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Concerted Actions of Multiple Transcription Elements Confer Differential Transactivation of HSP90 Isoforms in Geldanamycin-Treated 9L Rat Gliosarcoma Cells

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Abstract HSP90 chaperones are transducer proteins of many signaling pathways in cells. Using a highly specific inhibitor, geldanamycin (GA), an increasing number of the HSP90 client proteins have been identified. Nevertheless, there is little information on the differential transactivation of the two isoforms of the *hsp90* genes, *hsp90α* and *β*, in cells under stress conditions. Here, we demonstrate the differential expression of the HSP90 isoforms, HSP90α and β, in rat gliosarcoma 9L cells using a modified SDS–PAGE system that allowed us to distinguish the isoforms. We subsequently assessed the transcriptional controls involving the transcription elements located in the promoter regions of the *hsp90* genes. At the protein level, HSP90α is more responsive to GA in terms of rate of de novo synthesis and amount of accumulation, as shown by metabolic-labeling and Western-blotting analyses. Upregulation of the *hsp90* genes was demonstrated by real-time qPCR. The promoter elements *hsp90α*-HSE2 and *hsp90β*-HSE1 were also identified to be the major transcription elements involved in GA-activated gene expression, as shown by EMSA, whereas the results of supershift showed that the transcription factor HSF1 is also involved. Moreover, EMSA results of analysis of the GC box showed differences in both the initial amounts and inductive response of *hsp90*s transcripts, whereas analysis of the TATA box showed GA responsiveness in *hsp90α* only. Collectively, these results indicate that GA exerts its regulatory effects through transcription elements including heat-shock elements (HSEs), GC boxes and TATA boxes, resulting in differential transactivation of *hsp90α* and *hsp90β* in rat gliosarcoma 9L cells. J. Cell. Biochem. 104: 1286–1296, 2008.
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Key words: geldanamycin; heat shock protein 90; heat shock element; heat shock factor; transactivation

Heat-shock proteins (HSPs) are molecular chaperones responsible for maintaining cell homeostasis and promoting cell survival by facilitating protein folding [Takayama et al., 2003]. The subfamilies of HSPs are generally named after their apparent molecular weights; among them, the HSP90s and HSP70s are the most extensively studied [for reviews, see Csermely et al., 1998; Nollen and Morimoto, 2002; Neckers and Ivy, 2003; Takayama et al., 2003].

HSP90s are the most abundant chaperone proteins in cells. They account for 1–2% of total proteins in unstressed cells and up to 4–6%

Abbreviations used: GA, geldanamycin; HSP90, heat shock protein 90; EMSA, electrophoresis mobility shift assays; HSF, heat-shock factor; HSE, heat-shock element; real-time qPCR, real-time quantitative reverse-transcriptase polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction.

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in cells under stress conditions [Goetz et al., 2003]. In mammals, two isoforms, HSP90α and HSP90β (HSP86 and HSP84 for mouse homologs) have been identified and found to be highly conserved among different species. The results of sequence comparisons and phylogenic studies indicate that the *hsp90α* gene was derived from duplication of the *hsp90β* gene about 500 million years ago [Gupta, 1995]. It has also been proposed that the α and β isoforms of mammalian HSP90 may have different chaperone activities [Goetz et al., 2003]. Moreover, expression of HSP90 isoforms seems to be differentially regulated [Hansen et al., 1991; Chang et al., 2006b]. This was first demonstrated by subjecting human peripheral blood mononuclear cells to heat-shock conditions and mitogen treatment [Hansen et al., 1991]. Although both isoforms are constitutively expressed under normal condition, HSP90α expression is induced by a great variety of stimuli, whereas HSP90*β* expression is generally referred to as the constitutive isoform and is induced to a lesser significance by a few stimuli [Csermely et al., 1998].

The differential function or expression of HSP90 isoforms might be regulated by sequence differences. Sequence analysis has revealed that mammalian paralogs *hsp90α* and *hsp90β* less similarity in the amino acid sequences of their protein products (68% vs. 86% in rats) than in the 5′- and 3′-untranslated regions, the introns, and the regulatory 5′-flanking nucleotide sequences [Kumar et al., 2001]. The promoter regions of the *hsp90* genes in humans and mice contain many consensus heat-shock elements (HSEs) and other potential transcription/regulatory elements such as stimulatory protein-1 binding site (SP-1, GC box), activating protein-1 binding site (AP-1), CCAAT/enhancer-binding protein element, and cyclic AMP response elements [Hickey et al., 1989; Rebbe et al., 1989; Dale et al., 1996, 1997].

It is well documented that disruption of the HSP90 chaperone machinery by HSP inhibitors such as geldanamycin (GA), radicicol, or their analogs, leads to rapid degradation of the client proteins. Thus far, more than 100 client proteins of HSP90 have been identified and most are essential signaling molecules involved in cell proliferation, differentiation, and survival, and include hormone receptors, protein kinases, and transcription factors [Pratt and Toft, 2003]. GA is a benzoquinone antibiotic that binds specifically to HSP90s [An et al., 1997]. GA exerts its inhibitory activity by binding to the regulatory pocket in the N-terminal domain of HSP90 [Neckers et al., 1999]. Binding of GA to HSP90s blocks the assembly of HSP90–client protein complexes and destabilizes the preformed complexes, leading to the rapid degradation of the client proteins via a ubiquitin-dependent proteasomal mechanism [Mimnaugh et al., 2004]. However, little is known about how GA also modulates *hsp90* genes at the transcriptional level.

