The Phorbol Ester 12-O-Tetradecanoylphorbol 13-Acetate Enhances the Heat-induced Stress Response*

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Carina I. Holmberg‡§, Sirpa Leppä‡, John E. Eriksson‡, and Lea Sistonen‡

From the ‡Turku Centre for Biotechnology, University of Turku, Åbo Akademi University, and the §Department of Biochemistry and Pharmacy, Åbo Akademi University, FIN-20521 Turku, Finland

Induction of heat shock gene expression is mediated by specific heat shock transcription factors (HSFs), but the signaling pathways leading to activation of HSFs are poorly understood. To elucidate whether protein kinase C-responsive signaling pathways could be involved in the regulation of heat shock gene expression, we have examined the effects of the protein kinase C activator 12-O-tetradecanoylphorbol 13-acetate (TPA) on the heat-induced stress response in K562 cells. We demonstrate that TPA treatment markedly enhances heat shock gene expression during heat stress, although TPA alone does not induce the heat shock response. This TPA-mediated enhancement can initially be detected as an accelerated acquisition of DNA binding and transcriptional activity of HSF1 resulting in elevated Hsp70 protein concentrations. In the presence of TPA, the attenuation of HSF1 DNA binding activity during continuous exposure to heat shock occurs more rapidly and in concert with the appearance of newly synthesized Hsp70, which supports earlier studies on the autoregulatory role of Hsp70 in deactivation of HSF1. During heat stress, a correlation between the hyperphosphorylation of HSF1 and its transcriptional activity was observed, in both the presence and the absence of TPA. Our results show that the heat-induced stress response can be significantly modulated by activation of protein kinase C-responsive signaling pathways.

In eukaryotic cells, the common cellular response to heat shock and other types of stress is a rapid transcriptional activation of heat shock genes resulting in increased synthesis of the heat shock proteins (Hsps). The initial signaling events in this well characterized physiological process, also called the stress response, have not been identified. Hence, it is not known how cells sense stress and how the signal is conveyed to the transcriptional apparatus. However, it is well established that the transcriptional activity of heat shock genes in eukaryotes is regulated by specific pre-existing transcription factors (HSFs). Upon exposure to various forms of stress, HSFs bind to the heat shock element (HSE) located in the promoter regions of heat shock genes (for review see Ref. 1). The mechanisms regulating the DNA binding and transcriptional activity of HSFs are currently the focus of intense investigation.

In mammalian cells, two HSFs, HSF1 and HSF2, have been identified as regulators of heat shock gene expression (2–4). HSF1 is activated in cells exposed to elevated temperatures and other environmental stress conditions (5–7), whereas HSF2 appears to function in cells involved in processes of differentiation and development (8–10). In response to stress, HSF1 undergoes oligomerization from a non-DNA-binding monomer to a DNA-binding trimer and translocates into the nucleus to interact with HSEs of the heat shock gene promoters (5, 7, 11–14). In addition, mammalian HSF1 undergoes inducible phosphorylation upon activation (7, 15–17). The inducible phosphorylation has also been shown for the yeast Saccharomyces cerevisiae HSF, which unlike other eukaryotic HSFs is constitutively bound to the HSE but remains transcriptionally inactive prior to heat shock (18). Recent evidence suggests that the hyperphosphorylated state of HSF1 is not required for DNA binding activity but might be required for transcriptional activation. For example, treatment with the anti-inflammatory drugs sodium salicylate and indomethacin induces DNA binding activity of HSF1 but fails to activate transcription of heat shock genes (15, 19–21). In neither case is HSF1 hyperphosphorylated. In contrast, treatment with arachidonate leads to HSF1 DNA binding activity, hyperphosphorylation of HSF1, and activation of hsp70 gene transcription (22). Both activating and inactivating phosphorylation sites may be present on HSF1, because a study in the yeast Kluyveromyces lactis proposes that phosphorylation of HSF may also serve as a regulatory mechanism to inactivate HSF (23). Hence, there is substantial evidence indicating that phosphorylation is involved in the activation of HSF1, although the exact role of phosphorylation in the regulation of this transcription factor has not been established.

