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Sgt1 has co-chaperone properties and is up-regulated by heat shock

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ABSTRACT

The Sgt1 protein is a binding partner of heat shock proteins such as Hsp90, Hsp70 or Hsc70. In this work we show that the level of Sgt1 is increased in HEp-2 cells exposed to heat shock or radicicol. The citrate synthase aggregation assay shows that Sgt1 attenuates aggregation of the enzyme induced by increased temperature as efficiently as p23, a known co-chaperone of Hsp90. We have cloned two fragments of the human Sgt1 gene promoter (−708/+98 and −351/+98) into pGL3-luciferase vector and found that both fragments generated a 2-fold increase in luciferase activity upon heat shock. Furthermore, electrophoretic mobility shift assay revealed binding of the HSF-1 transcription factor to the heat shock element in the proximal (−42/−2) Sgt1 gene promoter fragment. These results indicate that Sgt1 is a co-chaperone protein with an expression pattern matching that of the well known heat shock proteins.

The Sgt1 protein was originally identified in yeast as a binding partner of Skp1 [1] and later found in other organisms including plants and mammals [2]. Based on the sequence homology between Sgt1 and CacyBP/SIP, its interaction with calcium binding proteins belonging to the S100 family was predicted and confirmed experimentally [3]. Currently the main scientific interest seems to be centered on the interaction of Sgt1 with chaperone proteins. The first chaperone family member shown to interact with Sgt1 was Hsp90 [4]. Later, studies performed by Lee et al. [5] showed that Sgt1 binds to human Hsp90 *in vitro* and emphasized the role of the CS domain of Sgt1 in this interaction. In our recent work we provided evidence that the Sgt1 protein interacts with Hsp70 both under the *in vitro* conditions and in the HEp-2 cell extract [6]. Moreover, we showed that the C-terminal part of Sgt1, termed the SGS domain, is important for the interaction between Sgt1 and Hsp70 or Hsp90. Accordingly, overexpression of a calcium binding protein, S100A6, which interacts with the SGS domain, decreased the amount of chaperones bound to Sgt1. Quite recently Noel et al. [7] showed that another chaperone protein, Hsc70, binds to Sgt1.

The interaction between Sgt1 and Hsp90 (or Hsp90 orthologs) seems to be conserved from yeast to humans [4,5,8]. In yeast, Sgt1 is supposed to be a client-adaptor protein that may draw substrates of Hsp90 to the chaperone complexes [9]. In human cells, the Sgt1–Hsp90 interaction guarantees proper kinetochore assembly and efficient cell division, while depletion of the Sgt1 protein causes hypersensitization of HeLa cells to 17-AAG, a Hsp90 inhibitor [10]. It was also shown that in stimulated human monocytic THP-1 cells, the Hsp90–Sgt1 complex controls the stability of NAPL3 and is crucial for activating the inflammasome during the innate immune response [11].

On the basis of all these data it can be hypothesized that Sgt1 is an essential component of the chaperone complexes. To check this hypothesis, in this work we examined if the level of Sgt1 changes after heat shock and performed citrate synthase assay to evaluate its co-chaperone properties. Since the transcription of many chaperone genes increases in response to stress conditions we examined whether the increase in Sgt1 expression might occur via the same mechanisms. The obtained results indicate that the Sgt1 gene promoter activity is elevated after heat stress and that this stimulation seems to be a result of HSF-1 activation.

Methods

Cell culture, heat stress, radicicol treatment, and extract preparation. HEp-2 cells were cultured as described by Spiechowicz et al. [6]. For heat shock, HEp-2 cells, 70–80% confluent, were washed in PBS and a serum-free medium was added. Culture dishes were sealed with parafilm and placed in a water bath at 43 °C or 37 °C (control) for 45 min. After the heat shock the serum-free medium was removed and

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSF, heat shock factor; PBS, phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBS, tris buffered saline; TBE, tris–borate–EDTA buffer.

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replaced with a fresh complete medium. Cells were allowed to recover in a 5% CO_2 incubator at 37 °C for the indicated times. When cells were treated with radicicol, the reagent was added in a fresh complete medium to a final concentration of 5, 10, 20 or 50 μM. After 90 min, cells were washed in PBS and maintained for 6 h in a fresh complete medium.

