Conventional and novel PKC isoenzymes modify the heat-induced stress response but are not activated by heat shock

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SUMMARY

In mammalian cells, the heat-induced stress response is mediated by the constitutively expressed heat shock transcription factor 1 (HSF1). Upon exposure to elevated temperatures, HSF1 undergoes several post-translational modifications, including inducible phosphorylation or hyperphosphorylation. To date, neither the role of HSF1 hyperphosphorylation in regulation of the transcriptional activity of HSF1 nor the signaling pathways involved have been characterized. We have previously shown that the protein kinase C (PKC) activator, 12-O-tetradecanoylphorbol 13-acetate (TPA), markedly enhances the heat-induced stress response, and in the present study we elucidate the mechanism by which PKC activation affects the heat shock response in human cells. Our results show that several conventional and novel PKC isoenzymes are activated during the TPA-mediated enhancement of the heat shock response and that the enhancement can be inhibited by the specific PKC inhibitor bisindolylmaleimide I. Furthermore, the potentiating effect of TPA on the heat-induced stress response requires an intact heat shock element in the *hsp70* promoter, indicating that PKC-responsive pathways are able to modulate the activity of HSF1. We also demonstrate that PKC is not activated by heat stress per se. These results reveal that PKC exhibits a significant modulatory role of the heat-induced stress response, but is not directly involved in regulation of the heat shock response.

Key words: HSF1, Heat shock response, PKC

INTRODUCTION

Stimuli associated with environmental stresses such as elevated temperatures and various physiological stress conditions induce a well-conserved cellular defense mechanism resulting in an increased synthesis of heat shock proteins (Hsps) (for a review, see Lindquist, 1988). Expression of heat shock genes is regulated at the transcriptional level by the specific heat shock transcription factors (HSFs), which upon activation bind to the heat shock element (HSE) present in the promoter region of heat shock genes (for a review, see Wu, 1995). To date, four HSFs (HSF1-4) have been cloned in vertebrates (Rabindran et al., 1991; Sarge et al., 1991; Schuetz et al., 1991; Nakai and Morimoto, 1993; Nakai et al., 1997), of which HSF1 is the predominant stress-activated factor (Baler et al., 1993; Sarge et al., 1993). In non-stressed mammalian cells, HSF1 is constitutively expressed as a non-DNA-binding monomer phosphorylated predominantly on serine residues (Larson et al., 1988, 1995; Sarge et al., 1993; Cotto et al., 1996; Chu et al., 1996; Kline et al., 1997). Although not all of the constitutive phosphorylation sites of HSF1 have been identified, recent studies suggest that constitutive phosphorylation of Ser-303/307 is involved in repressing the transcriptional activity of HSF1 under non-

stressful conditions and perhaps under recovery from stress (Chu et al., 1996; Knauf et al., 1996; Kline et al., 1997; Xia et al., 1998). Upon exposure to heat stress, HSF1 undergoes trimerization, localizes to the nucleus, and acquires DNA-binding and transcriptional activity (Baler et al., 1993; Sarge et al., 1993). Simultaneously, HSF1 becomes inducibly phosphorylated or hyperphosphorylated on serine residues (Larson et al., 1988, 1995; Jurivich et al., 1995; Chu et al., 1996; Cotto et al., 1996; Kline et al., 1997), but so far neither the kinase(s) involved in the regulation of HSF1 hyperphosphorylation nor the heat-inducible phosphorylation sites have been identified. Furthermore, the exact role of HSF1 hyperphosphorylation is unclear, although there is increasing evidence that HSF1 hyperphosphorylation may be involved in regulation of the transcriptional activity. For example, the yeast *Saccharomyces cerevisiae* HSF, which, unlike other eukaryotic HSFs, is constitutively bound to the HSE but transcriptionally inactive prior to heat shock, becomes hyperphosphorylated and transcriptionally active upon exposure to heat stress (Sorger and Pelham, 1988). In addition, oxidative stress induces HSF phosphorylation and HSF-dependent activation of the *Saccharomyces cerevisiae metallothionein* gene transcription (Liu and Thiele, 1996). In mammalian cells, treatment with arachidonate induces DNA-

binding activity, hyperphosphorylation of HSF1 and activation of *hsp70* gene transcription (Jurivich et al., 1994), whereas treatment with the anti-inflammatory drugs indomethacin or sodium salicylate induces HSF1 DNA-binding but fails to induce HSF1 hyperphosphorylation and transcription of *hsp70* (Jurivich et al., 1992, 1995; Lee et al., 1995; Cotto et al., 1996). Hence, these studies, supported by others (Larson et al., 1988; Green et al., 1995; Elia et al., 1996; Xia and Voellmy, 1997), show that the activation of HSF1 is a multistep process, where HSF1 hyperphosphorylation is not needed for DNA-binding activity, but may be involved in a subsequent step in the transcriptional activation of HSF1.

