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Biochemical and Biophysical Research Communications 311 (2003) 162–167
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The mechanism of αB-crystallin gene expression by proteasome inhibition☆

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Received 5 September 2003

Abstract

The mechanism of small heat shock protein/αB-crystallin gene expression by proteasome inhibition was investigated. Expression of αB-crystallin was induced efficiently only by proteasome inhibition and not by heat shock while expression of HSP27 was induced efficiently by both proteasome inhibition and heat shock. The promoter of the αB-crystallin gene contains two conserved heat shock elements, one located between −397 and −374 and the other between −57 and −37, relative to the transcription start site. Electrophoretic mobility shift assay (EMSA) revealed that proteasome inhibition induces binding of heat shock factors to both heat shock elements in the αB-crystallin gene promoter. However, a transient transfection assay using deletion constructs of the αB-crystallin gene promoter showed that the region between −373 and −58 plays an important role in promoter activity. These results indicate the presence of differential response mechanisms of αB-crystallin gene expression to proteasome inhibition and heat shock, and that the activation of heat shock elements is not sufficient for the efficient induction of the αB-crystallin gene by proteasome inhibition.

Keywords: αB-crystallin; Lactacystin; Gene expression

αB-Crystallin is a member of the small heat shock protein (sHSP) family, which is characterized by the presence of a highly conserved α-crystallin domain [1]. αB-Crystallin forms large hetero-oligomeric complexes with HSP27, another member of the sHSP family [2]. αB-Crystallin and HSP27 function as molecular chaperones [3] and can protect various cells against distinct stresses. For example, the adenovirus-mediated gene transfer of either αB-crystallin or HSP27 protects cardiomyocytes from ischemic cell death [4]. Protective effects of the overexpression of either αB-crystallin or HSP27 on TNFα-induced L929 fibroblast cell death have also been reported [5]. αB-Crystallin is expressed only in limited tissues including heart, skeletal muscle, and lens, while HSP27 is expressed relatively ubiquitously [6–8]. The expression of αB-crystallin as well as HSP27 is induced by various types of stress including heat shock, osmotic shock, or exposure to heavy metals [9–11].

Proteasome is involved in various cellular processes [12]. For example, proteasome degrades cyclins, which are essential for cell cycle progression [13]. Myogenic transcription factor MyoD is a short-lived protein that is degraded by proteasome [14]. Therefore, proteasome inhibition should induce cell cycle arrest and differentiation. Proteasome inhibition also induces the expression of HSP genes [15]. Since abnormal proteins are degraded mainly by the proteasome–ubiquitin pathway, proteasome inhibition causes an accumulation of abnormal proteins and induces cellular stress responses similar to heat shock. Indeed, it has been reported that proteasome inhibition leads to the activation of both heat shock factor (HSF)-1 and -2 and the subsequent

☆ *Abbreviations:* ALLN, acetyl–leucyl–leucyl-norleucinal; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; HSP, heat shock protein; HSE, heat shock element; HSF, heat shock factor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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doi:10.1016/j.bbrc.2003.09.186

induction of HSPs [16–18]. The induction of αB-crystallin gene expression by proteasome inhibition has also been reported [19], but the mechanism of induction has not been examined.

We searched for proteasome inhibition-responsive elements in the rat αB-crystallin gene promoter and found that multiple elements other than heat shock elements play essential roles in the induction.

Materials and methods

Materials. Lactacystin and acetyl–leucyl–leucyl-norleucinal (ALLN) were purchased from Kyowa Medicals (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. SJA6017 was kindly provided by Senjyu Pharmaceuticals (Kobe, Japan).

Anti-αB-crystallin antibody and anti-HSP27 antibody were purchased from StressGen (Victoria, BC, Canada) and Santa Cruz (Santa Cruz, CA, USA), respectively. Anti-HSF-1 and -2 antibodies were from Chemicon (Temecula, CA, USA).

