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Molecular characterization and expression of a heat-shock cognate 70 (Hsc70) and a heat-shock protein 70 (Hsp70) cDNAs in *Rana* (*Pelophylax*) *lessonae* embryos

Francesca Simoncelli, Lorena Morosi, Ines Di Rosa, Rita Pascolini, Anna Fagotti *

Dipartimento di Biologia Cellulare e Ambientale, Università degli Studi di Perugia, Via A. Pascoli 1, 06123 Perugia, Italy

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ABSTRACT

Heat-Shock Protein 70 (HSP70) is a class of highly conserved proteins which is involved in essential functions as molecular chaperones and in the acquired tolerance processes. In this work, two cDNAs encoding a constitutive Hsc70 and an inducible Hsp70 from the water frog *Rana* (*Pelophylax*) *lessonae*, belonging to the *Rana* (*P.*) *esculenta* complex of central Italy, have been isolated and characterized. The two cDNA clones, named *Rl*-Hsc70 and *Rl*-Hsp70, encode 646 and 640 amino acid proteins respectively, which present extremely conserved functional domains characteristic of cytosolic members of the HSP70 family. Comparative studies of the amino acid sequences showed that *Rl*-Hsc70 and *Rl*-Hsp70 had the highest homology with constitutive and inducible HSP70 members of other amphibian species. The phylogenetic analysis also demonstrated a separate clustering of the *Rl*-Hsc70 and *Rl*-Hsp70 with constitutive and inducible members from other vertebrate species. Heat-inducibility assays performed during embryogenesis showed that the two isolated mRNAs displayed different expression profiles. *Rl*-Hsp70 was induced only in heat shock-treated embryos, whereas *Rl*-Hsc70 transcript levels, which were constitutively modulated in non-stressed embryos, did not increase following the heat treatment. *In situ* hybridization experiments demonstrated that both transcripts showed a tissue-specific enrichment in the central nervous system and in the somites.

1. Introduction

Heat-Shock Proteins (HSPs) consist of several families of highly conserved proteins found in all living organisms from bacteria to humans (Lindquist and Craig, 1988). They are constitutively present in the cell with multiple chaperone functions and their expression can be up-regulated in response to different physiological perturbations or environmental stressors (Feder and Hofmann, 1999; Feige et al., 1996; Lindquist and Craig, 1988). HSPs have been classified into 3 major families according to their molecular weights: the high molecular weight HSP90s, the HSP70s, and the small HSPs. HSP70, the most abundant and conserved multigenic superfamily, has been studied extensively because of its role in protein chaperoning (Hartl, 1996; Gething and Sambrook, 1992; Mayer and Bukau, 2005), in developmental processes (Christians et al., 2003; Giudice et al., 1999; Heikkila et al., 1997; Krone et al., 2003) and in the acquisition of tolerance to chemical, physical and pathophysiological stresses (Lindquist, 1986; Lindquist and Craig, 1988; Macario and Conway de Macario, 2007). The HSP70 family includes constitutive and heat-inducible proteins, both with a cytosolic localization, the mitochondrial form Hsp75, the glucose-regulated protein Grp78 (BiP) that is located in the endoplasmic reticulum (Lindquist and Craig, 1988) and the Hsp110 and Grp170 proteins that are atypical Hsp70 homologs present in different cellular compartments (Easton et al., 2000).

Functionally, HSP70 members are required for protein folding, translocation of proteins between cellular compartments, degradation of misfolded proteins, prevention and dissolution of protein complexes and regulation of the heat-shock response (James et al., 1997; Mayer and Bukau, 2005).

