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Phosphorylation of Drosophila heat shock transcription factor

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Abstract The role that phosphorylation plays in regulating heat shock factor (HSF) function and activity has been the subject of several studies. Here, we demonstrate that Drosophila melanogaster HSF (DmHSF) is a phosphoprotein that is multiply phosphorylated at some sites and is dephosphorylated at others upon heat shock. However, the steady-state level of phosphorylation of Drosophila HSF remains unchanged after heat shock. Phosphoamino-acid analysis reveals that predominantly serine residues are phosphorylated for both the non-shocked and heat shocked molecules. Gel mobility shift assays using extracts from SL2 cells treated with a variety of phosphatase and kinase inhibitors show little or no effect on the heat shock induced DNA binding activity of HSF or on its recovery. We conclude that phosphorylation plays no significant role in regulating the heat induced DNA binding activity of Drosophila HSF.

INTRODUCTION

Elevated temperature, chemical and physiologic stresses all result in the heat shock response which involves the increased production of heat shock proteins (hsp) (Lindquist 1986; Lindquist and Craig 1988; Morimoto et al. 1994). Hsp gene expression in eukaryotes is regulated by heat shock transcription factor (HSF). In higher eukaryotes HSF1 is constitutively present in a non-activated, monomeric state and becomes activated by heat stress to a trimeric form capable of high affinity binding to a specific DNA response element (heat shock response element, HSE). Activation also leads to exposure of a transcriptional activation domain leading to increased transcription of hsp genes. The heat shock induced trimerization and acquisition of high affinity DNA binding appears to be separable from the heat shock induced activation of transcription (Hensold et al. 1990; Jurivich et al. 1992; Cotto et al. 1996). In budding yeast, the transcriptionally inactive form of HSF is substantially trimeric and bound to DNA; heat shock induces transcriptional activation (Jakobsen and Pelham 1988; Gross et al. 1990; Jakobsen and Pelham 1991). Exposure of higher eukaryotic cells to certain compounds such as antiinflammatory agents (indomethacin or salicylate) induces trimerization and high affinity DNA binding, but does not lead to increased transcription from hsp genes (Jurivich et al. 1992; Lee et al. 1995).

HSF has been cloned from numerous organisms including yeasts, plants, fruit flies, mouse, and human (reviewed in Wu 1995; Nover et al. 1996). The predicted protein size is quite variable, ranging from 301 amino acids for tomato HSF to 833 amino acids for *Saccharomyces cerevisiae*. All HSFs contain a highly conserved DNA binding domain near the amino terminus and an adjacent trimerization domain containing hydrophobic heptad repeats. In higher eukaryotes there is another conserved hydrophobic heptad repeat near the carboxy terminus. This carboxy terminal heptad repeat is proposed to negatively regulate oligomerization and maintain HSF in a monomeric and transciptionally inactive form under non-stressed conditions. The C-terminal transcriptional activation domains for HSF from several species have been identified including for *S. cerevisiae* (Nieto-Sotelo et al. 1990; Sorger 1990; Jakobsen and Pelham 1991; Bonner et al. 1992), tomato (Treuter et al. 1993; Nover et al. 1996), Drosophila (Rabindran et al. 1993; Wisniewski et al. 1996), and human (Shi et al. 1995; Zuo et al. 1995). These transcriptional activation domains are under negative regulation by other portions

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of HSF under non-stressed conditions (Nieto-Sotelo et al. 1990; Sorger 1990; Jakobsen and Pelham 1991; Bonner et al. 1992; Chen et al. 1993; Hoj and Jakobsen 1994; Green et al. 1995; Shi et al. 1995; Zuo et al. 1995; Wisniewski et al. 1996). Proposed mechanisms regulating the function of this activation domain include phosphorylation (Sorger and Pelham 1988; Nieto-Sotelo 1990; Hoj and Jakobsen 1994; Cotto et al. 1996; Knauf et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997) and interaction with molecular chaperone proteins leading to transcriptional repression (Boorstein and Craig 1990; Abravaya et al. 1992; Baler et al. 1992; Mosser et al. 1993; Schlesinger and Ryan 1993; Shi et al. 1998). There are also reports that overexpression of hsp can regulate the phosphorylation of HSF1 by activating protein phosphatases and inactivating protein kinase C activity (Ding et al. 1997 and 1998).

There is indirect evidence that there are factors present within eukaryotic cells that are required for suppressing the assembly of HSF trimers under non-stressed conditions. The intracellular environment affects the temperature at which heat shock induced activation of HSF occurs as demonstrated by the behavior of human HSF protein expressed in plant cells (Treuter et al. 1993), insect cells (Clos et al. 1993), and Xenopus oocytes (Baler et al. 1993; Zuo et al. 1994), as well as Arabidopsis HSF1 expressed in mammalian and insect cells (Hubel et al. 1995). Possibilities include interaction of HSF with hsp70 or another chaperone, or post-translational modification of HSF protein. Although HSF and hsp70 have been shown to associate under both non-stressed and heat-stressed conditions, overexpression of hsp70 accelerates HSF trimer dissociation (Price and Calderwood 1992; Mosser et al. 1993; Rabindran et al. 1994). In addition, recent evidence suggests that hsp70 and the cochaperone, Hdj1 interact with the transcriptional activation domain of human HSF1 to repress heat shock gene transcription (Shi et al. 1998).

The acquisition of stress induced high affinity DNA binding occurs via a monomer (non-stressed HSF form) to trimer transition (Perisic et al. 1989; Sorger and Nelson 1989; Westwood et al. 1991; Baler et al. 1993; Rabindran et al. 1993; Sarge et al. 1993; Westwood and Wu 1993; Sistonen et al. 1994). The trimeric form of HSF has a greater than 1000-fold increase in affinity for the HSE than the monomeric HSF DNA binding domain (Wu 1984; Wu 1985; Kingston et al. 1987; Sorger et al. 1987; Wu et al. 1987; Zimarino and Wu 1987; Taylor et al. 1991; Kim et al. 1995). Recent studies show that trimerization and DNA binding of purified Drosophila HSF can be directly and reversibly induced in vitro by high temperatures in the physiological range and by an oxidant, hydrogen peroxide, thus providing a physico-chemical mechanism for transduction of the heat stress signal (Zhong et al. 1998). In addition, hsp90 is also involved in maintenance of the monomeric state of human HSF1 (Zuo et al. 1998).