In contrast to the HSF-mediated regulation of heat shock gene transcription, the mechanisms by which the cells sense a rise in temperature leading to activation of the effector machinery of the stress response are not well understood. A number of in vitro studies conducted with recombinant HSF1 imply that the mechanism controlling conversion of monomers to trimers is inherent in the structure of the HSF1 polypeptide (Rabindran et al., 1993; Zuo et al., 1994; Goodson and Sarge, 1995; Farkas et al., 1998). However, whether HSF1 alone is sufficient to trigger the stress response or whether other proteins are involved in the physiological regulation of HSF1 is still unknown. An increasing number of studies indicate that protein phosphorylation might be involved in regulation of the stress response and, indeed, heat shock has been reported to activate several initiator and effector protein kinases of different signaling cascades, including stress-activated protein kinase or Jun N-terminal protein kinase (SAPK/JNK; Adler et al., 1995; Kim et al., 1997), p38/HOG1/Mpk2 (Rouse et al., 1994), mitogen-activated protein kinase (MAPK; Dubois and Bensaude, 1993; Kamada et al., 1995; Mivechi and Giaccia, 1995; Kim et al., 1997), protein kinase C (PKC; Wooten, 1991; Ritz et al., 1993), phosphatidylinositol 3-kinase (PI 3-kinase; Lin et al., 1997), RAC-protein kinase (RAC-PK/PKB/Akt; Konishi et al., 1996), and ribosomal S6 kinases (Jurivich et al., 1991). In addition, signaling pathways, involving Ras (Engelberg et al., 1994), phospholipases A_2 and C (Calderwood and Stevenson, 1993; Jurivich et al., 1996), and the epidermal growth factor receptor (EGFR; Lin et al., 1997) have been shown to be stimulated upon heat stress. How the activation of these different signaling pathways is coupled to the HSF1 activation and induction of the stress response remains to be elucidated.

We have previously shown that activation of the PKC-responsive pathways by the phorbol ester TPA markedly enhances the heat-induced stress response in human cell lines (Holmberg et al., 1997). Also other studies support involvement of PKC in regulation of the stress response (Erdos and Lee, 1994; Lee et al., 1994a,b; Yamamoto et al., 1994; Jacquier-Sarlin et al., 1995). However, it is not known whether PKC participates specifically in the pathway(s) leading to the heat shock response. Furthermore, involvement of the distinct isoenzymes of the PKC family in the heat shock response has not been characterized. The 11 members of the serine/threonine PKC family are divided into three groups: (1) the conventional PKCs (cPKC-α, -βI, -βII, -γ), which are regulated by calcium and diacylglycerol; (2) the calcium-independent but diacylglycerol-dependent novel PKCs (nPKC-δ, -ε, -η, -θ); (3) the atypical PKCs (aPKC-ζ, -ι/λ), which are unresponsive to diacylglycerol and calcium and, in contrast to

the cPKCs and nPKCs, do not respond to phorbol esters. The PKC-μ is distinctive from the other PKCs and shows features characteristic of the Ca^{2+}-calmodulin-dependent protein kinase family (for a review, see Newton, 1997).

In the current study, we have investigated the mechanisms by which TPA exerts its action on the heat shock response. Specifically, as there is substantial evidence indicating a relationship between the heat shock response and the PKC signaling pathways, we wanted to elucidate in detail whether heat shock per se activates the PKC-responsive signaling pathways and if these signals become amplified upon exposure to TPA, leading to enhancement of the heat shock response. Our studies show that PKC is not directly involved in the stress response but has a potent modulatory role.

MATERIALS AND METHODS

Cell culture, treatment conditions, and preparation of cell extracts

Human K562 erythroleukemia cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2. HeLa cervical carcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. K562 cells were seeded at 5×10^6 cells per 10-cm diameter plate prior to exposure to heat shock or other treatments. For heat shock treatments, plates were sealed with Parafilm and exposed to 42°C in a constant-temperature water bath. The cells were preincubated for 15 minutes with 1 μM bisindolylmaleimide I (LC Laboratories) when used in combination with heat shock or 12-O-tetradecanoylphorbol 13-acetate (TPA, Sigma). Whole cell extracts (Mosser et al., 1988), nuclear and cytosolic fractions (Dignam et al., 1983) for analysis of HSF1, and particulate and cytosolic fractions for analysis of PKC (Kass et al., 1989) were prepared as described previously.

Transfections

HeLa cells were transiently transfected with 20 μg of LSN-WT or LSPN-WT constructs containing a complete and an incomplete proximal HSE of the human *hsp70* promoter, respectively, fused to the *chloramphenicol acetyltransferase* (*CAT*) reporter gene (Williams et al., 1989; Williams and Morimoto, 1990), using the calcium phosphate method (Graham and van der Eb, 1973). Briefly, HeLa cells were plated at a density of 10^6 cells per 10-cm diameter plate and incubated overnight with DNA-calcium phosphate coprecipitates. Cells were washed twice with 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N,N′-tetraacetic acid (EGTA) in phosphate-buffered saline (PBS) and fresh medium was added. After 2 days the cells were subjected to heat shock in the presence or absence of TPA for 1 hour followed by 3 hours incubation at 37°C. A β-galactosidase-expressing plasmid (pSV-β-Galactosidase, Promega) or a CD20 DNA construct (pCDNA3-CD20, a kind gift from Dr T. P. Mäkelä, University of Helsinki, Finland) was used to monitor transfection efficiencies. CAT assays were performed on 50-200 μg of total cell extract and analyzed with thin-layer chromatography according to the Promega protocol. The β-galactosidase activities were measured spectrophotometrically as described previously (Vihinen et al., 1993), and CD20 was detected by incubating live cells with a fluorescein-conjugated mouse monoclonal antibody (Becton Dickinson).

Gel mobility shift assay

Whole cell extracts (15 μg) were incubated with a ^{32}P-labeled oligonucleotide corresponding to the proximal HSE of the human *hsp70* promoter, and the protein-DNA complexes were analyzed on a

native 4% polyacrylamide gel as described previously (Mosser et al., 1988). The synthetic oligonucleotide was ^{32}P-labeled with T4 polynucleotide kinase (Promega). The protein-DNA complexes were analyzed with a PhosphoImager (Bio-Rad).