Peroxidase-conjugated anti-rabbit and -goat IgG antibodies were purchased from Promega (Madison, MI, USA) and Sigma (St. Lois, MO, USA), respectively. All other reagents were commercially available.

Plasmids. The promoter of the rat αB-crystallin gene was amplified by PCR with primer A (5′-GGCTAGATGAGTGAGAGTCGGTTAGCCGGTCA-3′) corresponding to the region from +42 to +10 and primer B (5′-TGCTGTTGCGACTAGTAGCCCTGACCCAAGTG-3′) corresponding to the region from −1003 to −971 relative to the transcription start site. PCR was carried out with these primers and 500 ng of genomic DNA prepared from H9c2 cells using *pfu* DNA polymerase (Promega). Incubation conditions for PCR were 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C, repeated for 30 cycles. After PCR, the product was cloned into the *Sma*I site of pBluescript. The resultant plasmid was designated pαBpromoter. Then the plasmids were digested with *Sac*I and *Xho*I and the inserted fragment was cloned into the multicloning site of pGL3basic plasmid (Promega), which contains a luciferase gene. The resultant plasmid was designated pαB1003Luc. To create reporter plasmids containing a deletion mutant of the αB-crystallin promoter, PCR was carried out similarly for 20 cycles with pαBpromoter as a template, and the T7 primer and one of the following primers: 1–10. Primer 1 (5′-**AAACGCGT**AGCCCAGGAAGATTCCAGCC-3′, −396 to −377), primer 2 (5′-**AAACGCGT**CCCAGGCCCAAGATAGTTGC-3′, −373 to −354), primer 3 (5′-**AAACGCGT**ACCGTTCCAGAAGCTTCACA-3′, −57 to −38), primer 4 (5′-**AAACGCGT**GACTGCATATATAAGGGGCA-3′, −35 to −16), primer 5 (5′-**ACTACGCGT**GGACAGAGAGCTAGTG-3′, −231 to −214), primer 6 (5′-**GTGACGCGT**CACCGGCCAGCTCAGC-3′, −197 to −178), primer 7 (5′-**GCCACGCGT**TTCTCTTTTCTTAGCT-3′, −168 to −151), primer 8 (5′-**AGTACGCGT**TATGTGTCACCCTGCC-3′, −136 to −121), and primer 9 (5′-**ATCACGCGT**CCCCATGAACTGTCGG-3′, −104 to −87). Each primer contained an additional sequence, as indicated by bold letters, at the 5′ end to create an *Mlu*I site (underlined). The resulting PCR products were digested *Xho*I and *Mlu*I, and cloned into pGL3basic. The constructed plasmids were designated pαB396Luc, pαB373Luc, pαB57Luc, pαB35Luc, pαB231Luc, pαB197Luc, pαB168Luc, pαB136Luc, and pαB104Luc. To create a plasmid with deletion of HSE-2 on pαB373Luc, PCR was carried out with primer 2 and primer 10 (5′-**AAACGCGT**GATGTCAGGGGTTTTATTAT-3′, −57 to −76). The resulting PCR product was digested *Mlu*I and cloned into pαB35Luc. The resulting plasmid was designated pαB373Luc/HSE-2deletion. All plasmids were purified on QIAGEN-columns (Qiagen, Hilden, Germany) and the inserted sequences of the constructed plasmid were confirmed by dideoxy sequencing using an automated sequencer (Applied Biosystems, Foster City, CA, USA).

Cell culture and transient transfection assay. H9c2 cells were grown to about 70% confluence on 3.5 cm diameter dishes in DMEM supplemented with 10% FBS. All cell culture was performed at 37 °C under a 5% CO_2 atmosphere, except for cells subjected to heat shock (42 °C). The transfection was carried out by the calcium phosphate co-precipitation methods [20]. The cells were co-transfected with 5 μg of a reporter plasmid and 5 μg pSVβ-galactosidase plasmid (Stratagene, La Jolla, CA, USA). After 48 h of transfection, cell lysates were prepared and assayed for luciferase activities. Cell lysates were also assayed for β-galactosidase activities according to the standard methods [21] to normalize transfection efficiencies.