HSF is also known to be hyperphosphorylated on serine and threonine residues upon heat shock (Hoj and Jakobsen 1994; Cotto et al. 1996; Knauf et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997; Xia et al. 1998). Studies have demonstrated that for yeast and human HSF1 alterations in phosphorylation levels probably play no role in regulating trimerization or DNA binding (Sorger and Pelham 1988; Nieto-Sotelo et al. 1990; Cotto et al. 1996; Knauf et al. 1996; KlinE and Morimoto 1997; Xia and Voellmy 1997). However, a recent paper suggests that Arabidopsis HSF undergoes decreased DNA binding following phosphorylation by a specific cyclin dependent kinase (Reindl et al. 1997). The role of phosphorylation in regulating Drosophila HSF DNA binding activity has not been investigated.

Other reports suggest that phosphorylation of HSF may play a role in deactivation of transcriptional competence (Hoj and Jakobsen 1994; Mivechi et al. 1994; Mivechi and Giaccia 1995; Chu et al. 1996; Knauf et al. 1996; Kim et al. 1997; Kline et al. 1997; Chu et al. 1998; Xia et al. 1998). Several studies have demonstrated that serine/threonine protein kinase inhibitors (H7, staurosporine, 2-aminopurine, calphostin) inhibit hsp gene expression, whereas serine/threonine phosphatase inhibitors (okadaic acid and calyculin A) increase hsp gene expression in cells exposed to mild heat shock (Chang et al. 1993; Erdos and Lee 1994; Yamamoto et al. 1994; Xia and Voellmy 1997). It has been suggested that hyperphosphorylation directly leads to increased transcriptional activity of HSF (Xia and Voellmy 1997). However, a recent report indicated that heat shock induced dephosphorylation of a specific amino acid residue in HSF1 may be directly responsible for increased transcription (Xia et al. 1998).

In this study, we characterized the changes in phosphorylation between the non-stressed and heat stressed forms of DmHSF, and found that under steady-state conditions of ^{32}P labelling there is no heat shock-induced increase in phosphorylation of HSF in SL2 cells. Predominantly serine residues were phosphorylated in both non-shocked and heat shocked DmHSF, with very little threonine phosphorylation and no detectable tyrosine phosphorylation. Significant differences were found between the phosphopeptide maps of non-shocked and heat-shocked DmHSF. We also showed that numerous phosphatase and kinase inhibitors had little or no effect on the heat shock induced acquisition of HSF DNA binding or recovery following heat-shock. We conclude that phosphorylation and dephosphorylation of DmHSF plays no significant role in regulating the heat induced acquisition of DNA binding.

MATERIALS AND METHODS

Cell Culture and Harvest

Drosophila Schneider line 2 (SL2) cells were grown at room temperature (RT) (non-heat shocked), 21–25°C, in suspension in spinner flasks or adherent in T25 flasks using either modified Shields and Sang M3 medium (Quality Biological, Gaithersburg, MD) supplemented with 10% heat inactivated fetal bovine serum and gentamycin (20 μg/ml), or using serum-free H4Q-CCM 3 medium (Hyclone) and gentamycin (20 μg/ml). Cells were supplemented with 10–50% fresh medium 24 h prior to harvest. Cells in suspension were harvested at a density of between 0.5 and 1.5 × 10^7 cells/ml and adherent cultures were harvested at 75–90% confluency.

For SL2 cells used for inhibitor studies with spinner culture, 5 ml of cell suspension were placed in a 50 ml conical tube and 6× of the final concentration of compound or control vehicle was added in 1 ml of media for 0.75 to 3.5 h at RT on a rotating shaker. Cells were left at RT (non-shocked – NS) or placed at 36–37°C for 30 min (heat shock temperature – HS). Cells were either harvested or placed back on the shaker at RT for recovery to the non-shocked state and then harvested at the stated time points during recovery. Cell harvest involved placing the 50 ml tube on ice for 5 min followed by centrifugation at 4°C at 800 g for 6 min. The media was aspirated and 200 μl of whole cell extraction buffer added (10 mM HEPES, pH 7.9; 400 mM NaCl; 0.1 mM EGTA, pH 8.0; 0.5 mM DTT; 5% (v/v) glycerol; protease inhibitors 0.5 mM PMSF and/or 1 mM AEBSF; additional protease inhibitors (4 μg/ml aprotinin, 1 μg/ml leupeptin) were used in some experiments). The cell pellet was dispersed by pipetting up and down frequently for 5 min on ice. The samples were freeze–thawed on dry ice ×3 with pipetting between each cycle and the lysate centrifuged in a Beckman TL100 centrifuge (TLA 45 rotor) at 40 000 r.p.m. for 10 min at 4°C. The final whole cell extract (WCF) was removed from the pellet and stored at −70°C indefinitely (Zhong et al. 1996). Harvesting cells grown adherent to flasks involved placing the HS or NS flask on ice for 5 min, scraping the cells from the plate in media and placing them into 50 ml centrifuge tubes. The remainder of the steps were identical to those for cells grown in spinner flasks.