The cytosolic HSP70 members are constituted by heat-shock cognate proteins (Hsc70s) that have basal housekeeping functions and heat-shock proteins (Hsp70s) that have their primary task during cellular stress (Daugaard et al., 2007; Kabani and Martineau, 2008; Lindquist and Craig, 1988). All Hsc/p70 proteins share common structural features including a 44-kDa N-terminal ATP-binding domain, an 18-kDa peptide-binding domain and a 10-kDa C-terminal domain containing the highly conserved EEVD terminal sequence (Kiang and Tsokos, 1998). Eukaryotes have multiple genes encoding cytosolic HSP70 proteins. The requirement for these highly homologous Hsc/p70 isoforms is still an unresolved question, but their

Abbreviations: aa, amino acid(s); BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; bp, base pair(s); cDNA, DNA complementary to RNA; DNase, deoxyribonuclease; EDTA, ethylenediamine tetraacetic acid; Hsc70, heat-shock cognate 70; Hsp70, heat-shock protein 70; HSP, Heat-Shock Proteins; kDa, kilodalton(s); MOPS, 4-morpholinepropanesulphonic acid; NBT, nitro blue tetrazolium; nt, nucleotide(s); ORF, open reading frame; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pI, isoelectric point; 5′–3′RACE, Rapid Amplification of cDNA ends; RT, reverse transcriptase; UTR, unstranslated region(s).

* Corresponding author. Tel.: +39 0755855740; fax: +39 0755855736.
E-mail address: afagotti@unipg.it (A. Fagotti).

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individual and tissues-specific expression suggests that they have distinct biological functions (Daugaard et al., 2007; Kabani and Martineau, 2008). Many studies on cytosolic HSP70 member expression in different animal species have shown their physiological and ecological role in response to stress conditions. In particular, in the amphibians *Xenopus laevis*, *X.* (*Silurana*) *tropicalis*, *Pleurodeles waltl* and *Ambystoma mexicanum*, several heat-shock genes and proteins of HSP70 family have been isolated and characterized and their involvement during oogenesis, embryonic development and cellular response to stress has been demonstrated (Ali et al., 1996a,b, 1997; Angelier et al., 1996; Bienz, 1984; Delelis-Fanien et al., 1997; Heikkila et al., 1997; Heikkila, 2010; Lang et al., 2000; Lévesque et al., 2005).

The present study reports, for the first time, the identification and characterization of two cytosolic members of the HSP70 family, *Rl*-Hsc70 and *Rl*-Hsp70 cDNAs, in the water frog *Rana* (*Pelophylax*) *lessonae* belonging to the *Rana* (*Pelophylax*) *esculenta* complex of central Italy and the analysis of their expression in normal and heat shock-treated embryos. Whole mount *in situ* hybridization revealed that the accumulation of both transcripts was enriched in a tissue-specific manner.

2. Materials and methods

2.1. Maintenance and heat-shock treatment of Rana (P.) lessonae embryos

In this study *R.* (*P.*) *lessonae* embryos at the stages of gastrula (st. 11), neurula (st. 16) and tailbud (st. 17) were used. Embryos were obtained from natural matings in the laboratory, reared at 22 °C in water and staged according to Gosner (1960). Embryos collected for RNA isolation were frozen and stored at −80 °C. Samples destined for whole mount *in situ* hybridization, were fixed and stored at −20 °C. For heat-shock experiments, embryos were placed in Petri dishes containing water, sealed with parafilm and submerged in a water bath at 33 °C for 1 h. Control embryos were maintained at 22 °C.

2.2. RNA extraction

Total RNA was extracted from *R.* (*P.*) *lessonae* embryos with TRIzol reagent according to the manufacturer's manual (Invitrogen, CA, USA). To remove any remaining genomic DNA, all samples were then treated with 1 U/μg RQ1 DNAse (Promega, WI, USA). RNA concentration and quality were assessed by spectrophotometry and electrophoresis on 1% agarose gel under denaturing conditions.

2.3. Cloning strategy

Full-length *R.* (*P.*) *lessonae* Hsc70 and Hsp70 cDNAs, named *Rl*-Hsc70 and *Rl*-Hsp70 respectively, were obtained by using RT-PCR and 5′- and 3′-Rapid Amplification of cDNA ends (-RACE) methods. *Rl*-Hsc70 was isolated from total RNA of stomach tissue, whereas *Rl*-Hsp70 was isolated from total RNA of heat-shocked neurula stage embryos. The sequences of the degenerate and gene-specific primers used are listed in Table 1.