Northern blotting analysis. Total cellular RNA was prepared from cells using ISOGEN (Nippon gene, Osaka, Japan) according to the instruction mannual. Five micrograms of RNA was used for Northern blotting analysis and equal loading of RNA was confirmed by ethidium bromide staining of rRNA. The full length cDNA of rat αB-crystallin was labeled with [α-^{32}P]dCTP using a multiprime DNA labeling system (Amersham–Pharmacia, Buckinghamshire, UK) and used as a probe.

Immunoblotting analysis. Immunoblotting analysis was performed as described previously [22].

Electrophoretic mobility shift assay Nuclear extracts were prepared and Electrophoretic mobility shift assay (EMSA) was performed as described previously [23] with synthetic DNAs whose sequences are indicated in Fig. 3B.

Results

Proteasome inhibition induces the expression of αB-crystallin mRNA

We first examined whether proteasome inhibition leads to the expression of the αB-crystallin gene mRNA in a rat embryonic cardiomyocyte derived cell line, H9c2. Northern blotting analysis showed that both lactacystin and ALLN, inhibitors of proteasome [24,25], dose-dependently induce the expression of αB-crystallin gene mRNA (Fig. 1A). Time-dependent induction of the mRNA was also observed when cells were incubated with lactacystin (Fig. 1B). These findings indicate that

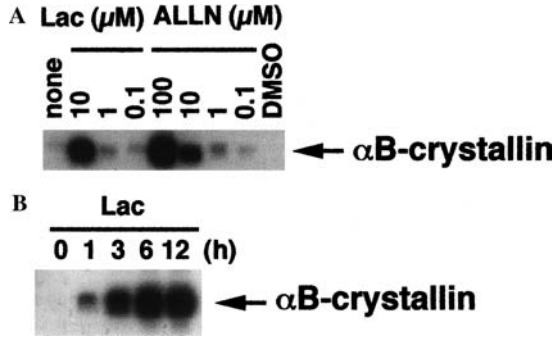

Fig. 1. Effects of proteasome inhibitors on the induction of αB-crystallin mRNA H9c2 cells were incubated (A) with the indicated concentrations of lactacystin (Lac) or ALLN for 12 h or (B) with 10 μM lactacystin (Lac) for the indicated time periods. Total RNA was extracted from cells and Northern blotting analysis was performed as described in 'Materials and methods.'

proteasome inhibition leads to the expression of the αB-crystallin gene at the mRNA level, as described for other HSPs [16–18].

Proteasome inhibition induces the expression of the αB-crystallin protein

We next examined whether the induction of mRNA by proteasome inhibition leads to the expression of the αB-crystallin protein. Immunoblotting analysis showed that both lactacystin and ALLN induce the expression of the αB-crystallin protein in both a dose- and time-dependent manner, while SJA6017, an inhibitor of calpain [26], has no effect (Figs. 2A and B). These results indicate that although ALLN is an inhibitor of both proteasome and calpain [25], the induction of the αB-crystallin protein is the result of proteasome inhibition. The HSP27 protein, another member of the sHSP family, is also induced by proteasome inhibition. Although the HSP27 protein was induced to the same extent by heat shock and proteasome inhibition, αB-crystallin was not efficiently induced by heat shock (Figs. 2A and B). These results indicate that the mechanism of the induction of αB-crystallin gene expression by proteasome inhibition is different from that by heat shock.