^{32}P Labeling of SL2 Cells and Harvesting of Whole Cell Extracts

SL2 cells were grown in suspension to a concentration of 1 × 10^7 cells/ml. Four to eight millilitres of cells were removed and washed ×4 with phosphate free media (either M3 without phosphate with 3% dialyzed serum or serum free IPC41 phosphate free insect media(GIBCO)). The cells were resuspended in phosphate free media for 1 h at RT with shaking. ^{32}P-orthophosphate (Amersham) was added to a final concentration of 0.5 mCi/ml and the tubes were incubated at RT (NS) with shaking for varying amounts of time. Steady-state phosphate levels appear to have been achieved by at least 1.5 h of incubation with ^{32}P phosphate (data not shown). Tubes were then heat-shocked (HS) for 15 min at 36–37°C or left at RT (NS). In experiments using ^{32}P orthophosphate, parallel tubes were treated exactly as above except unlabeled orthophosphate was added at the appropriate times. These extracts were used to test HS induced DNA binding using the gel mobility shift assay. WCEs were made as previously described except the harvest buffer contained phosphatase inhibitors and consisted of 10 mM HEPES, pH 7.9; 400 mM NaCl; 0.1 mM EGTA, pH 8.0; 0.5 mM DTT; 5% (v/v) glycerol; 0.5 mM PMSF; 1 mM AEBSF; 0.1 mM leupeptin; aprotinin (4 μg/ml); 50 mM NaF; 5 mM sodium pyrophosphate, pH 7.5; 2.5 mM sodium orthovanadate, pH 7.5 and 5 μM microcystin.

Western Blotting

Proteins fractionated by SDS gel electrophoresis (Laemmli 1970) were electroblotted onto nitrocellulose using a semidry transfer device (LKB 2117–250 Novablot Electrophoretic Kit) and following the manufacturers instructions. The filter was blocked for 1 h with 3% bovine serum albumin (BSA) in PBST (phosphate buffered saline, 20 mM K2HPO4/KH2PO4 – pH 7.5 and 150 mM NaCl, with 0.1% Tween 20) at RT with shaking. The primary antibody (943 – rabbit polyclonal anti-HSF was diluted 1:10,000 into 3% BSA in PBST and placed on the nitrocellulose for 1 h at RT with shaking. The nitrocellulose was washed ×3 with PBST at RT for 5 min each with shaking. The secondary antibody (donkey anti-rabbit conjugated to horseradish peroxidase, Amersham) was diluted 1:20 000 to 1:40 000 into 3% BSA in PBST and placed on the blot for 1 h at RT with shaking. The blot was washed with PBST. Chemiluminescent substrate from Amersham (ECL-Western Blotting Detection Reagents) was mixed 1:1 and 10 ml were added per piece of nitrocellulose for 5 min at RT with shaking. Excess substrate was removed, followed by exposure of the nitrocellulose to Kodak X-OMAT-AR film for 5–90 s. The nitrocellulose was rinsed in water and dried overnight. The blots could be stored indefinitely or reprobed with new antibodies. There was no need to strip these blots prior to reprobing for other proteins such as hsp70.

Gel Mobility Shift Assay

DNA binding of the non-shocked and heat-shocked DmHSF was performed as previously described

(Zimarino et al. 1990; Zhong and Wu 1996). In brief, ^{32}P 5′ end labeled double stranded oligonucleotides corresponding to a heat shock element (HSE) at 10–100 fmol were mixed with cell extracts (10–30 μg protein), 0.5 μg fragmented *Escherichia coli* DNA, 20 μg BSA in 10 μl of 15 mM Tris, pH 7.5, 0.1 mM EGTA, 10% glycerol, 0.5 mM dithiothreitol (DTT) and 120 mM NaCl. Samples were incubated on ice for 30 min and electrophoresed on a 0.8% agarose gel with 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA) at RT. The gel was dried onto DE81 paper (Whatman) and exposed to X-ray film.

^{35}S-Methionine Pulse-Chase Experiments

Fifteen millilitre aliquots of SL2 cells were washed 4 times with 10 ml each of methionine-free media (GIBCO) with 5% dialyzed serum. Cells were incubated in 6 ml of methionine-free media and ^{35}S-methionine (60 μCi/ml) at RT for 7 h with shaking (pulse). The cells were centrifuged, the ^{35}S-methionine containing media removed and 10 ml of fresh media containing unlabeled methionine was added (chase). Cells were harvested at varying times between 0 and 24 h after the addition of the unlabeled methionine. The cells were harvested in 300 μl of modified high salt RIPA buffer as described below. The HSF was immunoprecipitated as described below, the resulting purified HSF separated by SDS gel electrophoresis, a Western blot for HSF was performed in order to correct for variations in recovery of HSF by immunoprecipitation, and the Western blot was directly exposed to X-ray film. The resulting films were analyzed by the NIH Image computer program and the ratio of ^{35}S-methionine labeled HSF signal to HSF protein detected by ECL was plotted versus the time of chase and the half life of non-shocked HSF was determined from the graph to be between 8 and 10 h (data not shown).

Calculating the number of HSF molecules in SL2 cells

We determined that there are 32 000 molecules of HSF per SL2 cell by purifying HSF from a known number of SL2 cells (by the immunoaffinity purification method described below) and calculating the amount of HSF from these cells by quantitative Western blot analysis using internal standards of purified DmHSF overproduced in *E. coli* (data not shown).

Cross-linking antibody to Protein A beads

Two millilitre bed volume of Affiprep protein A beads (BioRad) were washed ×2 with PBS (40 ml each), the beads were centrifuged at 1500 g for 5 min between each wash. To 2 ml bead bed volume were added 25 ml PBS and 5 ml rabbit serum containing polyclonal antibodies against HSF (Ab 943) at 4°C for 1 h with mixing. The beads were washed ×3 with 10 vol. (20 ml) each of 0.2 M sodium borate (pH 9) at RT. The beads were resuspended in 10 vol. (20 ml) of 0.2 M sodium borate. Solid dimethylpalimidate (DMP) was added to a final concentration of 20 mM as a cross-linking agent. The pH was maintained near 9 to ensure crosslinking. This was mixed for 30 min at RT. The beads were washed ×1 with 10 vol of 0.2 M ethanolamine at pH 8. The beads were incubated for 2 h at RT in 0.2 M ethanolamine with gentle mixing. The beads were washed ×1 with ethanolamine and ×3 in PBS. The beads were stored in 10 vol. of PBS with 0.02% sodium azide at 4°C for up to 6 months. Crosslinking efficiency was tested by boiling an aliquot of beads before and after crosslinking in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, separating the proteins by SDS gel electrophoresis and staining the gel with Coomassie blue.

