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Molecules and Cells

Heat Shock Factor 1 Is a Transcription Factor of Fas Gene

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In mammalian cells, stress-induced expression of heat shock protein is controlled by heat shock factor 1 (HSF1). However, HSF1 functions as a regulator of additional genes. In this study, we observed that heat treatment effectively induced expression of Fas. Using bioinformatics, a high affinity and functional HSF1-binding element within the -1996/-1985 oligonucleotide of the 5′-flanking region of the Fas gene was found, and was determined by electrophoretic mobility shift assay and chromatin immunoprecipitation assay. Exogenous expression of a constitutively activative HSF1, induced Fas gene transcription and protein synthesis in the absence of heat stress. Moreover, RNA interference-mediated HSF1 gene-silencing attenuated Fas expression in a heat-induced model. Our results suggested that HSF1 is an important transcription factor of Fas gene.

INTRODUCTION

The death receptor Fas/APO-1 (CD95) is a key player in tumor formation, progression, and immunity as well as in tumor responses to anticancer drugs (Bhushan et al., 1998; Buhling et al., 2005; Eser et al., 2006; Peter et al., 2005). Corruption of this signaling pathway in tumor cells, for example by reducing Fas expression, can participate in tumor development and immune escape. There is a strong evidence demonstrating that reduced membranous Fas expression, as a mechanism of apoptotic resistance, plays an important part in carcinogenesis, which may predict poor survival (Viard-Leveugle et al., 2003). Restoration of Fas expression enhanced the immunological killing effect in tumor cells, which may overcome multi-drug resistance in clinical cancer chemotherapy.

The remarkably conserved heat shock protein response is one of the best characterized endogenous mechanisms of host defense and survival in prokaryotic and eukaryotic cells. Various physiological and cellular stresses trigger the expression of highly conserved heat shock proteins (Hsps) which are molecular chaperones that protect native proteins from unfolding, catalyze protein refolding and remove misfolded proteins, respectively (Bukau et al., 2006; Hartl and Hayer-Hartl, 2002). Stress-induced expression of Hsp genes is mediated primarily by a remarkably conserved transcription factor known as heat shock factor 1 (HSF1) (McMillan et al., 1998; Voellmy, 1994). Activated HSF1 can specifically bind to heat shock element (HSE) sequences that are present in Hsp genes. HSE sequences are composed of multiple nGAAn modules that are arranged in alternating orientation. In unstressed cells, HSF1 is present both in the cytoplasm and in the nucleus as an inactive monomer. In response to cellular stress, HSF1 converts to a DNA-binding homotrimer and translocates to the nucleus, where it acquires transactivation competence (Benjamin and McMillan, 1998; Chu et al., 1996). Recent studies have shown that HSF1 can also act as a regulator of certain apoptosis-related genes, including interleukin (IL)-1β, c-fos and TNF-α gene (Singh et al., 2002; Xie et al., 2002; 2003). It was reported that exposure to a mild heat stress rapidly sensitized Jurkat and HeLa cells to Fas-mediated apoptosis (Tran et al., 2003). Some researchers have found that HeLa cells are relatively insensitive to Fas-mediated killing; over-expression of a constitutively activated form of HSF1, (HSF1(+)), sensitized the cells to Fas-mediated killing (Xia et al., 2000). Strategies designed to up-regulate HSF1 activity in tumor cells, either by pharmacologic or gene-therapy approaches may provide a means for sensitizing tumors to the killing effects of chemotherapies whose effectiveness depends on activation of the Fas pathway.

This study addressed the question whether HSF1 regulates Fas expression. We described the presence of a high affinity HSF1 binding sequence within the 5′-flanking region of Fas gene, and demonstrated that HSF1 can bind to this sequence *in vitro* and *in vivo*. Our results revealed that hyperthermia enhanced Fas expression, and HSF1(+) produced a similar effect. HSF1 knockdown by RNA interference markedly suppressed the basal and heat-induced expression of Fas gene. These results indicate that HSF1 played a critical role in regulating Fas expression.

MATERIALS AND METHODS

Reagents and treatment

Anti-β-actin (A5316) antibody was purchased from Sigma. Anti-Fas (C20) antibody was purchased from Santa Cruz Biotechnology (USA). Anti-HSF1 (SPA-901) and anti-Hsp70 (SPA-810) antibodies were purchased from Stressgen Biotechnologies Corp (USA). For heat treatment, cell culture dishes were wrapped with

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Parafilm and immerged in a 42.5°C water bath for 30 min.

