Expression of *hsp90* mediates cytoprotective effects in the gastrodermis of planarians

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Abstract Heat shock proteins (HSPs) play a crucial role in the protection of cells. In the present study, we have identified an *hsp90*-related gene (*Djhsp90*) encoding a cytosolic form of HSP90 that is primarily expressed in gastrodermis of the planarian *Dugesia japonica*. *Djhsp90* becomes significantly induced after traumatic amputation or other stress stimuli, such as exposure to X-ray or ultraviolet radiations, heat shock, or prolonged starvation. When *Djhsp90* is silenced by ribonucleic acid interference (RNAi), planarians dramatically decrease in size, becoming unable to eat, and die in a few weeks. Our results indicate that this gene plays an essential cytoprotective role in the gastrodermis of planarians and suggest that this chaperone can be involved in autophagic processes that are activated by this tissue.

Keywords Heat shock protein 90 · Stress · Planaria · Intestine · Expression · RNAi

Abbreviations

<i>hsp90</i>	Heat shock protein 90
Gy	Gray
RT-PCR	Reverse transcription polymerase chain reaction
RNAi	RNA interference

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Introduction

The heat shock response is a common defensive mechanism that results in the increased expression of conserved heat shock proteins (HSPs) in a wide variety of cellular stresses. Under non-stressed conditions, HSPs play a central role as molecular chaperones, contributing to the proper folding and quality control of proteins (Latchman 1998). The 90 kDa HSPs are abundant proteins in eukaryotic cells. Specifically, HSP90 family consists of conserved cytosolic proteins implicated in the folding, stabilization, activation, and assembly of a wide subset of “client” proteins, related to cell proliferation, differentiation, and apoptosis events (Hahn 2009; Moser et al. 2009; Pearl and Prodromou 2006; Pearl et al. 2008; Sreedhar et al. 2004; Wandinger et al. 2008; Young et al. 2001). A fraction of HSP90 has also been identified at the cell surface, where it may play a role in tumor progression, eliciting a host immune response (Tsutsumi and Neckers 2007). Additional HSP90 analogues, which differ from the cytosol forms primarily by their targeting signals and the C-terminal endoplasmic reticulum (ER) retention signal, can be found in the ER and in the mitochondrial matrix (Picard 2002). *Hsp90* gene family experienced multiple duplications and also subsequent losses during its evolution. In a variety of species, such as budding yeasts and vertebrates, there are two *hsp90* genes coding for very similar cytosolic isoforms. Plants have even more than two *hsp90* genes, while the presence of a single *hsp90* appears to be a common feature of invertebrates (Gupta 1995; Picard 2002). HSP90 has been found to be essential for survival of several eukaryotic organisms (Latchman 1998; Picard 2002), and reduced levels of this protein can reveal previously hidden morphogenetic variations in the fruitfly *Drosophila melanogaster* (Rutherford and Lindquist 1998; Rutherford et

al. 2007). It has been suggested that HSP90 plays a pivotal role in the buffering of genetic variation, controlling the developmental networks that determine morphological stasis and change in evolution (Picard 2002; Rutherford et al. 2007).

Freshwater planarians are acoelomate, bilaterian worms of the phylum Platyhelminthes (Lophotrochozoa), characterized by a digestive system formed by a blind three-branched gut and a pharynx used for eating and for expelling waste substances. These animals are well known for their extensive potential for regeneration following trauma or as part of their reproductive strategy. In recent years, planarians have been increasingly recognized as an emerging model system for molecular studies of regeneration and stem cell function. Although hundreds of species with asexual, sexual, or mixed (sexual/asexual) modalities of reproduction exist, modern research on freshwater planarians has mainly focused on work in two species, in which asexual clonal strains have been obtained: *Schmidtea mediterranea* and *Dugesia japonica* (Oviedo et al. 2008). In addition to their extraordinary stem cell-based regenerative capacity, planarians are incredibly resistant to prolonged starvation during which they become markedly reduced in size, again growing back to the normal size after regular feeding (Bowen et al. 1976). A variety of perfectly orchestrated proliferative and cell death programs are responsible of this extraordinary body plasticity, contributing to adapt planarians to stress conditions (González-Estévez et al. 2007a, b; González-Estévez and Saló 2010; Pellettieri et al. 2010). A major surviving strategy to long periods of nutrient deprivation is activation of self-eating, autophagic processes that lead to the breakdown of non-vital components and the release of nutrients, ensuring vital functions for the whole organism. Autophagy is particularly important in nondividing cells (Mizushima et al. 2008), and, in planarians, food reserves from the gastrodermis and parenchyma cells are first recruited to tolerate long periods of starvation (González-Estévez 2008; González-Estévez and Saló 2010). A tightly regulated induction of HSPs also constitutes an important cell defense mechanism to protect these organisms from the effects of deleterious stress conditions. Recently, we have monitored the involvement of specific *hsp*-related genes in the adaptive response of planarian stem cells to stress conditions (Conte et al. 2009, 2010 in press). In this study, we analyze in *D. japonica* the expression of an *hsp90* ortholog (*Djhsp90*) under normal and stress conditions and investigate the impact of its ribonucleic acid interference (RNAi)-mediated inhibition. To our knowledge, *Djhsp90* is the sole member of HSP90 family found to be constitutively expressed in the intestinal tissue, playing a fundamental role in the functioning/survival of gastrodermal cells.