Immunoaffinity purification of HSF

For immunoaffinity purification of unlabeled or labeled HSF, the whole cell extraction buffer was changed to modified high salt RIPA (0.42 M NaCl, 0.1% NP40, 0.25% deoxycholate, 0.02% SDS, 5 mM EDTA, 40 mM Tris, pH 7.5, 1 mM AEBSF, 1 mM PMSF, 4 μg/ml aprotinin, 0.1 mM leupeptin, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, pH 7.5, 2.5 mM sodium orthovanadate, and 1.2 μM microcystin). Affiprep A beads crosslinked to antibody (15 μl bead bed vol.) were washed x2 with cold low salt modified RIPA without phosphatase inhibitors (0.15 M NaCl, 0.1% NP-40, 0.25% deoxycholate, 0.02% SDS, 1 mM EDTA, 40 mM Tris, pH 7.5, 1 mM AEBSF, 1 mM PMSF, 4 μg/ml aprotinin, 0.1 mM leupeptin). The beads were centrifuged at 6500 r.p.m. for 2 min at RT in a microfuge. The supernatants were aspirated using a 22 or 23 gauge needle. To the bead pellet were added 0.4 ml of sample in high salt RIPA with phosphatase inhibitors and 0.6 ml of low salt RIPA plus phosphatase inhibitors for 2 h with mixing at 4°C. The beads were centrifuged and the supernatant removed. The bead pellet was washed x2 with ice cold modified low salt RIPA with phosphatase inhibitors. The beads were transferred to a new 1.5 ml tube with 1 ml of Protein A wash buffer (0.15 M NaCl, 0.1% NP-40, 5% glycerol, 1 mM EDTA, 20 mM Tris, pH 7.5, 1 mM AEBSF, aprotinin (4 μg/ml), 0.1 mM leupeptin, 1 mM PMSF). The beads were centrifuged and the supernatant completely removed. Fifty microlitres of 1% SDS were added to the beads and the beads heated to 50°C for 5 min with occasional mixing. The beads were centrifuged and the entire supernatant (containing eluted HSF) was transferred to a second 15 μl bed vol. of beads that had been washed and resuspended in 1.25 ml low salt modified RIPA without SDS, with phosphatase

inhibitors at 4°C. This was mixed for 2 h at 4°C. The beads were washed x2 with low salt modified RIPA with SDS and phosphatase inhibitors, x1 with Protein A wash buffer, and x2 with 20 mM Tris, pH 7.5, and 1 mM EDTA. The last supernatant was completely aspirated. HSF was eluted from the beads using 0.11 ml of 0.1% trifluroacetic acid (TFA)/50% acetonitrile at RT for 20 min with multiple mixings. This sample could be directly placed on an HPLC or dried in a speedvac and resuspended in the buffer of choice. Based on Coomassie blue staining HSF was 50–70% pure and recovery of HSF based on western blot analysis was estimated to be between 40 and 80%.

HPLC separation of ^{32}P-labeled HSF following trypsin digestion

The ^{32}P labelled non-shocked and heat-shocked HSF molecules were purified by immunoprecipitation (autoradiographic exposure of SDS-PAGE showed HSF as the only ^{32}P-labeled protein recovered) as described above and the samples digested with trypsin by the same method as for phosphopeptide mapping. The samples were loaded in 0.1% TFA onto a C18 hydrophobic HPLC column (Vydac) attached to an Applied Biosystems HPLC. The buffer was stepped to 0.1% TFA/15% acetonitrile and a linear gradient from 15 to 55% acetonitrile/0.1% TFA was applied over 40 min followed by a step to 0.1% TFA/95% acetonitrile at a flow rate of 0.2 ml/min. Every drop (about 20 µl/drop) was collected separately and counted in a scintillation counter. The c.p.m. in each vial was divided by the total c.p.m. collected to determine the fraction of c.p.m. per vial. This was to correct for slight differences in the total amount of c.p.m. collected for the non-shocked and heat-shocked HSFs.

Phosphoamino acid analysis

The method used is exactly as described (Boyle et al. 1991). ^{32}P labeled HSF was purified by immunoprecipitation as described above. Ten micrograms of bovine serum albumin (BSA) was added to the sample in 0.1% TFA/50% acetonitrile. The sample was dried to completion using a SpeedVac and redissolved in 75 µl of ice-cold performic acid solution for 60 min (9 parts 98% formic acid and 1 part 30% hydrogen peroxide prepared 45 min prior to use and allowed to sit at RT. Four-hundred microlitres of ice cold H_2O was added, the sample frozen and lyophilized to dryness. The sample was resuspended in 60 µl of 5.7 M HCl and incubated at 110°C for 60 min. The sample was lyophilized in a Speed-Vac. Radioactivity was measured by Cherenkov radiation in a scintillation counter. The hydrolysate was redissolved in pH 1.9 buffer (formic acid (88%):glacial acetic acid:deionized water (50:156:1794)) which also contained 1 part in 16 of unlabeled phosphoamino acid standards (phosphoserine, phosphothreonine, phosphotyrosine – each at stock concentrations of 1 mg/ml). The samples (approximately 200 c.p.m. each) were loaded on thin layer chromatography (TLC) plates and electrophoresed in two dimensions using the Hunter thin-layer electrophoresis system (HTLE-7000; CBS Scientific). The first dimension electrophoresis was carried out with pH 1.9 buffer for 20 min at 1.5 kV at RT. The TLC plate was dried, rotated 90° counter clockwise, and electrophoresed in pH 3.5 buffer (glacial acetic acid:pyridine:deionized water [100:10:1890] and 0.5 mM EDTA) for 16 min at 1.3 kV. The plate was air dried and baked at 65°C for 10 min. The unlabeled phosphoamino acid standards were visualized by spraying the plate with 0.25% (w/v) ninhydrin in acetone and the plate placed back at 65°C for 15 min to develop the stain. The plate was then exposed to X-ray film for 2–5 days.