Significant progress has been made to define the signaling pathways that lead to transcriptional activation of eukaryotic gene expression. Although protein phosphorylation has been identified as a major post-translational mechanism responsible for regulating the activity of many known transcription factors, such as NF-κB, AP-1, and CREB (for review see Refs. 24–26), no specific protein kinases or protein phosphatases have yet been identified to be directly involved in the regulation of HSF1 activation or in the induction of the heat shock response. However, involvement of phosphorylation in the regulation of heat shock gene expression is supported by several studies. Various modifiers of kinase/phosphatase activities can modulate differ-
ent regulatory steps of the heat shock response (27–32). Heat stress per se has also been reported to activate major signaling processes, such as the mitogen-activated protein kinase (MAPK; Ref. 33), protein kinase C (PKC; Refs. 34 and 35), and Jun N-terminal kinase (Ref. 36). Furthermore, oxidative stress and heat shock have been reported to induce expression of the CL100 gene encoding a tyrosine/threonine-specific protein phosphatase (37, 38).

To elucidate the signaling pathways involved in the regulation of stress response, we have used the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) to examine the effects of PKC activation on heat-induced HSF1 activation and expression of the heat shock genes hsp70 and hsp90. Our results show that although TPA treatment alone does not induce the stress response in K562 cells, TPA treatment in combination with heat shock markedly enhances the stress response. This enhancement is detected initially on the transcriptional level, subsequently leading to increased levels of the hsp70 mRNA and protein. The TPA-mediated effect on the heat-induced stress response also shows gene specificity, because the effects on hsp70 and hsp90 gene expression are distinct.

EXPERIMENTAL PROCEDURES

Cell Culture, TPA Treatment, Heat Shock Conditions, and Preparation of Cell Extracts—Human K562 erythroleukemia cells and HeLa cervix carcinoma cells were grown in RPMI 1640 (HyClone) and Dulbecco’s modified Eagle’s medium (HyClone), respectively, supplemented with 10% heat-inactivated fetal calf serum (BioClear) in a humidified 5% CO₂ atmosphere at 37 °C. Cells were seeded at 5 × 10⁶ cells/10-cm diameter plate prior to exposure to TPA (Sigma), 4α-12-O-tetradecanoylphorbol 13-acetate (4α-TPA; LC Laboratories), or heat shock. For heat shock treatment, plates were scaled with Parafilm and immersed in a 42 °C water bath. Whole cell extracts were prepared as described previously (39), and the protein concentration was measured by a protein assay (Bio-Rad).

Gel Mobility Shift Assay —Whole cell extracts containing 15 µg of protein were incubated with γ-³²P-labeled HSE oligonucleotide corresponding to a sequence in the human hsp70 promoter and analyzed on a native 4% PAGE gel as described previously (39). For analysis of AP-1 DNA binding activity, a double-stranded TPA-responsive element-containing oligonucleotide was used (40). The synthetic oligonucleotides were ³²P-labeled with T4 polynucleotide kinase (Promega). Quantitative analyses of the HSE protein and TPA-responsive element-protein complexes were performed using a Fujix Bas 1000 PhosphorImager.

Transcriptional Nuclear Run-on Analysis —For in vitro run-on transcription reactions, isolated nuclei (approximately 5 × 10⁶ nuclei/react) were incubated with 100 µCi of [α-³²P]UTP (3000 Ci/mmol, Amersham Corp.) as described previously (41). The radiolabeled mRNA was hybridized with nitrocellulose-fixed plasmids for the following genes: human hsp70 (pH2.3; Ref. 42), human hsp90a (pUChSS801; Ref. 43), human hsc70 (pH7.6; Ref. 44), human grp78 (pHG23.1; Ref. 44), human hsp60 (SPD-920; StressGenes), human hsp27 (SPD-910; StressGenes), human β-actin (pHF/A-1; Ref. 45), rat GAPDH (pGAPDH, Ref. 46), and Bluscript vector (Stratagene). The hybridization and washing conditions were as described previously (10). The intensities of the radioactive signals were quantified with a Fujix Bas 1000 PhosphorImager.

