during heat stress (Yan et al. 1994). Hence, we studied the steady state level of the transcript of the receptor tyro-

sine kinase (RTK) gene during thermal stress. The RTK was recently cloned from *Geodia cydonium* (Schäcke et al. 1994b); it carries an extracellular, immunoglobulin domain (Schäcke et al. 1994a). Surprisingly, the level of RTK mRNA is down-regulated during thermal stress. This finding is an indication of alterations in the signal transduction pathways of *G. cydonium* during temperature shift.

The potential value of HSP70 expression as a tool to evaluate the impact of environmental stressors on *Geodia cydonium* has been demonstrated in the present *in vitro* studies. Field studies are ongoing to investigate conditions under which HSP70 is expressed as a response to natural and anthropogenic stressors. Attention will be given to the determination of threshold temperatures and/or pH levels in order to estimate if other environmental stressors affect the expression of HSP70.

Acknowledgements. This work was supported by grants from the Commission of the European Communities (93/Avi 001), MED-CAMPUS Project C034 and the 'Bundesministerium für Bildung und Forschung'. The sequence reported here is deposited in the EMBL/GenBank data base (accession no. X94985).

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This article was submitted to the editor

Manuscript first received: January 8, 1996
Revised version accepted: February 27, 1996