HSF1 Down-regulates XAF1 through Transcriptional Regulation

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Studies have indicated the role of HSF1 (heat-shock transcription factor 1) in repressing the transcription of some nonheat shock genes. XAF1 (XIAP-associated factor 1) was an inhibitor of apoptosis-interacting protein with the effect of antagonizing the cytoprotective role of XIAP. XAF1 expression was lower in gastrointestinal cancers than in normal tissues with the mechanism unclear. Here we showed that gastrointestinal cancer tissues expressed higher levels of HSF1 than matched normal tissues. The expression of XAF1 and HSF1 was negatively correlated in gastrointestinal cancer cell lines. Stress stimuli, including heat, hypo-osmolality, and H2O2, significantly suppressed the expression of XAF1, whereas the alteration of HSF1 expression negatively correlated with XAF1 expression. We cloned varying lengths of the 5′-flanking region of the XAF1 gene into luciferase reporter vectors, and we evaluated their promoter activities. A transcription silencer was found between the −592- and −1414-nucleotide region that was rich in nGAn/nTT/Cn elements (where n indicates G, A, T, or C). A high affinity and functional HSF1-binding element within the −862/−821-nucleotide region was determined by electrophoretic mobility shift assay and chromatin immunoprecipitation assay. Inactivation of this “heat-shock element” by either site-directed mutation or an HSF1 inhibitor, pifithrin-α, restored the promoter activity of the silencer structure. Moreover, pretreatment with antioxidants suppressed HSF1 binding activity and increased the transcriptional activity and expression of XAF1. These findings suggested that endogenous stress pressure in cancer cells sustained the high level expression of HSF1 and subsequently suppressed XAF1 expression, implicating the synergized effect of two anti-apoptotic protein families, HSF and inhibitors of apoptosis, in cytoprotection under stress circumstances.

Heat-shock proteins (HSPs) 3 are conserved molecules present in all prokaryotes and eukaryotes (1, 2). The expression of these proteins is very low under normal physiological conditions and can be induced by stress factors, including physiological (growth factors, oxidative stress, and hormonal stimulation), environmental (heat shock, heavy metals, and ultraviolet radiation), or pathological stimuli (inflammation and autoimmune reactions and viral, bacteriological, or parasitic infections) (3, 4). Stress factors, such as oxidative stress, have been considered as tumorigenic agents at low concentrations (5, 6). The main function of HSPs is to operate as an intracellular chaperone for aberrantly folded or mutated proteins and to provide cytoprotection against the stress conditions (31). For this reason, the presence of a cellular stress response in cancer cells reduces their sensitivity to chemical stress caused by insufficient tumor perfusion of chemotherapeutic agents (2).

Heat-shock transcription factors (HSFs or HSTFs) were originally characterized as regulators of the expression of the heat-shock protein, through binding to specific sequences (“heat-shock element” (HSE)), typically a pentanucleotide nGAn structure (where n indicates G, A, T, or C) oriented in inverted dyad repeats (7, 8). The HSF family consists of three members in humans, namely HSF1, HSF2, and HSF4. HSF1 is specifically responsible for the stress-mediated HSP induction. In unstressed cells, HSF1 is present in the cytoplasm either as a monomer or forming heteromeric complexes. Upon treatment with stress inducers, HSF1 homotrimerizes, translocates to the nucleus, and binds the HSE for its transactivation capacity (9, 10). Recent studies have shown that HSF1 can also act as a negative regulator of certain nonheat-shock genes, including IL-1β, c-fos, and TNF-α (11–13).

Inhibitors of apoptosis (IAPs) constitute another family of anti-apoptotic proteins. They were identified in baculoviruses where they function to prevent the death of infected host cells (14). XIAP is a potent member of IAPs that is expressed in all adult and fetal tissues with the exception of peripheral blood leukocytes. XIAP binds directly to caspases and functions as a competitive inhibitor of caspase catalytic function (15).

