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ORIGINAL ARTICLE

S100A6 mediates nuclear translocation of Sgt1: a heat shock-regulated protein

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Abstract Sgt1 was originally identified in yeast as a suppressor of the Skp1 protein. Later, it was found that Sgt1 is present in plant and mammalian organisms and that it binds other ligands such as S100A6, a calcium-binding protein. In this work we show that in HEp-2 cells Sgt1 translocates to the nucleus due to heat shock. We also found that in HEp-2 cells with diminished level of S100A6, due to stable transfection with siRNA against S100A6, such translocation occurred at a much smaller scale in comparison with cells expressing a normal level of S100A6. Moreover, translocation of Sgt1 was observed in HEp-2 cells treated with thapsigargin instead of heat shock. In contrast thapsigargin was ineffective in cells with diminished level of S100A6. Thus, our results suggest that increase in intracellular concentration of Ca^{2+}, transduced by S100A6, is necessary for nuclear translocation of the Sgt1 protein.

Keywords Sgt1 · S100A6 (calcyclin) · Heat shock proteins · Nuclear translocation

Abbreviations

AM	Acetoxymethyl ester
BAPTA	Bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular calcium concentration
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDAC1	Histone deacetylase 1
PBS	Phosphate buffered saline
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
TBS	Tris buffered saline

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Introduction

Sgt1 was originally identified in yeast cells where it was shown to be involved, via interaction with Skp1, in the CBF3 kinetochore complex and in the SCF ubiquitin ligase (Kitagawa et al. 1999). The results published later showed that Sgt1 binds several ligands and that its interacting partner, Hsp90, is important in plant and mammalian cells for the response to different stresses (Kadota et al. 2008; da Silva Correia et al. 2007; Mayor et al. 2007; Picard 2008; Shirasu 2009, Hubert et al. 2009). Hsp90 is a well-known chaperone endowed with ATPase activity (Nadeau et al. 1992, 1993). Its N-terminal ATPase domain binds to Sgt1 (Kadota et al. 2008) while Sgt1 interacts with Hsp90 by its middle (CS) and C-terminal (SGS) domain (Lee et al. 2004; Spiechowicz et al. 2007; Kadota et al. 2008). The SGS domain of Sgt1 binds also S100A6 and some other calcium-binding proteins from the S100 family (Nowotny et al. 2003). Moreover, depending on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) S100A6 attenuates the interaction between Sgt1 and Hsp90 (Spiechowicz et al. 2007). Recently, it was shown that the Sgt1 protein has co-chaperone properties and is up-regulated by heat shock (Zabka et al. 2008). It is well known that co-chaperone proteins

form complexes with chaperones (Meyer et al. 2004; Ali et al. 2006) and that those complexes are involved in proper folding and stabilization of proteins, in regulation of gene expression and in signaling pathways including those involved in response to different stresses (Sharma and Masison 2009) such as pathogen attack, radiation, hypoxia, oxidative or thermal stress (Arya et al. 2007). In response to stress the expression of co-chaperone proteins and chaperones is up-regulated (Lindquist 1986).

Since it is known that under stress conditions some proteins, including chaperones, translocate from the cytoplasm to the nucleus (Pratt et al. 2004; Daniel et al. 2008), in this work we examined the changes in subcellular localization of Sgt1 due to heat shock with special focus on its nuclear translocation. We found that in heat-shocked cells the amount of Sgt1 increases in the nucleus and that the nuclear translocation is specific. Furthermore, considering that heat shock may induce changes in $[Ca^{2+}]_i$ (Stevenson et al. 1986), we checked whether the calcium-binding protein, S100A6 (Leśniak et al. 2009), might have an effect on Sgt1 translocation depending on $[Ca^{2+}]_i$.

