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Hsp56 protein and mRNA distribution in normal and stressed *P. lividus* embryos

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Abstract — It was previously demonstrated that *Paracentrotus lividus* Hsp56 mitochondrial chaperonin is constitutively expressed during development, that it increases after heat-shock and cadmium treatment, and that it has a specific territorial distribution, both in normal and heat-shocked embryos, as shown by immunolocalization experiments. In this work, we analyzed by Western blot the territorial distribution of the protein in plutei exposed to heat-shock or sublethal cadmium concentrations, and we found that Hsp56 increases in both ectodermal and endodermal cells. Moreover, by "in situ" hybridization, we looked at Hsp56 mRNA during normal development and under stress conditions. We found that the territorial distribution of the messenger changes during development and that its amount is steadily increased in stressed embryos. Finally, by T1 RNase assay, we identified a cytoplasmic factor that binds to the region of Hsp56 messenger containing the 5'UTR.

Key words: cadmium, chaperonin, embryo development, heat shock, mitochondria.

INTRODUCTION

Heat shock proteins (Hsps) have an essential role in most organisms, participating in many basic processes, such as protein folding (Ellis and Hartl 1999). Mitochondrial Hsp60 chaperonin was studied in different organisms and it was shown to be involved in the folding of proteins imported into the mitochondrial matrix, assembly of protein complexes when required, and sorting of proteins to different locations in mitochondria, favoring the transport of imported proteins across membranes (Bukau and Horwich 1998; Soltys and Gupta 1999). As shown by sequence similarity at protein as well as at mRNA level, *P. lividus* mitochondrial Hsp56 is the homologue of vertebrate Hsp60 chaperonin.

We previously showed that Hsp56 is expressed at all stages of sea urchin development and, in heat-shocked embryos, the chaperonin concentration increases and it becomes evident also in the ectodermal layer, as shown by *in situ* (whole mount) immunoreaction (Roccheri *et al.* 2001). As previously reported (Roccheri *et al.* 2004), also sublethal cadmium concentrations have a clear effect on Hsp56 expression. In this paper, the territorial distribution of Hsp56 in embryos exposed to stress conditions was determined by Western analysis. We also analyzed, by "in situ hybridization", the Hsp56 mRNA localization, both in normal developing embryos and in gastrulae which underwent heat-shock. Finally, by T1 RNase assay, we looked for proteins able to bind to Hsp56 mRNA, possibly regulating its localization. It is indeed well known that post-trancriptional regulation of mRNA expression can be operated by proteins interacting with specific regions of messengers, often localized in the 3'-UTR (Derrigo *et al.* 2000; Colegrove *et al.* 2005; St Johnston 2005).

MATERIALS AND METHODS

Embryo culture and cell fractionation - Embryo culture and cell fractionation were carried out as described by Roccheri *et al.* (2001); ectodermal and endodermal cells were prepared from *P.lividus* plutei as reported by Roccheri *et al.* (1979).

Western analyses - Sample preparation, SDS-PAGE electrophoretic analysis and Western blot-

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ting were performed as described by ROCCHERI *et al.* (2001). Protein concentration was determined according to BRADFORD (1976). Protein markers, monoclonal anti-human Hsp60 antibodies and monoclonal anti-chicken actin antibodies were from Sigma. Autoradiographs and stained membranes were scanned and planimetries of scans were used to calculate, by ImageJ program, the relative abundance of proteins.

Whole mount in situ hybridization and digoxigenin labeled probes - Whole mount in situ hybridization was performed as previously described (GIANGUZZA *et al.* 1995). A 310 bp-long fragment of the coding region of Hsp56 cDNA (nt 1456-1788) was amplified by PCR using the 5'-GCAACCAGGGCTGCAGTAGAG-3' forward and 5'-AGATGCCACGCCCGATGCATC-3' reverse primers.

The product of the amplification was used in asimmetric PCR reactions (TAUTZ *et al.* 1992) to generate digoxigenin-labeled single-stranded DNA sense and antisense probes.