Northern blot analysis

Total RNA was isolated using RNAzol^{TM}B (Tel-Test Inc.). 10 μg of RNA was separated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond-N, Amersham), and hybridized with [α-^{32}P]dCTP-labeled probes for human *hsp70* (Wu et al., 1895) and rat *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*; Fort et al., 1985) according to the manufacturer's instructions. The plasmid DNA was ^{32}P-labeled using a nick translation kit (Promega). The levels of *hsp70* and *GAPDH* mRNA were quantified using a Bio-Rad PhosphoImager.

Western blot analysis and limiting-pore-size gel electrophoresis

Whole cell extracts (10-20 μg) were subjected to 8% or 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for analysis of HSF1 and heat shock proteins or of PKC, respectively, and transferred to nitrocellulose membrane (Schleicher & Schuell) by using a semi-dry transfer apparatus (Bio-Rad). The equal loading of proteins was checked by using Ponceau S staining (Sigma). Western blot analysis was performed using rabbit polyclonal antibodies to HSF1 (Sarge et al., 1993), mouse monoclonal antibodies to Hsp70 (4g4, Affinity Bioreagents, Inc.), and rat monoclonal antibodies to Hsc70 (SPA-815, StressGen). Antibodies to PKC were either polyclonal, raised against specific peptides in sheep against PKC-α, -βI, -βII, -δ, -ε, -γ, -η, -ι and -ζ (The Binding Site Ltd; Griffiths et al., 1996) or mouse monoclonal antibodies against PKC-α, -β, -δ, -ε and -θ purchased from Transduction Laboratories. Polyclonal antibodies against phosporylated cAMP-responsive element binding protein (PCREB) were a generous gift from Dr M. E. Greenberg (Harvard Medical School, MA, USA; Ginty et al., 1993). Horseradish peroxidase-conjugated anti-rabbit, anti-sheep, anti-mouse and anti-rat antibodies purchased from Promega, The Binding Site and Amersham, respectively, were used as secondary antibodies. The blots were developed by using an enhanced chemiluminescence method (Amersham). Limiting-pore-size gel electrophoresis was performed as described by Hardy and co-workers (1997). Briefly, 50 μg of whole cell extracts were resolved by 5%-20% gradient native PAGE in TGE (40 mM Tris, pH 7.4, 200 mM glycine, 2.4 mM EDTA, pH 8.0) at a constant voltage (13 V/cm) for 20 hours at 4°C. The native proteins were transferred to a nitrocellulose membrane and immunoblotted as described above.

RESULTS

Bisindolylmaleimide I inhibits the TPA-mediated enhancement of the heat shock response

We have previously reported that the heat-induced stress response can be markedly enhanced by TPA, although TPA alone does not induce the heat shock response in K562 and HeLa cells (Holmberg et al., 1997). To further elucidate the role of PKC activation in the heat shock response, we used the specific PKC inhibitor bisindolylmaleimide I (BIM; Toullec et al., 1991). K562 cells were pretreated with 1 μM BIM for 15 minutes and exposed to heat shock (42°C) or combined treatment with heat shock and TPA (3-10 nM) for various time periods, and the accumulation of Hsp70 was analyzed by western blot analysis. As shown earlier (Holmberg et al., 1997), combined treatment with TPA and heat shock induced an increased accumulation of Hsp70 as compared to the

accumulation of Hsp70 in cells exposed to heat shock alone (Fig. 1A). Here, we show that BIM abolished the TPA-mediated increase in heat-induced accumulation of Hsp70, returning it to a similar level as detected in heat-shocked cells (Fig. 1A). BIM neither induced Hsp70 accumulation nor affected the heat-induced accumulation of Hsp70 (Fig. 1A). To determine if BIM was affecting pre-translational steps in the TPA-mediated enhancement of the heat shock response, the steady-state levels of *hsp70* mRNA were analyzed using northern blotting. Fig. 1B shows that the enhanced increase in *hsp70* mRNA in TPA-treated heat-shocked cells was inhibited by BIM. In agreement with the protein data, BIM did not prevent the heat-induced increase in *hsp70* mRNA, which was induced to the same level as without pretreatment with BIM (Fig. 1B,C).

To investigate if the PKC inhibitor BIM abolished the TPA-mediated effect on the heat shock response at the level of HSF1 activation, the DNA-binding activity and the phosphorylation state of HSF1 were analyzed. The HSF1 DNA-binding activity, as measured by gel mobility shift assay, attenuated after 3 hours exposure to continuous heat shock, whereas HSF1 returned to its non-DNA-binding form by 3 hours in cells treated with both TPA and heat shock (Fig. 1D; Holmberg et al., 1997). This faster attenuation was partly abolished in the presence of BIM, since HSF1 DNA-binding activity was readily detectable at a 3-hour time point. Similar results were obtained in four independent experiments, i.e. the DNA-binding activity at 3 hours was consistently lower in cells pretreated with BIM and exposed to both TPA and heat shock than in cells exposed to heat shock alone. Treatment with BIM alone did not induce HSF1 DNA-binding activity (Fig. 1D). We next studied the hyperphosphorylation of HSF1, which can be detected as a band migrating slower on SDS-PAGE compared to HSF1 from untreated cells (Sarge et al., 1993; Cotto et al., 1996). In agreement with our previous results (Holmberg et al., 1997), the hyperphosphorylated form of HSF1 is predominant at earlier time points in cells exposed to both TPA and heat shock, than in cells exposed to heat shock alone, i.e. HSF1 hyperphosphorylation can be detected after 15 minutes of combined treatment with TPA and heat shock, whereas it is detected after 1 hour in only heat-shocked cells (Fig. 1E). Pretreatment with BIM prevented hyperphosphorylation of HSF1 induced by 15 minutes treatment with TPA and heat shock (Fig. 1E). Taken together, our results show that by using the specific PKC inhibitor BIM, the TPA-mediated enhancement of the heat shock response can be prevented, indicating the involvement of PKC in this modified heat shock response. Similarly, pretreatment with TPA for 6 hours before exposing the cells to heat shock abolished the TPA-mediated enhancement of the heat shock response (data not shown). Under these conditions PKC will be downregulated, suggesting that PKC downregulation would have the same affect as BIM.