Basal and inducible HSP expression are at least partially regulated by the heat-shock transcription factors (HSFs) [Sorger, 1991]. In response to various inducers, most HSFs undergo trimerization, become phosphorylated, and acquire DNA-binding activity to HSE, leading to transactivation of the heat-shock genes [Morimoto, 1993; Wu, 1995; Pirkkala et al., 2001]. Three isoforms of HSFs have been found in mammalian cells: HSF1, HSF2, and HSF4. It has been proposed that HSF1 is activated by various forms of stress, HSF2 is activated under developmental conditions, whereas expression of HSF4 is tissue-specifically regulated [Sistonen et al., 1992; Mezger et al., 1994; Pirkkala et al., 2001]. Interestingly, HSF1 is also a client protein of the HSP90 chaperones. This may explain the observation that treating cells with GA also leads to transactivation of the heat-shock genes including the *hsp90* genes [Bagatell et al., 2000; Katschinski et al., 2002; Shu et al., 2005]. Therefore, GA acts both as a specific antagonist for HSP90-protein function and as an inducer for the *hsp90* gene. To systematically assess the mechanisms by which GA induces HSPs, we have recently shown that the upstream signaling pathway(s) for GA-induced expression of stress genes involves reactive oxygen species as well as intracellular movement of calcium [Lai et al., 2003; Shu et al., 2005]. We found that disruption of intracellular calcium transport and protein kinase C activity suppresses the GA-induced expression of *hsp70* in a HSE-dependent manner [Shu et al., 2005]. Furthermore, reactive oxygen species and related signaling pathways are required for GA to affect the transactivation of *grp78* via the endoplasmic reticulum (ER) stress-specific elements ERSE1 and ERSE2 [Lai et al., 2003]. In this study, we aimed to determine the role of various transcription elements in the GA-induced transactivation of *hsp90* genes.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

Rat gliosarcoma 9L cells (9L cells) [Weizsaecker et al., 1981] were maintained in minimum essential medium Eagle with Earle's salts (MEM) (Sigma–Aldrich, Dublin, Ireland) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone, Logan, UT) in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were maintained in a logarithmic growth phase by routine passage every 2–3 days. Prior to each experiment, stock cells were plated in 25-cm^2 flasks or 6-well plates at a density of $4–6 \times 10^4$ cells/cm^2. Exponentially growing cells at 80–90% confluency were used for all experiments. Unless otherwise specified, the cells were treated with 0.5 μM GA for 6 h for protein analysis or 4 h for the preparation of mRNA and nuclear extracts.

Metabolic Labeling and SDS–PAGE

De novo protein synthesis was revealed by [^{35}S]-methionine labeling at a concentration of 20 μCi/ml. After treatment, the cells were labeled for 1 h, washed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and lysed in 2× sample buffer (0.1 M Tris–HCl, 20% glycerol, 288 mM β-mercaptoethanol, 2% SDS) [Laemmli, 1970]. Equal amounts of cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 9% gel at pH 8.0. After electrophoresis, the gels were fixed onto Whatmann 3-mm filter paper, dried and processed for autoradiography as described [Zhang et al., 2004]. Protein bands of interest were quantified by densitometric scanning (Molecular Dynamics Inc., Sunnyvale, CA). The relative synthesis rates of HSP90α and β were presented as the sum of pixel values of each band divided by that of actin in the same lane (internal control).

Western Blot Analyses

Whole cell extracts were resolved by SDS–PAGE and then transferred to nitrocellulose membrane in TBE buffer (90 mM Tris, 90 mM boric acid, and 1 mM EDTA, pH 8) by using a semidry transfer apparatus according to the manufacturer's protocols (OWL Scientific, Woburn, MA). After blocking with 5% nonfat milk in TTBS (0.5% Tween 20, 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl) for 90 min and washing once with TTBS, the membranes were incubated with a 1:1,000 dilution of respective antibodies against HSP90α and β (Lab Vision Co., Fremont, CA), and actin (Santa Cruz Biotech, Santa Cruz, CA) at 4°C for 8 h. Subsequently, the membranes were washed three times with TTBS, for 10 min each time, and incubated with a 1:5,000 dilution of anti-mouse or anti-goat secondary antibodies for 4 h. After washing three times for 5 min each time with TTBS, the immune complexes were detected by the ECL system (Perkin Elmer Life Sciences, Inc., Boston, MA) and quantified by densitometric scanning.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 9L cells according to methods of [Zhang et al., 2004]. The steady levels of *hsp90α* and *β* mRNAs were determined by using reverse transcription followed by PCR. Reverse transcription was carried out on 1 μg RNA of total RNA with M-MLV reverse transcriptase (Promega Co., Madison, WI). Three respective pairs of primers specific for *hsp90α*, *hsp90β* and *actin* were designed and synthesized according to their sequences: *hsp90α*: 5′-CAA GCC TGA AAT AGA AGA TG-3′ and 5′-GAA TGA AGA ATA CGG AGA G-3′; *hsp90β*: 5′-GAG GCA GAG GAA GAG AAA GG-3′ and 5′-CTG AAT AAG ACG AAG CCC AT-3′; *actin*: 5′-AGG CCA ACC GTG AAA AGA TG-3′ and 5′-CAG TGG TAC GAC CAG AGG CAT A-3′ (ScinoPharm, Taiwan Ltd., Taipei, Taiwan) as internal controls. Twenty-five cycles (94°C for 30 s, 58°C for 30 s, and 72°C for 30 s) were chosen for all PCR reactions with *Vio-Tag* DNA polymerase (Promega Co.). After amplification, the same volume of each PCR product was loaded onto 1.5% agarose gels prepared with TBE buffer. The gel was photographed following staining with ethidium bromide.

