Molecular cloning and characterization of Cpn60 in the free-living nematode *Plectus acuminatus*

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Abstract Heat shock proteins (Hsps) have provoked interest not only because of their involvement in human diseases but also for their potential as biomarkers of environmental pollution. Whereas the former interest is covered by numerous reports, the latter is an exciting new field of research. We report the isolation of the full-length *cpn60* messenger ribonucleic acid (mRNA) and partial genomic deoxyribonucleic acid from the free-living, environmental sentinel nematode *Plectus acuminatus*, a species used in classical ecotoxicity tests. Although the primary sequence displays high identity scores to other nematodes and human Cpn60 (75% and 70%, respectively), the intron-exon structure differs markedly. Furthermore, although mRNA levels remained constant after exposure to ZnCl₂ (0–330 µM) under laboratory conditions, protein levels increased significantly in a dose-dependent manner. In conclusion, this first account of molecular genetic similarities and differences of Cpn60 in a neglected nematode taxon provides a valuable insight into its potential uses in gene-based ecotoxicological risk assessment exercises.

INTRODUCTION

Nematodes are 1 of the most dominant and abundant groups of multicellular organisms. Bacterivorous nematodes play a major role in decomposition processes of organic matter by stimulating N mineralization and nutrient cycling (Anderson et al 1981). The most commonly studied member of the phylum Nematoda, class Chromadorea is undoubtedly *Caenorhabditis elegans*. With a fully sequenced and exquisitely annotated genome, a vast amount of information has consequently been rapidly accumulating. Unfortunately, there remains a multitude of neglected nematode species that are increasingly becoming underrepresented (because of the lack of data), despite their biological importance and value. For example, the nonparasitic soil-inhabiting bacterivorous nematode *Plectus acuminatus* is a member of the order Araeolaimida. It is highly abundant in the temperate regions of the world and has a life cycle that is notably different to *C. elegans*, namely an egg to egg period of approximately 3

weeks (opposed to 3 days) and an average life span of 3 months (opposed to 21 days) at 20°C.

Given that *Plectus* is a terrestrial bacterial feeder that inhabits the interstitial water of soil particles (Houx and Aben 1993), it is subjected directly to the dissolved fraction of contaminants in soils through contact or indirectly through the food source. It follows that these organisms in particular require efficient response pathways. Toxicity tests have shown that the effect levels to several contaminants was comparable with other invertebrates suggesting that *P. acuminatus* may be an ideal indicator species for exposure assessment and biomonitoring (Kammenga et al 1996). Although no genetic data is available for this species, the effects of metal toxicosis have been monitored at the protein level, using unspecific monoclonal antibodies to human stress proteins to evaluate the responses to increasing concentrations of cadmium and copper (Kammenga et al 1998).

Heat shock proteins (Hsps) are a multigene family first discovered to be activated by thermal stress but later found to be induced by a wide variety of other factors, including physical, chemical, and biological stress (Nover 1991). The ubiquitous 55–65 kDa Hsps have been found to occur almost exclusively in organelles of endosymbi-

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Fig 1. Identification of *cpn60* from the nematode *Plectus acuminatus*. An adapter ligated complementary deoxyribonucleic acid (cDNA) library was constructed from 7000 *P. acuminatus* individuals using the Marathon[®] cDNA Amplification Kit (CLONTECH Laboratories, Palo Alto, CA, USA). *Cpn60* was amplified, with degenerative primers, in a touchdown polymerase chain reaction and forthcoming bands purified, cloned (pGEM-T, Promega, Southampton, Hampshire, UK), and sequenced. The full-length *P. acuminatus cpn60* sequence was determined on both strands of the 5' fragment, the 3' fragment, and the 5'-3' overlap region resulting in a final contig of 2068 bp (European Molecular Biology Laboratory/Genbank accession no. AJ130877). The translation is given in panel A with the putative N-terminal mitochondrial targeting signal boxed. Homology searches using the BlastN and BlastP algorithms (Altschul et al 1990; Gish and States 1993) identified the sequence