Phosphopeptide Mapping of HSF

Sample preparation – 32p labeled HSF purified by immunoprecipitation as described above in 0.1% TFA/50% acetonitrile was completely lyophilized and then resuspended in 60 µl 8 M urea, 0.1 M ammonium bicarbonate and 20 mM methylamine. Dithiothreitol (DTT) was added to a final concentration of 6.2 mM. The sample was vortexed and placed at 50°C for 10 min and cooled to RT. Six microlitres of 0.1 M iodoacetamide in water was added for 10 min at RT. Three hundred seventy microlitres of 0.1 M ammonium and bicarbonate was added to bring the urea concentration to 1 M. One-half to one µl of trypsin at 1 mg/ml (Boehringer-Mannheim – sequencing grade) in 0.01% TFA was added overnight at 37°C. An additional aliquot of trypsin was added for an additional 2 h at 37°C. Eighty microlitres of 8 M urea in 0.1 M ammonium bicarbonate and 55 µl of 0.1% TFA/95% acetonitrile were added and the pH adjusted to approximately 2 with 10% TFA. The sample was applied directly to an Applied Biosystems HPLC using a Vydac C18 hydrophobic column. The sample was loaded at 0.1% TFA/10% acetonitrile and eluted with a 2 min gradient of 0.1% TFA/30–50% acetonitrile, followed by 1 min of 0.1% TFA/50–70% acetonitrile, 3 min of 0.1% TFA/70% acetonitrile, and finally 1 min of 0.1% TFA/70–95% acetonitrile. All eluted fractions were collected, lyophilized, resuspended and pooled into 400 µl of 1.9 pH buffer. After lyophilization, Cherenkov radiation was determined, and the final pellet resuspended in 8 µl 1.9 pH buffer. The phosphopeptides were separated in two dimensions on TLC plates (EM Science) as described (Boyle et al. 1991). The protein samples (1000 c.p.m.) were separated in the first dimension by

electrophoresis using the HTLE-7000 apparatus (CBS Scientific) in pH 1.9 buffer at 1000 v for 50–60 min at 18°C. The plates were dried and chromatography was performed in the second dimension in n-butanol:pyridine:acetic acid:deionized water (15:10:3:12) for 12–14 h. The plates were dried and visualized using autoradiography for 2–21 days.

RESULTS

Phosphorylation and dephosphorylation of HSF

Yeast and human HSF1 molecules have been shown to undergo heat-shock induced hyperphosphorylation (Hoj and Jakobsen 1994; Cotto et al. 1996; Knauf et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997). Our initial studies showed that DmHSF undergoes a rapid, 2–3-fold increase in phosphorylation when pulse-labeled with ^{32}P during a 15 min heat shock (data not shown). We demonstrated that SL2 cells contain approximately 32 000 HSF molecules per cell and that the half-life of non-shocked HSF protein in SL2 cells was 8–10 h (data not shown). We also showed that steady-state ^{32}P labeling was achieved within 1–1.5 h and that treatment of SL2 cells for up to 24 h resulted in no difference in the amount of ^{32}P incorporated into HSF (data not shown). To investigate changes in phosphorylation of DmHSF under steady state ^{32}P labeling conditions, SL2 cells were grown in the presence of ^{32}P–inorganic phosphate for 2 h followed by a 30 min heat shock. A DNA gel mobility shift assay using extracts from unlabeled SL2 cells treated identically, clearly showed induction of DNA binding activity of HSF after heat stress (Fig. 1A). Both non-shocked (N) and heat shocked (H) ^{32}P-labeled HSFs were highly purified from SL2 cell extracts by immunoprecipitation and analyzed by Western blotting (Fig. 1B) and autoradiography (Fig. 1C). The results revealed that there is no difference in the overall phosphorylation of heat-shocked (H) and non-shocked (N) HSFs under steady state conditions of ^{32}P labeling.

We determined which amino acid residues were phosphorylated for purified non-shocked and heat shocked DmHSF. Equal c.p.m.s of immunopurified non-shocked and heat shocked HSF were hydrolyzed, and the amino acids separated by 2 dimensional electrophoresis. Figure 1D shows that predominantly serine residues are phosphorylated for both non-shocked and heat shocked HSF. No tyrosine phosphorylation and only a very small amount of threonine phosphorylation were observed for both non-shocked and heat shocked HSF.

We next analyzed the pattern of serine phosphorylation of HSF before and after heat shock by two methods. The first method involved mapping of ^{32}P-labelled tryptic peptides. Purified HSF was treated with trypsin to completion and the peptide fragments were separated using