RNA Isolation and Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction (Real-Time qPCR)

Total cellular RNA was extracted from approximately 4×10^5 cells using Trizol reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Reverse transcription was

performed on each RNA sample (0.5 μg) using oligo-dT primer and M-MLV reverse transcriptase (Promega Co.). The mRNA levels of rat *hsp90α* and *hsp90β* mRNA were measured by SYBR GREEN PCR Master Mix and quantified using an ABI PRISM 7500 sequence detector system (Applied Biosystems, Foster City, CA). Primers were used to amplify a 175- and a 179-base fragment of the rat *hsp90α* and *hsp90β* mRNA (less than 30% similarity), respectively. PCR was performed in duplicate reactions with 4 ng cDNA and 0.1 μM of both primers in a final volume of 10 μl containing 1× SYBR Green PCR Master Mix (Applied Biosystems). Samples were preheated at 95°C for 10 min and performed for 40 cycles (94°C for 15 s and 60°C for 1 min) of amplification. Specificity was confirmed by electrophoretic analysis of the reaction products. The GAPDH gene was used as an internal standard to normalize the amount of total RNA present in each reaction and the comparative ΔC_t (threshold cycle number) ($\Delta\Delta C_t$) method were calculated to compare the expression levels among different samples [Livak and Schmittgen, 2001; Stavreva et al., 2004]. Primers were used to amplify a 175-base fragment of the rat *hsp90α*: 5′-CAA GCC TGA AAT AGA AGA TG-3′ (forward) and 5′-GAA TGA AGA ATA CGG AGA G-3′ (reverse); and 179-base of the mouse *hsp90β*: 5′-GAG GCA GAG GAA GAG AAA GG-3′ (forward) and 5′-CTG AAT AAG ACG AAG CCC A-3′ (reverse); and 118-base of the rat *GAPDH*: 5′-AGT TCA ACG GCA CAG TCA AG-3′ (forward) and 5′-TAC TCA GCA CCA GCA TCA CC-3′ (reverse).

Nuclear Extract Preparation

Nuclear extracts were prepared from 9L cells by a rapid fractionation protocol [Hennighausen and Lubon, 1987]. About 10^7 cells were trypsinized, collected by centrifugation at 100*g* for 8 min at 4°C, washed once with PBS, and centrifuged again as above. The pellet was resuspended in 0.5 ml of nuclear extraction buffer (10 mM Hepes-KOH pH 7.9, 0.5% Triton X-100, 0.5 M sucrose, 0.1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and then homogenized by three strokes with a Dounce grinder. The samples were briefly centrifuged at 10,000*g*, and the resulting pellet was lysed in 0.5 ml nuclear extraction buffer supplemented with 0.5 M NaCl and 5% glycerol at 4°C for 30 min. The samples were then centrifuged at 14,000*g* for 20 min, and the supernatant fractions were collected. The nuclear extracts obtained were dialyzed for 3–4 h against at least 50 volumes of dialysis buffer (10 mM Hepes-KOH, pH 7.9, 17% glycerol, 0.1 mM EDTA, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and kept frozen at −70°C.

Electrophoretic Mobility Shift Analysis (EMSA) and Supershift Assays

Double-stranded oligonucleotides corresponding to the respective transcription elements in the promoters of *hsp90α* and *hsp90β* were synthesized as listed in Table I (ScinoPharm, Taiwan Ltd., Taipei, Taiwan). For EMSA probes, 30 pmol of each of forward and reverse oligonucleotides corresponding to the proximal elements were heated at 94°C for 5 min and reannealed at room temperature for 1.5 h in a volume of 25 μl ddH_2O. The double-stranded products were further end-labeled with 20 μCi [γ-^{32}P] ATP (Amersham Biosciences, Piscataway, NJ) by using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled oligonucleotides were purified by

TABLE I. Double Stranded Oligonucleotides Used as Probes in the EMSA Experiments

Genes	Transcriptional elements	Sequences (forward)
hsp90α	HSE1	5′-GGA ATA GAT TCT TTG CAG GCA ACC TTC ACA AGC A-3′
	HSE2	5′-GGG GAG GGT TCT TCC GGA AGG TTC GGG AGG CTT C-3′
	HSE3	5′-GGC GGA GGG TCG GGG GCG AGG CGA ACG TTC GGC T-3′
	HSE4	5′-GGT CCG AAC GTC CTC CCG GAG GCG CGC ACA TGC GCC-3′
	GC box	5′-TGG TGG AGG GGC GGG GCC CGT CCA-3′
	CRE	5′-CAA AGT AAC GTC ATG ACA-3′
	TATA box	5′-GCC TCT ATA TAA GGC AGG CGC GGG GGG CG-3′
hsp90β	HSE1	5′-GAT TTG TAG GGA ACA TTC TAG TAA GAT CGG GTC T-3′
	HSE2	5′-TGG GAG CGC CTA CAT CGA ATT TTCTGC GCG AGT CCG TGG G-3′
	HSE3	5′-AGC TCA GAT TGT TCT GGA AGC CTC GGG CGC CCC G-3′
	GC box	5′-TCC CGC TCT TCC CCG CCC CCT CCC CG-3′
	CRE	5′-GCG AGC ATG ACG TCA AGG TGG G-3′
	TATA box	5′-TGT GCT TCG CCT TAT ATA GGG CGG-3′

Chroma Spin-10 column (BD, Franklin Lakes, NJ). In each reaction, 10 μg of nuclear extract were incubated with the labeled oligonucleotide for 20 min in binding buffer (25 mM Tris–HCl pH7.5, 1 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, 1 μg poly dI–dC). The DNA-protein complexes were then resolve by native 5% polyacrylamide gels (acrylamide/bisacrylamide 29:1 in 0.5× TBE buffer) using 0.5× TBE as running buffer, at 200 V for 1.5 h. After electrophoresis, the gels were prepared for autoradiography. In supershift assays, anti-rat HSF1 and anti-rat HSF2 monoclonal antibodies (Nventa Biopharmaceuticals Co., San Diego, CA) were incubated with the reaction mixtures at 4°C for 4 h before the addition of the HSE probes. The samples were then processed as for the EMSA experiments.