Yeast two-hybrid studies identified a XIAP-interacting N-terminal zinc finger protein designated XAF1 (XIAP-associated factor-1) (16). The incubation of recombinant XIAP with caspase-3 in the absence or presence of XAF1 demonstrated that XAF1 blocked the inhibitory activity of XIAP for caspase-3, and co-expression of XAF1 and XIAP inhibited XIAP-dependent caspase-3 suppression (17). XAF1 has been implicated as a tumor suppressor because its expression was lower in tumor cells than in normal tissues, and transient expression of XAF1 sensitized tumor cells to the pro-apoptotic effects of etoposide as well as tumor necrosis factor-related apoptosis-inducing ligand (17, 18). In gastrointestinal (GI) cancers, Byun et al. (23) reported gastric cancer tissues expressed lower levels of XAF1 than normal tissues. However, few studies have focused on the regulation of XAF1. In this study, we described the presence of a high affinity HSF1-binding sequence within the 5′-flanking region of the XAF1 gene. GI cancer cells expressed high levels of HSF1, which enhanced cell survival under stress stimulation, by negatively regulating XAF1 expression.
**Experimental Procedures**

**Primers, Oligonucleotides, and Probes—** All oligonucleotides were synthesized by Prologio, Singapore. Table 1 shows the sequences of each oligonucleotide used for reverse transcription-PCR, 5′-rapid amplification of 5′-cDNA ends (5′-RACE), electrophoretic mobility shift assays (EMSA), luciferase construction, site-directed mutagenesis, and chromatin immunoprecipitation (ChIP).

**Reagents—** Catalase, N-acetyl-cysteine (NAc), and pituitrin-α (PFA) were purchased from Sigma. Goat anti-human XAF1 (C-16), goat anti-human actin (I-19), normal goat IgG, goat anti-human HSF1 (C-19), and horseradish peroxidase-conjugated anti-goat IgG were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Tissue Specimens and Human Cell Lines—** Three gastric cancer and nine colon cancer specimens and their adjacent normal tissues were obtained from patients by surgical resection in the Nanfang Hospital (Guangzhou, China). All colon tissues were from sporadic colon cancer patients. Tissue specimens were snap-frozen in liquid N2 and stored at −70°C until used. Tissue slices were subjected to histopathological review, and tumor specimens consisting of at least 80% carcinoma cells were chosen for molecular analysis. Gastric cancer cell lines AGS and Kato-III and colon cancer cell lines SW1116, HT-29, Lovo, and Colo205 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Gastric cancer cell lines MKN45 and BCG823 were maintained by our laboratory and were described in a previous study (21). They were all maintained in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified incubator at 37°C with an atmosphere of 5% CO2.

For stress treatment, the cells were incubated in complete medium at 42°C (heat stress (HS)) or in hypo-osmotic (HO) medium for 30 min, followed by culture in normal medium at 37°C for 24 h. The hypo-osmotic medium contained 67% complete medium and 33% sterile double distilled water with the osmolarity of about 209 mosmol/kg. For oxidative stress, the cells were exposed to 200 μM of H2O2 for various time points.

**Transient Transfection—** For transient transfection, 4 μg of the pcDNA3.1 construct encoding HSF1 (pcDNA3.1/HSF1, kindly provided by Dr. R. E. Kingston) was mixed with 250 μl of serum and antibiotics-free medium containing 10 μl of LipofectAMINE2000 reagent for 20 min at room temperature. The mixtures were overlaid onto monolayers of cells seeded in a 6-well tissue culture plate preincubated under serum-free conditions. After 4 h of incubation at 37°C, the DNA-liposome complex was replaced with complete medium without antibiotics and cultivated at 37°C. Whole cell lysates were prepared 48 h later to evaluate the protein expression.

**Immunoblotting—** The whole cell lysates were prepared with lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). To prepare protein sample for tissue specimens, homogenization was performed in protein lysis buffer. The protein concentration was determined by the bicinchoninic acid assay (BCA protein assay kit, Pierce) with bovine serum albumin (Sigma) as the standard. Equal aliquots of total cell lysates (30 μg) were solubilized in sample buffer and electrophoresed on denaturing SDS-polyacrylamide gel (5% stacking gel and 12% separating gel). The proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Nonspecific binding was blocked with 10 mM Tris-HCl buffer saline, pH 7.6, plus 0.05% Tween 20 containing 2% skimmed milk. The blots were probed with primary anti-human XAF1 antibody for 1 h at room temperature followed by the horseradish peroxidase-conjugated anti-goat secondary antibody. Goat anti-human actin antibody (1:1000) was used as an internal control. Antigen-antibody complexes were visualized by the ECL system (Amersham Biosciences).