otic origin (mitochondria, chloroplasts) (Hemmingsen et al 1988; Cheng et al 1989) and are commonly referred to as Cpn60, Hsp60, stress-60, or chaperonins because of their function as molecular chaperones. Moreover, they facilitate the synthesis, folding, assembly and intracellular transport of proteins, reduce protein denaturation and aggregation, and aid in protein renaturation (Ellis and van der Vies 1991; Parsell and Lindquist 1993). They consist of large oligomeric structures composed of 2 stacked heptameric rings of identical or closely related rotationally symmetric 60-kDa subunits to form a central cavity (Hendrix 1979; Hohn et al 1979; McMullin and Hallberg 1988). The central cavity binds intermediately folded polypeptides and thereby prevents incorrect associations within and between polypeptide chains during protein folding and protects preexisting proteins under cellular stress. In most cases, adenosine triphosphate and a single heptameric ring of 10-kDa subunits, the cochaperonin Cpn10 (Hsp10, GroES), are required for folding of the polypeptide intermediates and release from the chaperonin (Martin et al 1993). Chaperonins are also involved in secretion and (membrane) translocation of a number of protein precursors and aid damaged and misfolded proteins to refold correctly to their native conformation or otherwise, making them sensitive to proteolytic digestion (Gething and Sambrook 1992; Parsell and Lindquist 1993). Its role in autoimmune diseases has been studied extensively (Wick et al 2001, 2004; Quintana et al 2002) and there is evidence for a possible involvement in prion diseases (Ranford and Henderson 2002).

This article not only aims to redress the lack of genetic information of a neglected nematode species by describing the molecular cloning of the full-length transcripts of the *P. acuminatus* Hsp *cpn60* and actin, but also highlights partial *cpn60* intron-exon structures and the measurement of differential transcriptional and translational responses of Cpn60 to zinc exposure.

RESULTS AND DISCUSSION

Identification of Cpn60 in *P. acuminatus*

Cpn60 is a member of the large and phylogenetically conserved family of stress proteins (Lindquist and Craig 1988). Consequently it was possible to isolate the messenger ribonucleic acid (mRNA) sequence of the *P. acuminatus* using degenerate *cpn60* primers. The final contig of a subset of independent rapid amplification of complementary

deoxyribonucleic acid (DNA) ends-polymerase chain reaction (PCR) yielded the full-length sequence of 2068 bp, comprising 96 bp 5' UTR and 229 bp 3' UTR. The sequence is accessible from the European Molecular Biology Laboratory (EMBL)/Genbank depositories under accession no. AJ130877. The 580 amino acid Cpn60 has a predicted weight of 61.8 kDa with an isoelectric point of 6.2. Using MitoProt II for Unix (Claros and Vincens 1996), it was possible to predict the mitochondrial signal sequence indicating, with a probability of $P = 0.96$, that Cpn60 is indeed exported to the mitochondria (see Fig 1, panel A). The presence of a putative targeting signal that is responsible for the translocation into the mitochondrial matrix has also been reported in Cpn60 of *Drosophila melanogaster* (Kozlova et al 1997), *Trypanosoma cruzi* (Giambiagi-de Marval et al 1993), *Culicoides variipennis* (Abdallah et al 2000) and *Paracentrotus lividus* (Gianguzza et al 2000) and others. The deduced amino acid translation displays a considerable sequence identity (75%) and similarity (87%) to the Cpn60s from *C. elegans* and *C. briggsae* (Fig 1, panel B). The phylogenetic conservation of Cpn60 is highlighted by the fact that the *P. acuminatus* sequence is remarkably conserved to the human paralog with an identity of 70% and a similarity 83% (Fig 1, panel B) and other organisms from vertebrates to plants (Fig 1, panel C).