Detection of Fas mRNA expression by RT-PCR
Total cellular RNA was extracted using Trizol (MRC, TR118) according to the instructions of the manufacturer. One microgram of total RNA was reverse-transcripted using AMV Reverse Transcriptase XL (Promega, USA) and random primers. The resulting cDNA was amplified by PCR using gene-specific primers. PCR primers for Fas gene were caagggattggaattgagga (sense) and gacaaagccaccccaagtta (antisense), for Hsp70 were agctgctgcaggacttcttc (sense) and cgttggtgatggtgatcttg (antisense), and for β-actin were accgtggagaagagctacga (sense) gtacttgcgctcagaaggag (antisense).

Viral infection
To generate adenovirus vectors, plasmid DNA containing human HSF1 mutants HSF1d202-316, which is HSF1(+) (Xia et al., 2000), were cut by enzyme and inserted into pShuttle-CMV vector (Stratagene, USA) following the instruction. Viral DNAs were generated using AdEasy adenoviral vector system (Stratagene) and viral particles were enriched as described previously (Fujimoto et al., 2005). Titers of virus stocks were $1\sim5 \times 10^8$ pfu/ml. Cells plated in 100-mm dishes containing 10 ml of medium were infected with Adenoviruses expressing HSF1(+) at a titer of 8×10^5 pfu/ml. After infection with the virus for 24-48 h, cells were harvested for assays.

Immunoblotting
Whole cell lysate was prepared using RIPA buffer (50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.25% sodium ortho-vanadate, 50 mM sodium fluoride, 1 mM EDTA, and 1μg/mL leupeptin). The protein concentration was determined using BCA protein assay kit (Pierce, USA). Equal aliquots of total cell lysates (10-15 μg) were electrophoresed on a 10% SDS-PAGE gel. The separated proteins were then transferred to polyvinylidene difluoride membranes (Millipore, USA) with a Bio-Rad tank system. Non-specific binding was blocked with 5% nonfat dry milk. The blot was probed with primary antibody for 2 h at room temperature followed by the HRP-conjugated secondary antibody. Antigen-antibody complexes were visualized by the enhanced chemiluminescence system (ECL, UK).

Construction of small interfering RNA plasmid vector
An HSF1-specific siRNA construct was designed to be homologous to human HSF1 mRNA (GenBank accession no. NM005526). Two complementary oligonucleotides (5′-caagtacttcaagcacaacaac*ttcaagaga*gttgttgtgcttgaagtacttttttttg-3′ and 5′-aattcaaaaaaaagtacttcaagcacaacaac*tctcttgaa*gttgttgtgcttgaagtacttgggcc-3′) (Yin et al., 2005) were annealed to generate double-stranded DNAs and ligated into the linearized empty vector psilencer1.0-u6. Sense or antisense strands are in bold letters and stem loop sequences are in italics. The siRNA encoded by the plasmids specifically targeted HSF1 mRNA and had no significant homology with other known genes. The nucleotide sequence of the plasmid was verified by automated DNA sequencing.

Electrophoretic mobility shift assay (EMSA) and super shift analysis
EMSA was performed on nuclear extracts from A549 cells. The extract preparation and binding reaction were performed as previously described (Shoshani et al., 2002). Biotin end-labeled double-stranded oligonucleotide (gtaacccaGAAttTTCtaagattat) containing a binding site for HSF1 was purchased from Invitrogen Life Technologies (USA). Specific binding was confirmed by competition experiments with 200-fold excess of unlabeled, identical oligonucleotides. The nuclear extracts were incubated with oligonucleotides before being separated on a non-denaturating PAGE gel. Target bands are detected by enhanced chemiluminescent (ECL) assay Kit (Pierce).

Chromatin Immunoprecipitation (ChIP) Assays
ChIP analysis was performed following a protocol provided by Upstate Biotechnology (USA). Precipitated DNAs were analyzed by PCR to detect the HSF1 binding site at position -2.0 kb within the Fas promoter (GenBank accession no. X87625). The sequences of the PCR primers used in the PCRs were: GTTTTCAAATTAAAGTAACCCAG (sense), 5′-CCCTACCCT-CTACCCAC-3′ (antisense).