**5′-RACE—** To extend the cDNA transcript, 5′-extension PCRs were performed by using SMART RACE cDNA amplification kit (Clontech) with Human Colon 5′-STRETCH PLUS cDNA Library (Clontech) as the template, as described previously (19). Briefly, the first round of touchdown PCR was performed using HotStart Taq polymerase (Qia-gen, Hilden, Germany) with AP1 (adapter primer 1) provided by the kit and the XAF1 GSP1 (gene-specific reverse primer 1). The PCR product was separated in a 1% gel. Because no intensified PCR product was found under UV light, a pair of nested primers was used to re-amplify the PCR product by using AP2 (adapter primer 2) provided by the kit and XAF1 GSP2. Both GSP1 and GSP2 were located at exon 2 of XAF1 gene. The sequences of primers are listed in Table 1. The conditions of touchdown PCR were as follows: 94°C for 30 min; 5 cycles at 94°C for 30 s and 72°C for 4 min; 5 cycles at 94°C for 30 s and 70°C for 4 min; and 25 cycles at 94°C for 30 s and 68°C for 4 min. The PCR product was separated in a 1% gel. DNA was isolated using a GFX PCR DNA and gel band purification kit (Amersham Biosciences) and cloned into a pGEMT-T cloning vector (Promega, Madison, WI). Plasmid DNAs were purified using a commercial kit (Promega) and sequenced using the ABI PRISM 377 DNA Sequencer (Applied Biosystems), according to the manufacturer’s instructions.

**Generation of XAF1-Promoter Luciferase Constructs—** Genomic DNA was isolated from cancer cells by proteinase K digestion and sequential phenol extraction. To locate the regulatory promoter of XAF1, five DNA segments that shared the same proximal site and different distal sites were obtained by PCR amplification. The distal sites were located at −1414, −920, −592, −254, and −107 nt, respectively, and the proximal primers were located at −42 to −20 bp of the XAF1 gene. The upstream nucleotide adjacent to the translation starting ATG codon is defined here as −1 (20). KpnI site was added into the 5′ terminus of all of the forward primers, and the XhoI site was added into the reverse primer. The primers used were listed in the Table 1. Genomic DNA of AGS cell was used as the template for PCR amplification with HotStart Taq polymerase. PCR products were visualized on 1% agarose gels by ethidium bromide staining and were purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences). After digestion of both the pGL3 basic vector (Promega) and the PCR products with KpnI and XhoI, the purified products were inserted in the forward orientation upstream of a luciferase reporter gene of pGL3 basic vector to generate pLuc-1414, pLuc-920, pLuc-592, pLuc-254, and pLuc-107 constructs.

**XAF1 Promoter-Luciferase Reporter Expression—** For luciferase assay, the cells were seeded into 24-well plates to 70–80% confluence and transfected with the various pLuc constructs by Lipofectamine 2000 as described previously (21). pRL-CMV (Promega) was used to normalize the reporter gene activity. 0.8 μg of pLuc plasmids and 0.008 μg of pRL-CMV vector were mixed with 50 μl of serum and antibiotics-free medium containing 4 μl of LipofectAMINE2000 reagent for 20 min at room temperature. The mixtures were overlaid onto monolayers of the various cell lines preincubated under serum-free conditions. After 4 h of incubation at 37°C, the DNA-liposome complex was replaced with complete medium without antibiotics and cultivated for an additional 48 h at 37°C. Cells were solubilized in 1× passive lysis buffer (Promega), scraped with a rubber policeman, and mixed with 50 μl of luciferase assay reagent (Promega). The firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega) with a model TD-20/20 luminometer (EG & G Berthold, Madison, WI).
Results—Gastric and Colon Cancer Expressed Higher Levels of HSF1 Than Normal Tissues—We detected HSF1 expression in three gastric cancer (Fig. 1A) and nine colon cancer (Fig. 1B) specimens and matched normal tissues by immunoblotting assay. All of the gastric cancer tissues and 7 of 9 colon cancer tissues expressed higher level of HSF1 than normal tissues. To evaluate the activity of HSF1, double-strand DNA probe consensus to the HSE sequence of human HSP70 promoter (HSE/consensus oligonucleotide was labeled with $^{32}$P; EMSA was carried out to detect its binding to the whole cell lysate of tissue specimens. This figure is representative of two independent experiments.

