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Induction of gene expression of the chaperones 14-3-3 and HSP70 by PCB 118 (2,3',4,4',5-pentachlorobiphenyl) in the marine sponge *Geodia cydonium*: novel biomarkers for polychlorinated biphenyls

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ABSTRACT: Polychlorinated biphenyls (PCBs) are ubiquitous industrial compounds found in almost every component of the terrestrial and marine ecosystem. Most of the PCB congeners bind to the aryl hydrocarbon receptor and in turn cause expression of stress response genes. Here we report for the first time that PCB 118 acts in the marine sponge *Geodia cydonium* as an inducer of 2 chaperones, the 14-3-3 protein(s) (a protein targeting molecule) and the heat shock protein HSP70 (a chaperone, primarily involved in folding of proteins). While the cDNA encoding the latter protein has been cloned previously, the *14-3-3* cDNA from sponges is reported in this study. The full-length cDNA clone of *G. cydonium*, *GC14-3-3*, has a size of 912 nucleotides (nt) and contains a 744 nt long potential open reading frame; the relative molecular weight (M_r) of the deduced aa sequence is 28 378 Da. The sponge polypeptide is closely related to the deduced polypeptides of the 14-3-3 sequences belonging to isoforms η and γ. Using the cDNAs, coding for the 14-3-3 and the HSP70 proteins as well as antibodies raised against these 2 proteins, it was demonstrated that neither chaperone can be detected in the absence of PCB. However, after incubation of sponge tissue with PCB 118 the transcripts of the 2 chaperones are detectable after 12 h, while the corresponding proteins appear after 1 d. Subsequently, the levels of the transcripts and of the proteins increase steadily. From these data we conclude that the 2 chaperones, 14-3-3 and HSP70, are useful biomarkers in sponges. Due to the broad cross-reactivity of their antibodies throughout the Metazoa, these chaperones may be useful biomarkers for monitoring environmental contaminants, as shown here for PCB 118, in all organisms.

KEY WORDS: *Geodia cydonium* · Sponges · Heat shock protein · 14-3-3 · HSP70 · Chaperones · Environmental stress · Biomarker

INTRODUCTION

Polychlorinated biphenyls (PCBs) have been used since the early 1930s in electrical equipment, in the manufacture of paints, plastics, adhesives and coating compounds, as well as pesticide extenders, flame retardants, and organic diluents (Safe 1990, Clark 1997). The widespread use of PCBs coupled with improper disposal practices has resulted in their ubiquitous introduction into the environment. Between 1977 and the mid-1980s the USA and most members of the European Union banned the production of PCBs. However, due to high bioaccumulation (Abramowicz 1995) and slow biotransformation (Jedamski-Grymlas et al. 1995) PCBs are still present in the environment. Their residues have been identified in the air, in water, in terrestrial and aquatic sediments, in fish, wildlife, and human adipose tissue, and in serum and milk (Voogt et al. 1990).

The effect of PCBs is mediated by activation of the aryl hydrocarbon (Ah) receptor (Ahlborg et al. 1992),

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which acts as a nuclear ligand-induced transcription factor that binds to the xenobiotic or dioxin responsive element located in the 5'-flanking regions of responsive genes (Safe 1995). Among these genes are those that encode the cytochrome P-450 proteins (CYP) (Shane et al. 1997). The inducibility of *CYP*, e.g. CYP1A, which can be determined by catalytic assays (Dehnen et al. 1973), immunodetection of proteins (Williams & Buhler 1984) and/or detection of mRNA synthesis (Heilman et al. 1988), has been used as a biomarker of environmental contamination with PCBs. CYP1A induction in fish appears to be one of the most useful tools for detecting PCB contamination (Stegeman et al. 1992). However, the data obtained for CYP-related enzymes in invertebrates have to be considered with more caution (Livingstone 1988). Therefore, other biomarker proteins are required to monitor the effect of PCBs in invertebrates, especially in sessile filter feeders such as sponges.

Sponges belong to the lowest metazoan phylum, the Porifera, and recent studies have revealed that these animals have many proteins which are characteristic of Eumetazoa (Müller 1995, 1998). Sponges are also provided with defense systems against xenobiotics similar to those found in higher invertebrates and in vertebrates (Müller & Müller 1998). Much emphasis has been put on those genes which are highly conserved, allowing the preparation of tools—cDNA and antibodies—which can be used for the detection of the respective transcripts and proteins of species belonging to all or to most metazoan phyla.

In our program we have focused on stress proteins such as heat shock proteins (HSPs) that can be used as biomarkers to monitor stressors of both anthropogenic and natural origin (Sanders 1990). In order to investigate those stress proteins ubiquitously present in invertebrates, the cDNA encoding the HSP70 stress protein from the marine sponge *Geodia cydonium* (Koziol et al. 1996) together with its associated protein DnaJ (Koziol et al. 1997a) were cloned. Furthermore, in order to determine the steady-state level of HSP70, antibodies against *G. cydonium* HSP70 were raised (this contribution). The fact that HSP70 is induced in both freshwater (*Ephydatia fluviatilis*) and marine (*G. cydonium*) sponges by temperature and pH stress (Koziol et al. 1996, Bachinski at al. 1997) or by non-ionic organic pollutants (Müller et al. 1995) renders this protein very useful as a biomarker.

HSPs are molecular chaperones which control and maintain the native, completely folded form of functional proteins (Becker & Craig 1994). A very different functional class of chaperone proteins consists of those that trap distinct proteins in a cellular compartment either to facilitate or to prevent their function. Among the few proteins of that class are the 14-3-3 proteins, encoded by different genes of the same size (Aitken et al. 1992), which (1) aid important cellular events such as transport of adrenodoxin precursor into the mitochondria (Alam et al. 1994), or (2) prevent the function of key molecules in cellular processes, e.g. the role of the BAD molecule in initiating apoptosis (Zha et al. 1996). Since these processes are essential to all Metazoa for tuned interactions of organelles within cells, e.g. mediated transport between cellular components (Verner & Schatz 1988), or homeostasis of cells within an organ or an organism, e.g. maintainance of the cell number with the precise differentiation state (Thompson 1995), their key molecules might be applicable as biomarkers for monitoring pollutants in different environments.