TPA induces translocation of conventional and novel PKC isoenzymes in heat-shocked cells

To further study the mechanism by which PKC is involved in regulation of the heat shock response, we examined which PKC isoenzymes were activated in the TPA-mediated enhancement of the heat shock response. First, we investigated which PKC isoenzymes are expressed in K562

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B

C

D
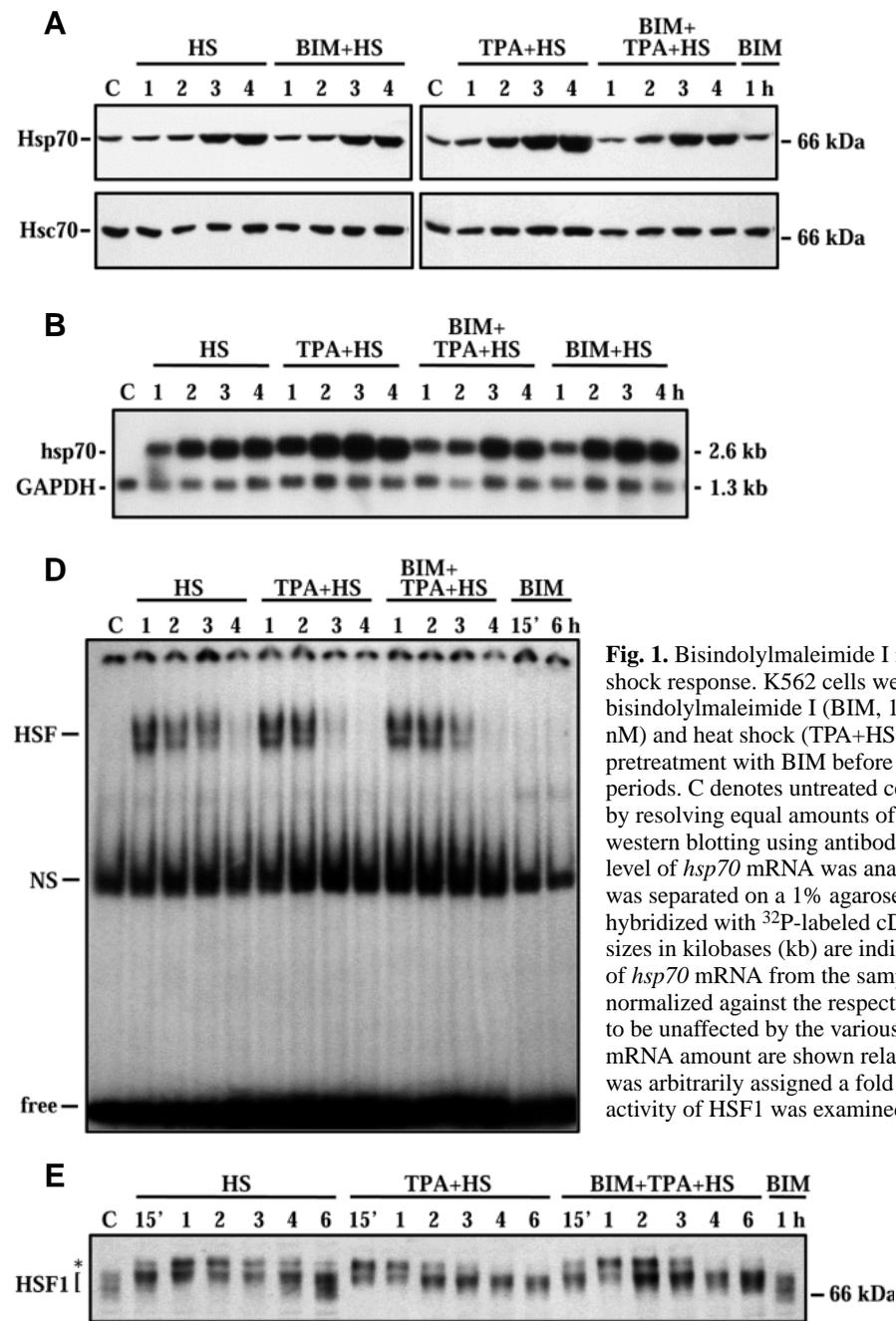
Fig. 1. Bisindolylmaleimide I inhibits the TPA-mediated enhancement of the heat shock response. K562 cells were exposed to heat shock (HS, 42°C), bisindolylmaleimide I (BIM, 1 μM), BIM and heat shock (BIM+HS), TPA (3 nM) and heat shock (TPA+HS), or BIM, TPA and heat shock (15 minutes pretreatment with BIM before exposure to TPA+HS) for the indicated time periods. C denotes untreated cells. (A) The accumulation of Hsp70 was analyzed by resolving equal amounts of protein (10 μg) by 8% SDS-PAGE followed by western blotting using antibodies against Hsp70 and Hsc70. (B) The steady-state level of *hsp70* mRNA was analyzed by northern blot analysis. Total RNA (10 μg) was separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled cDNA probes for *hsp70* and *GAPDH*. The mRNA sizes in kilobases (kb) are indicated on the right. (C) Quantification of the levels of *hsp70* mRNA from the samples shown in B. The *hsp70* mRNA values were normalized against the respective *GAPDH* mRNA values, which were presumed to be unaffected by the various treatments. Values for the fold induction of *hsp70* mRNA amount are shown relative to the amount in the control sample, which was arbitrarily assigned a fold induction value of 1. (D) The DNA-binding activity of HSF1 was examined by subjecting 15 μg of whole cell extracts to gel mobility assay using an HSF-specific ^{32}P-labeled oligonucleotide probe (HSE). HSF, the inducible HSF-HSE complex; NS, non-specific DNA-binding activity; free, unbound HSE oligonucleotide. (E) The phosphorylation state of HSF1 was analyzed by western blot analysis with antibodies against HSF1. The asterisk indicates hyperphosphorylated HSF1.