After recovery cells were washed with PBS, harvested and homogenized 25 times using a syringe with a needle (26-gauge; 0.45 × 12) in buffer containing 10 mM KCl, 0.1 mM EDTA, and protease inhibitor cocktail (Complete mini EDTA-free, Roche) in 10 mM Tris–HCl, pH 7.5. The homogenate was then centrifuged at 9600*g* for 30 min and protein concentration was estimated in the supernatants by the Bradford's procedure with BSA as a standard protein. 40 μg of total protein was subjected to SDS–PAGE.

SDS–PAGE and immunoblotting. Gel electrophoresis with 10% (w/v) polyacrylamide containing 0.1% SDS was performed by the method of Laemmli [12]. Proteins were then transferred electrophoretically onto nitrocellulose and identified using appropriate primary antibodies: mouse anti-Sgt1 monoclonal (BD Transduction Laboratories), rat anti-Hsp90 monoclonal and rabbit anti-Hsp70 polyclonal (Stressgen Bioreagents), rabbit anti-Raf-1 polyclonal and rabbit anti-HSF-1 polyclonal (Santa Cruz Biotechnologies), and mouse anti-GAPDH monoclonal (Chemicon International). After washing with TBS-T buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, and 0.05% Tween 20) the blots were allowed to react with appropriate secondary antibodies conjugated to horseradish peroxidase: goat anti-mouse IgG (Jackson Immunoresearch Laboratories), goat anti-rabbit IgG (Sigma) or rabbit anti-rat IgG (Stressgen Bioreagents). After three successive washes with TBS-T and two with TBS buffers the blots were developed with the ECL chemiluminescence kit (Amersham Biosciences).

Aggregation assay. Porcine heart citrate synthase (Sigma) was assayed for heat-induced aggregation as described by Buchner et al. [13]. The enzyme was prepared in 40 mM Hepes, pH 7.5. Sgt1 was prepared as described by Nowotny et al. [3]. The p23 protein, which was a gift from Dr. A. Żylicz (International Institute of Molecular and Cell Biology, Warsaw) was used as a positive control. Non-chaperone control proteins: BSA and lysozyme, were purchased from Sigma. Aggregation induced by incubation at 43 °C was monitored by measuring the optical density at 360 nm. The light scattering signal was recorded every 5 s for 22 min. Measurements were performed using a Shimadzu RF-5301 spectroflourometer and the Hyper RF spectroscopy software.

Searching for the Sgt1 gene promoter region and in silico analysis of its sequence. To map the human Sgt1 gene promoter sequence a multiple alignment of twelve human Sgt1 mRNA sequences currently available in the GenBank to the human genome sequence at the UCSC genome browser (http://genome.ucsc.edu/) was performed using the Clustal W (1.83) software. A 3 kb fragment of the putative Sgt1 gene promoter sequence (chr13: 52122002–52125001) was then screened by the MatInspector software tool (Genomatix portal) to identify putative transcription factor binding sites.

Cloning of the Sgt1 gene promoter fragments and luciferase assay. Two Sgt1 gene promoter fragments, corresponding to positions −708/+98 (chr13: 52124294–52125099) and −351/+98 (chr13: 52124651–52125099) relative to the transcription start site assigned as +1 (chr13: 52125002) were cloned into the pGL3-luciferase basic vector (Promega) at the XhoI and HindIII restriction enzyme sites. The fidelity of cloning was verified by DNA sequencing (GATC Biotech, Germany). HEp-2 cells were transfected with these vectors and the pRL-SV40 vector (Promega), as an internal control reporter, using Lipofectamine 2000 (Invitrogen) and the heat shock was applied 24 h post-transfection. The luciferase assay was performed after additional 6 h using the dual-luciferase reporter assay system (Promega).

Results

The level of Sgt1 changes due to heat shock or radicicol treatment

To check if the level of Sgt1 changes in response to stress conditions, we subjected HEp-2 cells to heat shock and examined the protein extracts prepared at different times after treatment. As it can be seen in Fig. 1 significant changes in the Sgt1 level were seen 6 h after heat shock and this elevated amount of Sgt1 remained unchanged until 48 h. When cells were incubated for 90 min with the Hsp90 inhibitor, radicicol, and harvested after 6 h recovery the level of Sgt1 was also increased and the maximal changes were observed at 20 μM concentration of the drug (Fig. 1). In both cases the changes in the level of Sgt1 were very similar to those observed for Hsp70 and Hsp90. On the contrary, the amount of Raf-1 kinase, a client protein of Hsp90, decreased proportionally to the applied concentration of radicicol. Immunoblots developed with anti-GAPDH antibody served as controls of the amount of protein loaded on the gel.