The aim of this study was to identify 14-3-3 protein(s) in the marine sponge *Geodia cydonium* using antibodies raised against the conserved region of the 14-3-3 isoforms isolated from higher Metazoa, and to determine whether the expression of 14-3-3 is upregulated after incubation of sponges with the model compound PCB 118 (2,3',4,4',5-pentachlorobiphenyl), which belongs to the group of mono-ortho-substituted chlorobiphenyls. In order to determine the expression on the level of transcription, the cDNA encoding 14-3-3 from *G. cydonium* was isolated, characterized and subsequently used as a probe. The biological effects of PCBs are due not only to the interaction with the Ah receptor (Ahlborg et al. 1992) but also to estrogenic or antiestrogenic influences (Krishnan & Safe 1993). To the best of our knowledge the upregulation of HSP70 in response to PCB treatment has not yet been reported.

MATERIALS AND METHODS

Materials. Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were from Stratagene (Heidelberg, Germany), QIAGEN (Hilden, Germany) and USB (Cleveland, OH, USA); corn oil (C8267) from Sigma (Deisenhofen, Germany), TRIzol Reagent from GibcoBRL (Grand Island, NY, USA), disodium 2-chloro-5-{4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)-tricyclo(3.3.1.1^{3,7})decan]-4-yl} phenyl phosphate (CDP) from Boehringer (Mannheim, Germany). The polyclonal antibody (rabbit IgG; cat. no. PC70) against the conserved aa residues of the 14-3-3 protein family and the 14-3-3 peptide aa_{221} to aa_{242} was obtained from Calbiochem/Oncogene (Cambridge, MA, USA). PCB 118 was obtained from Dr Ehrenstorfer GmbH (Augsburg, Germany).

Sponge. Live specimens of *Geodia cydonium* (Porifera, Demospongiae, Geodiidae) were collected near Rovinj, Croatia, from a depth of 25 m at 17°C from their habitat, the muddy sand bottoms.

Exposure of *Geodia cydonium* to PCB 118. Tissue samples of 40 g were injected with 1 ml of PCB 118 (0.1 mg ml^{-1} in corn oil). The samples were incubated in seawater for up to 6 d in 20 l aquaria at 17°C under continuous aeration; the water was changed once at Day 2. Aliquots of tissue (approximately 200 mg each) were taken at time zero, or after incubation periods of 0.5, 1, 3, 5 and 6 d. These aliquots were immediately frozen in liquid nitrogen and stored at –80°C.

Extracts. Extracts to determine the levels of 14-3-3 and HSP70 were obtained by grinding frozen tissue samples in 3 times their volume of phosphate-buffered saline, supplemented with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After centrifugation the supernatant was collected and protein content was determined (Lane 1957).

RNA was extracted from liquid-nitrogen pulverized sponge tissue with TRIzol Reagent (GibcoBRL) as recommended by the manufacturer.

Isolation of *Geodia cydonium GC14-3-3* cDNA. The complete sponge *GC14-3-3* cDNA was cloned by polymerase chain reaction (PCR) from a *Geodia cydonium* cDNA library in lambda ZAP Express™ (Pfeifer et al. 1993). The forward primer 5'-GTKGCCTACAARAAY-GTGGT-3' (where K = G/T, R = A/G and Y = C/T) in conjunction with the ZAPII 3'-end vector-specific primer T7 was used. The degenerate primer was designed against the conserved aa sequence in human 14-3-3 proteins (14-3-3 human-η aa_{47} to aa_{53}; VAYKNVVG). The PCR reaction was carried out using a GeneAmp 9600 thermal cycler (Perkin Elmer) at an initial denaturation at 95°C for 3 min, then 35 amplification cycles at 95°C for 30 s, 58°C for 45 s, 74°C for 1.5 min, and a final extension step at 74°C for 10 min. The reaction mixture of 50 µl included 20 pmol of the degenerate primer and 10 pmol of the primer T7, 200 µM of each nucleotide, 1 µl of the cDNA library (approximately 10^7 plaque-forming units, pfu), buffer and 2.5 units of *Taq* DNA polymerase (Boehringer). A fragment of ~670 bp (base pairs) was obtained by primer walking (Ausubel et al. 1995). The longest insert obtained had a size of 912 nucleotides (nt) [excluding the poly(A) tail]. The clone was termed *GC14-3-3* and was sequenced using an automatic DNA sequenator (Li-Cor 4200).

Sequence comparisons. Sequence was analyzed using the computer programs BLAST (Altschul et al. 1990) and FASTA (Lipman & Pearson 1985). Multiple alignment was performed with CLUSTAL W Ver. 1.6 (Thompson et al. 1994), and the graphic presentation was prepared with GeneDoc (Nicholas & Nicholas 1997).