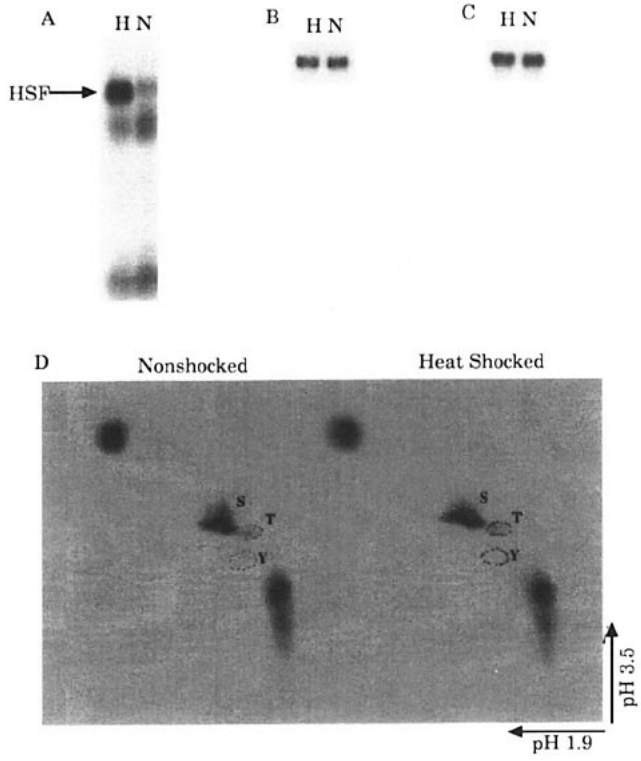

Fig. 1 DmHSF shows equal phosphorylation under non-shocked and heat-shocked conditions. (A) Gel mobility shift assay demonstrates heat inducible DNA binding of HSF using WCEs from non-shocked (N) and heat-shocked (H) SL2 cells grown in suspension under identical conditions to the ^{32}P labeled cells, except unlabeled inorganic phosphate was used. Heat shock was for 30 min at 37°C. (B) Western blot analysis of purified ^{32}P-labeled non-shocked (N) or heat shocked (H) HSFs from SL2 cells were separated by SDS-PAGE and (C) the accompanying autoradiograph of this same Western blot demonstrated a single radiolabeled band and no difference in overall phosphorylation under steady state ^{32}P-labeling conditions (2 h prior to heat shock). (D) Phosphoamino acid analysis of non-shocked and heat-shocked ^{32}P labeled and immunopurified HSF from SL2 cells demonstrating predominantly serine phosphorylation. The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards are indicated. The direction and pH of the electrophoresis are indicated.

gel electrophoresis in the first dimension and chromatography in the second dimension. The resulting phosphopeptide maps are shown for non-shocked HSF (Fig. 2A) and heat shocked HSF (Fig. 2B). Peptides common to both forms of HSF are labeled A to Q. If peptides A, B, and C are used as internal standards, then peptides D, E, F, G, and O all appear to show increased intensity following heat shock, while peptide M shows a decrease. In addition, newly phosphorylated peptides can be identified following heat shock (Fig. 2, numbers 1 to 8). By contrast, peptides 9 and 10 appear on the non-shocked phosphopeptide map, but disappear after heat shock. These results indicate that several new sites on HSF become phosphorylated following heat shock and that at least a few sites become dephosphorylated as well.

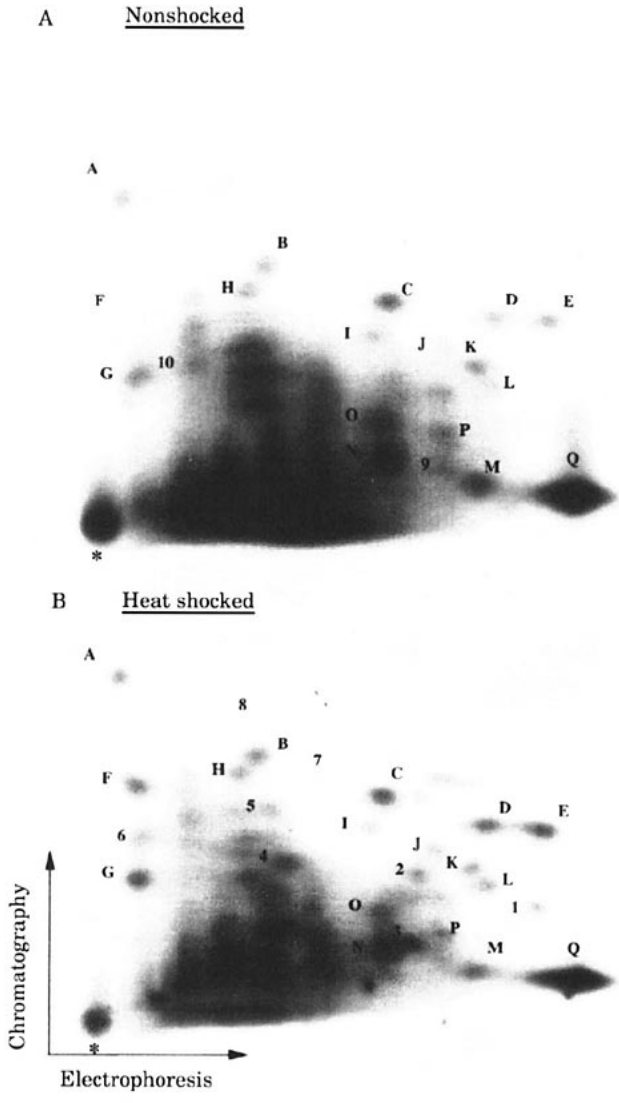

Fig. 2 Phosphopeptide analysis of purified non-shocked and heat-shocked DmHSF. Non-shocked (A) or heat-shocked (B) immunopurified ^{32}P-labeled HSFs from SL2 cells were trypsin digested and the phosphopeptides separated in two-dimensions. The origin for each phosphopeptide map is marked with a (*) and the direction of electrophoresis and chromatography are marked by arrows. Peptides common to both non-shocked and heat-shocked HSF are labeled A to Q. New peptides identified following heat-shock include 1 to 8 and peptides 9 and 10 disappear following heat-shock.

The second method to determine differences in the phosphorylation pattern between non-shocked and heat shocked HSF involved separating phosphopeptides by HPLC. Phosphopeptides of HSF prepared from trypsin digestion were separated on a C18 reverse phase HPLC column. As shown in Figure 3A, substantial differences in the intensity of ^{32}P labeled peptides can be observed between non-shocked and heat shocked HSF. The non-shocked HSF profile was directly subtracted from the profile for heat shocked HSF to generate Figure 3B, which shows that there are at least 11 prominent ^{32}P peaks present for the heat shocked HSF (peaks above the horizontal of Fig. 3B). Similarly there are at least 4 prominent peaks present in the non-shocked HSF (below the horizontal of Fig. 3B). The HPLC data are consistent with the 2 dimensional maps of Figure 2 and confirm that new serine residues on HSF are phosphorylated after heat shock, while some sites are dephosphorylated following heat shock.