Proteasome inhibition induces binding activities to heat shock elements in the rat αB-crystallin gene promoter

In order to learn the molecular mechanism of αB-crystallin gene expression by proteasome inhibition, we performed EMSA analysis. The rat αB-crystallin gene promoter contains two conserved heat shock elements (HSEs), that are conserved among human, rat, and mouse [27]. One of these HSEs is located between −397 and −374, and the other is located between −57 and −37 (Fig. 3A). We designate distal HSE as HSE-1 and proximal HSE as HSE-2. EMSA analysis using HSE-1 and HSE-2 as probes showed the induction of binding to both HSE-1 and HSE-2 by proteasome inhibition as well as by heat shock (Figs. 4B and C), as observed when the HSE-consensus sequence was used as a probe (Fig. 4A). EMSA analysis using an Sp1 probe shows equal loading of nuclear extracts (Fig. 4D). To examine whether HSF-1 and/or -2 bind to HSEs during proteasome inhibition, we performed antibody super-shift assays. As reported previously [16–18], the binding to HSEs induced by proteasome inhibition is the result of binding of both HSF-1 and -2 (Fig. 4E). These results show that proteasome inhibition induces binding of both HSF-1 and -2 to heat shock elements in the αB-crystallin gene promoter.

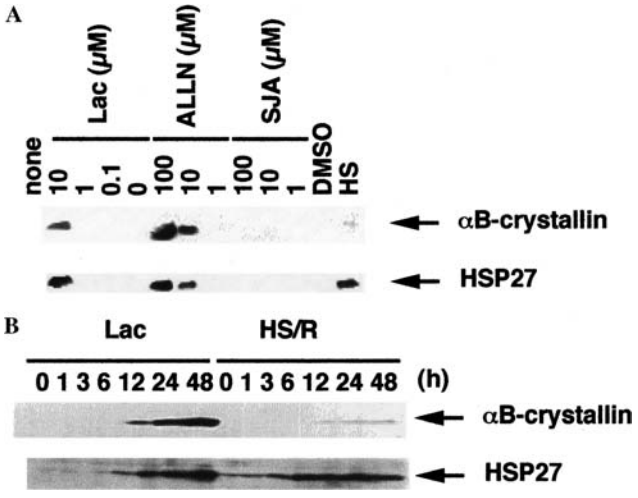

Fig. 2. Effects of proteasome inhibitors and heat shock on the induction of αB-crystallin and HSP27 proteins. H9c2 cells were (A) incubated with the indicated concentrations of lactacystin (Lac), ALLN, or SJA6017 (SJA) for 24 h, or subjected to heat shock (HS: 42 °C) for 1 h followed by 24 h of recovery (37 °C), or (B) incubated with 10 μM lactacystin for the indicated times or allowed to recover for the indicated times from 1 h of heat shock (HS/R). Total cellular proteins were prepared from H9c2 cells and equal amounts of proteins (40 μg) were separated by SDS–PAGE, blotted, and probed with antibodies against αB-crystallin or HSP27.

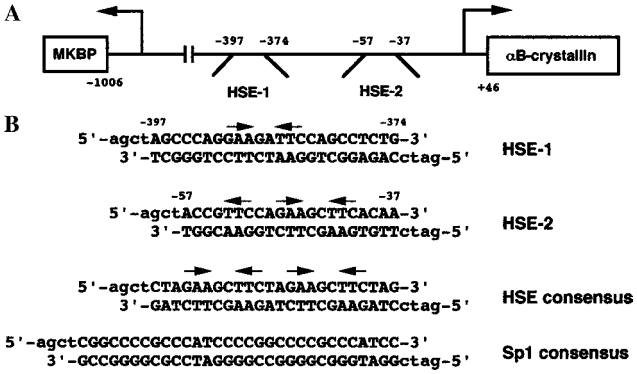

Fig. 3. Promoter structure of the αB-crystallin gene and the nucleotide sequences of the probes for EMSA. (A) The αB-crystallin gene and the myotonic dystrophy kinase-binding protein (MKBP) gene, another member of the sHSP family [31], are aligned in a head-to-head manner [27]. The locations of two conserved HSEs are indicated. (B) The sequences of the oligonucleotides used as probes for EMSA are indicated. Each oligonucleotide contained an additional sequence, which is indicated by small letters, at the 5′ end. The arrow indicates GAA repeats characteristic of HSE. The HSE consensus sequence and the Sp1 consensus sequence were adapted from the HSP70 promoter and the herpes simplex virus immediate-early 3 gene promoter, respectively.