Northern blot. RNA was extracted from liquid-nitrogen pulverized sponge tissue with TRIzol Reagent (GibcoBRL) as recommended by the manufacturer. An amount of 5 µg of total RNA was electrophoresed through formaldehyde/agarose gel and blotted onto Hybond N^+ membrane following the manufacturer's instructions (Amersham). Hybridization experiments were performed sequentially with 2 probes, firstly with the 762 bp long *HSP70* cDNA [EMBL accession number X94985 (Koziol et al. 1996); obtained by PCR amplification with the forward primer 5'-ACCAA-GGGTCCGTGTCGAGTA-3' and the reverse primer 5'-GTCCTGGAGAAGTTTCTGA-3'] and subsequently with the 529 bp probe *GC14-3-3* from *Geodia cydonium* (forward primer 5'-GTGGCCTACAAGAACGT-GGT-3'; reverse primer 5'-TCAGTAGTTGCATGAT-GAGAGT-3') as described (Eisel 1995). These probes were labeled with digoxigenin (DIG-11-dUTP) by the DIG DNA labeling kit. Hybridization was performed with the antisense DIG-labeled probes at 42°C overnight using 50% formamide, containing 5 × SSC (sodium chloride), 2% blocking reagent (Boehringer), 7% (w/v) SDS (sodium dodecyl sulphate) and 0.1% (w/v) N-lauroylsarcosine, following the instructions of the manufacturer (Boehringer). After washing, DIG-labeled nucleic acid was detected with anti-DIG Fab (fragment, antigen-binding) fragments (conjugated to alkaline phosphatase) and visualized by chemiluminescence technique using CDP, the chemiluminescence substrate of alkaline phosphatase, according to the instructions of the manufacturer (Boehringer). For quantitation of the signals of Northern blots (Stanley & Kricka 1990) the screen was scanned with the GS-525 Molecular Imager (Bio-Rad).

Preparation of monoclonal antibodies against sponge HSP70. The cDNA encoding the *Geodia cydonium* HSP70, *GCHSP70* (Koziol et al. 1996; accession number X94985) was subcloned into the *Bam*HI and *Sal*I sites in the bacterial 6-His tag expression vector pQE32 (QIAGEN). Expression was performed in *Escherichia coli* strain XL1-blue MRF', and the recombinant protein (termed rHSP70-polypeptide) was purified under denaturing conditions by metal-chelate affinity chromatography using Ni-NTA-agarose resin according to the manual (QIAGEN).

Monoclonal antibodies (MAb) against the recombinant rHSP70-polypeptide were raised in Balbc/A mice. Purified rHSP70-polypeptide (20 to 30 µg per treatment) was injected into mice at 4 wk intervals. After 3 boosts, spleen cells from immunized mice were fused with Ag8653 cells. The resulting hybridomas were tested by enzyme-linked immunosorbent assay, using rHSP70-polypeptide bound to the solid phase, selecting for positive Ab-producing clones according to standard procedures (Harlow & Lane 1988). The MAb selected for these studies, termed MAb-HSP70 is of the IgG class, as detected by an Ouchterlony test using class-specific goat anti-mouse antibodies (Sigma).

In control experiments 100 µl of the MAb-rHSP70 was adsorbed with 50 µg of rHSP70-polypeptide (30 min; 4°C) prior to its use.

Western blotting. Gel electrophoresis was performed in 10% polyacrylamide gels containing 0.1% $NaDodSO_4$ (PAGE). Proteins were stained with Coomassie brilliant blue. 20 µg of protein per lane was applied. Electrotransfer was performed according to Kyhse-Andersen (1984) onto PVDF (polyvinylidene difluoride)-Immobilon. Membranes were processed (Bachmann et al. 1986) and incubated either with mouse MAb-HSP70 (dilution 1:500) or with rabbit polyclonal antibody (PAb) against 14-3-3 protein (dilution 1:500). After blocking the membranes with 3% bovine serum albumin, the immune complexes were visualized by incubation with anti-mouse (in the experiments with mouse MAb-HSP70) or with anti-rabbit (PAb-14-3-3 protein) alkaline phosphatase-conjugated IgG in the presence of the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

The PAb-14-3-3 was adsorbed with 14-3-3 peptide aa_{221} to aa_{242} as described above. To quantitate the signals of Western blots the chemiluminescence procedure was applied (see above).

RESULTS

Cloning of the *GC14-3-3* cDNA and its deduced aa sequence from *Geodia cydonium*

One degenerate oligonucleotide primer, corresponding to the nt sequence of the conserved peptide present in the human 14-3-3 protein-η sequences (see 'Materials and methods') was used to detect and isolate the corresponding cDNA from the sponge cDNA library. The fragment obtained contained the deduced aa sequence characteristic of the C-terminal sequence of the different isoforms of 14-3-3 (Aitken et al. 1992). The complete cDNA obtained by primer walking was 912 nt [excluding the poly(A) tail] in length and comprised a potential open reading frame (ORF) extending from nt 82 to 825, coding for 248 aa (Fig. 1A). (The sequence reported here is deposited in the EMBL/GenBank database, accession no. Y15900.) The putative AUG initiation site displays the strong consensus sequence A_{-3}/G_{+4} (Kozak 1991) and reads AAGAUGA (the putative translation initiation site is underlined).

The deduced aa sequence of *GC14-3-3*, termed GC_1433, has a putative size (relative molecular weight, M_r) of 28378 Da and an isoelectric point (pI) of 4.63 (PC/GENE 1995). It shows the characteristic annexin-like domain (Wheeler-Jones et al. 1996), which ranges from aa_{124} to aa_{141} (Fig. 1A). As noted earlier by Ichimura et al. (1988), the acidic 14-3-3 proteins can be subdivided into 2 stretches, a less acidic N-terminal region and an extremely acidic C-terminal region, the putative binding segment to the respective ligand. In the deduced sponge sequence the pI values of the 2 regions are 5.46 (aa_1 to aa_{178}) and 3.83 (aa_{179} to aa_{248}), respectively (Fig. 1A).

Northern blot analysis was performed with the sponge *GC14-3-3* clone as a probe; one prominent band of approximately 1.0 kb was obtained, confirming that a full length cDNA was isolated (Fig. 2B).