E

cells by western blot analysis using specific antibodies against the distinct PKC isoenzymes. Our analysis revealed that K562 cells expressed PKC-α, -βII, -δ, -ε, -ι, -θ and -ζ (Fig. 2). The detection of PKC-α, -βII, -ε, -ι and -θ is in accordance with earlier reports (Hocevar et al., 1992; Baier et al., 1994; Zheng et al., 1994; Murray and Fields, 1997), but PKC-δ and -ζ have not been detected previously in K562 cells (Zheng et al., 1994; Murray and Fields, 1997). The difference in detection is probably due to the use of different antibodies. No expression of PKC-βI, -γ or -η was detected in K562 cells (data not shown; Hocevar et al., 1992; Zheng et al., 1994).

To examine activation of PKC, translocation of PKC isoenzymes between the cytosolic and particulate fractions was determined by western blot analysis. As shown in Fig. 3, PKC-βII, PKC-ε and a major fraction of PKC-α were detected in the cytosolic fraction in untreated and heat-shocked cells. Upon exposure to TPA alone, or to both TPA and heat shock for 15 minutes, these isoenzymes were translocated to the particulate fraction. PKC-δ and PKC-θ were predominantly localized to the particulate fraction both in untreated and treated cells. TPA treatment for up to 6 hours did not affect expression of the cPKCs and nPKCs, but the combined treatment with TPA and heat shock decreased the level of these PKCs (data not shown). Localization and expression of the TPA-non-responsive aPKCs, PKC-ι and PKC-ζ, were not affected by treatment with TPA, heat shock or the combined treatment with TPA and heat shock (Fig. 3).
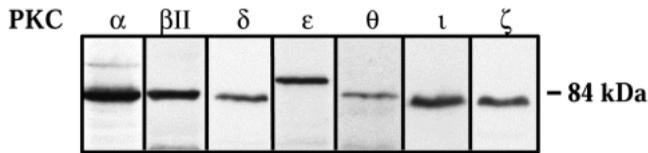
Fig. 2. Expression of PKC isoenzymes in K562 cells. Equal amounts of K562 cells (2×10^5 cells) in Laemmli buffer were subjected to 10% SDS-PAGE followed by western blot analysis with specific antibodies against the distinct PKC isoenzymes. Note that the blots are not equally exposed.

Enhancement of the heat shock response by TPA is mediated through HSE

Although we could not detect any significant activation of PKC isoenzymes upon exposure to heat shock (Fig. 3) or inhibit the heat-induced stress response by the specific PKC inhibitor BIM (Fig. 1), there is a marked TPA-mediated enhancement of the heat shock response (Fig. 1; Holmberg et al., 1997). Therefore, we wanted to establish the mechanism by which the effect of TPA is coupled to the heat shock response, i.e. if the TPA-mediated enhancement involves the specific HSF-binding sequence HSE found in the promoters of heat shock genes (Amin et al., 1988; Xiao and Lis, 1988). In addition to the HSE, the *hsp70* promoter contains several consensus elements (e.g. CCAAT, GC, TATA), which mediate the basal *hsp70* gene expression and the induction of *hsp70* in response to non-classical stress stimuli such as serum and adenovirus E1a (Wu and Morimoto, 1985; Morgan et al., 1987; Morgan, 1989; Williams et al., 1989). To investigate whether the effect of TPA on the heat shock response is mediated via HSE or via the basal elements, HeLa cells were transiently transfected with different constructs of the *hsp70* promoter fused to the *CAT* reporter gene. The LSN-WT construct contains the *hsp70* promoter up to −188, whereas the LSPN-WT-construct ends at −100, lacking part of the proximal HSE located at −91 to −105, and is therefore not responsive to elevated temperatures
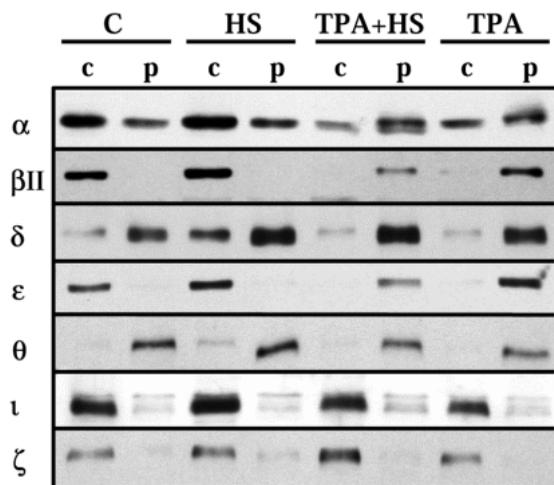
Fig. 3. Translocation of cPKCs and nPKC upon combined treatment with TPA and heat shock. Cytosolic (c) and particulate fractions (p) were prepared from K562 cells exposed for 15 minutes to heat shock (HS, 42°C), TPA (TPA, 10 nM) or combined treatment with TPA and heat shock (TPA+HS). The C above the fraction labels of lanes 1 and 2 corresponds to samples from untreated cells.
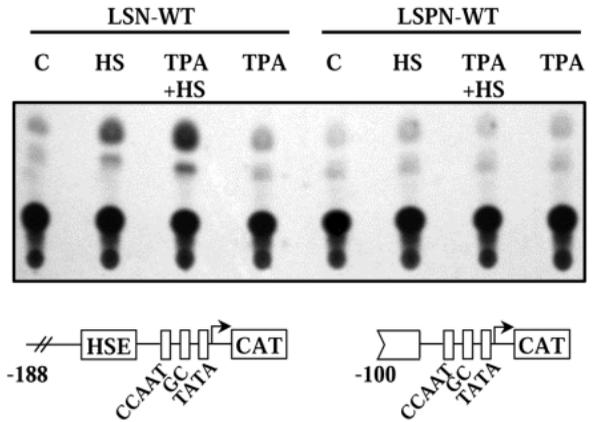