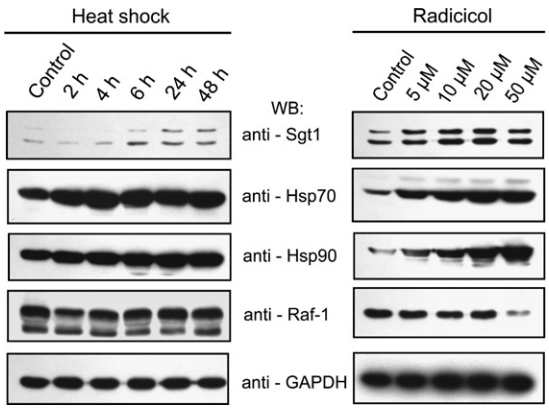

Fig. 1. Changes in the level of stress response-related proteins in HEp-2 cells exposed to heat shock or cultured in the presence of radicicol monitored by Western blot analysis. Cells were heat-shocked at 43 °C for 45 min and harvested after indicated times of recovery at 37 °C (left panel) or were cultured for 90 min in a medium containing indicated concentration of radicicol and then harvested after 6 h of recovery (right panel). Forty micrograms of total protein from each cell extract were loaded on the gel.

Sgt1 functions as a co-chaperone in the citrate synthase aggregation assay

Since Sgt1 binds to chaperone proteins and its level increases after heat shock or radicicol treatment we checked whether Sgt1 might have co-chaperone properties. To test that we performed citrate synthase aggregation assay in the presence of Sgt1 or control proteins at 1:1 molar ratio to the enzyme. As it can be seen in Fig. 2, Sgt1 attenuates citrate synthase protein aggregation as efficiently as the p23 co-chaperone. This result strongly suggests that Sgt1 has co-chaperone properties.

Higher level of Sgt1 is due to increased activity of the Sgt1 gene promoter

To check whether, upon stress conditions, the Sgt1 expression is induced at the transcriptional level we cloned and characterized

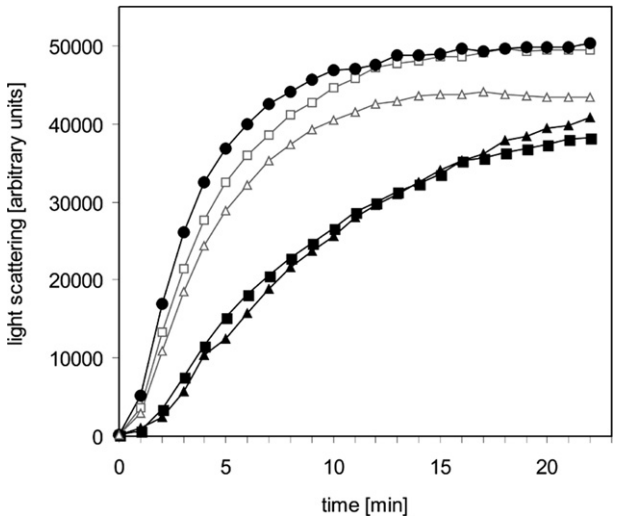

Fig. 2. The Sgt1 protein is able to suppress the citrate synthase thermal aggregation process. Light-scattering measurements of solutions containing citrate synthase (black circles), citrate synthase in the presence of Sgt1 (black squares), citrate synthase with p23 (black triangles), citrate synthase with BSA (white triangles) or citrate synthase with lysozyme (white squares) were performed at 43 °C for 22 min; values collected at 1 min intervals are marked. Citrate synthase and the examined proteins were mixed at 1:1 molar ratio. Data of a typical experiment (out of three performed) are presented.

the Sgt1 gene promoter sequence. We observed that five Sgt1 mRNA sequences (DN995623, AY321358, AJ344097, BC000911, and NM006704) had the same 5′-end corresponding to chr13: 52125002 and thus we assigned this position as a putative transcription start site (+1). According to this assignment the start of translation (chr13: 52125057) corresponds to position +56. The sequence upstream of the transcription start site was considered to be the sequence of the Sgt1 gene promoter. To check whether this sequence is indeed able to initiate and support transcription we cloned two fragments corresponding to positions −708/+98 (chr13: 52124294–52125099) and −351/+98 (chr13: 52124651–52125099) into a luciferase reporter vector. As it can be seen in Fig. 3 both constructs had a substantial transcriptional activity (assayed as luciferase activity) over the pGL3-basic vector which means that the cloned DNA contains all sequence elements necessary for a functional promoter within the −305/+98 fragment. Most importantly the activity of both Sgt1 gene promoter fragments was higher in cells exposed to heat shock. Similar stimulation range (about 2-fold) suggests that the increase in activity may arise from a shared regulatory element contained in the proximal promoter fragment.