Sequence comparison of sponge 14-3-3 with related molecules from other organisms

Until now at least 7 distinct isoforms of 14-3-3 proteins have been identified (Isobe et al. 1991, Aitken et al. 1992). Selected sequences from each isoform were chosen and aligned with the sponge sequence (Fig. 1); these are isoform β from rat (RAT-beta, accession number S83440), γ from bovine and rat (BOV-gamma, P29359; RAT-gamma, S55305), η from human, mouse, rat and bovine (HOMO-eta, X80536; HOMO-etaB, X78138; MUS-eta, 1354808; RAT-eta, D17445; BOV-eta, 112692; BOV-etaB, J03868), Θ from rat and mouse (RAT-theta, 402509; MUS-theta, U56243), ξ from sheep (SHEEP-zeta, 112696) and σ from human (STRATIFIN) (HOMO-sigma, 398953), related 14-3-3 polypeptides from *Xenopus laevis* (XENLA-D2, 214097) as well as human and mouse phospholipase A2 (HOMO-pla2, M86400; MUS-pla2, D78647), human HS1 gene product (HOMO-hs1, X57346), *Drosophila melanogaster* 14-3-3 like protein (Leonardo protein) (DROME-LP, 112683), the deduced protein cds4 from the nt sequence F52D10 from *Caenorhabditis elegans* (CAEL-cds4, Z66564), the BMH1 gene coding for a protein kinase C from *Saccharomyces cerevisiae* (YEAST-bmh1, X66206), a protein kinase C inhibitor homologue from *Spinacia oleracea* (SSOLE-pkc, X62837), and the *Zea mays* regulatory protein GF14 (ZEA-gf14, M96856).

Based on the close phylogenetic relationship of the *Geodia cydonium* sequence to the 14-3-3 isoforms γ and η (Fig. 1B), the sponge aa sequence GC_1433 was operationally termed GEODIA-ge (γ, η). On aa level, the putative 14-3-3 protein of the sponge showed the following degrees of identity (similarity) to the sequences included in this comparison (Fig. 1): isoforms γ from rat and bovine 64% (77%), η from human (eta and etaB), mouse, rat and bovine (etaA and etaB) 65% (76%), human and mouse phospholipase A2 as well as ξ from sheep 62% (77%), the 14-3-3 related polypeptides from *Xenopus laevis*, human HS1 gene product and β from rat 63% (77%), Θ from rat and mouse 60% (74%), *Drosophila melanogaster* 14-3-3 like protein (Leonardo protein) (DROME-LP) 62% (76%) and the

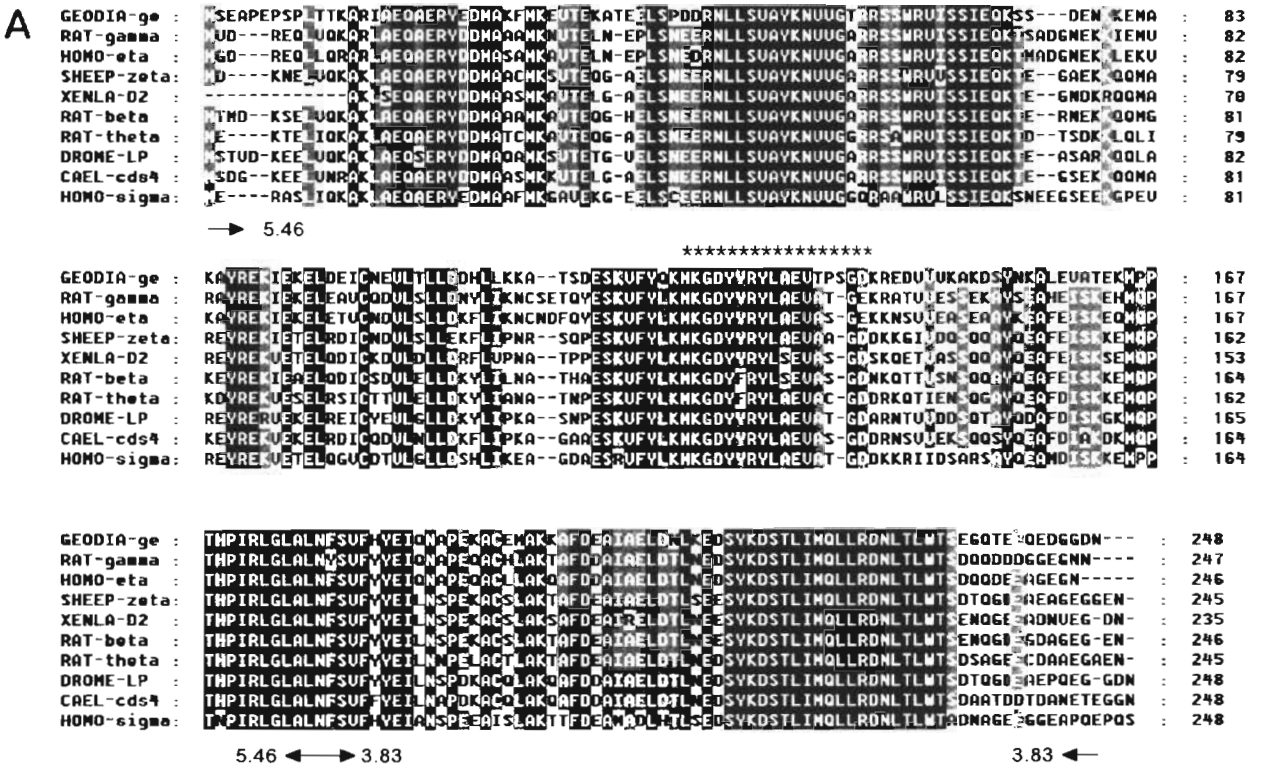
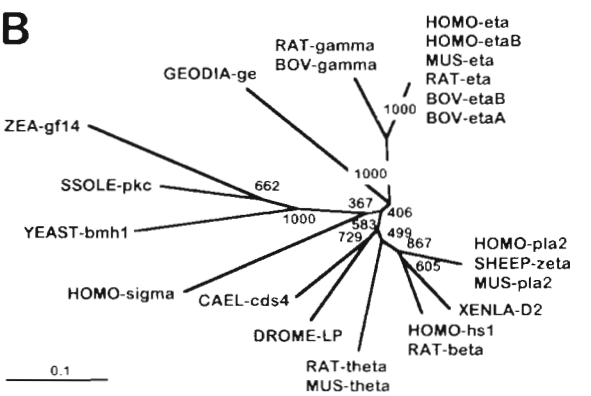