Fig. 4. The TPA-mediated enhancement of the heat shock response requires an intact HSE. HeLa cells were transiently transfected with 20 µg of LSN-WT or LSPN-WT constructs, containing a complete and an incomplete proximal HSE of the human *hsp70* promoter, respectively, fused to the *chloramphenicol acetyltransferase* (*CAT*) reporter gene as schematically shown. The transfected cells were exposed to heat shock (HS) or combined treatment with TPA and heat shock (TPA+HS) for 1 hour at 42°C followed by 3 hours at 37°C, or exposed to TPA alone (TPA, 10 nM) for 4 hours. C denotes untreated transfected cells. CAT assays were performed and a thin-layer chromatogram from one representative experiment is shown.

(Williams et al., 1989; Williams and Morimoto, 1990). Upon exposure to heat shock, an increase in the CAT activity was detected in cells transfected with LSN-WT (Fig. 4). The increase was further enhanced in cells treated with both TPA and heat shock, whereas treatment with TPA alone did not induce CAT activity above the control level. In cells transfected with the LSPN-WT construct, no increase in the CAT activity was detected upon exposure to the various treatments, showing that the TPA-mediated enhancement of *hsp70* gene expression requires an intact HSE (Fig. 4).

## TPA neither induces trimerization nor nuclear localization of HSF1

Having demonstrated that the TPA-enhanced heat shock response is mediated through interaction between HSF1 and HSE, we wanted to examine how TPA affects HSF1. We have previously shown that treatment with TPA alone does not induce HSF1 DNA-binding activity (Holmberg et al., 1997), but it is possible that TPA affects the oligomerization state or the intracellular localization of HSF1, thereby facilitating the activation of this transcription factor. Analysis of the oligomeric state of HSF1 using limiting-pore-size gel electrophoresis and immunoblotting with antibodies against HSF1 revealed, however, no difference in the size of HSF1 in TPA-treated cells as compared to control cells (Fig. 5A). Upon exposure to heat shock or to combined treatment with TPA and heat shock, similar high molecular mass complexes, corresponding to trimerized HSF1 (Baler et al., 1993; Sarge et al., 1993; Hardy et al., 1997), were detected (Fig. 5A).

For the localization studies, cytosolic and nuclear fractions from K562 cells exposed to the various treatments were prepared and analyzed by western blot analysis. In agreement with previous reports (Baler et al., 1993; Sarge et al., 1993; Sistonen et al., 1994), our results show that the major part of

A

B
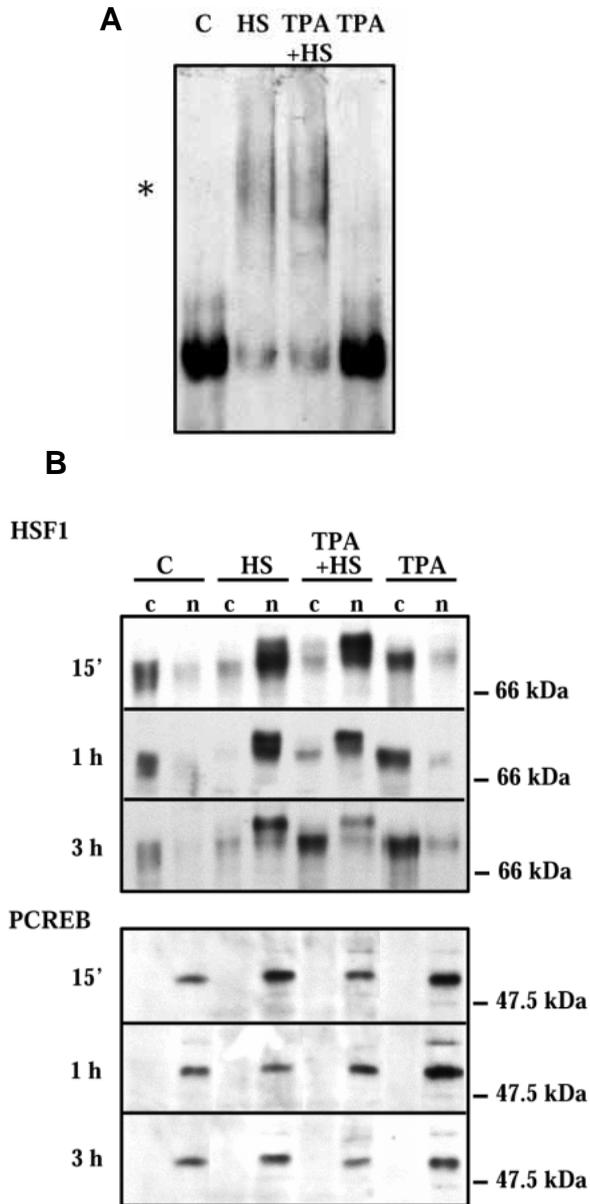
Fig. 5. TPA alone does not affect oligomerization or intracellular localization of HSF1. (A) The oligomeric state of HSF1 was analyzed by limiting-pore-size electrophoresis. Whole cell extracts (50 μg) from untreated K562 cells (C) or from cells exposed to heat shock (HS, 42°C), TPA (TPA, 10 nM), or combined treatment with TPA and heat shock (TPA+HS) for 15 minutes were resolved by 5%-20% gradient native PAGE and analyzed by western blotting with antibodies against HSF1. The asterisk indicates the trimeric state of HSF1. (B) To examine the localization of HSF1, cytosolic (c) or nuclear fractions (n) were prepared from cells exposed to the various treatments described in A for the indicated time periods. Equal amounts of protein (12 μg) were analyzed by western blotting using antibodies against HSF1 (upper panel) and PCREB (lower panel).