Effect of the deletion of HSE on αB-crystallin gene promoter activity

Finally we constructed a reporter plasmid, pαB1003Luc, that contains a 5′-flanking region of the αB-crystallin gene to search for elements responsible for the induction of αB-crystallin gene expression by proteasome inhibition. When H9c2 cells were transfected with this construct, the cells showed the induction of luciferase activity following proteasome inhibition. To clarify the element(s) responsible for the induction by

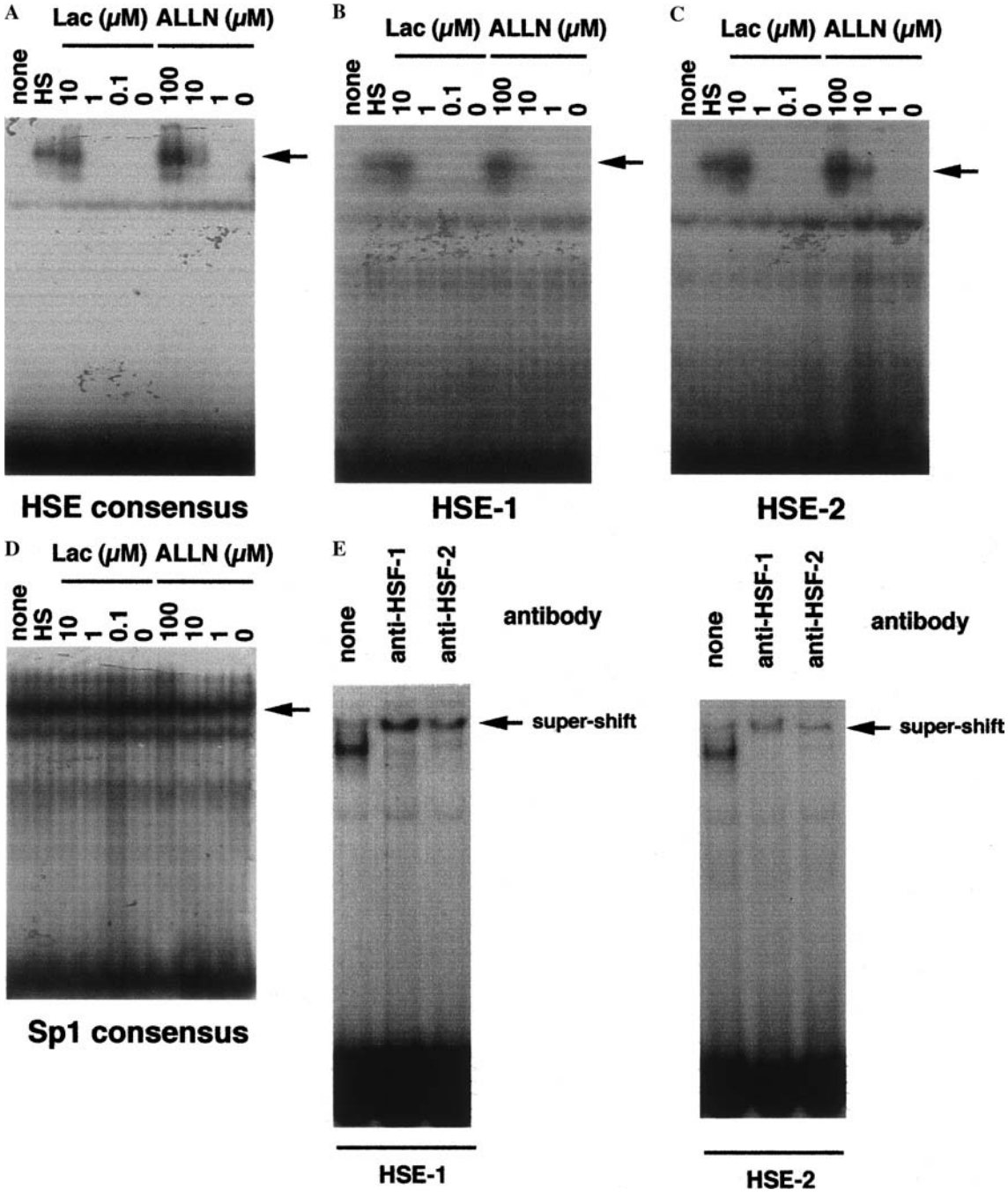

Fig. 4. Effect of proteasome inhibition and heat shock on HSE-binding activity. (A–D) H9c2 cells were incubated with the indicated concentrations of lactacystin (Lac) or ALLN for 12 h, or subjected to heat shock (HS) at 42 °C for 1 h followed by 12 h of recovery at 37 °C. Equal amounts (16 μg) of nuclear extract prepared from cells were incubated with the indicated ^{32}P-labeled probe DNA, whose sequences are shown in Fig. 3B. The retarded bands are indicated by arrows. For antibody super-shift assay (E), 0.1 μg of anti-HSF-1 or -HSF-2 antibody was added to nuclear extracts prepared from cells incubated with 10 μM lactacystin for 12 h. The super-shift bands are indicated by arrows.

proteasome inhibition, we constructed plasmid containing truncated αB-crystallin gene promoter. Luciferase activity driven by the promoter truncated at −373 (pαB373Luc) was efficiently induced by proteasome inhibition, suggesting that the distal HSE (HSE-1) is not so important (Fig. 5A). Further deletion to −57 (pαB57Luc) strongly reduced promoter activity (Fig. 5A), though pαB57Luc contains proximal HSE (HSE-2). These results indicate that element(s) responsible for the induction by proteasome inhibition resides between −373 and −58. To examine whether the fragment spanning from −373 to −58 can mediate the induction independently of HSE-2, we constructed a plasmid deleted HSE-2 from pαB373Luc (pαB373 Luc/HSE-2 deletion). Deletion of HSE-2 from pαB373Luc reduced the induction by proteasome inhibition considerably but not completely,