RESULTS

Differential Transactivation of HSP90 Isoforms in GA-Treated 9L Cells

De novo protein synthesis in GA-treated cells was monitored by metabolic labeling with [^{35}S]methionine to measure the inducibility of HSP90α and β isoforms. As shown in Figure 1, synthesis of HSP90α and HSP90β was increased in cells treated with GA in a concentration-related manner. Six hours of treatment with 0.1 μM GA had no effect on expression of the HSP90 isoforms, but 6 h of treatment with 0.5 μM markedly increased the expression level. At higher concentrations, the expression levels of both HSP90 isoforms were significantly reduced (Fig. 1).

When the GA concentration was fixed at 0.5 μM, synthesis of the HSP90 isoforms was upregulated in a time-dependent manner. Induction of HSP90 isoforms peaked after 6 h of treatment and the expression levels then gradually reduced (Fig. 2A, upper panel). Quantitative analysis of the autoradiographs showed that maximal expression levels of HSP90α and HSP90β were approximately 3.7- and 2.4-fold, respectively, compared with control levels (Fig. 2A, lower panel), indicating that HSP90α is the more inducible isoform. Western-blotting assays were used to verify the identities and changes in protein levels of the two HSP90 isoforms (Fig. 2B, upper panel). It was found that the protein level of HSP90α was increased by eightfold, whereas that of HSP90β was only increased by fourfold (Fig. 2B, lower panel). The different induction and protein accumulation rates indicate that the translational efficiency and half-life of HSP90 isoforms are also affected by GA treatment.

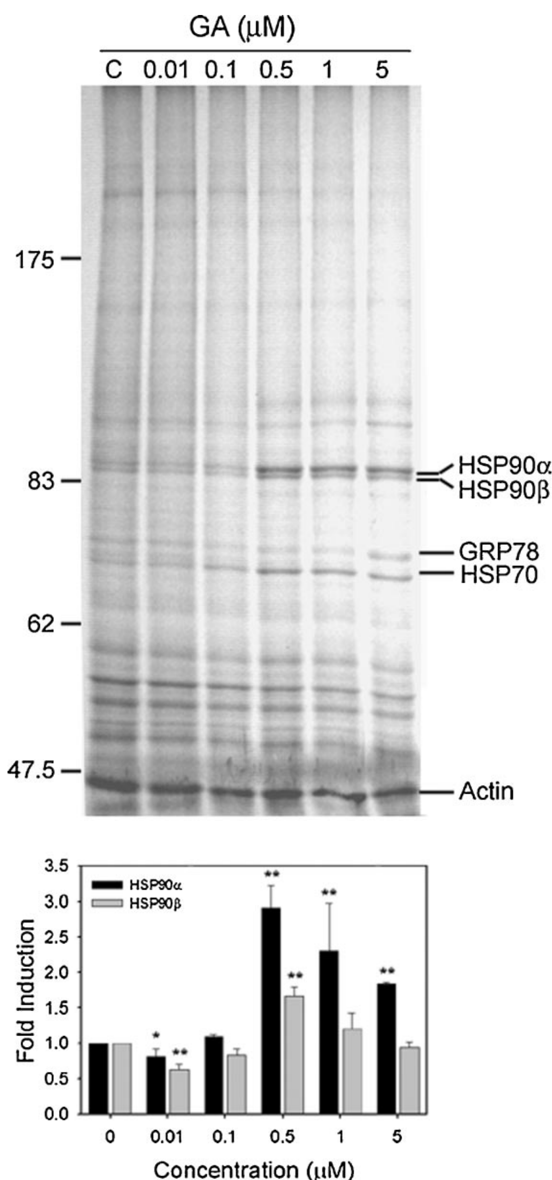

Fig. 1. Dose-dependent induction of HSP90α and HSP90β by GA in 9L cells. Cells were treated with 0–5 μM GA for 6 h. The cells were metabolically labeled with [^{35}S]-methionine for 1 h before harvested and the cell lysates were resolved by 9% SDS–PAGE. Protein bands were visualized by autoradiography (**upper panel**). The autoradiographs were quantified by densitometric scanning and the relative synthesis rates of HSP90α and β were presented as fold-induction represents the amount of HSP90α and β, corrected for internal control (actin), relative to that without GA-treated cells. Each data point indicates the mean ± standard deviation of the relative synthesis rate of the HSP90s from at least three independent experiments, and statistical significance compiled by Student's *t*-test. *$P < 0.05$, **$P < 0.01$.