RESULTS

Heat induces Fas gene expression in A549 cell line
A previous study demonstrated that hyperthermia enhances Fas mediated killing (Tran et al., 2003). In this study, we investigated whether heat treatment affect Fas mRNA transcription and protein expression. Cultures were heat-treated (42.5°C for 30 min) and were allowed to recover at 37°C for different time periods, or were not heat-treated. Fas expression was analyzed by RT-PCR. As shown in Fig. 1A, the level of Fas mRNA was significantly elevated in heat-treated cells compared with the cells maintained at normal growth temperature (37°C). This observation indicates that the Fas gene is a molecular target of heat treatment. Increased Fas mRNA level also correlated with increased level of Fas protein as shown by Western-blot (Fig. 1B) and Immunostaining (Fig. 1C).

HSF1 directly binds to HSEs in the Fas gene promoter
Heat stress has been shown to activate heat shock response genes in many different experimental models (Morimoto, 1998; Pirkkala et al., 2001). This evolutionarily conserved phenomenon is primarily mediated by HSF1. The high affinity binding sequence for HSF1 comprises a minimum of two nGAAn/nTTCn elements arranged as an inverted dyad repeat (Fig. 2A). In order to understand whether HSF1 is involved in heat-induced Fas expression, 5′-flanking region of the Fas gene was analyzed to find HSE by TRANSFAC search (http://transfac.gbf.de/TRANSFAC). Based on DNA sequence analysis, one putative HSE, which is rich in pentanucleotide nGGAn (nTTCn) sequences, is present between -1996- and -1985-nt region of the Fas 5′-flanking region.

To test the binding capacity of each nGAAn/nTTCn-containing sequence in the regulatory promoter region of the Fas gene, EMSA was performed. -1996/-1985 oligonucleotide was biotin end-labeled and used as a probe to test the nuclear extracts from A549 cells. As is shown in Fig. 2B, DNA-protein complexes were detected in extract of heat-treated cells (lanes 2 and 4) but essentially not in the extract of not-heated cells (lane 1). It was found that the binding was completely blocked by the excessive unlabeled cold probes (-1996/-1985 oligonucleotide in Fas promoter), indicating the specificity of binding reaction. Furthermore, these DNA-protein complexes could be specifically supershifted by antibody against HSF1 (Fig. 2B, lane 5). These results suggest that a high affinity HSE (HSF1/HSE) exists in the -1996/-1985 region.

To substantiate the activity of this HSE *in vivo*, we performed ChIP assay by using specific antibody against HSF1. Normal goat IgG was used as the negative control. DNA associated with the chromatin immunoprecipitated by these antibodies was