Binding of the HSF-1 transcription factor to the Sgt1 gene promoter

The MatInspector software tool (core/matrix sim: 0.75) predicted over 500 potential transcription factor binding sites within a 3 kb-long sequence of the human Sgt1 gene promoter (chr13: 52122002–52125001), among them several heat shock elements (HSE, marked by triangles) (Fig. 4A). Two of the HSE sequences are located in the proximal promoter region, shared by the cloned Sgt1 gene promoter fragments, while the remaining ones are more distant from the transcription start site. To examine binding of the HSF-1 transcription factor to the heat shock elements in the Sgt1 gene promoter, two promoter fragments (marked by white squares) corresponding to positions −42/−2 (Hsf) and −2432/−2395 (Hsf-bis), each containing two predicted overlapping HSE elements, were chosen. We first checked whether the level of HSF-1 increases in the nuclear fraction (prepared as described in the Supplementary Material) in cells exposed to heat shock and found a robust accumulation of HSF-1 in the nucleus under stress conditions (Fig. 4B). As it can be seen in Fig. 4C (lanes 1 and 2), EMSA (performed as described in the Supplementary Material) revealed that a protein–DNA complex with the Hsf probe is formed only in the case of nuclear extracts from heat-shocked cells. This interaction is specific since it is quenched by a 100-fold excess of a non-labeled Hsf probe (lane 4) and of the HSE probe (lane 5) but not by the unspecific DNA (lane 6). Furthermore, addition of the anti-HSF-1 antibody (lane 3) to the nuclear extract destabilized the protein–DNA complex thus identifying its protein component as HSF-1. The non-labeled Hsf-bis probe decreased the intensity of the band corresponding to protein–DNA complex only slightly, suggesting that it is a weak competitor of the Hsf probe (lane 7). Essentially the same results were obtained in EMSA experiments using the nuclear extracts from radicicol-treated cells (Fig. 4D). In both cases when EMSA was performed with a radiolabeled Hsf-bis probe no signal corresponding to the protein–DNA complex was observed (data not shown). This negative result was probably due to the fact that the sequence of the Hsf-bis probe is less similar to the consensus defined as 5′-TTCnnGAAnnTTC-3′ (see Table 1) than the sequence of the Hsf probe. All these observations suggest that the HSF-1 transcription factor selectively binds to the −42/−2 fragment of the Sgt1 gene promoter region which makes it a good candidate for being a factor engaged in the regulation of Sgt1 gene transcription.

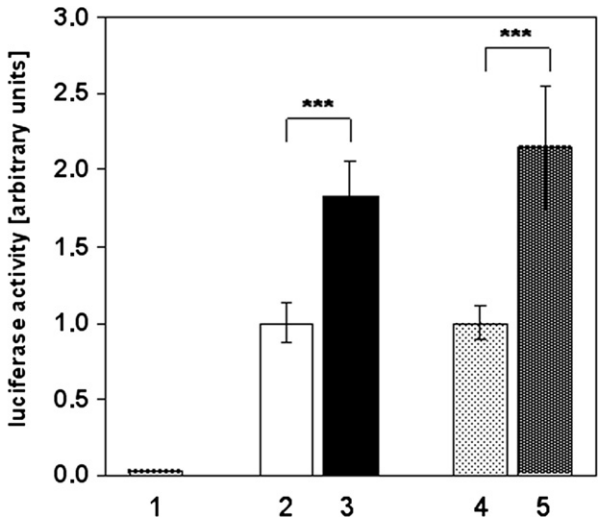

Fig. 3. Luciferase activity assay. Twenty-four hours post-transfection HEp-2 cells were exposed to heat shock and lysates were prepared after a 6 h long recovery. Luciferase activity in cells transfected with the promoterless pGL3 vector (1), the pGL3 vectors containing luciferase gene under control of the −351/+98 Sgt1 gene promoter fragment (marked by white and black bars, 2 and 3) or under control of the −708/+98 Sgt1 gene promoter fragment (marked by white dotted and black dotted bars, 4 and 5). White and dotted white bars (2 and 4) represent luciferase activity (arbitrary units) in control cells kept at 37 °C while black and dotted black bars (3 and 5) represent the activity in cells exposed to heat stress (45 min at 43 °C). Statistical analysis was performed using the Student's *t*-test. All data are means ± SD of three independent experiments. $^{***}p \leqslant 0.001$.