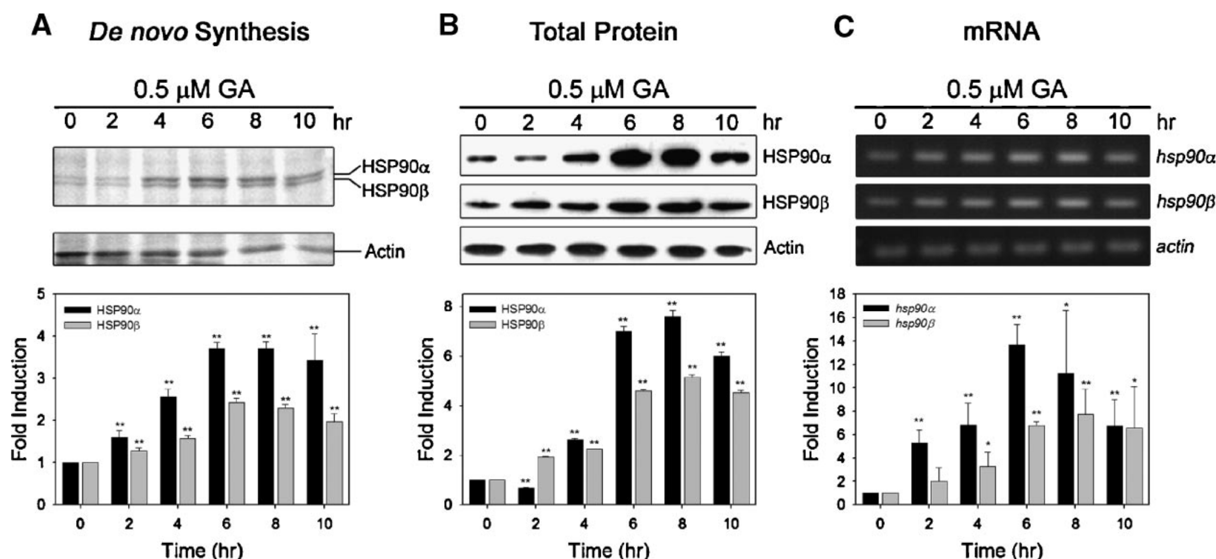

Fig. 2. Time-dependent changes on protein and mRNA levels of HSP90α and β in GA-treated 9L cells. Cells were treated with 0.5 μM GA and harvested at the indicated time intervals. **A**: De novo synthesis protein metabolically labeled as mentioned in Figure 1. **B**: Total cellular proteins were also resolved by 9%, pH 8.0 SDS–PAGE and the amount of the protein of interest was estimated by the Western blotting technique using monoclonal antibodies against HSP90α, HSP90β, and actin (loading control) as the primary antibodies. Fold-induction represents the amount of HSP90α and β, corrected for internal control (actin), relative to that without GA-treated cells. **C**: In parallel experiments, total RNA was extracted from the treated cells and the mRNAs of interest were amplified by RT-PCR (**upper panel**), and monitored by real-time qPCR (**lower panel**). Fold-induction here represents the amount of *hsp90α* and *β* mRNA, corrected for internal control (*gapdh*), relative to that without GA-treated cells. Each data point indicates the mean ± standard deviation (n = 3) and statistical significance compiled by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

To determine whether GA-induced expression of the HSP90 isoforms is transcriptionally regulated, the steady accumulation of *hsp90α* and *hsp90β* mRNA in GA-treated cells was measured semi-quantitatively by RT-PCR. Two isoform-specific primer pairs corresponding to cDNA encoding exon 4 of *hsp90α* and exon 3 of *hsp90β* were used to amplify 175- and 179-bp fragments, respectively. Both *hsp90α* and *hsp90β* mRNAs were induced by GA treatment. The initial amount of total *hsp90β* mRNA was slightly higher than that of *hsp90α* mRNA and the maximal level of the individual mRNAs peaked after 6–8 h of treatment (Fig. 2C, upper panel). Subsequently, the results of real-time quantitiative PCR indicated that *hsp90α* mRNA is more sensitive to GA treatment in terms of both induction amplitude (13.6-fold increase) and time for up- and down-regulation. By contrast, *hsp90β* mRNA responded to GA showed lower induction in amplitude (7.1-fold) and slower in time (Fig. 2C, lower panel).

Responsiveness of Specific HSEs to GA Treatment

Computer-assisted identification of the putative HSEs (set at a cutoff score of >85% identity) were performed using MOTIF (available at http://motif.genome.jp/), and four and three putative HSEs were identified in the promoter regions of rat *hsp90α* and *hsp90β*, respectively (Fig. 3). The involvement of these HSEs and corresponding transcription factors in GA-enhanced transcriptional activities of the *hsp90* genes were further characterized. Binding activities of nuclear extracts prepared from cells treated with or without 0.5 μM GA for 4 h were monitored by EMSA, using double-stranded DNAs corresponding to individual HSEs as probes (sequences of probes are listed in Table I). Interestingly, all HSEs interacted with nuclear factors, and shifted bands were observed in both untreated and treated cells (Fig. 4). The binding specificities were confirmed by competition assays with unlabeled probes of the HSEs (data not shown). Of the HSEs in *hsp90α* GA treatment caused the most significant changes in DNA binding of HSE2 (Fig. 4, left panel). However, the binding activities of the *hsp90β* HSEs did not change after GA treatment, the only exception being a slight increase in *hsp90β* HSE1 (Fig. 4, right panel). To determine whether HSF1 or HSF2 was involved in the process, supershift assays involving monoclonal antibodies against HSF1