Materials and methods

Animals and treatments

Asexual specimens of *D. japonica* (GI strain) were maintained at 18°C in autoclaved stream water, fed weekly with chicken liver. Animals of similar body size were used for experiments after 7–10 days of starvation. Planarians starved for 30 days were used for starvation analysis. Regenerating fragments were produced by transverse amputation at the prepharyngeal level. Some intact worms were exposed to a lethal dose (30 Gy) of X-rays (200 keV, 1 Gy/min), using a Stabilipan 250/L instrument (Siemens, Gorla-Siama, Milan, Italy) equipped with a Radiation Monitor 9010 dosimeter (Radcal, Monrovia, CA, USA). Irradiated animals were then sacrificed after 4 or 10 days for subsequent experiments. For heat shock experiment, intact planarians were maintained at 28°C for 20 h before they will be harvested for RNA extraction. Based on preliminary experiments performed in our laboratory, exposure to ultraviolet (UV-A) light was carried out by placing ten planarians in uncovered Petri dishes containing wet 3 mm paper, for 60 min. Irradiation was performed with a dose of 11.6 W/m² (UV-A 320–400 nm), delivered to planarians from a mercury vapor lamp (UV-A TLD 15 W10, Philips, Italy). Gene expression was evaluated by triplicate groups of three specimens, sacrificed after 3 h from treatment. Triplicate groups of specimens maintained under standard laboratory conditions were used as controls.

In all experiments, total RNA was extracted from whole planarians with NucleoSpin RNAII kit (Macherey Nagel). Superscript first-strand synthesis system kit (Invitrogen) was used for cDNA synthesis. RNA extraction and reverse transcription were performed as described by Conte et al. (2009).

Sequence analysis of *Djhsp90*

Four partially overlapping expressed sequence tags (EST: 32901745, 32903586, 32901418, 32903639) from *D. japonica* (Mineta et al. 2003) were annotated as homologues of *hsp90* genes and allowed us to amplify a gene that perfectly matches the nucleotide sequence of a full-length cDNA for 90 kDa HSP from *D. japonica*, deposited in GenBank under the accession number FJ628362. The *Djhsp90* homologue, *Smedhsp90*, was also identified in the *S. mediterranea* database (<http://smedgd.neuro.utah.edu/index.html>; contig 005595.02.01). Clustal W was used to align the predicted open reading frames (ORFs) of DjHSP90 and SmedHSP90. Similarity search was conducted with <http://www.ncbi.nlm.nih.gov/BLAST/>. Motif scan was performed using http://myhits.isb-sib.ch/cgi-bin/motif_scan and <http://smart.embl-heidelberg.de>. The hydro-

philicity was predicted using <http://www.expasy.ch/cgi-bin/protscale.pl>. The potential subcellular localization was predicted on the server <http://psort.ims.u-tokyo.ac.jp/form2.html>.