HSF1 was found in the cytosolic fraction of the untreated cells, and upon exposure to heat shock HSF1 was localized to the nucleus (Fig. 5B). Similarly, combined treatment with TPA and heat shock induced nuclear localization of HSF1. However, unlike heat-shocked cells, where HSF1 was still nuclear after 3 hours exposure to heat shock, HSF1 was relocalized to the
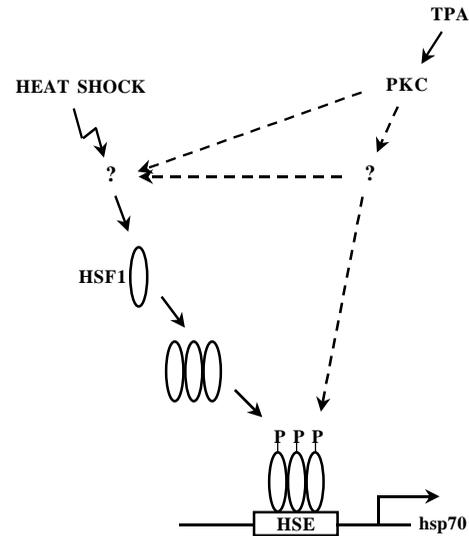
Fig. 6. Model of the synergistic action of HSF1 and PKC. Upon exposure to heat shock, a signal, which does not involve PKC, triggers the activation machinery of HSF1. However, the activation of PKC-responsive signaling pathways, for example by TPA treatment, is able to enhance the heat shock response. TPA does not affect the trimerization of HSF1, but enhances its transcriptional activity, resulting in increased expression of the *hsp70* gene. The dotted lines indicate putative routes for the TPA-mediated effect, and P indicates hyperphosphorylated HSF1.

cytosol by 3 hours of continuous treatment with TPA and heat shock (Fig. 5B). The faster relocalization of HSF1 detected in TPA-treated heat-shocked cells shows a close correlation with the attenuation of DNA-binding activity observed in these cells (Fig. 1D). Upon nuclear localization, the hyperphosphorylated state of HSF1 was detected after 1 hour of heat shock, whereas HSF1 hyperphosphorylation was already predominant after 15 minutes in cells exposed to both TPA and heat shock (Fig. 5B). Simultaneously with relocalization to the cytosol, HSF1 was dephosphorylated to a form migrating slightly slower than the control form, but faster than the hyperphosphorylated form, indicating the existence of a putative intermediate phosphorylation state of HSF1. According to the results from the fractionation experiments, hyperphosphorylated HSF1 was found only in the nuclear fraction and not in the cytosolic fraction. In addition, an intermediate phosphorylated state of HSF1 was detected in the nuclear fraction from cells exposed to heat shock for 15 minutes. Treatment with TPA alone neither induced nuclear localization nor hyperphosphorylation of HSF1; however, a putative intermediate phosphorylated form of HSF1 was detected in TPA-treated cells (Fig. 5B; Holmberg et al., 1997). To monitor the possible leakage of soluble nuclear proteins into the cytosolic fractions during the biochemical fractionation, the nuclear localized transcription factor PCREB (Ginty et al., 1993) was used as a control. As shown in Fig. 5B, PCREB was found only in the nuclear fractions.

DISCUSSION

Results from several studies employing agents that affect PKC activity have indicated that PKC-responsive signaling

pathways might be involved in regulation of the heat shock response (Erdos and Lee, 1994; Lee et al., 1994a,b; Yamamoto et al., 1994; Jacquier-Sarlin et al., 1995; Holmberg et al., 1997). However, the specific relationship between PKC activation and induction of the heat shock response has not been investigated in these published studies. We have previously shown that the phorbol ester TPA markedly enhances the heat-induced stress response (Holmberg et al., 1997). As TPA is a well-established PKC activator (for a review, see Kuo, 1994), a possible explanation for the enhancement is that TPA stimulates a heat-induced PKC activity. Alternatively, the TPA-mediated PKC activity could stimulate some yet undefined heat-induced signaling pathways. Both alternatives could plausibly give rise to the enhanced transcription of the *hsp70* gene and subsequent accumulation of Hsp70 (Fig. 1; Holmberg et al., 1997).