Preparation of in vitro transcribed RNAs for T1 RNase assay - A region of the *P.lividus* Hsp56 cDNA (GIANGUZZA *et al.* 2000; Genbank accession n° AJ249625) was amplified by PCR and cloned between KpnI and EcoR1 sites of Bluescript (+) SK plasmid. The oligonucleotides used for PCR amplification were:

5'-GCGCGGTACCGGCACGAGCTCAGCCGTA-3' forward and

5'-GCGCGGGAATTCTCTCCTGCCTCCTCGTTG-3' reverse (fragment 54-510);

T1 RNase protection assay - T1 RNase protection assay was carried out according to the method described by ZAIDI and MALTER (1994), as modified by IZQUIERDO and CUEZVA (1997). Briefly, cell extracts (15µg of total proteins) were incubated for 10 min, at 30°C, with 1 x 10^6 cpm (specific activity: 1-2 x 10^7 cpm/pmol of RNA) of *in vitro* transcribed, radiolabeled RNA. Samples were then incubated for 30 min at 37°C, with 100 units of T1 RNase (Roche) to degrade the whole of the RNA except the regions protected by bound proteins; the extracts were finally exposed to a spectroline UV (254 nm) lamp (Aldrich Chemical Co., Inc.), for 20 min at 4°C, to cross-link RNA to proteins. The covalent radioactive complexes were analyzed by denaturing electrophoresis on sodiumdodecylsulfate (SDS)-polyacrylamide slab gels (PAGE), according to LAEMMLI (1970) and the gels were directly exposed to x-ray film for autoradiography, with intensifying screens, for 12-18 h, at –70°C. At the end of exposure, the gels were stained with Coomassie brilliant Blue R-250 (Sigma) to confirm the loading of equal amounts of proteins per lane.

RESULTS

It was previously shown by immunolocalization that Hsp56 has a characteristic distribution in *P.lividus* embryos, being apparently more abundant in the archenteron and mesenchimal cells of gastrulae and in the archenteron of plutei, and that the protein increases in heat-shocked embryos (ROCCHERI et al. 2001). It is worth noting that Hsp56 concentration is higher in embryos grown in the presence of cadmium than in control embryos (unpublished results).

Moreover, it was also shown by Western blot (ROCCHERI *et al.* 2001; ROCCHERI *et al.* 2004) that Hsp56 protein is more abundant in *P.lividus* gastrulae grown in stress conditions. To test the levels and localization of the protein in stressed whole plutei, and in their ectodermal and endodermal cells separately, after fertilization**,** *P. lividus* embryos were divided into two batches, one of which was incubated with 0.1mM $CdCl_2$: both batches were grown at physiological temperature (20°C) until pluteus stage. For heat-shock experiments, a batch of normally developed plutei was kept for 90 minutes at 31°C and then collected. To evaluate the increase of Hsp56 protein in different regions of the stressed embryos, ectodermal and endodermal cells were isolated from control, heat-shocked and cadmium-treated plutei, according to the method suggested by ROCCHERI *et al.* (1979). Whole plutei and isolated cells were used to prepare mitochondrial protein extracts. The Western analysis reported in figure 1a (upper panel), carried out using an anti-Hsp60 monoclonal antibody, shows a clear increase of Hsp56 in heat-shocked- (lane 2) and cadmium-treated- plutei (lane 3), respect to normally grown plutei (lane 1). Moreover, in control plutei, the amount of the Hsp56 protein in endodermal cells (lane 4) is slightly higher than in ectodermal cells (lane 7). After heat-shock (lane 5, endodermal cells; lane 8, ectodermal cells), or cadmium treatment (lane 6, endodermal cells; lane 9, ectodermal cells), there is an increase of Hsp56 both in the ectodermal- and endodermal-layer of stressed plutei. The blots were also incubated with an anti-actin antibody, to normalize for the amounts of loaded proteins (figure 1a,