Analysis of *Djhsp90* expression

Both whole mount in situ hybridization and in situ hybridization on paraffin-embedded sections (6 μ m) were carried out as described by Conte et al. (2009). Digoxigenin-labelled riboprobe was prepared using digoxigenin-labelling kit (Roche). To detect *Djhsp90* hybridization signal, chromogenic exposure was performed for approximately 3 h. Red-brown color development of the alkaline phosphatase-conjugated anti-DIG-antibody was executed with a mixture of BCIP/NBT (Sigma). To assess gene expression of *Djhsp90* in different stress conditions, the transcript level of stressed animals was compared to that of unstressed planarians. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed at least 3 times with independent RNA samples. Specific sense and antisense oligonucleotides (forward: 5'GAAGACAGTGTAAATAGGAATA3'; reverse: 5'CAACAAATGCAGAA TGACATAC3') were generated using NetPrimer software. SYBR Green chemistry-based RT-PCR was carried out on a Rotor-Gene 6000 real-time PCR (Corbett Research). After an initial denaturation step (5 min at 95°C), 45 cycles of amplification were performed as follows: denaturation: 95°C, 15 s; annealing: 60°C, 20 s; extension: 72°C, 40 s. Melt analysis was performed at the end of the run using the Rotor-Gene software. The mRNA levels were compared with controls using planarian *elongation factor 2* (*Djef2*) as reference gene to normalize RNA input (Rossi et al. 2007). The expression level of this gene did not change under the different stress conditions used (not shown).

Djhsp90(RNAi)

The *Djhsp90* sequence region, amplified using 5' ATGATGTTAGTCAAGAAGATTATGCT3' forward primer and 5'CATCACCCTCGAAGTTGAATAAA3' reverse primer, was utilized to synthesize double-stranded RNA (dsRNA) according to Sánchez Alvarado and Newmark (1999). About 10^{10} – 10^{11} molecules of dsRNA were injected in each specimen with a Nanoject microinjector apparatus (Drummond). Injections schedule was as reported in Mannini et al. (2004). Amputated fragments were analyzed during the second round of regeneration. Intact animals were analyzed after 20 days from the first injection. The reduction of the endogenous transcripts after RNAi was assessed by real-time RT-PCR (not shown). *Djhsp90* (RNAi) animals and controls were photographed in vivo at the same magnification. For morphometric analysis, the

areas of 30 *Djhsp90*(RNAi) planarians and 30 water-injected controls were measured from specimens photographed at the same magnification after ethanol fixation, using Nikon ACT-2U imaging software.

Results and discussion

Characterization of *Djhsp90*

We have cloned a sequence with high similarity to *hsp90* in the planarian *D. japonica*. The sequence appears identical to the full-length cDNA sequence for 90 kDa HSP from *D. japonica*, deposited in GenBank. The deduced amino acid sequence of *Djhsp90* cDNA appears highly homologous to HSP90 of vertebrate and invertebrate species and almost identical (92% amino acid identity) to that deduced from *Smedhsp90*, a *S. mediterranea* gene identified by in silico analysis of the genome. As a sole *Djhsp90* ortholog has been identified in *S. mediterranea* genome and no other genes with similar levels of identity could be detected, we hypothesize that, as in other invertebrates, *hsp90* is a single-copy gene in planarians.

Both planarian ORFs (715 amino acids) encode bona fide HSP90 members with the conserved core architecture defined by the N-terminal adenosine triphosphate (ATP) binding domain (6–233), followed by the middle domain (278–531), and the C-terminal domain (532–715; Fig. 1). In particular, five motifs that identify the HSP90 family appear well conserved in DjHSP90 and SmedHSP90: NKEIFLR ELISNSSDALDKIR (29–49), LGTIA[K/R]SGT (96–104), IGQFGVGFYS[A/S]YLV[A/S]D (120–135), IKLYVRRVFI (345–354), and GVVDSIDLPLNISRE (371–385). A highly charged region, characteristic for HSP90s of eukaryotic organisms, joins the N-terminus to the middle domain (234–277) and in position 651–664 of the planarian HSP90; the sequence YETSLSSGFTLED seems well conserved and very similar to the HSP90 novobiocin-binding region, where the second HP90 C-terminal ATP-binding site has been identified (Söti et al. 2002). The putative existence of a second nucleotide binding site, demonstrated by Ishiwarari-Hayasaka et al. (2003) in porcine HSP90, appears also preserved in the planarian deduced protein, starting at amino acid 170. Moreover, as all cytosolic HSP90 proteins (Gupta 1995), planarian HSP90 ends with the pentapeptide MEEVD at the C-terminus. The presence of structural and functional domains that are typically conserved in HSP90 proteins, as well as the preservation of the MEEVD motif at the C-terminus in the deduced amino acid sequence, indicates that the planarian *hsp90* gene represents a functional cytosolic member of this family, sharing high homology to the counterparts in other organisms.