Experimental procedures

Cell-based experiments

HEp-2 (human epidermoid carcinoma) cells were obtained from ATCC and cultured in a complete medium consisting of DMEM (Sigma), 10% FBS (Gibco), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (Sigma) in 5% CO_2 at 37°C. Cells stably transfected with the pSilencer 2.1-U6 hygro vector (Ambion) encoding short hairpin RNA comprising either a 19-bp-long S100A6 siRNA sequence [HEp-2-S100A6(−) cells] or a control siRNA sequence [HEp-2-S100A6(+) cells] were obtained as described by Słomnicki et al. (2009). Cells were grown in the medium described above supplemented with 200 μg/ml of hygromycin B. Cells were passaged when confluent and used for experiments 24 h later. For heat shock, 70% confluent cells were washed in PBS and kept in DMEM with 5% of serum without antibiotics for 45 min at 43°C. After heat shock the complete medium was added and cells were allowed to recover for 1, 2, 4, 6 or 24 h. Cells were then harvested and protein extracts were prepared. When cells were treated with thapsigargin, they were incubated for 1.5 h in HBS buffer (20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, 25 mM glucose, 1 mM $MgCl_2$, 1% BSA, pH 7.4) containing thapsigargin at a final concentration of 1 or 2 μM and $CaCl_2$ at a final concentration of 2 mM. In control experiments BAPTA/AM instead of thapsigargin was used. In that case cells were incubated for 1.5 h in HBS buffer supplemented with EGTA and BAPTA/AM at a final concentration of 1 mM and 2 μM, respectively. In experiments with leptomycin B, the agent at a final concentration of 10 ng/ml was applied for 24 h.

Immunocytochemistry

Glass cover slips were coated with poly-L-lysine (50 μg/ml) for 30 min and then washed three times with PBS. HEp-2 cells were cultured on these slips in complete medium for 24 h and then used for appropriate experiments. After treatment (heat shock, thapsigargin, BAPTA/AM) cells were fixed with 3% paraformaldehyde in ICCH buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM $MgCl_2$, pH 7.0) for 20 min at RT. In the next step cells were treated with 50 mM NH_4Cl for 10 min at RT to reduce aldehyde groups and then permeabilized with 0.1% Triton X-100 for 4 min at 4°C and finally incubated in ICCH buffer containing 5% of BSA. All steps described above were preceded by a double washing in PBS. To detect proteins of interest cells were stained with primary mouse anti-Sgt1 monoclonal antibodies (BD Transduction Laboratories) (diluted 1:500) in 2.5% BSA in ICCH buffer, overnight at 4°C. Next day cells were washed five times in PBS and incubated with secondary donkey anti-mouse antibodies conjugated with Alexa Fluor 555 (Molecular Probes) diluted 1:1,000 in 2.5% BSA in ICCH buffer for 1.5 h at RT. After that cells were washed three times in PBS and twice with water. Then, cover slips were mounted on slides using the Vectashield mounting medium (Vector Laboratories) and examined with a Leica confocal laser fluorescence microscope (Leica TCS SP5 Spectral Confocal with STED or Leica TCS SP2 Spectral Confocal, Germany).

Preparation of protein extracts, SDS-PAGE and immunoblotting

To prepare protein extracts cells were washed in PBS, collected in 1 ml of PBS and centrifuged at 68 × *g* for 5 min. Cells were suspended in 1.5 ml of buffer A (20 mM HEPES, pH 8.0, 0.2% IGEPAL CA-630, 1 mM EDTA, 1 mM DTT), vortexed and kept on ice for 10 min. Next, cells were centrifuged at 1,700 × *g* for 10 min at 4°C and after washing in buffer A, the nuclear pellet was resuspended in 150 μl of buffer B (20 mM HEPES, pH 8.0, 420 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM DTT) and kept on a rotating wheel for 30 min at 4°C. Nuclear protein extract was obtained after centrifugation at 10,600 × *g* at 4 °C for 10 min. Protein concentration in the cytoplasmic and nuclear extracts was determined by Bradford reagent. In each case 30 μg of protein from the extract was loaded on the SDS gel (Laemmli 1970). Then proteins were separated and transferred onto nitrocellulose