Northern Blot Analysis —Total RNA was isolated by acid guanidium thiocyanate and phenol-chloroform extraction as described previously (47). 10 µg of total RNA was separated on a 1% agarose gel, fixed to a nylon membrane (Hybond-N, Amersham Corp.), and hybridized with a ³²P-labeled DNA probe containing either the human hsp70 gene (pH2.3), human hsp90 gene (pUChSS801), or rat GAPDH gene (pGAPDH). The hybridization and washing conditions were as specified by the manufacturer (Amersham Corp.). The plasmid DNA was radiolabeled using a nick translation kit (Promega).

Metabolic Labeling with [³⁵S]S-Methionine and Western Immunoblot Analysis —1 × 10⁶ cells/ml were washed with methionine- and cysteine-free medium, resuspended in 0.5 ml of methionine-free medium containing 10% dialyzed fetal calf serum, and exposed to treatment. During the last 30 min of treatment, 50 µCi of [³⁵S]methionine (1190 Ci/mmol, TRAN³⁵S-LABEL; ICN) was added. Whole cell extracts containing 15 µg of protein were analyzed by 8% SDS-PAGE and fluorography. For Western blot analysis, whole cell extracts (10 µg) were subjected to an 8% SDS-PAGE and transferred to nitrocellulose membrane by using a semi-dry transfer apparatus as specified by the manufacturer (Bio-Rad). After blocking for 90 min in 3% nonfat dry milk in phosphate-buffered saline, the filters were incubated with rabbit polyclonal antibodies with HSF1 or HSF2 (1:10,000 anti-rat immunoglobulin G (1:20,000 dilution; Promega), goat anti-mouse immunoglobulin G (1:20,000 dilution; Promega), goat anti-μ mouse immunoglobulin G (1:20,000 dilution; Amersham Corp.) were used as secondary antibodies. The detection was performed by using the ECL system (Amersham Corp.).

RESULTS

TPA Treatment during Continuous Heat Shock Results in Accelerated Acquisition and Attenuation of HSF1 DNA Binding Activity —To examine the effect of TPA on the activation of HSF1 DNA binding activity, K562 cells were exposed to heat shock at 42 °C, 100 nM TPA, or to both TPA and heat shock for various time periods extending to 6 h. Whole cell extracts were prepared and analyzed by gel mobility shift assay. As shown earlier (48), the HSE binding activity of HSF1 was transiently activated in cells exposed to a continuous heat shock at 42 °C (Fig. 1). The maximum level (about 6-fold induction above the control level) of heat-induced HSF1 HSE binding was obtained within 1 h, after which the binding activity decreased to the basal level within 4 h. TPA treatment alone did not induce any HSF1 DNA binding activity (Fig. 1), although TPA was obviously effective in these cells as reflected by a 2–3-fold stimulation of AP-1 DNA binding activity upon TPA treatment over a time period of 15 min to 4 h. To our surprise, TPA treatment during continuous heat shock markedly affected the kinetics of HSF1 HSE binding activity. The maximum HSF1 HSE binding activity (approximately 7-fold induction) was detected within 15 min followed by a rapid attenuation of HSF1 HSE binding activity that returned to the pre-heat shock level by 3 h. Furthermore, a moderate increase (approximately 1.5-fold induction) in the level of HSF1 HSE binding was detected within the first 60 min of incubation in TPA-treated heat-shocked cells. To ensure that the TPA-mediated effect was not due to down-regulation of PKC, a lower concentration (10 nM) of TPA was used and identical results, including kinetics, were obtained. 2 Furthermore, the TPA-mediated enhancement of the heat-induced HSF1 HSE binding activity occurs too fast to be accounted for by down-regulation of PKC. The faster attenuation of HSF1 HSE binding activity in TPA-treated heat-shocked cells appears not to be a cell-specific phenomenon, because similar effects were observed using HeLa cells. 2 None of the treatments had any effect on cell viability as determined by trypan blue exclusion.