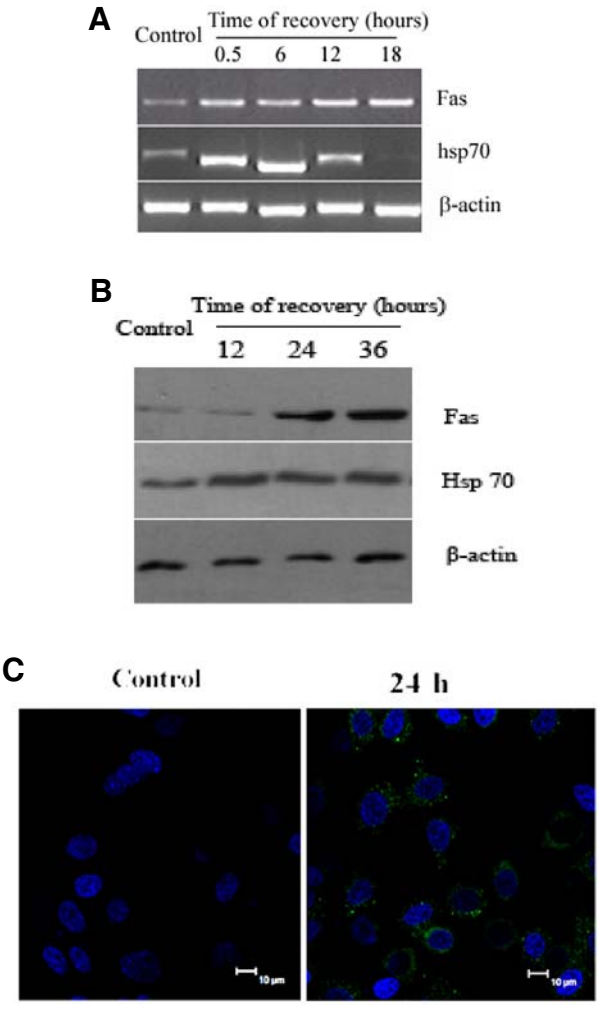

Fig. 1. Hyperthermia (42.5°C, 30 min) up-regulates mRNA and protein expression of Fas gene in A549 cells. (A) mRNA expression of Fas gene was determined by semiquantitative RT-PCR. RT-PCR with β-actin-specific primers was used to document equal amounts of cDNA. Total mRNA was obtained from A549 cell untreated (control) or heat-treated, followed by 0.5, 6, 12, and 18 h recovery at 37°C. (B) Protein expression of Fas was determined by Western-blot analysis. Whole cell extracts were obtained from A549 cells untreated (control) or heat-treated, followed by 12, 24, and 36 h recovery. (C) Immunofluorescence staining for Fas (green) in heat treated cells showed that increase of membranous receptor levels compared to untreated cells. A549 cells were untreated or heat-treated, followed by 24 h recovery at 37°C. This figure was representative of three independent experiments.

then amplified by PCR with primers specific for the putative HSE region of the Fas promoter. As expected, no DNA fragments were detected when normal IgG was used (Fig. 2C). In contrast, anti-HSF1 antibody specifically precipitated the Hsp70 and Fas promoter fragment (Fig. 2C). In addition, we showed that HS increased the amount of immunoprecipitated DNA. These findings together with EMSA results strongly support that the -1996/-1985 sequence of Fas gene contains a high affinity HSE for HSF1.

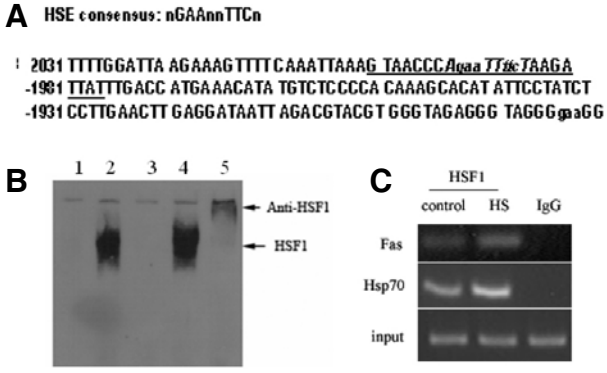

Fig. 2. HSF1 binds to the HSE sequence in Fas promoter. (A) Consensus sequence of HSE. One nGAAn (nTTCn)-rich sequence presented in the 5′-flanking region (-1995/-1986) of the Fas gene. The putative HSF1-binding elements are underlined, and the nGAAn (nTTCn) motifs are italicized. (B) Double-strand oligonucleotides consensus to the -1995/-1986 sequence of Fas promoter were labeled with biotin and bound to the nuclear extract from heat treated after 6 h recovery (lanes 2 and 4) or untreated (lane 1) A549 cells. Nuclear extract were incubated with an anti-HSF1 super-shifting antibody (lane 5), non-specific antibody (lane 4), or 200-fold excess of unlabeled HSE oligonucleotide of Fas promoter (lane 3). The complex of HSF1-HSE is indicated by arrow (HSF1). (C) ChIP analysis of HSE/Fas and HSE/Hsp70 elements from untreated (lane 1), and heat induced after 6 h recovery (lane 2) A549 cells using antibody specific for HSF1 (lanes 1 and 2) or goat IgG control (lane 3). These figures were the representative of two to three independent experiments.

Over-expression of the constitutively activated HSF1 mutant enhances Fas expression

Our next goal was to test whether HSF1 regulated the expression of endogenous Fas gene. In our experiments, HSF1(+), the constitutively active HSF1 mutant, was over-expressed in A549 cells using an adenoviral expression vector. The capability of Ade-HSF1(+) infection was determined by immunoblot analysis. With a deletion in HSF1, HSF1(+) migrated faster on SDS-PAGE compared with the endogenous wild-type HSF1 (Fig. 3B). As shown in Fig. 3B, the levels of Hsp70 were increased in Ade-HSF1(+) infected samples compared with Ade-ß-gal control. Also, infection of Ade-HSF1(+) alone increased both Fas mRNA and protein levels (Figs. 3A, 3B, and 3C). This was similar to that observation with heat shock treatment.