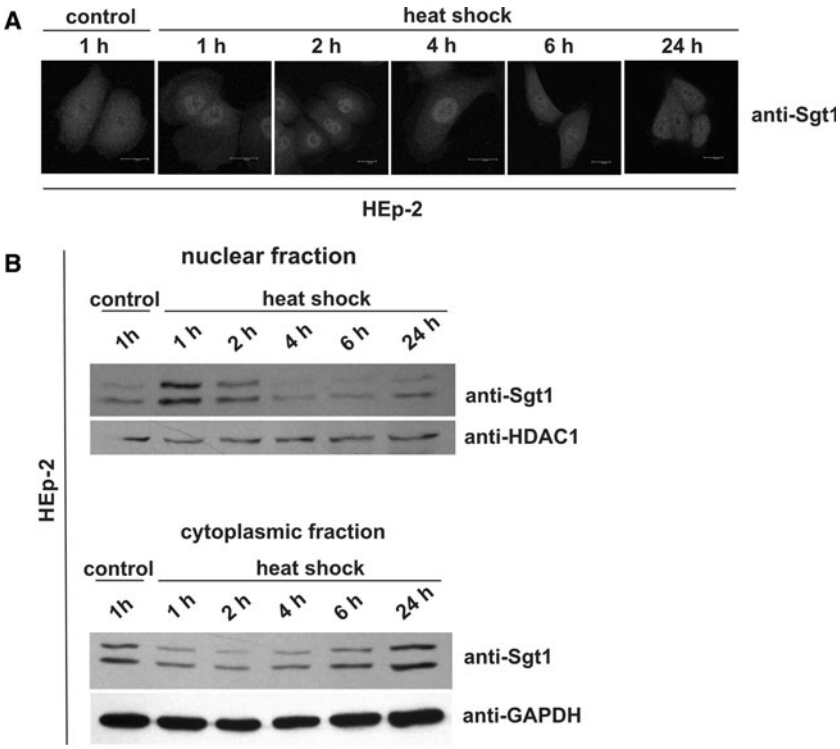

Fig. 1 Subcellular localization of Sgt1 in HEp-2 cells exposed to heat shock. **a** Cells were stained with anti-Sgt1 antibodies after the indicated time and analyzed by use of a confocal laser microscope. *Bar* 20 μm. **b** Western blot analysis showing the level of Sgt1 in the nuclear (*top*) and cytoplasmic (*bottom*) fractions. Staining with anti-HDAC1 and anti-GAPDH antibodies show that each lane contains a similar amount of nuclear or cytoplasmic proteins, respectively

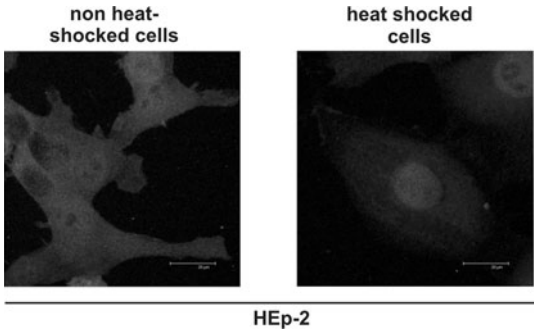

Fig. 2 Subcellular localization of Sgt1 in HEp-2 cells incubated in the presence of leptomycin B. HEp-2 cells were either left untreated or heat shocked. After 24 h cells were stained with anti-Sgt1 antibodies and analyzed by confocal laser microscopy. *Bar* 20 μm

filter. Examined proteins were identified using appropriate primary antibodies: mouse anti-Sgt1 monoclonal diluted 1:1,000 (BD Transduction Laboratories), mouse anti-GAPDH monoclonal diluted 1:5,000 (Chemicon International) and rabbit anti-HDAC1 polyclonal diluted 1:5,000 (Santa Cruz Biotechnologies). After washing with TBS-T buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, and 0.05% Tween 20) the blots were incubated with goat anti-mouse IgG (Jackson Immunoresearch Laboratories) or goat anti-rabbit IgG (Sigma) secondary antibodies conjugated to horseradish peroxidase. After three washes with TBS-T and two washes with TBS (50 mM Tris–HCl, pH 7.5, 200 mM NaCl), blots were developed with the ECL kit (Amersham Biosciences).