The genomic organization of *cpn60*

A 506-bp fragment of genomic DNA was amplified from the central region *cpn60* gene, cloned, and sequenced on both strands to provide an insight into the genomic organization of *P. acuminatus*. It should be noted that although it was never the primary aim to extract extensive sequencing data of the *cpn60* genomic locus, the fragment obtained nevertheless provided valuable information. In contrast to the single intron encountered in *C. elegans* and *C. briggsae*, the *Plectus cpn60* contains 2 introns. However, the combined intronic size of 139 bp of both *Plectus* introns (86 and 53 bp, respectively) is nevertheless significantly shorter than the single intron in *C. elegans* (1365 bp) and *C. briggsae* (717 bp). Although there is an approximate 2-fold difference in size of both *Caenorhabditis* intronic sequences, the splice sites perfectly match each other. In contrast, *Plectus* introns are spliced at different locations (Fig 2, panel A). Although this only provides a diminutive view of the general genomic organization, the lack of

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to be closely related to 2 further nematodes (panel A). Pairwise comparisons of the deduced amino acid sequence were calculated from 3 nematode species and the human Cpn60, where the first number represents percent identity and the second, percent similarity scores (panel B). A ClustalW multiple alignment was used to construct a Cladogram of the *P. acuminatus* Cpn60 amino acid sequence and 12 further Cpn60s from a variety of species to highlight phylogenetic relationships (panel C).

Fig 2. Identification of nonconserved splicing in *Plectus acuminatus* Cpn60. Amplification of *cpn60* from *P. acuminatus* genomic deoxyribonucleic acid identified intron-exon structures that markedly differed to the boundaries encountered in *Rhabditidae* nematodes (panel A). The cartoon is drawn to scale and numbers in italics represent amino acid identifiers. Note that the amplified region of *P. acuminatus cpn60* contains 2 short introns, compared with 1 in the *Caenorhabditis* species, with the *C. elegans* intron being significantly larger. Correct splicing was confirmed in silico (panel B), where splice sites are annotated by filled triangles.

“splice site conservation” highlights the evolutionary distance of the 2 classes, both belonging to the phylum Nematoda.

cpn60/Cpn60 expression in *P. acuminatus*

Because Cpn60 operates in a multifactorial manner, its transcriptional activation is equally complex and is activated by numerous triggers, ranging from heat shock to exposure to heavy metals. Direct evidence of this statement is provided by a report that demonstrated an induction of HSP60 and HSP70 protein levels after exposure to cadmium and copper in *P. acuminatus* (Kammenga et al 1998). Although, at the time, limited by the lack of genetic information available, the study not only highlighted the conserved nature of the protein family but also the potential uses of cross-reactive mammalian antibodies in environmental and toxicological testing regimens.

With the molecular genetic composition of *P. acuminatus cpn60* elucidated (see above), it was feasible to design specific primers and measure transcriptional responses by reverse transcriptase (RT)-PCR. In a semiquantitative approach, it was possible to verify that the transcription of *Plectus cpn60* is indeed responsive to temperature stress, in a marginal but clearly detectable manner (Fig 3, panel A), an observation that could be confirmed by fully quantitative PCR (data not shown). This, in itself, is of course not a significant, nor novel, finding, but merely a confirmatory statement that supports the many publications that *cpn60* expression is temperature dependent (Yoshida et al 1995; Martin et al 2002; Kingsley et al 2003).

Because protein levels have been shown previously to be sensitive to exposure of cadmium and copper (Kammenga et al 1998), a zinc exposure regimen (ranging from 0 to 330 μ M Zn) was devised, and nematodes subsequently split to allow the measurement of mRNA and protein levels on the same pool of animals. Consequently,