Statistical Analysis—Results obtained from triplicate luciferase experiments were expressed as the mean ± S.D. RLU with different treatments were compared using a two-tailed Student’s t test and were considered significant if the p value was less than 0.05.
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Because the HSF1 expression and activity reflected cellular stress status, these results inferred that cancer cells have encountered higher stress pressure than normal cells.

XAF1 Expression Inversely Correlated with HSF1 in GI Cancer Cells—To elucidate the correlation between XAF1 and HSF1 in cancer cells, we first checked their expression in GI cancer cell lines by immunoblotting. As shown in Fig. 2A, negative correlation was found between these two proteins in 6 of 7 cell lines except gastric cancer cell line AGS. Second, to confirm the down-regulation of XAF1 by HSF1, we next transfected AGS and Lovo cells with pcDNA3.1-HSF1 expressing vector and detected XAF1 expression. We showed that overexpression of HSF1 down-regulated XAF1 expression in both cell lines (Fig. 2B). Third, we suppressed HSF1 expression by RNA interference (Fig. 2C). These findings indicated that the low level expression of HSF1 in cancer cells might be attributed to the high expression of HSF1 and stress pressure.

To test the effect of HSF1 activator (stress stimuli) on XAF1 expression, we then treated gastric cancer cell Kato-III, which constitutively expresses XAF1, with oxidation (200 μM of H2O2), HO (for 30 min), or HS (42 °C for 30 min). We showed that stress stimuli up-regulated XAF1 expression (Fig. 2D).

Location of the Regulatory Promoter of XAF1 Gene—To investigate the putative role of HSF1 in down-regulation of XAF1 expression through transcriptional regulation, the transcription starting site (TSS) of XAF1 gene was determined by 5'-RACE assay. No visible band was found after the first round of touchdown amplification using AP1 and GL2 primers, whereas a 96-bp fragment was obtained from nested PCR using AP2/GSP2 primer pair. After cloning the 96-bp PCR product into pGEM-T, 12 clones were sequenced using the vector primers, SP6 and T7 promoter sequences. Only three clones contained the adapter sequence but no adapter sequence, and for these clones mapped the 5'-end of the mRNA to an adenine 26 nucleotides upstream of the ATG initiator codon. A schematic diagram of the 5'-untranslated region of XAF1 gene was shown in Fig. 3A. No typical TATA box was found within this region. Other clones contained the XAF1 sequence but no adapter sequence, and for that reason we did not believe that these contained the true 5' ends. The phenomenon was most likely the result of incomplete reverse transcription. Based on the above findings, we believed that XAF1 has a single TSS located 26 bp upstream of the ATG initiator codon.

To determine the regulatory promoter region of the XAF1 gene, truncated 5'-flanking sequences extending up to −1414 nt of the XAF1 gene (Table 1 and Fig. 3B) were inserted in forward orientation upstream of a luciferase reporter gene (pGL3 vector) to generate pLuc constructs. The fold of RLU induced was evaluated after transient transfection into AGS/SW1116 cells. As shown in Fig. 3C, the RLU induction of pLuc−1414, pLuc−920, pLuc−592, pLuc−254, and pLuc−107 were 8.3 ± 1.2, 2.9 ± 1.4, 2.3 ± 0.4, 29.2 ± 2.9, 10.2 ± 0.9, and 16.2 ± 1.2, respectively. The highest RLU was observed for pLuc−592, indicating the presence of cis-enhancing element(s) between −26 to −592 nt. However, transcription of the longer XAF1 5'-flanking sequences, pLuc−920, resulted in a significant decrease in transcription activity, thus implicating the presence of a potential repressor element(s) between −592 and −1414 nt. Both cell lines have a similar pattern of transcription activities with different values for individual constructs.

Identification of HSF1-binding Sequence in XAF1 Promoter—The high affinity binding sequence for HSF1 comprises a minimum of two nGAAAn/TTTCa elements arranged as an inverted dyad repeat (Fig. 4A).
TABLE 1
List of the oligonucleotide primers for amplification and mutation.