Fig. 1. (A) Alignment of the deduced aa sequences of the 14-3-3 protein from *Geodia cydonium* (GC_1433: GEODIA-ge) with the following isoforms: γ from rat (RAT-gamma), η from human (HOMO-eta), ξ from sheep (SHEEP-zeta), the 14-3-3 related polypeptides from *Xenopus laevis* (XENLA-D2, 214097), β from rat (RAT-beta), Θ from rat (RAT-theta), *Drosophila melanogaster* 14-3-3 like protein (Leonardo protein) (DROME-LP), the deduced protein cds4 from *Caenorhabditis elegans* (CAEL-cds4), and σ from human (STRAT-IFIN) (HOMO-sigma). Residues conserved in all sequences are shown in inverted type; those present in at least 5 sequences are shaded. The annexin-like domain (*) and the gaps (–) are marked. The numbers at the end refer to the aa residues of the respective sequence. The regions of different charges, with isoelectric point (pI) values 5.46 and 3.83 are indicated. (B) Phylogenetic tree using sponge 14-3-3 and the sequences listed in (A) inferred by neighbour-joining. In addition, the following sequences have been added: γ isoform from bovine (BOV-gamma), η from human, mouse, rat and bovine (HOMO-etaB, MUS-eta, RAT-eta, BOV-eta, BOV-etaB), human and mouse phospholipase A2 (HOMO-pla2, MUS-pla2), human HS1 gene product (HOMO-hs1), the BMH1 gene coding for a protein kinase C from *Saccharomyces cerevisiae* (YEAST-bmh1), a protein kinase C inhibitor homolog from *Spinacia oleracea* (SSOLE-pkc) and the *Zea mays* regulatory protein GF14 (ZEA-gf14). Numbers at the nodes refer to the level of confidence as determined by bootstrap analysis (1000 bootstrap replicates). Scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence



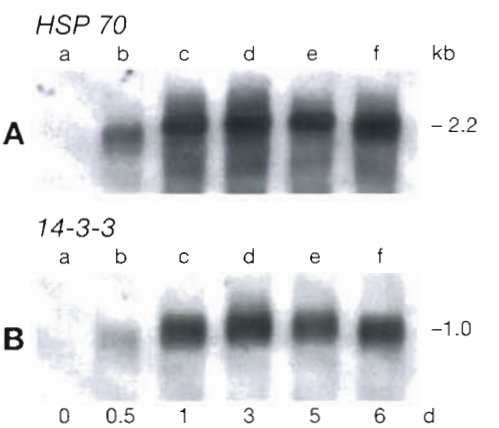


Fig. 2. Northern blot analysis to determine the size of the transcripts of the mRNA encoding (A) the HSP70 and (B) the 14-3-3 protein. RNAs from sponge samples of a specimen treated for 0 to 6 d with PCB 118 were used for the experiments. After blot transfer the hybridization was performed first with the (A) *HSP70* probe and subsequently with (B) the *GC14-3-3* probe, both isolated from *Geodia cydonium*. 5 µg of total RNA, each, were analyzed. The duration of stress is given in days (d)

deduced protein cds4 from *Caenorhabditis elegans* (CAEL-cds4) 64% (76%). The remaining aa sequences, including the plant [*Spinacia oleracea* (SSOLE-pkc) and *Zea mays* (ZEA-gf14)] and the yeast [*Saccharomyces cerevisiae* (YEAST-bmh1)] 14-3-3 protein homologs as well as human σ showed a lower degree of similarity at <58% (<74%).

The robustness of the inferred phylogenies was tested by bootstrapping and revealed that the grouping of the sponge 14-3-3 GEODIA-ge to the isoforms γ and η are of high significance (100%) (Fig. 1B).

Expression of *14-3-3* in *Geodia cydonium* after treatment with PCB 118

Level of transcription

The 1.0 kb large transcript of *14-3-3* could not be detected by Northern blotting in untreated *Geodia cydonium* tissue with the methods used (Fig. 2B). After incubation with PCB 118 for 12 h and up to 6 d the intensity of the band increased; the strongest signal was observed after 3 d of incubation.

Quantitative analysis of the bands revealed that the initial signal of the 14-3-3 mRNA appears after 12 h. The peak level is reached after 3 d with a value 2.3-fold higher than that measured after 1 d of incubation (Fig. 3).

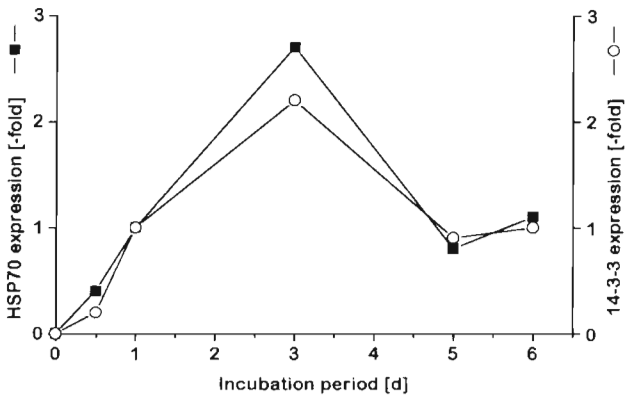


Fig. 3. Quantification of expression of both the *HSP70* gene and the gene encoding the *Geodia cydonium* 14-3-3 protein, *GC14-3-3*. After the indicated periods of incubation of tissue from *G. cydonium*, samples were taken and RNA was extracted, size separated and hybridized to the 2 cDNA probes, *HSP70* and *GC14-3-3*. The intensities of the 2.2 kb band, representing the transcript of the *HSP70* gene (Fig. 2A) and the 1 kb band (Fig. 2B) of the mRNA of the *GC14-3-3* transcripts, were analyzed with the GS-525 Molecular Imager. The intensities of the *HSP70* and *14-3-3* transcripts after 1 d of incubation with PCB 118 were set arbitrarily to 1-fold; the other intensities correspond to this value. Left ordinate: *HSP70* expression (■); right ordinate: expression of *14-3-3* (○). The means of 4 parallel experiments are given; SD does not exceed 15%