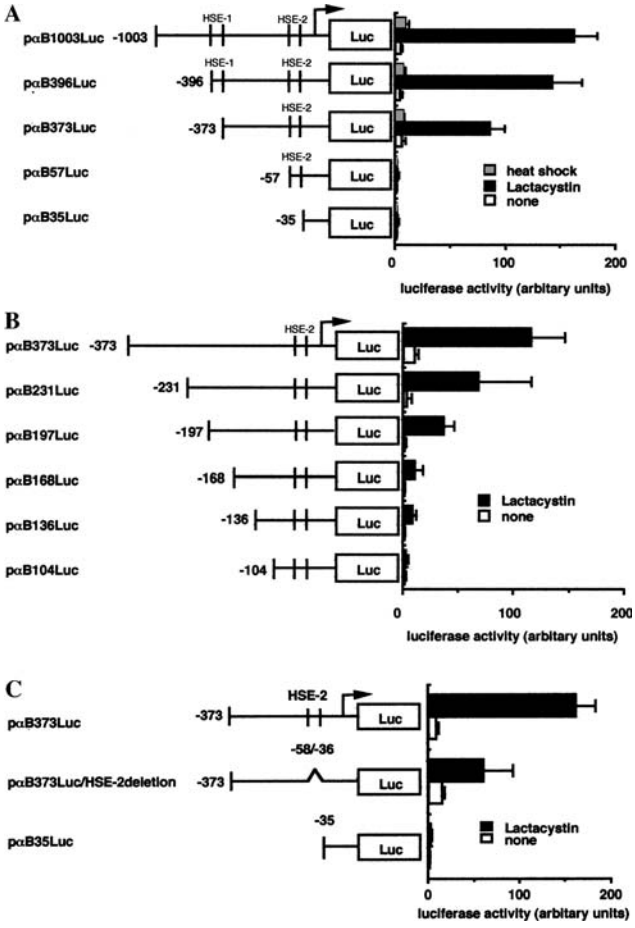

Fig. 5. Promoter activities of deletion mutants of the αB-crystallin gene promoter The 5′-truncated promoters of the rat αB-crystallin gene are presented schematically and the luciferase activities driven by these promoters in H9c2 cells are shown. Cells were incubated with 10 μM lactacystin (Lac) for 24 h or subjected to heat shock (HS) at 42 °C for 1 h followed by 24 h of recovery at 37 °C. Transfection efficiencies were normalized to the β-galactosidase activities. The relative luciferase activities were calculated by setting the luciferase activity from pαB35Luc (A and C) or pαB104Luc (B) as unity. Data are presented as averages and standard deviations of three samples.

suggesting that proteasome inhibition induces the αB-crystallin gene expression in both a HSE-dependent and -independent manner (Fig. 5C). To determine the elements responsible for proteasome inhibition that resides between −373 and −58, we created plasmids containing promoters truncated at −231, −197, −168, −136, or −104. Deletion to −232, −198, −169, or −137 gradually reduced promoter activity (Fig. 5B), suggesting that multiple elements between −373 and −105 are involved in the promoter activity induced by proteasome inhibition.

Discussion

The present study demonstrates that in rat embryonic cardiomyocyte-derived H9c2 cells, αB-crystallin gene expression is efficiently induced by proteasome inhibition but only slightly by heat shock, although HSP27 is efficiently induced by both proteasome inhibition and heat shock. Although EMSA revealed that proteasome inhibition induces binding activity to HSEs in the αB-crystallin gene promoter, transient transfection analysis using reporter plasmids driven by a set of deleted promoters of the αB-crystallin gene showed that HSE is not sufficient for the induction of promoter activity by proteasome inhibition. Efficient induction of the αB-crystallin gene by proteasome inhibition requires multiple elements between two HSEs.

Extensive studies by Piatigorsky et al. have demonstrated multiple tissue-specific regulatory elements in the murine αB-crystallin gene promoter. The enhancer activities of αBE-1 (−407 to −397), αBE-2 (−360 to −327), αBE-3 (−317 to −288), and MRF (−300 to −288) are important for the expression of αB-crystallin in both skeletal and cardiac muscle cells [28], while αBE-4 (−394 to −368), which shares sequences with HSE-1, is a cardiac-specific enhancer [29]. Since deletions between −373 and −232 reduce promoter activity (Fig. 5B), these elements are involved in the induction by proteasome inhibition. In addition to these muscle-preferred enhancers, two lens-specific regulatory regions (LSR1 and LSR2) are determined between −147 and −118 and between −78 and −46 [30]. Considering that multiple elements between −373 and −105 are involved in the induction by proteasome inhibition, these tissue-specific elements might also have contributed to the expression of the αB-crystallin gene by proteasome inhibition.

In conclusion, the present study reveals that the efficient induction of αB-crystallin gene expression requires not only HSE but also other elements. These results suggest some systems other than the HSF/HSE system play important roles in HSP gene expression by proteasome inhibition.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan, Yamanouchi Foundation for Research on Metabolic Disorders, The Inamori Foundation, The Kowa Life Science Foundation, Takeda Science Foundation, The Mochida Memorial Foundation for Medical, and Pharmaceutical Research, Japan Heart Foundation Research Grant, NOVARTIS Foundation (Japan) for the Promotion of Science, Research Program for Special Promotion of the Venture Business Laboratory, Yamaguchi University, Osaka Cancer Research Foundation, The Old Age Research Foundation of Yamaguchi, and Yamaguchi Industrial Promotion Foundation (for Y.M.).

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