| Experiment               | Name       | Position or orientation | Sequence (5′–3′)                  |
|--------------------------|------------|-------------------------|-----------------------------------|
| Luciase construction     | Reverse    | −42 to −20              | CCCGCTCGAGTTTCGTTGAGTTCTTCTTCG    |
|                          | Forward 107 | −90 to −107             | GGGGTTACCGATTTTCCCTCTTCCCTGAA    |
|                          | Forward 254 | −235 to −254            | GGPG TTACCCAGCGTGGAGGAAGTATG    |
|                          | Forward 592 | −592 to −60             | GGPGTTACCGGTTCTGAGAAAACCTAAGGAC |
|                          | Forward 920 | −920 to −896            | GGPGTTACCGTTAATAGGGATAAAAAGCGA |
|                          | Forward 1414| −1414 to 1391           | GGPGTTACGGTTATAGGAGAGGGTCTCC    |
| Site-directed mutagenesis| Wild type  | Sense                   | AAACATAGGAAACCAATGTGGAAACAGCTTTTTTCTTCCT   |
| Mutant 1                 | Sense       | 862/821                 | AACATAGGAAACCAATGTGGAAACAGCTTTTTTCTTCCT   |
| Mutant 2                 | Sense       | −1008/−982              | AAACATAGGAAACCAATGTGGAAACAGCTTTTTTCTTCCT   |
| EMSA                     | Sense       | −862/−821               | AAACATAGGAAACCAATGTGGAAACAGCTTTTTTCTTCCT   |
| RACE                     | Forward     | 821                     | CCATCTCTAACTAGCCTACTACAAAGGCGG |
|                          | Reverse     | 892                     | ACTCTAGTACCAAGGTCGACAGCGG |
|                          | Reverse     | 862                     | GACCTCGGAAACAGGACAGGAGAAAC |
|                          | Reverse     | 982                     | CATGGAAGGTGAAATGTCGAAAGACAGACT |
| Reverse transcription-PCR | XAF1       | Forward                 | GCTCCAGAGTCCTGACTCG              |
|                          | Reverse     | 821                     | ACTCTAGTACCAAGGTCGACAGCGG |
|                          | Reverse     | 862                     | GACCTCGGAAACAGGACAGGAGAAAC |
|                          | Reverse     | 982                     | CATGGAAGGTGAAATGTCGAAAGACAGACT |
|                          | Reverse     | 862                     | GACCTCGGAAACAGGACAGGAGAAAC |
|                          | Reverse     | 982                     | CATGGAAGGTGAAATGTCGAAAGACAGACT |
| ChiP                     | Forward     | −1021/−779              | TCTCTGGCTCCATTCTTCTTT |
|                          | Reverse     | 862/821                 | GAGAAAGCTGTGTGTTGCTT |

AGS cells exposed to 42 °C for 30 min as a source of HSF1. It was found that −862/−821 but not −1008/−982 oligonucleotide blocked HSF1-specific binding. Competition for binding by −862/−821 oligonucleotide was as complete as that of a comparable concentration of HSE/consensus itself (Fig. 5A). Second, to confirm that HSF1 bound with high affinity to the −862/−821 sequence, we repeated the EMSA analysis using each −1008/−982 and −862/−821 oligonucleotide as a radiolabeled probe (Fig. 5B). Of the XAF1 sequences studied, typical doublet bands were only observed with −862/−821 oligonucleotide and completely blocked by the excessive unlabeled cold probe, indicating the specificity of binding reaction. Third, to define further the role of the −862/−821 sequence in stress response, nuclear extracts of AGS cells with or without stress stimulations were extracted and bound to the radiolabeled −862/−821 oligonucleotide. As shown in Fig. 5C, HS, HO, and oxidative stress increased the binding capacity of the −862/−821 probe, and specific bands were completely blocked by unlabeled oligonucleotides. Therefore, we concluded that a high affinity HSE existed in the −862/−821 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 associates with the chomatin immunoprecipitated by these antibodies was then amplified by PCR with primers specific for the putative HSE region of the XAF1 promoter. As expected, no DNA fragments were detected when normal IgG was used (Fig. 5D, lanes 1, 4, 7, and 10). In contrast, DNA fragments with the expected size were detected using anti-HSF1 antibody in AGS cells (Fig. 5D, lane 2). In addition, we showed that HS, HO, and oxidative stress increased the amount of immunoprecipitated DNA (Fig. 5D, lanes 2, 5, 8, and 11). These findings suggested that the −862/−821 sequence of XAF1 gene contained a high affinity HSE (XAF1) for HSF1.