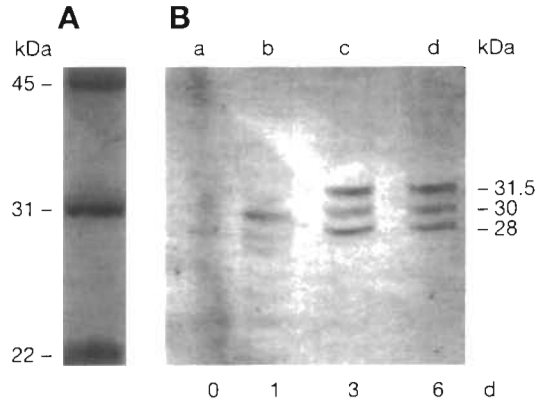


Fig. 4. Level of protein, cross-reacting with the PAb-14-3-3, after incubation of sponge tissue with PCB 118 for 1 to 6 d. The M_r markers are given in (A). In (B) the levels of the putative 14-3-3 isoforms in the sponge samples (31.5; 30 and 28 kDa), treated with PCB 118, are shown

Level of translation

From parallel samples the protein level of the 14-3-3 protein(s) was determined by Western blotting (Fig. 4). As expected, the PAb-14-3-3, raised against the conserved region of all human isoforms recognised more than one protein specimen in the sponge extract. Three putative isoforms were identified, with apparent sizes of 31 500, 30 000 and 28 000 Da (Fig. 4B). The first signals in the Western blot were seen after 1 d of incubation and increased further during the 6 d incubation period.

The quantitative analysis revealed that at time zero no signal was seen, while the 3 putative isoforms increased steadily in intensity during the 6 d incubation period chosen (Fig. 5B). The 31.5 kDa species is the dominant 14-3-3 isoform present in PCB 118-treated tissue. In control experiments it was established that the PAb-14-3-3, which was adsorbed with the 14-3-3 peptide, showed no immunoreaction with any protein in the extract (not shown).

Expression of *HSP70* in *Geodia cydonium* after treatment with PCB 118

The second biomarker, HSP70, was analyzed in parallel to 14-3-3, in response to treatment of the sponges with PCB 118.

Level of transcription

As shown in Fig. 2A and summarized in Fig. 3, the steady-state level of the *HSP70* transcript increased

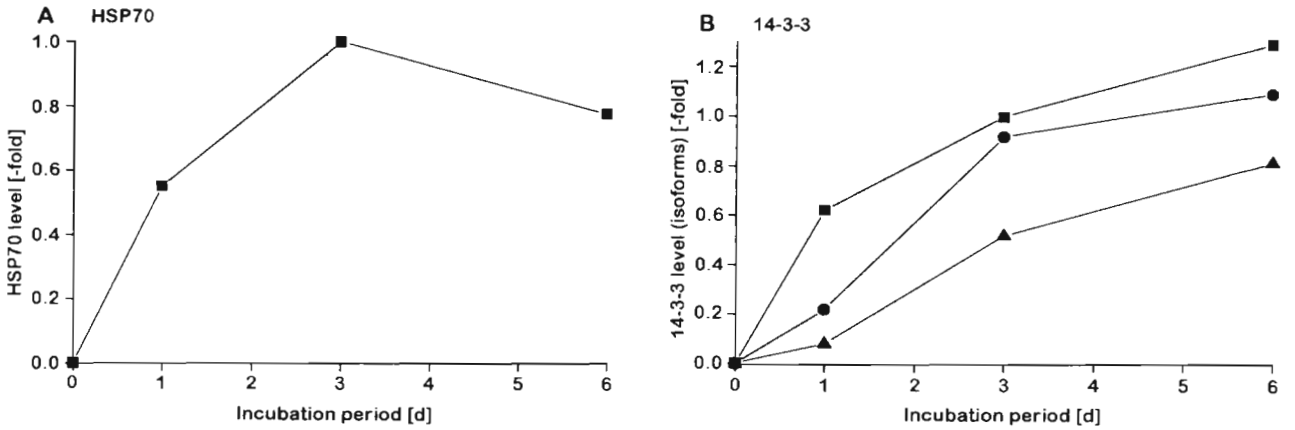


Fig. 5. Relative levels of the chaperone proteins, (A) HSP70 and (B) 14-3-3, in tissue samples from *Geodia cydonium* treated with PCB 118 for the indicated period of time. (A) In the series to determine the level of HSP70 protein the value for the steady-state level after 3 d was set to 1-fold. (B) The levels of 14-3-3 protein(s) were quantified by setting the level of the dominant 31.5 kDa isoform reached after the 3 d incubation period arbitrarily to 1-fold; the other values are correlated to it; 31.5 kDa isoform (■), 30 kDa isoform (●) and 28 kDa isoform (▲). The means of 5 parallel experiments each are given; SD does not exceed 15%

strongly during a period as short as 12 h. The maximal level of the transcript was measured after a 3 d incubation with the chemical. After prolonged incubation the level dropped by 60% on Day 6.

Level of translation

An increase of the HSP70 translation product is first seen by the newly raised antibody against homologous HSP70, MAb-HSP70, after a 1 d incubation period with the chemical (Fig. 6B). Some additional bands besides the one corresponding to HSP70 are seen on the blot (Fig. 6B), suggesting that some proteolytic fragments are present in the samples. However, as described earlier (Bachinski at al. 1997), further protein species are found in sponge tissue which are recognised specifically by antibodies against HSP70. It is interesting to note that the HSP70 protein is already visible in the gel, stained with Coomassie brilliant blue, as expected after 1 d (Fig. 6A). The relative intensities of the HSP70 bands seen in the immunoblots were quantitated by the chemiluminescence procedure (Fig. 5A); highest levels were measured after the 3 d incubation with PCB 118. Again it was established in control experiments that the MAb-HSP70 fails to detect any protein in the extract after adsorption with recombinant HSP70 (not shown).