Fig 3. Quantification of *Plectus acuminatus* Cpn60 transcript and protein levels. The *cpn60* response at the messenger ribonucleic acid (mRNA) level was determined by quantitative polymerase chain reaction (PCR) (LightCycler, Idaho Technology, Idaho Falls, ID, USA). Conditions were set and optimized as described by Stürzenbaum et al (1998) and Stürzenbaum (1999), and every PCR product was assessed for its amplification specificity by real-time melting curve analysis (Ririe et al 1997), ensuring that only true amplification products were included in the quantification. All amplifications were normalized to actin, which was isolated as described in Figure 1 (accession no. AJ012665). Cpn60 protein levels were determined using the mouse monoclonals anti-human Cpn60 and anti-*Globodera rostochiensis* tropomyosin as an internal control on Western blots as described in De Boer et al (1996). Gray values of the blot bands were measured by densitometric image analysis (Applied Imaging, Tyne & Wear, UK) and processed using the MIDAS software (Applied Imaging). All measurements (at the mRNA and protein level) were taken after a 24-hour exposure period, mean values were calculated from 4 independent replicate analyses and assessed for statistical significance by one-way analysis of variance. Note that at the transcriptional level, a distinct response was obtained to heat shock (panel A) but not to zinc exposure (panel B). In contrast, a zinc exposure dependent translational response was clearly visible (panel B).

it was surprising to find that mRNA levels remained remarkably constant (after signal normalization to actin) upon exposure to the zinc concentration gradient, particularly because protein levels were seen to rise significantly ($P = 0.001$) with increasing supplementation of zinc (Fig 3, panel B).

However, it should be noted that each family of Hsps comprises multiple closely related isoforms (Sanders 1993), and several Cpn60 isoforms have been detected in *P. acuminatus* (Kammenga et al 1998). Given that we know very little about the interspecific cross-reactivity of the commercial antibody used in this study, it is important to state that the *cpn60* mRNA measured by quantitative RT-PCR may indeed not represent the identical pool of Cpn60 measured at the protein level. Nevertheless, it does highlight the notion that mRNA levels-responses may not always equal protein levels-responses. Particularly with the family of Hsps, this may be an ill-defined assumption. Differential degradation, stability or posttranslational modifications may well influence the abundance or ratio of mRNA and protein levels. For example, Ornatsky et al

(1995) report that whereas *cpn60* mRNA levels did not alter in chronically stimulated rats skeletal muscles, Cpn60 protein level increased 3.2-fold. In contrast, *T. cruzi* *cpn60* mRNA levels were responsive to temperature exposure, whereas the protein level remained essentially constant (Sullivan et al 1994).

It is doubtful that the documented differential responses of mRNA and protein expression are entirely independent from each other, but rather a transiently elevated response on a different time scale, where the protein response may lag behind the mRNA response but ultimately sustain for considerably longer periods. This is exemplified in human proximal tubule cells, where cadmium-induced elevation of *cpn60* mRNA returned to the control levels within 8 hours but proteins levels required 24 hours after removal of cadmium before returning to basal levels (Somji et al 2000). Similarly, changes in *hsp70* transcription was more sensitive than stress protein accumulation in the gray garden slug *Deroceras reticulatum* exposed to cadmium- or zinc-enriched food (Köhler et al 1998). Given that the measurements were taken after a

24-hour exposure period, it is conceivable that the mRNA levels may have surpassed the transcriptional upregulation and returned to basal level expression, resulting in an apparent lack of response. Alternatively, a hypersensitive response to laboratory setup or handling may have resulted in a continuous elevation of *cpn60* transcripts—even in control exposures. In any case, either of the above possibilities warrants further investigations.

Overall this first account of the molecular genetic makeup of a neglected nematode phylum of direct environmental relevance highlights clear similarities and differences of the Cpn60 family. The primary sequence may be highly conserved, but the intron-exon structure proves to be more complex compared with *Caenorhabditis*. The marked difference between Cpn60 mRNA and protein levels in response to zinc exposure provides a valuable insight into the potential exploitation of *cpn60* in gene-based ecotoxicological risk assessment exercises, namely the results shown here beg for caution when using *cpn60* mRNA responses as a stress marker.

The sequence data reported in this article have been submitted to the EMBL/Genbank libraries under the accession numbers AJ012665, AJ130877, and AJ130947.

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