HSF1 knock-down decrease Fas expression in A549 cells

We investigated whether HSF1 knockdown could affect expression of the Fas gene in A549 cells. A psilencer1.0-u6 construct expressing a specific siRNA targeting HSF1 transcripts (psilencer-HSF1) was generated. Sub-confluent A549 cells were transfected with psilencer-HSF1 construct or empty vector. Sixty hours after transfection, cells were either incubated at 37°C, or subjected to heat treatment, and then allowed to recover at 37°C. Cell extracts were analyzed for mRNA or protein expression analysis. Heat-induced expression of Fas mRNA was markedly suppressed by HSF1 knockdown (Fig. 4A), and the expression level of Fas protein was also decreased (Fig. 4B, lane 3) or impaired to heat induction (Fig. 4A, lane 4).

These results strongly suggest that stress-induced Fas expression is mediated by HSF1.

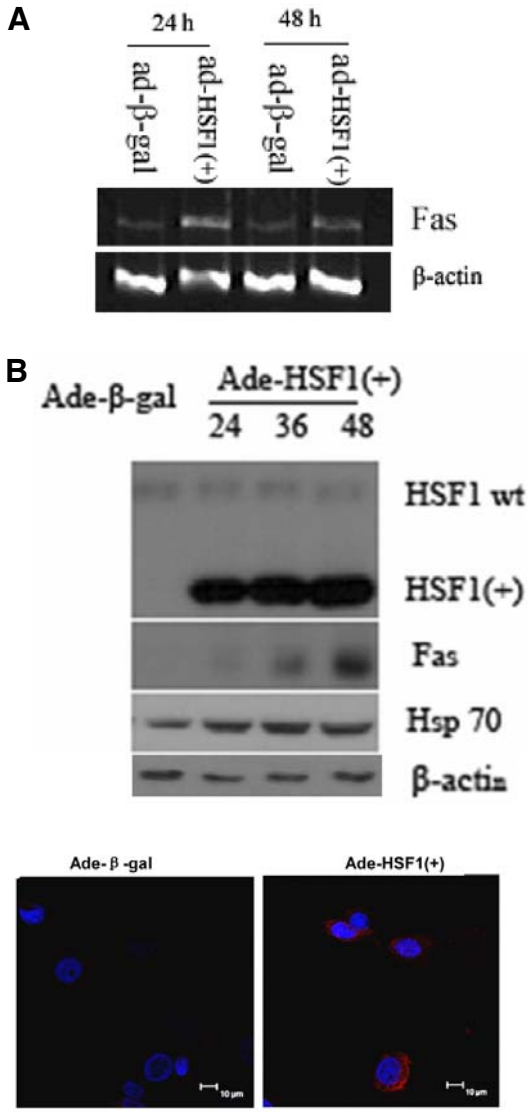

Fig. 3. Over-expression of HSF1(+) enhances Fas expression. (A) Fas mRNA was determined by RT-PCR after cells were infected with Ad-HSF1(+) for 24 or 48 h. (B) Increase of Fas protein levels after adenovirus infection was determined by Western-blot. β-actin was considerd as an loading control. After 24, 36 and 48 h infection, cells were collected and lysated for immunblotting. (C) HSF1(+) increases surface Fas protein levels (red). A549 cells were infected with adenovirus. After 36 h infection, cells were collected for indirect immunofluorescence. This experiment was repeated twice in A549 cells and the result was repeatable.