Results

Subcellular localization of Sgt1 in HEp-2 cells after heat shock

In HEp-2 cells the level of Sgt1 is high when compared to other cell lines such as neuroblastoma NB2a or NIH3T3 fibroblasts (data not shown). Under normal conditions the Sgt1 protein is mainly present in the cytoplasm. To check whether Sgt1 changes its subcellular localization in cells exposed to heat shock, HEp-2 cells were analyzed by immunocytochemistry and western blot at different time points after stress. As it is shown in Fig. 1a, 1 h after heat shock the Sgt1 protein starts to concentrate in the nucleus in comparison with untreated cells, analyzed after the same time, where it is predominantly seen in the cytoplasm. A clear nuclear localization of Sgt1 is observed up to 1–2 h after heat shock. However, 24 h after heat shock the Sgt1 protein is re-located to the cytoplasm. In control cells Sgt1 remained in the cytoplasm throughout the 24-h experiment (data not shown). The same results were obtained by subcellular fractionation and Western blot analysis. As it can be seen in Fig. 1b, the level of Sgt1 in the nuclear fraction of heat-shocked cells is higher after 1–2 h than in non heat-shocked cells. In agreement with these data are the results showing that the level of the examined protein decreases in the cytoplasmic fraction.

To check if nuclear translocation of Sgt1 occurs specifically in response to heat shock we carried out an