Effects of Inhibitors on DNA Binding Activity of HSF

To evaluate whether phosphorylation or dephosphorylation regulates the ability of DmHSF to bind to its specific DNA element (HSE), we treated SL2 cells with a variety of phosphatase and kinase inhibitors under non-shocked, heat shocked, and recovery conditions, and analyzed the DNA-binding activity of HSF by the gel mobility shift assay. Okadaic acid (Ishihara et al. 1989; Cohen et al. 1990; Hardie et al. 1991) and calyculin A (Ishihara et al. 1989), are potent inhibitors of protein phosphatases-1 and 2A (PP1 and PP2A – see Table 1). Staurosporine (Tamaoki et al. 1986; Schachtele et al. 1988; Tischler et al. 1990) can inhibit a number of kinases with a preference for protein kinase C (PKC) at low concentrations and at higher concentrations can inhibit several other protein kinases (Table 1). Vanadate has been used as a general inhibitor of tyrosine phosphorylation (Gordon 1991). Each compound was used in the initial study at concentrations at least 5-fold higher than reported Ki values (Table 1). SL2 cells grown in suspension were treated with these compounds for 1 h in fresh media.

As shown in Figure 4A, treatment of cells with okadaic acid had no significant effects on the DNA binding activity of HSF when compared with untreated, non-shocked or heat shocked cells (control). The DNA:HSF protein complex from the okadaic acid treated SL2 extracts migrates further into the native gel than the untreated complex, consistent with hyperphosphorylation of HSF. When okadaic acid-treated cells are heat shocked and allowed to recover, a slight delay in the time of recovery is also observed. The Western blot in Figure 4B for this set of samples shows that approximately equal amounts of HSF are analyzed for non-shocked and heat shocked samples. All of the okadaic acid-treated samples show slower migration of HSF on SDS-PAGE, again consistent with hyperphosphorylation of HSF.

Similar results were observed when SL2 cells were treated with calyculin A, staurosporine, or vanadate (Figs 4A & B). There was a slight delay in recovery time to the non-shocked state for the staurosporine treated cells compared to controls (Fig. 4A). Therefore, okadaic acid, calyculin A, staurosporine, and vanadate do not induce the DNA binding activity of HSF in non-shocked SL2 cells,

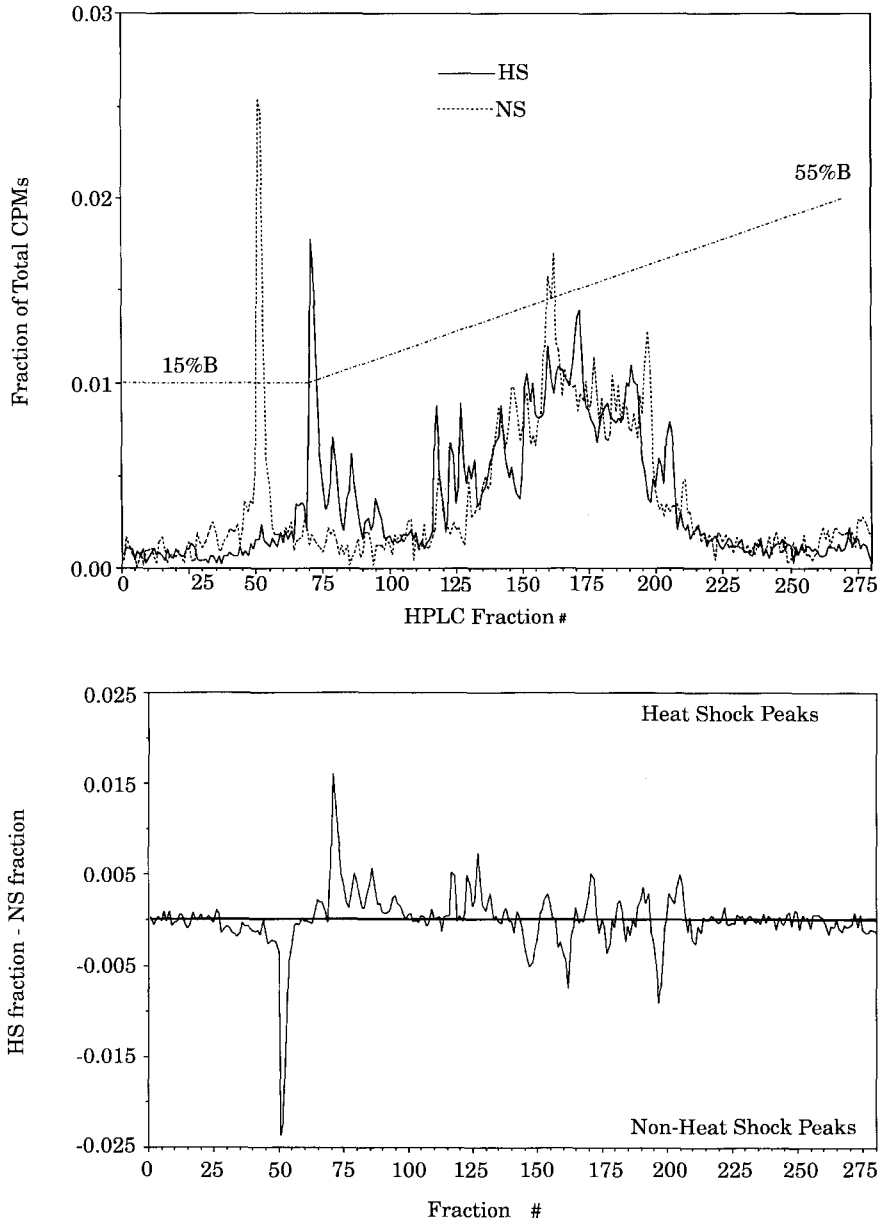

Fig. 3 HPLC analysis of trypsin-generated phosphopeptides from purified ^{32}P labeled non-shocked (dashed profile in A) or heat-shocked (solid profile in A) HSFs. A C18 Vydac HPLC column was used to separate the phosphopeptides generated by complete trypsin digestion of purified ^{32}P labeled HSF. Solvent A was 0.1% TFA and solvent B was 0.1% TFA/95% acetonitrile. A gradient (graphically indicated as the dash/dot line) from 15% to 55% solvent B was used to separate the phosphopeptide fragments from each other. (A) Each fraction was counted and the raw cpms were converted to the fraction of total cpm (cpm/total cpm recovered) for each HSF form in order to correct for slight differences in total cpms loaded on the column for each HSF form. The calculated non-shocked fraction was directly subtracted from the heat-shocked fraction resulting in the plot in (B) showing discrete peaks above zero representing heat-shock induced phosphopeptides and peaks below zero representing phosphopeptides present only in the non-shocked HSF.

and do not prevent the induction of DNA binding activity following heat shock. These compounds also do not prevent the recovery of the heat shocked, DNA binding form of HSF back to the non-shocked non-DNA binding state.