We next studied the phosphorylation states of HSF1, based on its migration on SDS-PAGE. By using specific anti-HSF1 antibodies in Western blot analysis, a slower migrating band on an 8% SDS-PAGE could be observed in cell extracts from heat-shocked K562 cells compared with the HSF1 band from untreated cells (Fig. 2). The slower migration is presumably due to increased phosphorylation of HSF1 as has been previously suggested (7, 15). Upon exposure to heat shock, a minor retardation of HSF1 migration could be observed at 15 min, whereas the major slower migrating HSF1 band was detected at 1–2 h, after which it returned back to the control form by 6 h (Fig. 2, upper panel). In comparison, simultaneous treatment with TPA and heat shock induced hyperphosphorylation of HSF1 more rapidly, because the slower migrating HSF1 form

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was predominant already at 15 min. By 3 h, the slower migrating HSF1 form in TPA-treated heat-shocked cells shifted back to a faster migrating form (called the putative intermediate form, see below; Fig. 2, upper panel), which seems to be distinct from the control form. Consequently, the hyperphosphorylated form of HSF1 appears and disappears in parallel with the acquisition and attenuation of HSF1 DNA binding activity, respectively (Fig. 1). Consistent with the studies on DNA binding activity, TPA treatment by itself did not induce the hyperphosphorylated form of HSF1. However, in TPA-treated samples, a slightly slower migrating form of HSF1 as compared with HSF1 in control sample was detected from 15 min to 6 h of incubation, suggesting that a putative intermediate phosphorylation state of HSF1 can be induced by TPA (Fig. 2, upper panel).

For Western blot analysis, HSF2 was used as an internal control, because no change in HSF2 migration on SDS-PAGE upon treatment with heat or hemin has been detected (7, 49). Indeed, heat shock did not affect the migration of HSF2, but TPA treatment seemed to decrease the amount of HSF2 after 4–6 h (Fig. 2, lower panel).

**Fig. 2. Examination of the phosphorylation states of HSF1 by Western blot analysis.** 10 μg of whole cell extracts from untreated K562 cells (C), cells exposed to heat shock (HS, 42°C), TPA treatment (TPA, 100 nM), and combined treatment with TPA and heat shock (TPA + HS) for the indicated time periods were run on an 8% SDS-PAGE. Antibodies against HSF1 and HSF2 were used for Western blot analysis. The distinct phosphorylation states of HSF1 were detected as slower migrating complexes in the gel (see text for further details).

**Fig. 3. Examination of the effects of 4α-TPA on the HSF1 DNA binding activity and on the phosphorylation states of HSF1.** A, K562 cells were exposed to heat shock (HS, 42°C), 4α-TPA treatment (4α-TPA, 100 nM), or combined treatment with 4α-TPA and heat shock (4α-TPA + HS) for the indicated time periods. Whole cell extracts were subjected to gel mobility shift assay as described in the legend to Fig. 1. CHBA, constitutive HSF DNA binding activity; free, unbound HSE oligonucleotide; C, extracts from untreated cells. B, Western blot analysis with antibodies against HSF1.