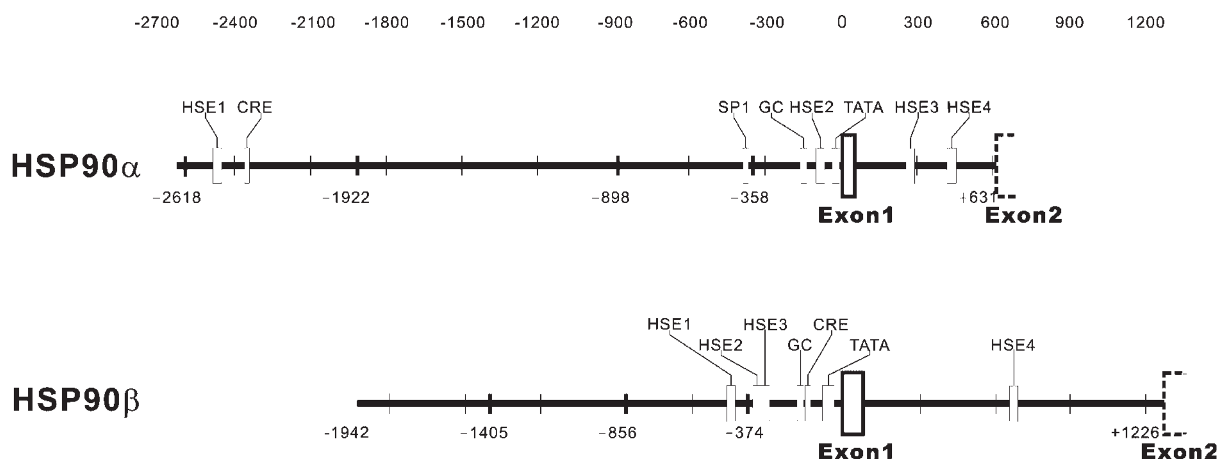

Fig. 3. Schematic putative regulatory elements in the promoter regions of rat *hsp90α*, *hsp90β*. DNA sequences of rat *hsp90α* (−2,618 to +633) (accession number AJ297736) and *hsp90β* (−1,942 to +1,333) (accession number DQ022068) were from our own work. Regulatory elements of interest including the HSEs, TATA box, CRE and GC box were represented by open boxes at their approximate positions.

and HSF2, respectively, were performed. A monoclonal antibody against HSF1, but not HSF2, was able to supershift DNA–protein complexes (Fig. 5), indicating that HSF1 is involved in the GA-induced transactivation of *hsp90α* and *hsp90β* in 9L cells.

Responsiveness of TATA Box, GC Box, and CRE Transcription Elements to GA Treatment

The roles of several well-known basal transcription elements including TATA box, GC box (Sp1), and cAMP response element (CRE) in the transcriptional regulation of *hsp90α* and *β* in GA treatment were also investigated by EMSA (Fig. 6). The gel shift patterns of GC boxes changed with GA treatment. The *hsp90α*-GC box showed a strong binding shift after cells were treated with GA for 4 h. By contrast, the *hsp90β*-GC box showed strong and consistent shift bands regardless of GA treatment. In addition, TATA-box binding was stronger for *hsp90α* than *hsp90β* but neither binding

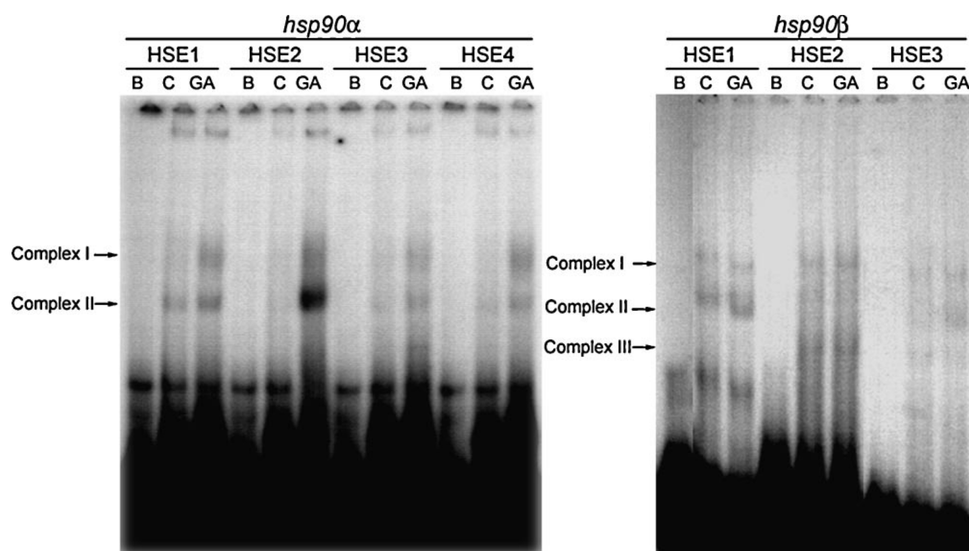

Fig. 4. Binding activities of the nuclear extracts from GA-treated cells toward various HSEs in the promoter regions of rat *hsp90α* and *β* genes. Nuclear extracts were prepared from untreated and treated cells as described in Materials and Methods Section. Radioactive double-stranded oligonucleotides prepared according to their specific sequences HSE1 to HSE4 (as specified in Table I and respectively denoted in Fig. 3 for *hsp90α* and *β* genes) were labeled and used as probes in the EMSA studies. B: Blank, probe only; C: Nuclear extracts from untreated cells; GA: Nuclear extracts from cells were treated with 0.5 μM GA for 4 h. The arrows indicate specific binding bands (Complex I, II, and III).