Up-regulation of XAF1 Expression by Inactivation of HSF1 Binding— To clarify the function of the −862/−821 sequence in the repression of XAF1 transcription, we abrogated this binding activity of HSE/XAF1 by introducing the GAA to CCC and TTC to GGG mutation into pLuc–920. As the typical HSF1-binding element consisted of inverted dyad repeats of the nGAAn/nTTCn motif, two mutant constructs were generated with type 1 mutating the outer pair of GAA/TTC elements and type 2 mutating the inner pair of GAA/TTC elements (Fig. 6A). After verifying the prospective mutation by DNA sequencing, the mutant...
pGL3 constructs were transiently transfected into AGS and SW1116 cells. Transcription activity was evaluated and compared with the wild type construct. As indicated in Fig. 6B, RLU induction of wild type, type 1, and type 2 mutant pLuc-920 constructs were 3.04 ± 0.52, 2.74 ± 0.36, and 13.54 ± 0.36 in AGS cell and 3.15 ± 0.22, 3.08 ± 0.48, and 16.44 ± 0.33 in SW1116 cells. Type 2 but not the type 1 mutation increased the transcription activity of pLuc-920 significantly (p < 0.05 compared with the wild type control). To define further the binding capacity of the inner GAA/TTC sequence, EMSA was carried out to examine the specific binding of wild type and type 2 mutant oligonucleotides (Fig. 6C). Wild type but not the type 2 mutant probe bound to the nuclear extract of AGS cells effectively. These findings indicated that the inner GAA/TTC sequences in −862/−821 region contributed to HSF1 binding and repression of XAF1 transcription.

Moreover, PFA, a novel defined inhibitor of HSF1 (24), was applied to examine its effect on the transcription activity of truncated XAF1 promoter constructs. SW1116 cells were transiently transfected with pLuc-592, pLuc-920, and pLuc-1414 followed by incubation with 15 μM of PFA. Cells were lysed and assayed for luciferase activity. As shown in Fig. 6D, treatment with PFA did not or only slightly changed the transcription activity of pLuc-592 constructs. However, it increased the transcription activity of pLuc-1414 and pLuc-920 significantly (p < 0.05). This finding proved the role of HSF1 binding in repression of XAF1 transcription.
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Anti-oxidants Overcome the Suppression of XAF1 Expression—ROS has been implicated as an etiologic factor in numerous diseases, including cancer. ROS can originate exogenously from agents that generate oxygen free radicals and originate endogenously, for example, as a result of normal cellular metabolism, such as mitochondrial oxidative phosphorylation (25–27). It has been reported that H$_2$O$_2$ could stimulate binding of HSF1 to the HSE (28–30). To verify the role of HSF1 in suppressing XAF1 expression, AGS and SW1116 cells were exposed to 200 μM H$_2$O$_2$ in the presence or absence of antioxidants, NAc (20 mM) or catalase (CAT) (1000 units/ml), for 12 h. Nuclear extracts were incubated with radiolabeled –862/–821 probe. This figure was the representative of two independent experiments.

DISCUSSION

Bcl-2, HSP, and IAPs are three anti-apoptotic family proteins. The cross-talk between HSP and IAPs was confirmed by the finding that HSP90 positively modulated the expression and function of survivin in cancer cells via binding to the conserved baculovirus IAP repeat structure (32). Our present study provides new insight into the interaction between HSF and IAPs that HSF1 down-regulated IAP-interacting protein, XAF1, through transcription regulation.

It was well known that cancer cells have encountered higher level of stress pressure, both exogenous and endogenous (25, 26). Many cancers like sporadic colon cancers might have originated from inflammation such as inflammatory bowel diseases in which higher oxidative metabolites were produced by both infiltrated neutrophils and colon epithelial cells (33). The subsequent induction of stress-associated proteins, including HSPs and mitogen-activated protein kinases, will promote cell transformation (34, 35). As the pivotal transcription factor that stimulates HSP proteins, the expression profile of HSF1 in GI cancers has seldom been studied. By using matched normal and cancerous gastric and colon tissues, we showed cancer tissues expressed higher level of HSF1 than normal tissues. This result was consistency with the findings of Cen et al. (36) that HSF1 expression was increased in 86% (30/35) of sporadic colon cancer patients demonstrated by cDNA microarray assays. EMSA showed cancer tissues possessed stronger HSF1 binding activity than their normal counterparts. These data indicate that GI cancer cells had a higher level and active form of HSF1 protein than normal tissues.
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Inhibitor of apoptosis proteins, which is partly attributable to adaptive stress response, is commonly found in cancer cells (1–3). XAF1, a newly identified antagonist of XIAP, has been identified as a tumor suppressor. Expression of XAF1 in cancer cells, including gastric cancers, was lower than that of normal tissues (16, 22). Our findings that HSF1 as well as oxidative, hypo-osmotic, and heat stress down-regulated XAF1 expression in GI cancer cell lines suggested that XAF1 was a stress-associated gene with its expression being negatively regulated during stress response.