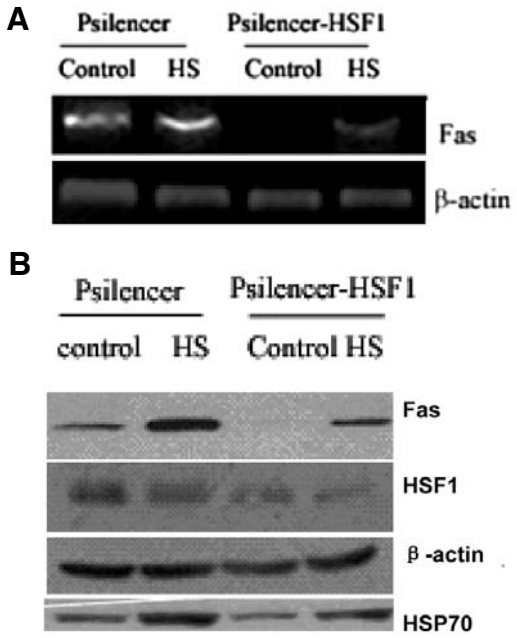

Fig. 4. HSF1 knockdown suppresses Fas gene expression. (A) After transfected with psilencer1.0-u6 or psilencer-HSF1 for 60 h (lane1: psilencer1.0-u6, lane 3: psilencer-HSF1), cells were then heat-treated and allowed to recover for 12 h at 37°C (lanes 2, 4). mRNA of all samples were extracted and subjected to detection of Fas expression through RT-PCR. (B) A549 cell was transfected with psilencer (lanes 1, 2) or psilencer-HSF1 (lanes 3, 4) for 60 h, HSF1, HSP70 and Fas expressions were detected by immunoblotting with β-actin as the internal control (lanes 1, 3). After 60 h transfection, cells were heat-treated followed by a recovery at 37°C for 24 h, and then Fas, HSF1 and HSP70 expression were detected by immunoblotting (lanes 2, 4). This experiment was repeated twice with consistent finding.

DISCUSSION

Systemic and regional hyperthermia has been considered a therapeutic tool for patients with malignant diseases and has been used as a direct treatment or an adjunct to classical radio/chemotherapy protocols since the 1970s (Hildebrandt et al., 2002; Wust et al., 2002). In this study, we demonstrated that heat stress effectively induced Fas gene expression, and we have shown that HSF1 is a transcription factor of Fas gene. Also, hyperthermia, but not hyperthermia alone (date not shown), may enhance Fas-mediated killing in A549 cells. Previous reports have demonstrated that heat stress or over-expression of HSF1 sensitized cells to apoptosis through Fas pathway (Tran et al., 2003; Xia et al., 2000). Our results provide an insightful explanation to the results of these two reports, *i.e.* the sensitization to Fas-mediated apoptosis is most probably due to the increased expression of Fas gene by HSF1. These data indicate that induced expression of the Fas antigen may be involved in triggering apoptosis in these cells.

High temperature may induce multiple actions on cell structure and physiology, the most remarkable of which are the pathways involved in activation of the stress protein response. This evolutionarily conserved phenomenon is mediated by the activation of a pivotal transcriptional factor called HSF1 in many different experimental models. As a key stress-associated transcription factor, HSF1 exerts both activating and suppressing effects on different target genes. HSF1 can specifically bind to so-called heat shock element (HSE) sequences which are arrays of sequence motifs nGAAn arranged in alternating orientation. We found that there is a segment rich in nGAAnnTTCn contigs between the -1996- and -1985-nt region of Fas promoter. EMSA demonstrated the -1996/-1985 segment has a high affinity binding activity to HSF1. ChIP assay indicated that HSF1 potentially associates with HSE located in the promoter of Fas *in vivo*.

Expression of many heat shock proteins, particularly heat shock protein 70, is primarily regulated at the level of transcription. The transcription of heat shock protein involves interactions be-

tween the transcription factor HSF1 and HSEs in the promoter regions of heat shock proteins. Consistent with this concept, the expression of Fas gene in response to heat shock also appears to be regulated at the level of transcription. This assertion is supported by the observation that Fas mRNA were induced in cells transfected with a HSF1 mutant HSF1(+), and the protein level of Fas was also increased. To elucidate the role of activated HSF1 to the Fas pathway, we over-expressed a constitutively activated form of HSF1 (HSF1(+)) in A549 cells. HSF1(+) mimics heat-induced activation of HSF1 without subjecting cells to heat stress. We detected an induction of Fas after infection of HSF1(+). To validate that HSF1 mediated up-regulation of Fas expression, we suppressed HSF1 expression by RNA interference. Fas expression was down-regulated both in unstressed cells and in heat-stressed cells by interference of HSF1. As a constitutively active HSF1 and heat stress increased Fas expression in A549 and in Hela cells (date not shown), we concluded that expression of Fas was positively regulated by HSF1. For the first time, our findings define Fas as a novel HSF1 regulated gene.

In summary, the present study has identified Fas as a novel molecular target of HSF1. It has been proposed that tumor cells expressing Fas upon induction of anticancer therapy may become susceptible targets for killer cells. Up-regulation of Fas may target the tumor cells for elimination by the immune system using a Fas-dependent pathway. Cancer patients with tumors that express Fas protein exhibited significantly longer survival times than patients with Fas-negative carcinomas. Therefore, our findings, which demonstrate that HSF1 up-regulates Fas expression and augments Fas-mediated apoptosis in cancer cells, may have important clinical implications for the prevention and treatment of cancer.

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