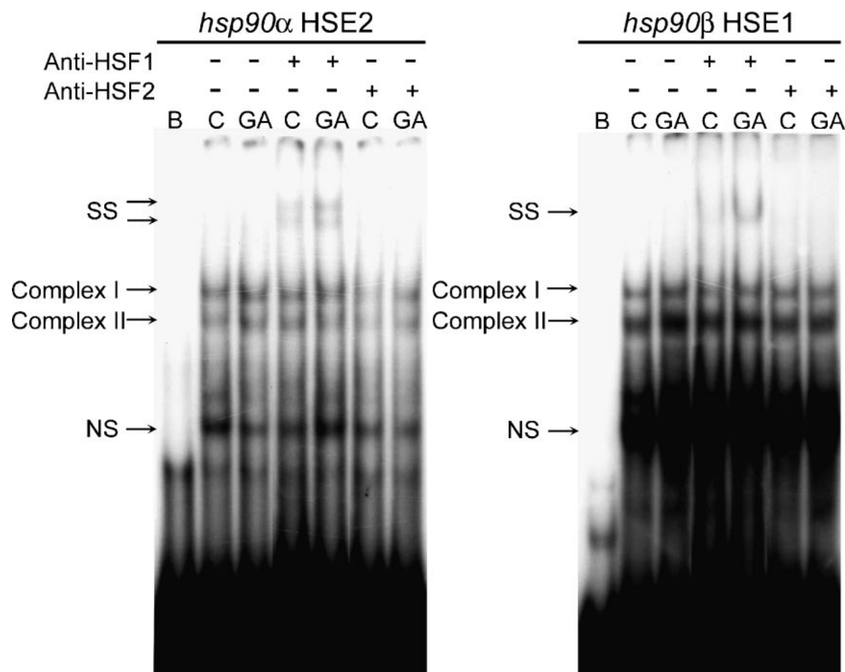

Fig. 5. Identification of HSF1 as the major binding factor for *hsp90α* HSE2 and *hsp90β* HSE1. Cells were treated with 0.5 μM GA for 4 h. After treatment, nuclear extracts were mixed with *hsp90α* HSE2 and *hsp90β* HSE1 (Table I) probes. The protein-DNA complexes were allowed to react with specific antibodies against HSF1 and HSF2, respectively, at 4°C for 4 h. The samples were then subjected to EMSA analysis. B: Blank, probe only; C: Nuclear extracts from untreated cell; GA: Nuclear extracts from cells were treated with 0.5 μM GA for 4 h. The arrows indicate the specific binding bands (Complex I and II), supershift bands are labeled as SS, and non-specific binding as NS.

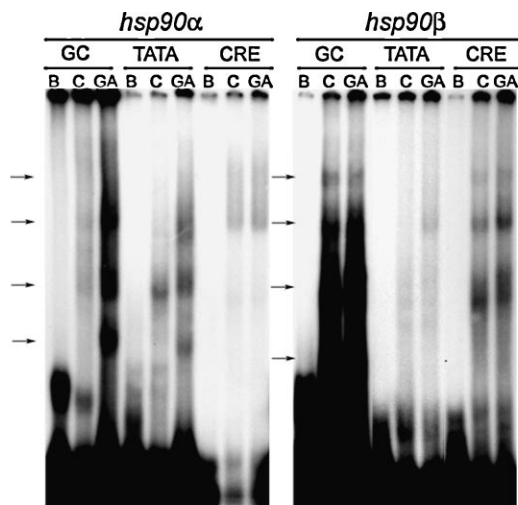

Fig. 6. Binding activities of the nuclear extracts from the GA-treated cells toward various basal transcription elements, GC box, TATA box, and CRE found in the promoter regions of rat *hsp90α* and *β* genes. Nuclear extracts were prepared from untreated and treated cells as described in Materials and Methods Section. Radioactive double-stranded DNA, prepared according to their specific sequences and labeled as GC box, TATA box, and CRE (as specified in Table I and *hsp90α* and *β* genes) were used as probes in the EMSA studies. B: Blank, probe only; C: Nuclear extracts from untreated cells; GA: Nuclear extracts from cells were treated with 0.5 μM GA for 4 h. The arrows indicate the specific binding bands.

interaction was as strong as those of the GC box. Finally, the gel-shift patterns of CREs were unchanged in both genes, with or without GA treatment. Taken together, these data indicate that TATA boxes are the basal transcriptional elements involved in the induction of *hsp90α* and *hsp90β*. In the case of GC boxes, stronger complex binding was observed for *hsp90β* than *hsp90α* in the untreated sample (in Fig. 6, lanes labeled as "C"), which might account for the differences in the basal levels of the *hsp90* transcripts. On the other hand, GC-box binding was associated with greater induction differences in *hsp90α* compared with *hsp90β* suggesting that the GC box might cooperate with previously described transcription elements in the overall responsiveness of *hsp90α* in GA-treated cells. The CRE element does not seem to be involved in the transactivation processes.

DISCUSSION

Our previous studies indicated that GA, which is a specific inhibitor of HSP90s, could be an effective inducer of many stress proteins such as the glucose-regulated proteins (GRPs) and HSPs, including the HSP90s. The effects

were concentration-dependent and might be cell-type-specific [Lai et al., 2003; Shu et al., 2005; Chang et al., 2006a,b]. Owing to their similar molecular weights, HSP90α and HSP90β have not been readily distinguished in most studies of this particular chaperone, and have therefore often been treated as a single entity and referred to as the HSP90(s). Using SDS–PAGE with lower SDS concentrations (9% instead of 10% or above) and lower pH values (8.0 instead of the usual pH 8.8), we were able to easily separate and distinguish HSP90α and HSP90β (Fig. 1). Induction of HSPs by GA in 9L cells could be achieved at a relatively low concentration (0.5 μM) in this study, which was approximately one-tenth of the GA concentration required for induction of GRPs (5.0 μM) [Lai et al., 2003; Sun et al., 2006]. These observations indicate that the induction of HSPs is more specific and sensitive compared with that of GRPs. Furthermore, the effects of GA on HSP90s induction are mainly at the mRNA stage, relative to de novo protein synthesis and total HSP90 amounts, as shown by metabolic-labeling and Western-blotting assays, respectively. In addition, the changes in mRNA levels of *hsp90α* and *hsp90β* are sensible-induced and dose-dependent. It should also be noted that the basal level and the induced level of *hsp90α* mRNA synthesis were consistently lower than those of *hsp90β*, which is in contrast to the observations at the protein level (as shown by de novo synthesis rates and total protein amounts). This phenomenon was reported in our recent study [Chang et al., 2006b] and it is believed that there is differential expression between HSP90α and HSP90β in rat 9L cells after exposure to GA, owing to distinct mRNA profiling and translation processes. However, the magnitude of the induction of HSP90α and HSP90β after GA treatment from mRNA to protein levels showed a consistent twofold difference. We believe that both translational and transcriptional levels are involved in differential expression of HSP90 isoforms.