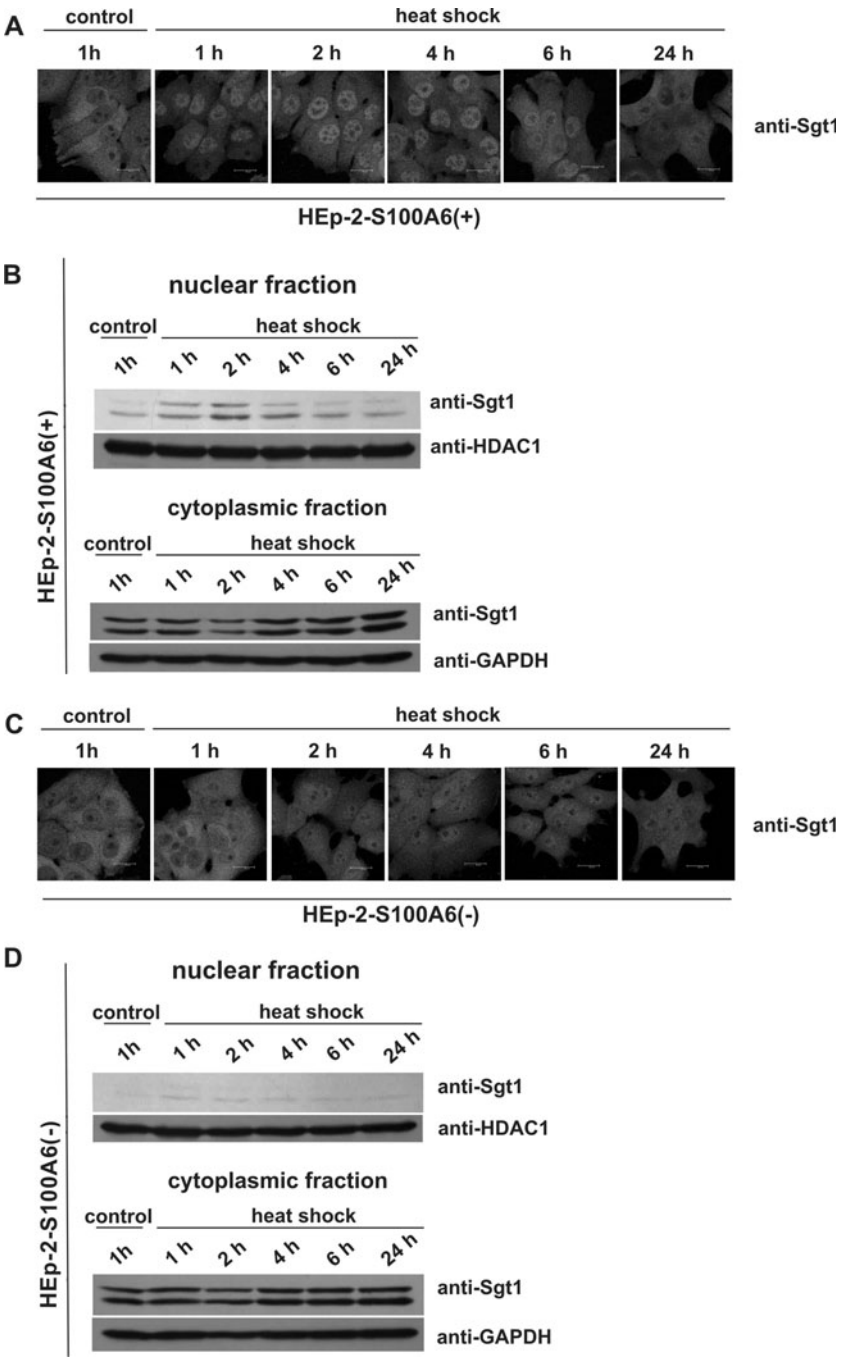

Fig. 3 The influence of S100A6 on Sgt1 translocation during heat shock in HEp-2 cells. HEp-2-S100A6(+) cells (**a**, **b**) or HEp-2-S100A6(−) cells (**c**, **d**) were exposed to heat shock. **a**, **c** After the indicated times cells were stained with anti-Sgt1 antibodies and then analyzed by use of a confocal laser microscope. *Bar* 20 μm. **b**, **d** Western blot analysis showing the level of Sgt1 in the nuclear and cytoplasmic fractions. Staining with anti-HDAC1 and anti-GAPDH antibodies show that each lane contains a similar amount of nuclear or cytoplasmic proteins, respectively

experiment, in which cells were treated with leptomycin B, an inhibitor of exportin. As it can be seen in Fig. 2, the immunofluorescent staining with anti-Sgt1 antibodies shows that in non heat-shocked cells no accumulation of Sgt1 in the nucleus is seen after 24 h, while in cells which were exposed to heat shock, the accumulation of Sgt1 in the nucleus after this time is significant.

Nuclear translocation of Sgt1 in HEp-2 cells with various levels of S100A6

Since HEp-2 cells have a high level of S100A6, a protein which interacts with Sgt1 depending on $[Ca^{2+}]_i$, we examined the influence of S100A6 on Sgt1 translocation during heat shock. For that we used stably transfected cells with a diminished [HEp-2-S100A6(−)] and unchanged