2.4. Reverse transcription-PCR

To obtain fragments in the coding region of each gene, degenerate primers, designed on conserved regions of Hsc70 and Hsp70 amphibian proteins, were initially used. First strand cDNAs were synthesized from 4 μg of each total RNA samples using an Oligo $dT_{(18)}$ primer and Improm II™ Reverse Transcriptase (Promega, WI, USA) according to the manufacturer's protocol. The first-strand cDNAs were used as templates to amplify a conserved region (aa 20–180) from both final *Rl*-Hsc70 and *Rl*-Hsp70 sequences in the presence of the degenerated primers P1 and P2. The reactions were performed in a 50 μL volume of PCR Master Mix (Promega, WI, USA) with 50 μM of each primer. The 484 bp amplification products were obtained with the following thermal profile: an initial denaturation (94 °C, 2 min), followed by 40 cycles of denaturation (94 °C, 30 s), annealing (53 °C, 45 s), and extension (72 °C, 1 min) and a final extension step (72 °C, 10 min).

In the cloning strategy of *Rl*-Hsp70, a second conserved region (aa 120–478 in the final *Rl*-Hsp70 sequence) was also amplified as follows: cDNA synthesis was carried out by using the degenerate primer P3 and PCR amplification was primed by the degenerate reverse primer P5 and the gene-specific sense primer P4 designed on the basis of *Rl*-Hsp70 partial sequence obtained above. The reaction was carried out under the following conditions: an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 50 °C for 45 s, 72 °C for 2 min and a final step at 72 °C for 10 min.

The PCR products were electrophoresed on 1.5% agarose gel and the bands of appropriate size were gel-purified by using the Concert Rapid Gel Extraction System (Invitrogen, CA, USA) and cloned in pGEM®-T Easy Vector (Promega, WI, USA). Several recombinant clones were submitted to BMR Genomics (Padova, Italy) for sequencing from both ends.

Table 1
Degenerate and specific primers used for *Rl*-Hsc70 and *Rl*-Hsp70 cloning and semi-quantitative RT-PCR.

Primer	Direction	Sequences (5′–3′)
RT-PCR		
P1	F	GGNGTNTTYCARCAYGG
P2	R	GCNGCNGTNGGYTCRTT
P3	R	ACYTCYTCDATNGTNGG
P4	F	TCCTCTATGGTTCTGGTG
P5	R	GTNACYTCDATYTGNGG
5′–3′ RACE		
5′_HSC70_1	R	ATTGAGCCCAGAGATGGAG
3′_HSC70_1	F	AAAGGATGCCGGCACCATC
3′_HSC70_2	F	TCTCTGGGCTCAATGTACTAC
5′_HSP70_1	R	CCATCGCTCACCACCTG
5′_HSP70_2	R	CAAAGACGGTGTTCTGG
3′_HSP70_1	F	TTCCTACCAAGCAGACTCAGGT
3′_HSP70_2	F	CGGTGTCCTCATCCAGGTG
Semi-quantitative RT-PCR		
HSC70_R	R	TTTCAGGCAGATGGAGTGG
HSC70_F	F	AATGAGGTCATTGCTTGGC
HSP70_R	R	CCAGGTCAATGCCAATCG
HSP70_F	F	AGAGAAGAGAGCGGATACAC
Actin_R	R	TGTTCACTGACCTCTTGC
Actin_F	F	GGACTTGCCCTTATGTTC

2.5. Rapid Amplification of cDNA ends (5′–3′ RACE)

To obtain full-length *Rl*-Hsc70 and *Rl*-Hsp70 cDNAs, specific primers were designed based on the fragment sequences obtained above, and used for 5′ and 3′ RACE experiments according to Frohman et al. (1990).

In the 5′ RACE experiments, the 5′ end of *Rl*-Hsc70 cDNA was synthesized using primer Oligo $dT_{(18)}$, whereas *Rl*-Hsp70 cDNA was obtained by using the specific primer 5′_HSP70_1. Both cDNAs were then 3′-tailed using a terminal deoxynucleotidyl transferase (Promega, WI, USA) and deoxyguanosine triphosphate. The resulting polyG-tailed cDNAs were used as templates in the presence of the primer Oligo $(dC)_{15}$ and the gene-specific reverse primer 5′_HSC70_1 for cloning *Rl*-Hsc70, and 5′_HSP70_2 for cloning *Rl*-Hsp70. The PCR conditions were: initial denaturation at 94 °C for 2 min, followed by