Here, we extend our previous findings to show that the induction of HSP90s by GA is isoform-specific—the transactivation of *hsp90α* and *hsp90β* in the presence of GA may result from different regulatory mechanisms. As a first step, we demonstrated the involvement of the HSEs and other transcriptional elements, notably the GC and TATA boxes, during GA-induced expression of the *hsp90* genes.

Transactivation of HSE-containing heat-shock genes primarily involves HSE–HSF complex formation [Bienz and Pelham, 1986]. In general, HSF exists in an inactive state and is activated to cooperate with HSE and allow transcription of heat-shock genes in cells under physiological stresses [Kingston et al., 1987; Abravaya et al., 1991]. The HSEs are characterized by nGAAn five-nucleotide motifs in a 'head-to-head' or 'tail-to-tail' alignment [Cunniff et al., 1991]. As the upstream sequences of the *hsp90* genes were identified, several HSEs were identified in the promoter region and intron 1 (Fig. 3). Various HSEs may have different levels of involvement in the basal and induced expression of heat-shock genes. Using oligonucleotides based on the sequences of *hsp90α* HSE1–4 and *hsp90β* HSE1–3 as the EMSA probes, we have shown that HSE–nuclear-protein complexes can form in cells with or without GA treatment. This observation supports the hypothesis that HSE–HSF formation is required for basal and induced expression of the *hsp90* genes. Comparisons of nuclear extracts from cells with or without GA treatment showed that the changes in binding activities to *hsp90α*-HSE2 (two HSE, −108/−99 and −95/−86) were greater than those to other HSEs in *hsp90α* (Fig. 4, left panel). This result indicates that the alterations in binding activities of *hsp90α*-HSE2 are more significant than those to other *hsp90α* HSEs. However, it should be noted that the differences in binding activity to HSEs of *hsp90β* were less marked, and only binding to HSE1 (−447/−438) was greater than those of other HSEs. Therefore, GA treatment would be expected to affect binding of the transcription factors to *hsp90α*-HSEs to a greater extent than binding to *hsp90β*-HSEs. These results lead us to conclude that the *hsp90α*-HSE2 and *hsp90β*-HSE1 may have more important roles than the other HSEs in the GA-induced transactivation of the *hsp90* genes.

It is known that two mammalian HSFs, HSF1 and HSF2, interact with HSEs in heat-shock genes under stress or differentiation conditions, leading to large increases in the transcription of these target genes [Rabindran et al., 1991; Sarge et al., 1991, 1993; Morimoto, 1998]. For instance, in human K562 erythroleukemic cells, HSF1 binding is activated more by heat than by hemin treatment, whereas HSF2 binding is only activated by hemin treatment and not by heat [Trinklein et al., 2004]. Among heat shock

factors, HSF1 is expressed ubiquitously in the highest amount and its activation is most sensitive to heat and other stressors in all mammalian cells [Morimoto, 1998]. Upon activation, HSF1 undergoes a multi-step process that involves conversion from a monomer to a trimeric nuclear protein complex, followed by binding to the HSEs of the heat-shock genes, thus triggering their transactivation [Pirkkala et al., 2001]. Most recently, we have also demonstrated that treatment of human non-small-cell lung cancer H460 cells with GA leads to phosphorylation-activation of HSF1, which in turn stimulates HSP70-1/2 [Shu et al., 2005; Chang et al., 2006a]. In the present study, the results of supershift assays also indicate that HSF1 is the transcription factor that is activated in the GA-induced transactivation of the *hsp90* genes in rat 9L cells.

We have also studied the possible involvements of three other transcription elements – GC box, CRE, and TATA box—in the differential transactivation of the *hsp90* genes by GA. Comparisons of the binding activities of the nuclear extracts from untreated and GA-treated cells to the DNA probes specified above showed that the GC box may be involved in both basal and GA-induced expression of the *hsp90* genes. In both *hsp90α* and *hsp90β*, there was greater binding to the GC box in cells treated with GA than in the untreated cells. In addition, it was evident that, on the whole, the GC box in *hsp90β* in both GA-treated and untreated cells displayed greater binding activities than that in *hsp90α*, which in turn indicates that the GC box may have a more critical role in the expression of *hsp90β*. However, binding to the TATA boxes showed greater GA-dependent changes in *hsp90α* than in *hsp90β*, so may also have additional roles in transactivation of the *hsp90* genes. Finally, it seems that the CRE elements have no involvement in transactivation of the hsp90 genes. Thus, the regulation plasticity of GA treatment involves similar transcription elements of the HSEs, GC boxes and TATA boxes, which together control the differential transactivation of the *hsp90α* and *hsp90β* genes in rat gliosarcoma 9L cells.

In summary, although the two copies of HSP90 are highly homologous at the protein level, the regulatory regions of these genes are different, and fewer studies have investigated their individual transactivation. In this report, we have shown that, in GA-treated rat gliosarcoma cells, the differentially induced expression of HSP90α and HSP90β after transactivation of the genes results from the cooperative actions of multiple transcription elements and presumably their corresponding transcription factors.

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