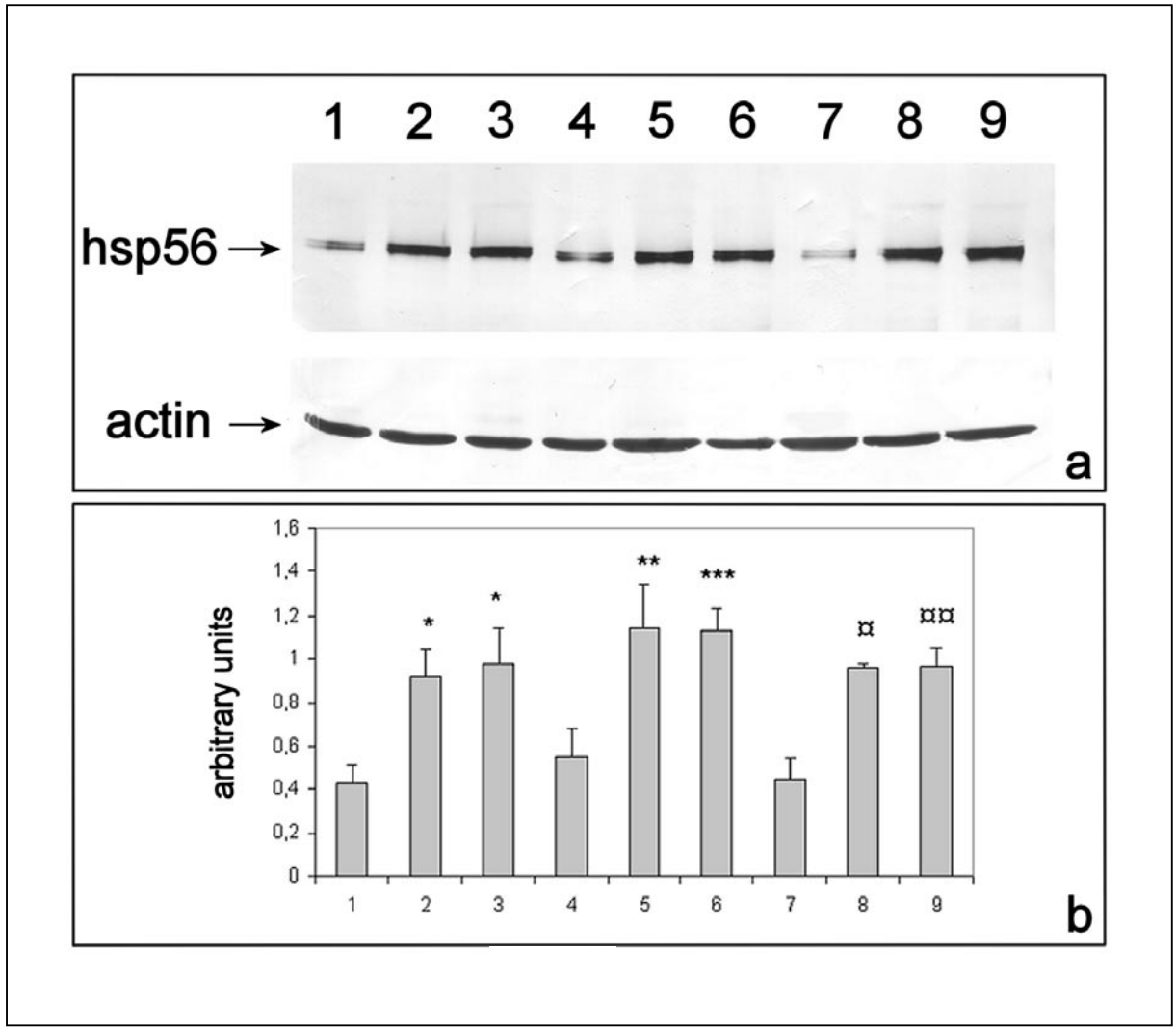

Fig. 1 — Western blot analysis of Hsp56 in *P.lividus* plutei. **a**) 15 μg of proteins from mitochondrial extracts prepared from 1) control plutei; 2) heat-shocked (Hs) plutei; 3) cadmium-treated (Cd) plutei; 4) endodermal control cells; 5) endodermal Hs cells; 6) endodermal Cd cells; 7) ectodermal control cells; 8) ectodermal Hs cells; 9) ectodermal Cd cells, reacted with anti-Hsp60 antibody (upper panel), or with anti-actin antibody (lower panel). **b**) graphic representation of Western analyses data (mean of three experiments). Bars indicate mean values for each sample. Standard deviations are indicated. *p<0, 025 when compared to sample 1; **p<0, 05 and ***p<0, 025 when compared to sample 4; ¤p<0, 0125 and ¤¤p<0, 025 when compared to sample 7.

lower panel). The graphic representation in figure 1b summarizes the results of 3 independent experiments analyzed by Student t test, and the significance calculated for each group of samples.