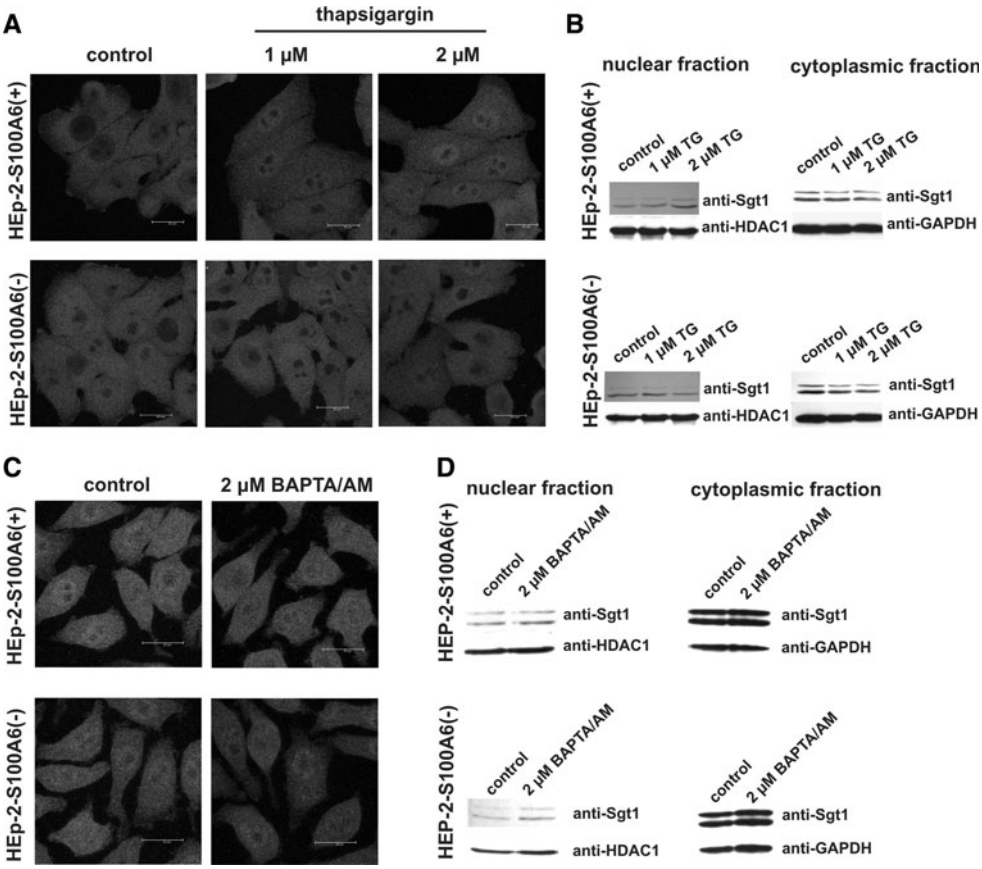

Fig. 4 The changes in subcellular localization of Sgt1 after incubation of HEp-2-S100A6(+) and HEp-2-S100A6(−) cells with 1 and 2 μM thapsigargin (*TG*) (**a**, **b**) or 2 μM BAPTA/AM (**c**, **d**). **a**, **c** Immunostaining of cells with anti-Sgt1 antibodies and analysis by use of confocal laser microscopy. *Bar* 20 μm. **b**, **d** Western blot analysis showing the level of Sgt1 in the nuclear and cytoplasmic fractions. Staining with anti-HDAC1 and anti-GAPDH antibodies show that each lane contains a similar amount of nuclear or cytoplasmic proteins, respectively

[HEp-2-S100A6(+)] level of S100A6. As it can be seen in Fig. 3a, b, in HEp-2-S100A6(+) cells, a clear nuclear localization of Sgt1 is observed within 2 h after heat shock. At the same time the level of Sgt1 in the cytoplasmic fraction decreases. In contrast, in HEp-2-S100A6(−) cells (Fig. 3c, d), Sgt1 translocation is significantly less substantial and does not change with time. This suggests that S100A6 deficiency interferes with nuclear accumulation of Sgt1 after heat shock.