In control series of experiments, during which sponges were injected with sea water only, specimens did not express increased levels of HSP70 or 14-3-3.

DISCUSSION

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds are known to be strong Ah receptor agonists (reviewed in Nebert & Gonzalez 1987). Many PCBs exhibit TCDD-like activity, e.g. the mono-ortho coplanar congeners (like PCB 118) which com-

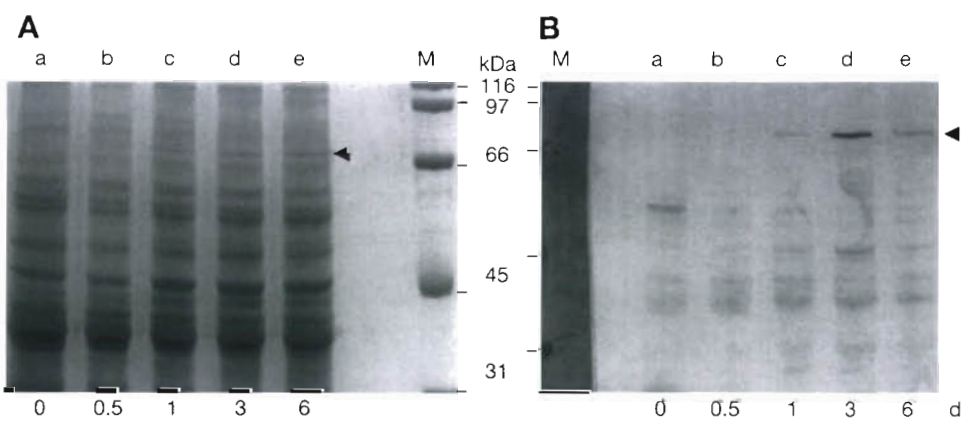


Fig. 6. Protein level of HSP70 in response to the PCB 118 treatment. (A) Sponge extracts from samples of tissue, treated for 0 to 6 d with the chemical, were size-separated and the gel was stained with Coomassie brilliant blue. (B) The proteins were subsequently blotted and probed with MAb-HSP70. Further details are given in 'Materials and methods'. M: protein size markers; arrowheads point to the bands representing the HSP70

petitively displace TCDD from the cytosolic Ah receptor (Parkinson et al. 1983), resulting in the induction of CYP 1A1 and CYP 1A2. Besides the expression of the *CYP* genes, via activation of Ah receptor, other non-*CYP* genes [e.g. those encoding UDP glucuronyltransferase and NAD(P)H:menadione oxidoreductase] are induced in response to PCBs or TCDD (reviewed in Okey 1990).

Here we show that PCB 118 induces 2 proteins hitherto not known to be induced in response to this toxin, the 14-3-3 protein(s) and the stress protein HSP70. The family of 14-3-3 proteins was initially identified by Moore & Perez (1967) in the frame of a systematic analysis of brain proteins. At least 7 mammalian isoforms have been identified (Aitken et al. 1992). Originally the 14-3-3 proteins were described as functioning as regulators of protein kinase C and of tryptophan and tyrosine hyroxylases (reviewed in Aitken et al. 1992). More recently, however, after application of the 2-hybrid system, 14-3-3 has been found to bind a series of crucial signaling proteins, including Raf-1 (see Muslin et al. 1996), an oncogene which is expressed after treatment of rats with PCB (Jenke 1998). 14-3-3 (including members from different isoforms) undergoes homo- and heterodimerization *in vivo* and *in vitro* (Muslin et al. 1996) generating 2 ligand binding sites (Fig. 7). The ligand(s) bind, after phosphorylation which is mediated by Ser/Thr kinase(s), through the RS × S × P motif to the 14-3-3 dimer (Muslin et al. 1996). After binding, this complex remains in a distinct cell compartment (Zha et al. 1996), preventing the realization of the function of the respective molecule. With respect to the topic here it is interesting to note that 14-3-3 binds not only to signaling proteins, but also to molecules involved in protein folding, e.g. HSP90 (Xu & Lindquist 1993), and key factors controlling apoptosis. It has been demonstrated that 14-3-3 binds to BAD, a distantly related member of the BCL2 family, and thereby prevents the induction of the apoptotic pathway (Zha et al. 1996). A second molecule, A20, an inhibitor of tumor necrosis factor-induced apoptosis, also binds to 14-3-3 and inhibits the function