Discussion

The results presented in this work reveal that the level of Sgt1 is elevated in HEp-2 cells due to stress conditions such as heat shock or radicicol treatment. The changes we observed were similar to those seen for the Hsp90 and Hsp70 proteins. Thus, our finding suggests that Sgt1 is not a client protein but a co-chaperone. The citrate synthase assay confirmed that Sgt1 is able to protect proteins from thermal aggregation as efficiently as the p23 protein, a known co-chaperone of Hsp90. These results may serve as an additional evidence that Sgt1 is a component of the chaperone complexes and that it might protect proteins that tend to form aggregates at an elevated temperature. Heat shock proteins, known also as molecular chaperones, minimize the consequence of cell damage caused by different stress factors [14–16] acting in large protein complexes. The role of molecular chaperones as well as co-chaperones, e.g., Hsp90, Hsp70, Hsp60, Hsp25 or p23 is also to prevent protein aggregation—they selectively recognize and bind non-native proteins that tend to aggregate [17,18]. During the heat shock response their expression is up-regulated and their role is to restore proper protein folding or direct misfolded proteins for the degradation in proteasome [19,20].

The results showing that the Sgt1 level increases after heat shock prompted us to examine whether this increase occurs at the transcriptional level as in the case of other heat shock proteins [21]. Up to now, no experimental data concerning regulation of the Sgt1 gene promoter activity have been published but, based on an *in silico* analysis, the Sgt1 gene has been classified into a group of 46 putative heat-induced genes that contain HSE elements in their promoter regions [22]. In this work we have cloned for the first time the Sgt1 gene promoter and showed that its activity increased 2-fold after heat shock. HSE sequences, present also in the Sgt1 gene promoter, can be recognized by heat shock factors 1 (HSF-1) and 2 (HSF-2). These two factors are the main transcriptional regulators of the heat stress-induced expression of genes encoding