**TPA Treatment during Continuous Heat Shock Enhances**
hsp70 and hsp90 Gene Expression—Because simultaneous treatment of K562 cells with TPA and heat shock affected the DNA binding activity of HSF1, we wanted to analyze the consequences of this effect on the expression of HSF1-regulated heat shock genes. First, we examined transcription by performing nuclear run-on analysis on nuclei isolated from K562 cells that were exposed to heat shock, TPA, or a combination of TPA and heat shock. As shown in Fig. 4, the maximal, 20-fold induction of hsp70 transcription was obtained after 60 min of heat shock. At this time point, a gradual attenuation of hsp70 transcription occurred, with transcription returning to its basal level after 6 h of continuous exposure to 42 °C. Upon exposure to TPA and heat shock, an additional 3-fold increase in the transcription rate of hsp70 was detected after 15 min (Fig. 4). This difference between TPA-treated heat-shocked cells and cells exposed only to heat shock was maintained until the 2-h time point. Hence, the induction of hsp70 transcription was both accelerated and enhanced by the TPA treatment during a continuous heat shock as compared with the induction of hsp70 transcription by heat shock alone (Fig. 4B).

hsp90, another HSF1-regulated heat shock gene, has earlier been shown to be transcriptionally induced upon heat shock (10, 43, 51), but the extent of heat-induced transcription of hsp90 was usually less dramatic relative to that of hsp70. In this study, the maximal increase of about 3-fold in hsp90 transcription was detected during the first hour at 42 °C (Fig. 4). In analogy with the enhanced hsp70 transcription upon the combined treatment with TPA and heat shock, the transcriptional activity of hsp90 was further increased by approximately 2-fold at 15 min of treatment as compared with cells exposed to heat shock alone. In addition, a moderate increase in the transcriptional activity of hsp60 and hsc70 genes was detected (Fig. 4).

TPA treatment by itself did not induce heat shock gene transcription, which is consistent with the results of DNA binding and phosphorylation state of HSF1 in TPA-treated cells. However, transcription of β-actin was transiently induced by TPA (Fig. 4) as has been previously shown (52, 53). The lack of TPA-induced β-actin gene transcription in TPA-treated heat-shocked cells is presumably due to down-regulation by heat shock, as has been previously shown (54).

Following the analysis of transcriptional activities, we examined the steady-state levels of hsp70 and hsp90 mRNA by Northern blot analysis (Fig. 5). Upon heat shock, the amount of hsp70 mRNA was induced by approximately 80-fold reaching the maximum level at 2–3 h of treatment. In cells exposed to both TPA and heat shock, an additional 4-fold increase in hsp70 mRNA amount above the levels in heat-shocked cells was detected within the first hour of treatment (Fig. 5). Likewise, following 1–6 h of incubation, the steady-state levels of hsp90 mRNA were approximately 2-fold higher in TPA-treated heat-shocked cells than in cells exposed to heat shock alone (Fig. 5). At the attenuation phase, a clear difference between the decline of hsp70 and hsp90 mRNA was observed. After the peak at 2–3 h exposure to heat shock in both the presence and the absence of TPA, the hsp70 mRNA levels steadily decreased up to 6 h of incubation, whereas the maximally induced levels of hsp90 mRNA were maintained throughout this time period. Taken together, our results indicate that the TPA-mediated increase in the steady-state levels of hsp70 mRNA in heat-shocked cells is mainly due to transcriptional induction of hsp70.

TPA Treatment during Continuous Heat Shock Causes a
Multifold Increase of Hsp70 Synthesis—Because it had been earlier implied that heat shock gene expression can be regulated at multiple levels and that increased transcription does not necessarily result in increased protein synthesis (22), we wanted to examine the effect of TPA on heat-induced expression of heat shock proteins. The rate of Hsp70 and Hsp90 synthesis was examined by labeling K562 cells with \[^{35}S\]methionine during the last 30 min of treatment with heat shock, TPA, or TPA and heat shock together. A 4-fold induction in Hsp70 synthesis was detected in cells exposed to the combined treatment with TPA and heat shock for 1 h, as compared with the increase in Hsp70 synthesis during a 1-h heat shock (Fig. 6, A and B). The difference in the induction of Hsp70 between TPA-treated heat-shocked cells and cells subjected to heat shock decreased at later time points. In contrast to Hsp70, no similar enhancement in Hsp90 synthesis was detected in cells exposed to both TPA and heat shock (Fig. 6, A and B). TPA treatment alone did not induce synthesis of Hsp70 or Hsp90.