While PKC has been implicated to affect the stress response, it has not been determined whether PKC is directly involved in the activation of heat shock gene expression or whether its role is modulatory. To determine whether PKC has a direct or indirect role in regulation of the heat shock response, we examined the TPA-induced effects in greater detail, including experiments with the specific PKC inhibitor BIM. Our results show that BIM inhibits the enhanced accumulation of *hsp70* mRNA and Hsp70 protein in TPA-treated heat-shocked K562 cells. Furthermore, the inhibitory effect of BIM can be detected at the transcriptional level, i.e. BIM prevents the faster attenuation of HSF1 DNA-binding activity and the faster hyperphosphorylation of HSF1 induced by TPA in heat-shocked cells. These results corroborate the assumption of a PKC-mediated modulation of the stress response. However, although BIM abolishes the TPA effect on the heat shock response, this PKC inhibitor does not affect the heat-induced stress response. Analysis of PKC activation, based on translocation of the seven distinct PKC isoenzymes expressed in K562 cells, reveals that TPA activates several PKCs, such as the cPKCs, PKC-α and PKC-βII, and PKC-ε belonging to the nPKC group, both in the presence and absence of heat shock. In contrast, we were not able to detect translocation of any PKC upon exposure to heat stress alone. Taken together the results from our different experiments show that: (1) the heat shock response is not inhibited by BIM; (2) TPA alone does not induce the heat shock response (Holmberg et al., 1997); (3) heat shock does not induce translocation of PKC isoenzymes between cytosolic and particulate fractions. We conclude that PKC is not activated upon heat shock in K562 cells and that PKC activation is not required for a heat shock response. Hence, the role of PKC seems to be modulatory rather than direct.

It is of interest to determine the target of PKC activation in the initiator, mediator or effector machinery of the heat shock response. We have recently shown that TPA neither induces HSF1 DNA-binding activity nor heat shock gene transcription in K562 or HeLa cells, whereas TPA markedly enhances the heat-induced stress response at the transcriptional level (Holmberg et al., 1997). In the current study, we demonstrate that the potentiating effect of TPA on the heat shock response is not mediated by the basal consensus elements present in the *hsp70* promoter, but requires an intact HSE. As the HSE functions as the binding site for HSF and the transcriptional activity of HSF1 is mediated through interaction between

HSF1 and HSE (for a review, see Wu, 1995), it is most likely that TPA-stimulated PKC signaling pathway(s) affects the heat shock response at the initiator level, i.e. through HSF1. In contrast to our results, there are studies showing that the heat-induced accumulation of *hsp70* mRNA could be prevented by various kinase inhibitors (e.g. H-7, staurosporine, calphostin C; Lee et al., 1994b; Yamamoto et al., 1994). This difference is likely to be a consequence of variability in the specificity of the used inhibitors and in the experimental set-up.

Activation of HSF1 is a multistep process, involving trimerization, nuclear localization, acquisition of DNA-binding activity and hyperphosphorylation (Larson et al., 1988, 1995; Baler et al., 1993; Sarge et al., 1993; Jurivich et al., 1995; Chu et al., 1996; Cotto et al., 1996; Kline et al., 1997). These steps are independent since acquisition of DNA-binding activity does not necessarily lead to transcriptional activity of HSF1 (Jurivich et al., 1992, 1995; Lee et al., 1995; Cotto et al., 1996). In this study, we show that activation of PKC by TPA does not affect the oligomeric state or the intracellular localization of HSF1. Instead, a putative intermediate phosphorylated state of HSF1 occurs in TPA-treated cells (Fig. 5B; Holmberg et al., 1997), suggesting that PKC activation might affect and modify HSF1, without being able to activate this factor. There is increasing evidence supporting a role of hyperphosphorylation as a regulator of HSF1 transcriptional activity (Larson et al., 1988; Sorger and Pelham, 1988; Jurivich et al., 1994, 1995; Lee et al., 1995; Cotto et al., 1996; Elia et al., 1996; Liu and Thiele, 1996; Xia and Voellmy, 1997). Heat stress has been reported to induce putative intermediate phosphorylated states of HSF1 before the fully hyperphosphorylated state is obtained (Elia et al., 1996; Holmberg et al., 1997), implying that hyperphosphorylation of HSF1 might be a multistep process involving several protein kinases. This is supported by reports showing that both MAPK and glycogen synthase kinase 3 (GSK3) are involved in phosphorylating some of the known constitutively phosphorylated sites of HSF1, resulting in repression of HSF1 transcriptional activity at control temperatures (Chu et al., 1996; Knauf et al., 1996; Kline et al., 1997; Xia et al., 1998). It is tempting to speculate that PKC-responsive pathways might affect the activity of some of the putative HSF1 protein kinases, although it is also possible that pathways responding to PKC affect other putative HSF1 regulatory proteins than protein kinases. So far, the only reported HSF1 regulatory proteins are Hsp70 and the cochaperone Hdj1. These proteins function as negative regulators of the heat shock response, as they by binding directly to HSF1 repress the *hsp70* gene transcription during the attenuation phase of the heat shock response (Shi et al., 1998).

In Fig. 6, we propose a model of the relationship between PKC activation and induction of the heat shock response. While our results clearly show that PKC is not directly involved in activation of HSF1, simultaneous activation of the PKC-responsive pathways and the effector machinery involved in regulation of HSF1 activity leads to an enhanced heat shock response. Whether there is cross talk between these two signaling pathways or whether they separately affect HSF1 remains to be established. With respect to the physiological significance of our model system, it might mimic situations when a faster and more vigorous synthesis of the molecular chaperone Hsp70 is required for protection against stress

damage. This response could be beneficial for actively growing cells or cells undergoing differentiation, in which PKC is activated (for a review, see Glazer, 1994), to cope with the deleterious effects of misfolded and denatured proteins caused by various stress stimuli.

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