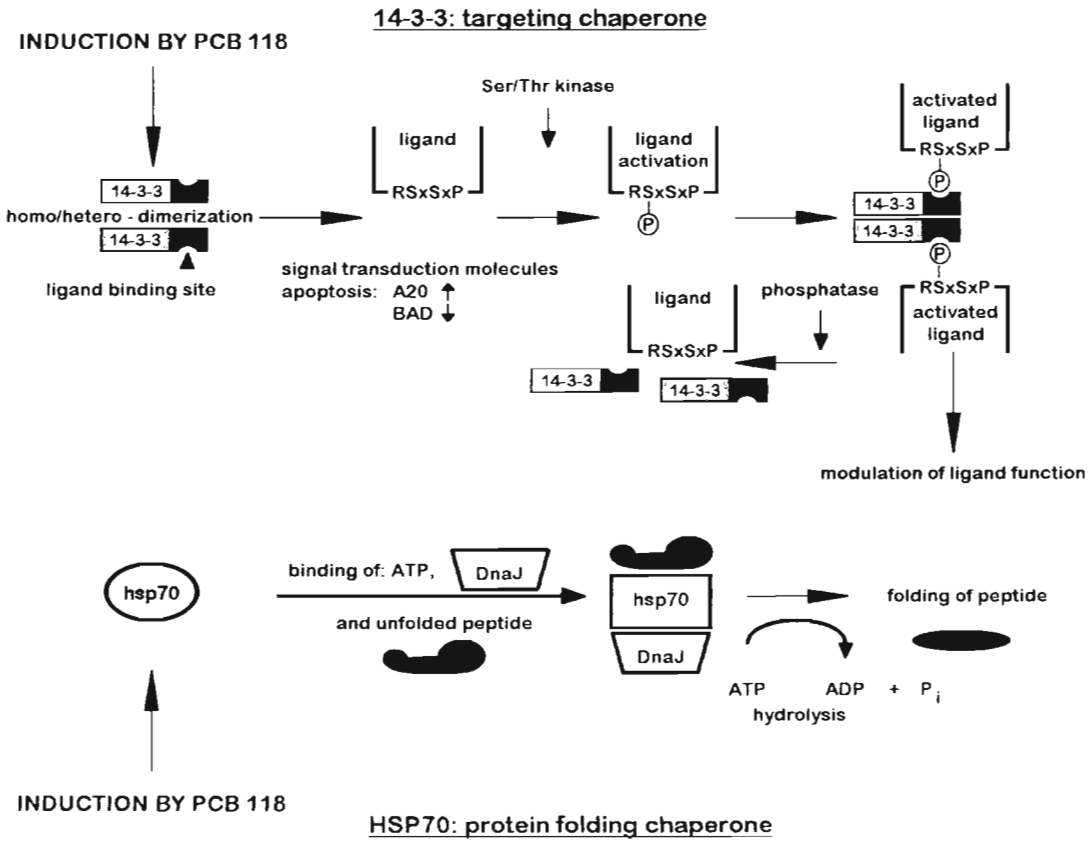


Fig. 7. Schematic representation of the roles of 14-3-3, the targeting chaperone, and HSP70, the protein-folding chaperone, in cell metabolism. The model is based on the data obtained with sponges. As outlined in the text, 14-3-3 undergoes dimerization, allowing ligands provided with the RS × S × P motif to bind. Complex formation is promoted by phosphorylation and prevented by dephosphorylation. Besides signaling proteins, the function of heat shock proteins and of apoptosis-controlling factors, e.g. A20 or BAD, are also modulated by 14-3-3 via targeting the respective molecule to a distinct cell compartment. In contrast, HSP70 binds to the target protein to control primarily its correct folding

of A20 (Vincenz & Dixit 1996). These examples show that 14-3-3 molecules act as chaperones (Vincenz & Dixit 1996, Zha et al. 1996) by guiding distinct proteins to selected cell compartments. The dissociation of the 14-3-3/factor complex is initiated by dephosphorylation of the RS × S × P motif (Dent et al. 1995).

The presented data unequivocally show that the sponge *Geodia cydonium* responds to exposure to PCB 118 with a drastic expression of the gene coding for the *GC14-3-3*. This conclusion can be drawn from the determination of the steady-state level of the mRNA and of the protein level; quantitation experiments revealed that the level of 14-3-3 had already increased many-fold after 1 d. In the present study an antibody raised against the highly conserved aa residues 221 to 242 of the 14-3-3 protein family cross-reacted with 3 protein species of M_r 28 000 to 31 500 Da, in the sponge, suggesting the presence of at least 2 more isoforms of this family. Experiments with an exposure period of longer than 6 d, as chosen here, are under way.

Phylogenetic analysis of the 14-3-3 molecule from *Geodia cydonium* showed that this molecule forms the base of the metazoan 14-3-3 γ and η isoforms. The η isoform influences hydroxylases, the Ca^{2+}/calmodulin-dependent kinases, as well as the Ca^{2+}/phospholipid-dependent protein kinase C (Lefflers et al. 1993), all of which are key pathways controlling apoptosis (Wang & Studzinski 1997, Wright et al. 1997). Hence, we suggest that the upregulation of the 14-3-3 protein(s) in *G. cydonium* parallels the regulatory mechanisms initiating or preventing apoptosis. However, at present it is only known that some of the isoforms, e.g. isoform β, interact with Raf-mediated mitogenesis and differentiation (Fantl et al. 1994).

Also interesting is the fact that one member of the 14-3-3 family, the isoform Θ, is abundant especially in male germ cells (Perego & Berruti 1997), suggesting that at least this isoform might be used in the future to monitor those PCBs provided with estrogenic or antiestrogenic properties. It should be stressed at this point that no biomarker proteins have been identified in lower invertebrates which can be used to monitor such effects caused by PCBs. In vertebrates, vitellogenin serves as a suitable biomarker for environmental estrogens (Heppell et al. 1995).

Heat shock proteins, the 'classic' stress proteins, have the potential to act as biomarkers for general stress (Sanders 1990). We have shown previously that sponges serve as suitable bioindicator organisms for a series of stressors using the biomarker HSP70 (Koziol et al. 1996, 1997a, b). In the present study, the level of HSP70 was greatly elevated in sponges treated with PCB 118; the mRNA level as well as the protein level increased several-fold. In contrast to 14-3-3, HSP70 controls the folding state of a given protein (Fig. 7).

Taken together, the data reported in this study demonstrate for the first time that PCB 118 is able to induce 14-3-3 protein(s) and HSP70, 2 different types of chaperones. It can be assumed that the main function of 14-3-3 is the prevention of PCB-induced apoptosis. The impact of these findings might be seen in the fact that the use of these 2 proteins in environmental monitoring is strongly recommended because these proteins are highly conserved and hence antibodies can be used which cross-react with the corresponding antigens from sponges to humans. This is very different from the use of cytochrome P450s in monitoring of PCBs because of their high divergence (Gonzales 1989); hence different substrates have to be used in the monitoring programs.

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