To examine the effects of simultaneous treatment with TPA and heat shock on the kinetics of Hsp70 accumulation, whole cell extracts prepared from K562 cells treated for various time periods up to 6 h were analyzed by Western immunoblotting. As shown in Fig. 6C (top panel), simultaneous treatment with TPA and heat shock resulted in markedly elevated levels of Hsp70 already after 2–3 h of incubation. These levels were comparable with the amount of Hsp70 detected in cells that were subjected to a 6-h heat shock in the absence of TPA (Fig. 6C, top panel). The kinetics of Hsp70 accumulation correlate closely with HSF1 dephosphorylation and attenuation of HSF1 DNA binding activity, as seen in Figs. 1 and 2. Consistent with the results on Hsp90 synthesis, as presented above, no difference in the kinetics of Hsp90 accumulation was observed between TPA-treated heat-shocked cells and cells exposed to heat shock alone (Fig. 6C, middle panel). Hsc70 (Fig. 6C, bottom panel) was used as an internal control for equal loading because no significant induction in Hsc70 accumulation occurs upon exposure to heat shock. Taken together, our results on the synthesis and accumulation of Hsp70 and Hsp90 suggest that the expression of the corresponding genes is, at the translational level, differentially regulated upon TPA treatment during continuous heat shock.

DISCUSSION

The results from the present study show that a remarkable enhancement of the heat shock response can be achieved by stimulating the TPA-responsive signaling pathways during continuous heat shock. The observed amplification in heat shock gene expression is mainly a consequence of effects on the transcriptional level, because the induction of both DNA binding activity of HSF1 and transcription of the HSF1-regulated target genes are accelerated and enhanced. Subsequently, treatment with TPA during continuous heat shock leads to elevated synthesis and accumulation of Hsp70. Furthermore, the deactivation or attenuation of HSF1 DNA binding activity during continuous heat shock is accelerated in the presence of TPA. Because this faster attenuation is tightly correlated with the accelerated effects on transcription and protein synthesis, it is likely that some of the downstream effects turn off the initial response. The TPA-induced signal is therefore not only sufficient to amplify the heat shock response but will also accelerate both induction and suppression of the response.

TPA per se is not sufficient for induction of the heat shock response. Hence, an initial triggering signal by heat shock is required to obtain the TPA-induced amplification. Because considerable evidence exists that phorbol esters function principally by persistent stimulation of PKC (for review see Ref. 55), the results from our study imply that a PKC-regulated path-

Fig. 6. Analysis of Hsp70 and Hsp90 synthesis and accumulation kinetics by metabolic labeling and Western immunoblotting, respectively. A, K562 cells exposed to heat shock (HS, 42°C), TPA treatment (TPA, 100 nm), or combined treatment with TPA and heat shock (TPA + HS) for the indicated time periods were labeled with 50 µCi of \[^{35}S\]methionine during the last 30 min of each treatment. Whole cell extracts were run on an 8% SDS-PAGE followed by fluorography. C indicates sample from untreated cells, and the indicated molecular mass markers are in kilodaltons. B, quantitative analysis of Hsp70 and Hsp90 synthesis in heat-shocked and TPA-treated heat-shocked cells. Note the difference in scales between Hsp70 and Hsp90. C, Western blot analysis with antibodies against Hsp70, Hsp90, and Hsc70.