The effect of thapsigargin on nuclear translocation of Sgt1

Since it was reported that $[Ca^{2+}]_i$ increases during heat shock (Stevenson et al. 1986) and that interaction between S100A6 and Sgt1 is Ca^{2+}-dependent (Nowotny et al. 2003), we checked whether nuclear translocation of Sgt1 takes place in cells which were treated with thapsigargin instead of being exposed to heat shock. For that, HEp-2-S100A6(+) and HEp-2-S100A6(−) cells were incubated with thapsigargin (an agent which inhibits the SERCA pump and causes increase in $[Ca^{2+}]_i$) or BAPTA/AM (a chelator of Ca^{2+}) as described in the "Experimental procedures". As it can be seen in Fig. 4a, b, the results of an immunostaining experiment and Western blot analysis indicate that incubation of HEp-2-S100A6(+) cells with thapsigargin induces nuclear translocation of Sgt1. The effect is best seen at 2 μM concentration of this agent. In contrast, nuclear translocation of Sgt1 was not observed in HEp-2-S100A6(−) cells. As it was expected, when cells were incubated with BAPTA/AM, the nuclear translocation of Sgt1 was not observed (Fig. 4c, d).

Discussion

Under normal conditions the Sgt1 protein is present mainly in the cytoplasm, although there are some reports showing that during mitosis Sgt1 is seen in the nucleus, where it is involved in proper kinetochore assembly (Steensgaard et al. 2004). In this work we show for the first time that due to heat shock the Sgt1 protein concentrates in the nucleus. The nuclear level of Sgt1 is highest within 1–2 h after heat shock, similar to what was reported for some heat shock proteins (Perdew et al. 1993; van Bergen en Henegouwen et al. 1987). Since heat shock leads to inhibition of DNA synthesis and growth arrest (Khandjian and Rose 2000), we can speculate that during heat shock nuclear Sgt1 might play a different role in the nucleus than the one documented during cell division. This hypothesis might be

supported by the fact that Sgt1 has co-chaperone properties (Zabka et al. 2008), and that Sgt1 stabilizes some nuclear proteins (Martins et al. 2009).

Since we found that Sgt1 translocates to the nucleus after heat shock, in our work we focused on the factors that may induce or inhibit this translocation. We show that in cells with diminished S100A6 level translocation of Sgt1 is significantly reduced when compared to cells with normal S100A6 content. As the interaction between Sgt1 and S100A6 is Ca^{2+}-dependent (Nowotny et al. 2003) and $[Ca^{2+}]_i$ increases after heat shock (Stevenson et al 1986), it was interesting to check whether thapsigargin might have a similar effect on Sgt1 translocation. We found a significant accumulation of Sgt1 in the nucleus after thapsigargin treatment in cells with normal level of S100A6. In cells with diminished level of S100A6 thapsigargin had no effect on Sgt1 translocation. This indicates that the Ca^{2+} bound form of S100A6 is necessary to induce nuclear translocation of Sgt1. Since it was shown previously that S100A6 competes with Hsp90 for the binding with Sgt1 (Spiechowicz et al. 2007) and that Hsp90 inhibits nuclear translocation of its ligands (Catelli et al. 1985; Passinen et al. 2001) the effect of Ca^{2+}/S100A6 on Sgt1 nuclear translocation conforms with these observations (Spiechowicz et al. 2007). Regardless of the signal that induces Sgt1 translocation, it is interesting how this protein can enter the nucleus. Most steroid receptors which possess nuclear localization signal (NLS) translocate to the nucleus very rapidly (half time of about 5 min) (Galigniana et al. 2004). Since up to now, neither NLS nor nuclear export signal (NES) for Sgt1 have been identified and indeed we found that Sgt1 translocation is relatively slow when compared with that of steroid receptors, one can speculate that nuclear translocation of Sgt1 might occur either by diffusion, which is possible for molecules up to 60 kDa (Knudsen et al. 2009), or by selective non-diffusional transport (Silver 1991). Our results obtained by incubation of cells with leptomycin B, an inhibitor of exportin, suggest that Sgt1 translocation occurs specifically due to heat shock since Sgt1 was accumulated only in the nuclei of heat-shocked cells. Moreover, these results suggest that the export of Sgt1 from the nucleus is specific and depends on exportin. To summarize, findings of this work show that the heat shock-induced nuclear translocation of Sgt1, a protein involved in many complexes including those important for cell survival, depends on Ca^{2+}-bound form of S100A6.

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