Afterwards, the distribution of the Hsp56 mRNA in whole embryos at different stages of development, as well as in heat-shocked gastrulae, was visualized by "in situ" hybridization assays, using as a probe a digoxigenin-labeled fragment of the Hsp56 cDNA (nt 1456-1788). Figure 2 shows an example of the mRNA localization in the embryos. In 2-cell embryos (b), the messenger is quite evenly distributed, while in 4-cell embryos (d) it is more concentrated at the periphery of the cells, just under the plasma membrane. In embryos at blastula (not shown) and gastrula (f) stages, the mRNA distribution reflects that of the protein: its concentration is apparently higher in the vegetative region of blastulae (not shown) and in the endodermal layer of gastrulae (f), and increases in all territories of the heat-shocked gastrulae (g). The

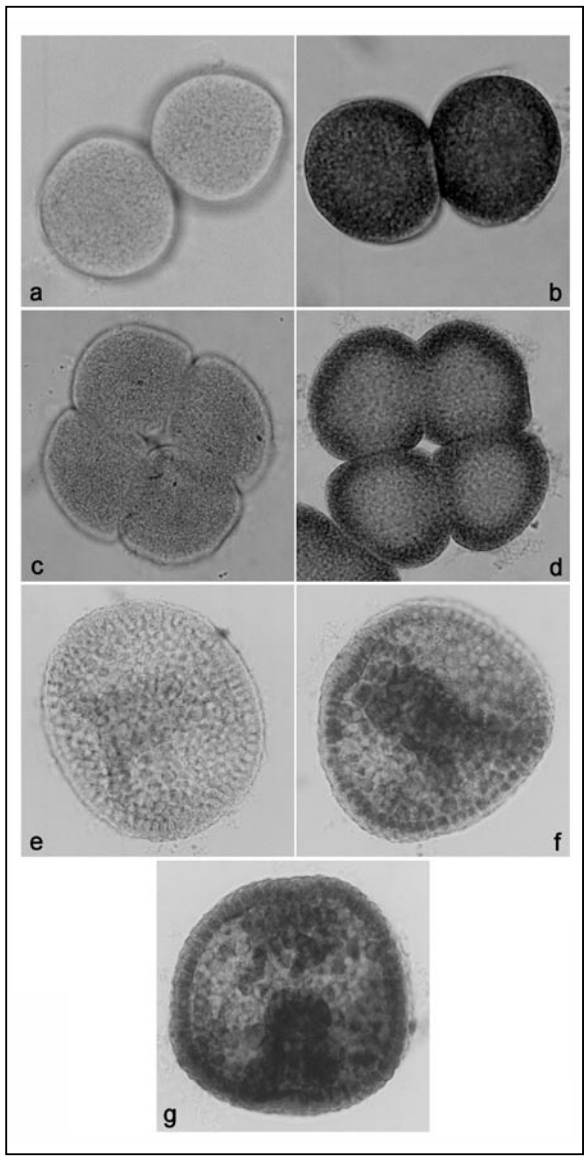

Fig. 2 — Whole mount *in situ* hybridization of *P.lividus* embryos with digoxigenin-labeled Hsp56 single stranded DNA probes. **a**) 2 blastomeres embryo hybridized with Hsp56 sense probe; **b**) 2 blastomeres embryo hybridized with Hsp56 antisense probe; **c**) 4 blastomeres embryo hybridized with Hsp56 sense probe; **d**) 4 blastomeres embryo hybridized with antisense Hsp56 probe; **e**) control (20°C) gastrula hybridized with sense Hsp56 probe; **f**) control (20°C) gastrula hybridized with antisense Hsp56 probe; **g**) heat-shocked gastrula (31°C) hybridized with antisense Hsp56 probe.

distribution of Hsp56 mRNA suggests that its localization could be regulated, possibly through interaction with RNA-binding proteins. To inquire on such hypothesis, different regions of the Hsp56 cDNA were *in vitro* transcribed, and the corresponding radioactive transcripts were incubated with cytoplasmic extracts from *P. lividus* embryos and analyzed by T1 RNase protection assay. As shown in figure 3, the initial region of the messenger (nt 54-510 of the cDNA) binds a protein, present in the post-nuclear extracts from blastulae and gastrulae (BL; GA). The protein complex has an apparent mass of about 90 kDa and its concentration increases during development. As it can be seen in the first lane (No), where the RNase T1-digested riboprobe was loaded as control, no band is formed in the absence of extract.