way appears to be operative in regulation of the heat shock response. This is in agreement with an earlier report showing that treatment with the PKC inhibitors H-7 and calphostine C...
inhibits hsp70 mRNA induction in cells exposed to heat shock (56). These observations are important in revealing a new mechanism involved in regulation of the heat shock response. It is also significant from the point of view that PKC-mediated signaling pathways are involved in regulation of a multitude of different cellular processes (for review see Ref. 57). The TPA-induced effects on the heat shock response could be mediated directly by PKC or by some of the downstream signaling cascades activated by PKC. In concordance with previous studies (58), our data show that TPA causes a significant increase in AP-1 DNA binding activity, suggesting possible activation of a MAPK cascade (for review see Ref. 26). The different MAPKs embrace a central position in the interactive signaling pathways regulating transcription. For example, extracellular signal regulated kinase 1/2, the stress-activated protein kinase, also called Jun N-terminal kinase, and p38 kinase, all members of the MAPK family, have been shown to participate in the regulation of c-fos and c-jun transcription, although their activities are clearly initiated by different signals (for review see Refs. 26 and 59). Considering the preceding documentation demonstrating MAPKs as key elements in transcriptional regulation, it will be of major interest to elucidate the role of MAPKs in the regulation of the heat shock response.

Previous studies have indicated that the transcriptionally active HSF1 is hyperphosphorylated, as reflected by the appearance of slower migrating forms of HSF1 on SDS-PAGE (7, 15). In this study, a close correlation between the activation-specific, slower migrating form of HSF1 and the DNA binding activity of HSF1 was observed, coupled with the transcriptional induction of hsp70 and hsp90 genes. These data support the previously raised hypothesis that phosphorylation may play an important role in the regulation of heat shock gene expression (7, 15, 16, 17). The accelerated activation and attenuation of the HSF1 DNA binding in TPA-treated heat-shocked cells correlate well with HSF1 hyperphosphorylation and dephosphorylation. Earlier studies have shown that the process of HSF1 activation potentially involves multiple steps, including various post-translational modifications of HSF1, such as trimerization, nuclear localization, and hyperphosphorylation (for review see Ref. 1). These steps can be uncoupled because certain stimuli, such as the nonsteroidal anti-inflammatory compounds sodium salicylate and indomethacin, activate the heat shock response only partially so that HSF1 acquires DNA binding activity without induction of heat shock gene expression (15, 20, 21). In contrast to the fully activated heat-inducible form of HSF1, the drug-activated HSF1 does not undergo hyperphosphorylation. Recently, two studies have indicated that HSF1 is phosphorylated on serine residues upon heat shock, whereas the stress-induced serine-directed phosphorylation cannot be induced by the anti-inflammatory agents (15, 19). Furthermore, Cotto and co-workers (15) have shown that acquisition of the trimeric DNA binding state of HSF1 occurs independently of hyperphosphorylation and that HSF1 trimerization precedes the inducible phosphorylation of HSF1. The inducible phosphorylation of HSF1 is likely not required for the acquisition of DNA binding activity but may be required for the transcriptional activation. Although evidence is accumulating in support for the hypothesis of a multistep activation of HSF1, the exact phosphorylation sites of the inactive and active forms of HSF1, as well as the kinases and/or phosphatases regulating these sites remain to be identified.

Several reports have indicated that heat shock proteins themselves may be involved in the activation and deactivation of HSF1 by an autoregulatory loop (60–65). Hsp70 has been implicated in the negative regulation of HSF1 in mammalian cells, and this autoregulatory mechanism has been proposed to affect the DNA binding and oligomerization of HSF1. According to Mosser and co-workers (64), constitutive overexpression of hsp70, as obtained by transfection techniques, results in a reduction in the level of HSF1 activation following heat shock. In their study, the DNA binding activity of HSF1 was regulated in a dose-dependent manner so that the extent of inhibition was greater in cells expressing higher levels of Hsp70. Furthermore, Kim and co-workers have shown that overexpression of Hsp70 accelerates the recovery of heat-shocked mammalian cells through its modulation of HSF1 (65). The autoregulatory role of Hsp70 is further supported by the observations that Hsp70 binds to the active form of HSF1 and that excess exogenous Hsp70 prevents the activation of HSF1 in vitro (60, 61). However, the autoregulatory model is contradicted by a finding that Hsp70 and Hsc70 associate to similar extents with both the latent non-DNA-binding form and the active DNA-binding form of HSF1 and that the induction of HSF1 DNA binding is not affected by overexpression of Hsp70 (66). In our study, extensive genetic manipulation to obtain overexpression of Hsp70 was avoided, and the results show that the gradual HSF1 dephosphorylation and attenuation of HSF1 DNA binding activity follow the kinetics of elevated Hsp70 accumulation. Although we have not determined whether the accumulated Hsp70 exists free or is bound to a substrate, our results give further support to the hypothesis that Hsp70 plays a role in the attenuation of HSF1 DNA binding activity. Moreover, the close correlation of increased Hsp70 synthesis with decreased phosphorylation of HSF1 suggests that Hsp70 may have a role not only in the regulation of HSF1 DNA binding activity but also in the regulation of HSF1 phosphorylation.