Fig. 1 Graphic depiction of planarian HSP90 domain structure. Amino acid numbers of domain boundaries are marked. Highly conserved regions (*N*, *M* and *C* domains) are shown in dark gray, light gray and spotted areas, respectively. The highly charged domain between the N-terminal and C-terminal domains is indicated

Constitutive expression of *Djhsp90*

Whole mount in situ hybridization in intact planarians shows that *Djhsp90* transcripts are restricted primarily to the intestinal epithelium (gastrodermis), although a weak hybridization signal could also be detected in the mesenchymal tissue (parenchyma; Fig. 2a, b; Fig. 3a). The three-branched planarian intestine is made up of two different cell types: large columnar or gastrodermal digestive cells, with a basal nucleus and smaller goblet cells with a variable shape, depending on the nutritive state of the animal. Additional experiments, performed on wax sections, confirmed that *Djhsp90* transcripts were present in gastrodermal cells (Fig. 2c–f). In addition, parenchymal expression of *Djhsp90* could be detected in some small cells of variable morphology, scattered among the intestinal branches. We postulate

that this expression can be associated with determination or commitment of these cells to intestinal cells. Indeed, gastrodermal cells are short-lived cells (Baguña and Romero 1981) and are continuously being replaced by differentiating neoblasts, as have been extensively documented by light and electron microscopy (Hori 1986). HSP90 is one of the most abundant proteins in the cytosol of eukaryotic cells even in the absence of stress, being required for final maturation of different substrates (Csermely et al. 1998; Nathan et al. 1997), and its distribution pattern (ubiquitary or tissue-specific) varies in different organisms (Krone and Sass 1994; Li et al. 2009a, b). The constitutive *hsp90* expression pattern, detected in *D. japonica* and also conserved in *S. mediterranea* (data not shown), seems to be a typical feature of planarians. Amputation dramatically enhances the expression level of *Djhsp90*. In fact, upregulation of *Djhsp90* was observed in the gastrodermis area localized at the injured stump during the first days of regeneration (Fig. 2b), probably providing protection in the intestinal cells exposed to adverse environment of the wound.

Stress-responsive expression of *Djhsp90* mRNA and phenotypic effects of *Djhsp90* RNAi

The cells of the intestinal epithelium are regularly exposed to potentially harmful substances of dietary origin. Express-

Fig. 2 Expression of *Djhsp90* in *D. japonica*. **a** An intact planarian and **b** a tail fragment, amputated at the prepharynx level and sacrificed after 3 days of regeneration, as detected by whole mount in situ hybridization. Three independent experiments were performed. About 20 specimens were used in each experiment. After amputation, all regenerating fragments showed increased *hsp90* mRNA accumulation at the stump site. Asterisk indicates accumulation of *Djhsp90* transcripts. *ph* pharynx. All whole mount preparations show dorsal view, anterior is up. **c–f** In situ hybridization on transverse wax

sections, dorsal is up. **c** Transverse section at the eye level (*e*) showing hybridization signal in the gut branches. The ventral cephalic ganglia (*cg*), representing the planarian brain, are indicated by a dotted line. **d** Enlarged view of a labelled gut branch (*g*). Some *hps90*-expressing cells can be also detected in the surrounding parenchyma (arrows). **e** Transverse section at the postpharynx level, showing hybridization signal in the gut branches. **f** Enlarged view of some labelled gastrodermal cells. Scale bar: 500 μ m in **a** and **b**; 20 μ m in **d**, 100 μ m in **c** and **e**; 50 μ m in **f**

Fig. 3 Analysis of *Djhsp90* expression after stress. **a–c** In situ hybridization in 30 Gy-irradiated planarians. **a** An unirradiated planarian. **b** 4 days after irradiation, **c** 10 days after irradiation. **d–g** Real-time RT-PCR. Expression levels are indicated in relative units, assuming a value of 1 for untreated specimens (*K* control). Values are expressed as mean $\pm$ S.D. of three independent samples, collected at each experimental condition and analyzed in triplicate. Samples were compared using the unpaired *t*-test. ***P* < 0.001, **P* < 0.05. **d** 30 Gy-irradiated planarians. **4d-irr** 4 days after irradiation. **e** UV-A-irradiated planarians. **f** Heat-shocked planarians. **g** Starved planarians. **30d** 30 days of starvation. Scale bar 500 μ m in **a**, **b** and **c**

sion of *Djhsp90* in this tissue, as well as its up-regulation during regeneration, may involve a protective mechanism specifically developed by intestinal cells. As the stress response can be induced by different detrimental condi-