Stress-activated survival response included the induction of anti-apoptotic proteins. Yet, if the exposure to a specific stress is excessive, cell death will occur, either by necrosis or apoptosis (3, 37–38). It is reported that when cells are exposed to low H2O2 concentration, they develop resistance to subsequent challenges with high concentrations of the same agent that would otherwise be lethal (38). In this study, the concentration of H2O2, the temperature of HS, and the osmolarity used were all within the tolerable or physiological range (39–41). Their effect on repression of XAF1 and/or induction of other IAP proteins such as survivin reflects a novel survival mechanism of cancer cells.

As a key stress-associated transcription factor, HSF1 exerts both an activating and suppressing effect on different target genes. Although the consensus HSF1-binding DNA sequence within the promoter of HSPs is the conserved inverted dyad repeat of pentanucleotide nGAn (HSE) (22), the capacities of the cis-formed dyad repeats of nGAn or nGAn/nTTCn binding to HSF1 have been confirmed in all genes repressed by HSF1. These genes include IL-1β, c-fos, TNF-α, and RANK ligand (11–13). The HSE in RANK ligand is located in the −1275- to −2-kb region.

To search for the transcription regulatory element, we first determined the transcription initiation site of the XAF1 gene by 5′-RACE. It is located in the −266 nt adenosine upstream of the ATG initiator. By searching the DBTSS data base (dbtss.hgc.jp/index.html) where most genes possessed multiple TSS because of the different assay other than RACE utilized, we found the XAF1 transcription starting region was located at −4 to −40 nt. Thus, we cloned the 5′-flanking sequence containing part of 5′-untranslated region sequence and defined them as the regulatory promoter of XAF1. A putative repressor or silencer sequence was eventually located between −592 and −920 nt by dual luciferase reporter assay. Two segments rich in nGAn/nTTCn contigs were located at the proposed transcription silencer region. On the other hand, this region contained two pairs of inverted dyad repeats of nGAn/nTTCn motifs. Further site mutagenesis strategy implicated the inner pair of the nGAn/nTTCn motif as the functional HSF1-binding element (HSE/XAF1) that is responsible for repression of XAF1 transcription.

To validate further the HSF1-binding mediated suppression of XAF1 transcription, we used an HSF1 inhibitor (24), pifithrin-α, to inactivate cytosolic HSF1 protein. Pifithrin-α can also suppress p53 activity; however, no typical p53-binding element is determined within the 5′-flanking region of XAF1. We showed that pretreatment with this inhibitor eliminated the effect of the transcription repressor within the XAF1 regulatory promoter in unstressed cancer cells, suggesting endogenous intracellular stress pressure maintained the transcriptional inhibition of the XAF1 gene in cancer cells.

ROS was the predominant endogenous stressor of cancer cells. Bittinger et al. (42) reported that melanoma cells produced large amounts of superoxide anions without stimuli, as implicated in metastasis by promoting endothelial injury. ROS also plays a central role in the modulation of HSF1 activation because it was not only one of the stressors that could activate HSF1 but could also be increased by many other cellular stresses that lead to HSF activation (28, 30, 43). Therefore, we evaluated the effects of antioxidants on XAF1 regulation. We found both N-acetyl-L-cysteine and catalase were able to suppress HSE/XAF1 binding activity, to abrogate transcription inhibition, and to induce XAF1 expression. Because XAF1 was a pro-apoptotic gene and its overexpression suppressed cell growth (data not shown), our findings were consistent with previous observations that a moderate level of intracellular ROS was important to maintain the appropriate redox balance and to stimulate cancer cell proliferation (44, 45).

In summary, GI cancer cells expressed high levels of HSF1. It mediated stress stimuli-induced down-regulation of XAF1 via interaction with an HSE within the 5′-flanking region. This mechanism may contribute to the low expression of XAF1 in cancer cells and prevent them from apoptosis. For the first time, our findings define XAF1 as a novel stress-associated gene that negatively modulates cancer cell growth.

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