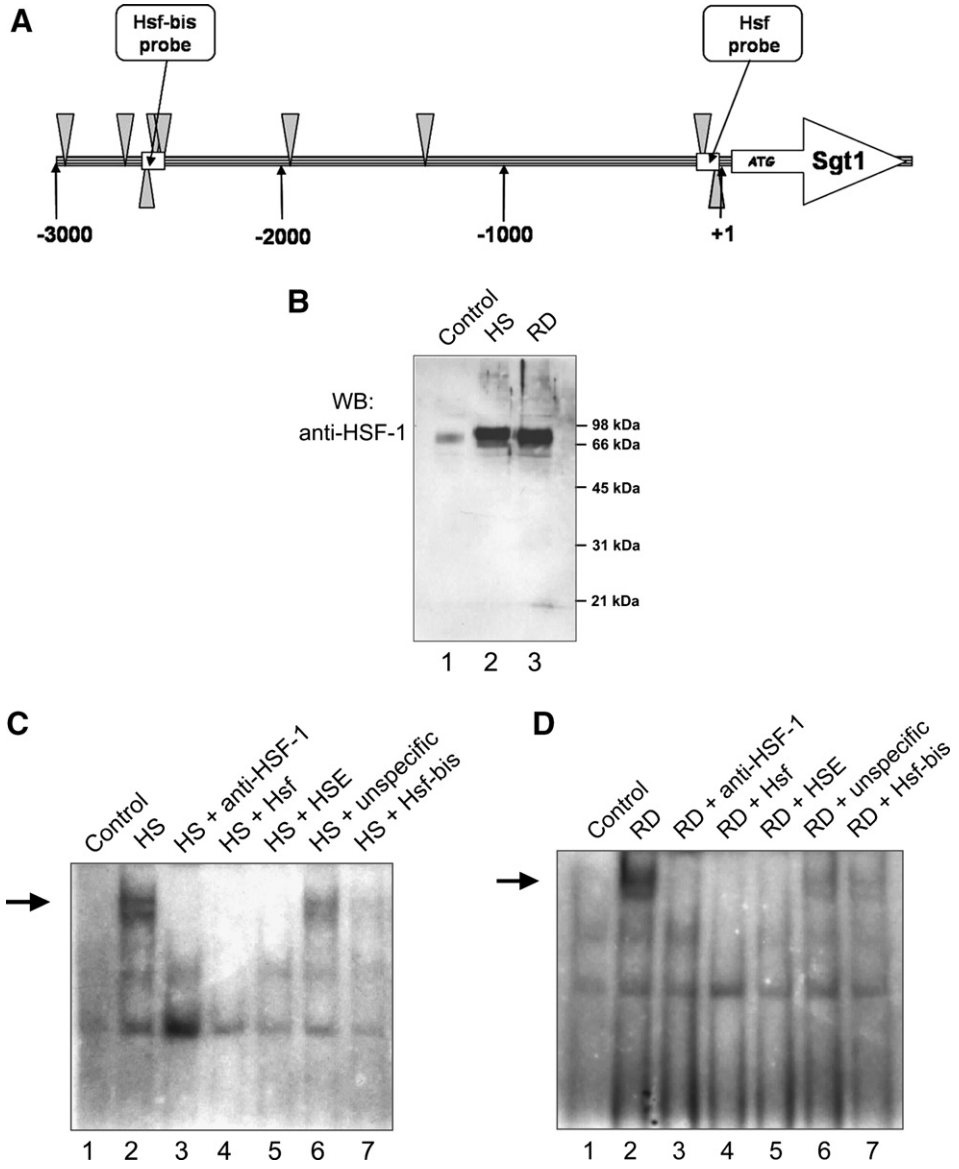

Fig. 4. (A) Schematic presentation of the Sgt1 gene promoter region. The predicted binding sites for the transcription factors of the HSF family (HSE elements) and the localization of the −42/−2 (Hsf) and −2432/−2395 (Hsf-bis) probes used in EMSA are indicated by gray triangles and white squares, respectively. (B) Western blot showing the level of HSF-1 in nuclear fraction. Lanes 1, 2 and 3 represent 50 μg protein of the nuclear extracts prepared from control, heat-shocked (HS) or radicicol (RD) treated cells. The positions of the molecular weight standards are indicated on the right. The amount of protein was controlled by staining the nitrocellulose membrane with Ponceau S solution. (C) and (D) show the results of the EMSA performed using a radiolabeled −42/−2 fragment of the Sgt1 gene promoter (Hsf probe) and 25 μg protein of nuclear extracts from heat-shocked (HS) or radicicol (RD) treated HEp-2 cells, respectively. The non-labeled competitors were added at a 100-fold excess. Lane 1 shows the nuclear extract of control cells and lanes 2–7 represent the nuclear extracts of treated cells. Lanes 1 and 2, no additions; lane 3, anti-HSF-1 antibody; lane 4, non-labeled Hsf probe; lane 5, non-labeled HSE-probe; lane 6, non-labeled unspecific DNA probe; lane 7, non-labeled Hsf-bis probe.

Table 1
Oligonucleotide sequences of EMSA probes

Oligonucleotide	Sequence	Genomic position
Hsf	5′-ggttggtgttctccagaagtttccccctttgggcggtggt	chr 13: 52124961–5000
Hsf-bis	5′-cctgtgcggtgattggaagcttcctgaggcctctgcaa	chr 13: 52122487–2524
HSE	5′-gcctcgattgttcgcgaagtttcg	—
Unspecific	5′-gctccccttatccaatcacgtgtcacga	—

HSF-1/HSF-2 binding sites are underlined.
The genomic position of Hsf and Hsf-bis probes is given according to the UCSC genome browser.

the heat shock proteins [23–25]. The HSF-1 factor is activated in eukaryotic cells after exposure to elevated temperature, treatment with azecytidine, cadmium sulfate or with proteasome inhibitors [26] whereupon it becomes phosphorylated, forms homo- or heterotrimers with HSF-2 and translocates into the nucleus. Our EMSA results revealed that, *in vitro*, HSF-1 recognizes and binds to the proximal (−42/−2) region of the Sgt1 gene promoter implying that this sequence is a functional heat shock element (HSE). This binding was transient and was not observed 6 h after heat shock (not shown). Since the observed interaction correlates in time with the translocation of HSF-1 to the nucleus, we suggest that HSF-1 might regulate the Sgt1 expression also *in vivo*. It should be underlined that the proximal HSE (−42/−2) seems to be evolutionary conserved since an identical sequence is found in a similar location in DNA of many other species (rhesus monkey, mouse, dog, and armadillo) while another HSE studied in our EMSA experiments (−2515/−2478) is shared only with the rhesus monkey genome. The involvement of HSF-1 in transcriptional regulation of Sgt1

would guarantee its expression pattern to be well synchronized with that of heat shock proteins.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.055.

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