tions, planarians were exposed to distinct stresses and the expression level of *Djhsp90* was then compared with that of unstressed animals. Some specimens were irradiated with X-rays (30 Gy). According to our previous results, a significant transcriptional induction of *Djhsp90* was elicited by this treatment, although the expression pattern remained confined to the gastrodermis (Rossi et al. 2007; Fig. 3a, b, d). As 30 Gy represents a lethal X-ray dose (i.e., all irradiated animals die in 10–12 days), *Djhsp90* signal was strongly depressed around 10 days after irradiation in dying animals (Fig. 3c). We used real-time RT-PCR to assess *Djhsp90* expression level also after exposure to UV-A radiation (Fig. 3e), heat-shock (Fig. 3f) or starvation (Fig. 3g). We found that all experimental treatments caused significant *hsp90* up-regulation, indicating that this gene is very sensitive at the transcriptional level to detrimental

Fig. 4 Bright-field images of *Djhsp90*(RNAi) phenotypes. **a** Morphometric analysis of the ratio between area of *Djhsp90*(RNAi) and control planarians (*K*). Values are average $\pm$ S.D. from 30 samples. Samples were compared using the unpaired *t*-test. ***P* < 0.001. **b** A water-injected planarian. **c** A *Djhsp90*(RNAi) planarian. **d** A *Djhsp90*(RNAi) planarian: its intestine ruptured after feeding. A total of 100 *Djhsp90*(RNAi) planarians were injected in three independent experiments. In order to visualize food ingestion, all specimens were fed with chick liver mixed with red food dye. Dorsal view, anterior is up. Scale bar 400 μ m

situations. The transcriptional up-regulation of *Djhsp90* is consistent with that observed in a variety of organisms after exposition to different stressors (Farcy et al. 2007; Gao et al. 2007; Lei et al. 2009; Li et al. 2009a; Manchado et al. 2008; Tokalov et al. 2007; Zhang et al. 2009) and indicates that this gene encodes an inducible HSP90 form. To assess the function of *Djhsp90* in intestinal cells, we used RNA interference (RNAi). We observed that many *Djhsp90* dsRNA-injected planarians (63/100) became very small with respect to the controls (Fig. 4a–c). Regeneration potential did not seem affected and, after amputation, all fragments formed a typical blastema (data not shown). In addition, when planarians were fed with liver added with red food dye at the end of RNAi treatment (i.e., after 20 days from the first injection), we observed that these animals were unable to eat and died within 4–5 weeks. Only some *Djhsp90* dsRNA-injected specimens (10/100; probably mild phenotypes) showing body size similar to the controls tried to eat, but after food ingestion, their intestine ruptured and planarians died after a short time (Fig. 4d). These phenotypes were never observed in the controls, which underwent only a slight decrease in the body size due to starvation and, upon regular feeding, grew back to the former dimensions. Our results suggest that a reduced amount of HSP90 seriously impairs fundamental functions in the gastrodermal cells. Although we cannot exclude that HSP90 function may be only related to protect gastrodermis tissue against apoptotic insults and pathogens, we are attempting to speculate that the constitutive *hsp90* expression in these cells is associated with the planarian capability to survive to long periods of starvation. It has been demonstrated that autophagy may play cytoprotective effects (Moreau et al. 2009). Induction of autophagy has been demonstrated during the early neonatal starvation in mice (Kuma et al. 2004). Mutant yeast cells that have a reduced autophagic capability rapidly perish in nutrition-deficient conditions (Tsukada and Ohsumi 1993) and a tight control of autophagy is also required for survival of *Hydra* and *C. elegans* after starvation-induced stress (Chera et al. 2009; Galliot 2006; Sigmond et al. 2008). HSP90 is a key component of the chaperone-mediated autophagy (CMA) mechanism, with a possible inhibitory role in the molecular chaperone complex (Dice 2007; Majeski and Dice 2004). CMA could be constitutively active in the planarian gastrodermal cells to buffer against starvation, i.e., rapidly supply these cells with the amino acids required for the synthesis of essential proteins by the selective degradation of cytosolic proteins. Due to the possible inhibitory function of HSP90 in CMA, RNAi-mediated *Djhsp90* knockdown could affect this process, with a dramatic increase of proteolysis in the intestinal cells of planarians. Further analyses will give us crucial clues to pinpoint the mechanism and regulation of CMA in planarians.

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