We demonstrate that the expression of two classic heat shock genes, hsp70 and hsp90, is differentially regulated upon simultaneous treatment with TPA and heat shock. Although hsp70 is known to be the most highly stress-inducible heat shock gene, our experiments show that the transcription of both hsp70 and hsp90 genes is markedly stimulated in response to heat stress and that this transcriptional activation is further enhanced by the combined treatment of heat and TPA. In the case of Hsp70, the transcriptional enhancement results in accelerated and increased protein synthesis and accumulation, whereas no such additive effects can be detected on Hsp90. The distinct effects of TPA and heat shock on the expression of hsp70 and hsp90 genes raise the question of whether Hsp90 has a role in the autoregulatory model of Hsp70. Assuming that both hsp70 and hsp90 are transcriptionally activated by a common factor, HSF1, as earlier suggested (51), it would be important to know whether Hsp90, in analogy to Hsp70, can form a complex with the DNA-bound form of HSF1 and thereby inhibit the DNA binding and transcriptional activities of HSF1. According to Rabinovitch and co-workers (66), the association of Hsp90 with HSF1 cannot be detected in a co-immunoprecipitation assay. The distinct features of Hsp70 and Hsp90 interactions with HSF1 lead to another question, i.e. what is the mechanism behind attenuation of the inducible hsp90 transcription during continuous exposure to heat stress? Although the synthesis and accumulation of Hsp90 are not further enhanced by combined treatment with TPA and heat shock as compared with heat shock alone, the induction and attenuation of hsp90 transcription are equally accelerated as in the case of hsp70, suggesting that Hsp70 could also be involved in the regulation of other HSF1 target genes, in addition to hsp70.

The complex regulation of eukaryotic heat shock gene transcription is reflected by the presence of binding sites for transcription factors other than HSFs in the heat shock gene promoters. For example, the hsp70 promoter contains several consensus elements such as TATA, GC, CCAAT, AP-2, and
activating transcription factor- or AP-1-like elements (67, 68, 69). These elements are involved in basal expression of the hsp70 gene and mediate the induction in response to nonclassical stress stimuli such as serum (70) and adenovirus E1a (69). In addition, the basal promoter elements have been shown to be required for maximal stress-induced transcription of the hsp70 gene (71). The involvement of basal transcription factor activities in the TPA-enhanced heat shock response cannot be excluded, although TPA alone is not capable of inducing transcription of heat shock genes in K562 cells. The question of a synergism between the basal transcription factors and HSFI1 in TPA-treated heat-shocked cells needs to be addressed in the further studies.

Although activation of PKC in the present study has been achieved by artificial means, the results obtained are highly relevant in terms of the wide spectrum of cellular processes regulated by PKC. PKC-mediated signaling pathways are used by a number of different cell membrane receptors that can trigger either cell growth or differentiation in various cellular systems. It is plausible that some of these processes could involve a modified stress response as a consequence of activated PKC-responsive signaling pathways. It is well established that PKC is a family of several different isoenzymes with specific regulatory functions, and the specificity is likely to be achieved by the differential activation of PKC isoenzymes. It remains to be determined which PKC isoenzyme(s) is involved in the regulation of the heat shock response.

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