DISCUSSION

We analyzed the territorial expression of Hsp56 in *P.lividus* normal and stressed plutei, and we found that stress conditions induce a sharp increase of the expression of the protein, in whole embryos as well as in isolated endodermal and ectodermal cells, confirming previous results. "*In situ*" hybridization assays show that the level of the mRNA increases in shocked gastrulae, thus confirming results obtained by northern experiments (DI LIEGRO and RINALDI 2007). These results suggest that the stress-induced regulation of *hsp56* gene expression is mainly played at the transcriptional level. As we recently isolated and characterized the *hsp56* gene (Genbank accession n° DQ464426) from a *P.lividus* genomic library, experiments are now in progress aimed at studying regulation of the gene promoter.

A further finding concerns the peculiar distribution of Hsp56 mRNA in the embryo, which suggests that its localization might be regulated, possibly through interaction with RNA-binding proteins. Indeed, we found two proteins that interact with different regions of Hsp56 mRNA, forming at least two complexes. One of the complexes, of about 90 kDa, is formed between a region of the mRNA including the 5'UTR and a protein present in the cytoplasmic extract (fig. 3); another complex, of about 40 kDa (DI LIEGRO and RINALDI 2007), involves the most proximal region of the 3'UTR of Hsp56 messenger and a protein present in the mitochondrial fraction. Formation of these two complexes might be responsible for localization of Hsp56 mRNA. As the identified proteins interact with opposite regions of the messenger it could be hypothesized that their function is based on formation of some specific mRNA structure that involves both ends of mRNA.

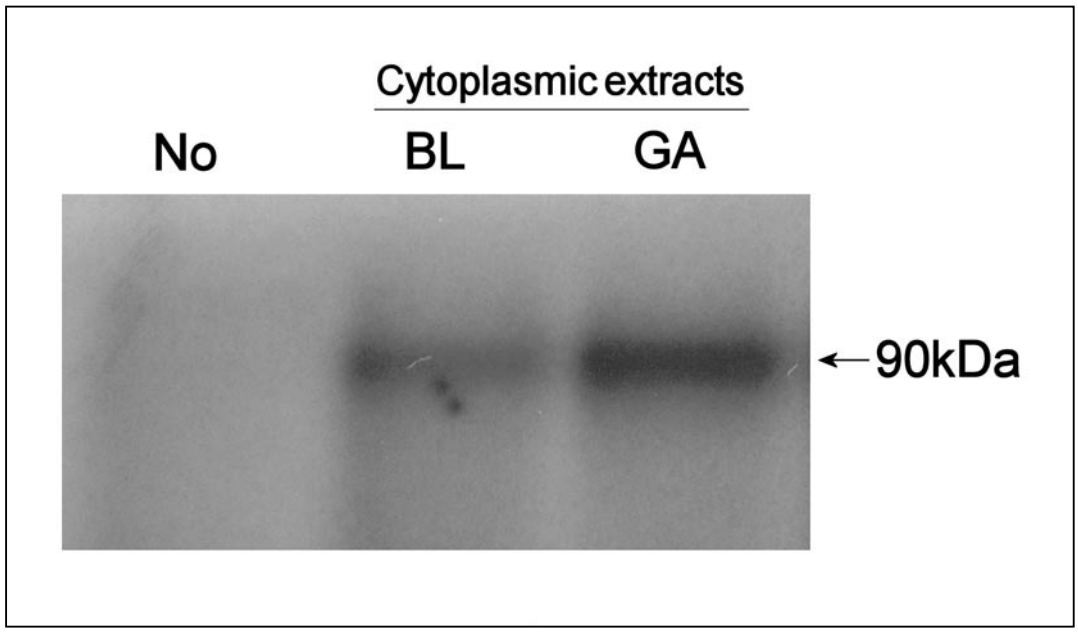

Fig. 3 — Identification of an Hsp56 RNA-binding protein in *P. lividus* embryos by T1 RNase protection assay. An Hsp56 cDNA region (nt 54-510), was *in vitro* transcribed, incubated with post-nuclear extracts, prepared from blastulae (BL) and gastrulae (GA), and treated with RNase T1. 1 x 10^6 cpm of radiolabeled riboprobe, treated with T1 RNase in the absence of proteins, were loaded on first lane (No), as control.

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