Both okadaic acid and calyculin A led to slight delays in recovery of HSF to the non-DNA binding form. Therefore, we tested the possibility that okadaic acid could lead to the induction of DNA binding of HSF at a lower temperature than required for untreated cells. As shown by the gel retardation assay (Fig. 5A), a slightly increased induction of the DNA binding activity of HSF in the okadaic acid treated cells was observed at 27.5 C compared to the untreated controls, but at all other temperatures no significant differences in the induction of

Table 1 Summary of compounds analyzed

Phosphatase Inhibitors (ser/thr)	PP1	Ki (nM) PP2A
Okadaic acid	200	< 1
Calyculin A	2	< 1

Phosphatase Inhibitor (tyr)	
sodium orthovanadate	General inhibitor, working concentration 1–10 mM

Kinase Inhibitors	Ki (μM)			
	PKC	PKA	PKG	MLC
Staurosporine	0.0007	0.007	0.009	0.001
K252b	0.02	0.09	0.1	0.15
Chelerythrine	0.7	170	–	–
Iso-H7	49	–	–	–
KT5720	> 2	0.06	> 2	> 2
KT5823	4	> 10	0.23	> 10
KT5926	0.72	1.2	0.16	0.02

Kinase Activator	
Phorbol ester (PMA/TPA)	PKC activator, usual working concentration 30 nM

The above compounds were added to SL2 cells 1 h prior to treatment with or without heat shock followed by +/– recovery to the non-shocked state. The concentrations of each compound used in a given experiment are indicated in the text and figure legends. These compounds were purchased from LC Laboratories and/or Sigma. Ki values, the concentration where 50% of enzyme activity is inhibited, are from the references cited in the text and from the LC Laboratory catalog. Abbreviations include: serine/threonine (ser/thr); tyrosine (tyr); protein phosphatase 1 (PP1); protein phosphatase 2A (PP2A); protein kinase C (PKC); cAMP-dependent protein kinase (PKA); cGMP-dependent protein kinase (PKG); myosin light chain kinase (MLC). The phorbol ester used is PMA (phorbol 12-myristate 13-acetate).

DNA binding activity were observed. Western blot analysis shows approximately equal recovery of HSF protein under all conditions with HSF from okadaic acid-treated cells migrating slower and more diffusely than the HSF from control cells (Fig. 5B). Therefore, okadaic acid treatment of SL2 cells does not significantly facilitate activation of HSF to a DNA binding state.

We also investigated whether cells under okadaic acid treatment could be reactivated by a second heat shock after recovery. As shown in Fig. 5C, SL2 cells under treatment with okadaic acid were able to induce the DNA binding activity of HSF upon a second heat shock, despite hyperphosphorylation as revealed by the faster migration of the HSF:HSE complex on the native gel. Western blot analysis (Fig. 5D) shows approximately equal recovery of HSF protein for all samples.

Since the above studies were performed using SL2 cells grown in suspension, we also tested whether SL2 cells grown in flasks (adherent cells) showed the same response to okadaic acid and calyculin A. Adherent SL2 cells were treated with okadaic acid (200 nM), or calyculin A (100 nM) for 1 h and then either left at non-shocked (N) temperature or heat-shocked (H), and allowed to recover as indicated (Fig. 6A). We found that recovery to the non-DNA binding state for untreated adherent SL2 cells was much slower (greater than 2 h) compared to cells maintained in suspension, presumably due to metabolic differences between adherent and suspended cell cultures. Okadaic acid and calyculin A slightly delayed recovery to the non-DNA binding state as compared to controls, as was previously observed for cells grown in suspension (Fig. 4). As shown in Figure 6B, there was approximately equal recovery of HSF protein for all samples.

The compounds examined above are some of the best characterized phosphatase (okadaic acid, calyculin A, and vanadate) and kinase (staurosporine) inhibitors. We next tested a number of other kinase inhibitors and kinase activators. The enzymes or pathways inhibited or activated by these compounds and their reported Ki values are summarized in Table 1. Besides DMSO as a control, the compounds included calyculin A (100 nM), staurosporine (100 nM), K252b (0.5 μM) – a broad spectrum protein kinase inhibitor (Yasuzawa et al. 1986), KT5926 (0.5 μM) – an inhibitor of myosine light-chain kinase (Nakanishi et al. 1990), KT5823 (2.5 μM) – an inhibitor of cGMP – dependent protein kinase (Kase et al. 1987), and KT5720 (1 μM) – an inhibitor of cAMP-dependent protein kinase (Kase et al. 1987). As can be seen in Figure 7A none of these compounds induced DNA-binding at non-shock temperatures, prevented the heat-shock induced increase in DNA-binding, or had any effect on the recovery of HSF back to the non-DNA binding state. The recovery of HSF protein as assayed by Western blot analysis was approximately equal for all compounds tested (Fig. 7B).

Finally we investigated whether protein kinase C (PKC) plays any role in regulating HSF activation to the DNA binding state. We used four known PK/C inhibitors (staurosporine at 10 nM, K252b at 0.1 μM, chelerythrine chloride at 10 μM [Herbert et al. 1990], and iso-H7 at 200 μM [Quick et al. 1992] and a potent PKC activator (PMA/TPA at 60 nM [Boutwell 1974]). Staurosporine and K252b, as previously shown, had no effect on activation of DNA binding of HSF (Fig. 7C). Chelerythrine treatment of cells significantly delays recovery of the heat shocked HSF back to the non-shock state (Fig. 7C). However, chelerythrine does not induce DNA binding without heat shock and does not completely prevent recovery to the non-shock state. Iso-H7 treatment of cells leads to a modest induction of DNA binding by non-shock HSF and retards complete recovery to the non-shock state. H7 has been

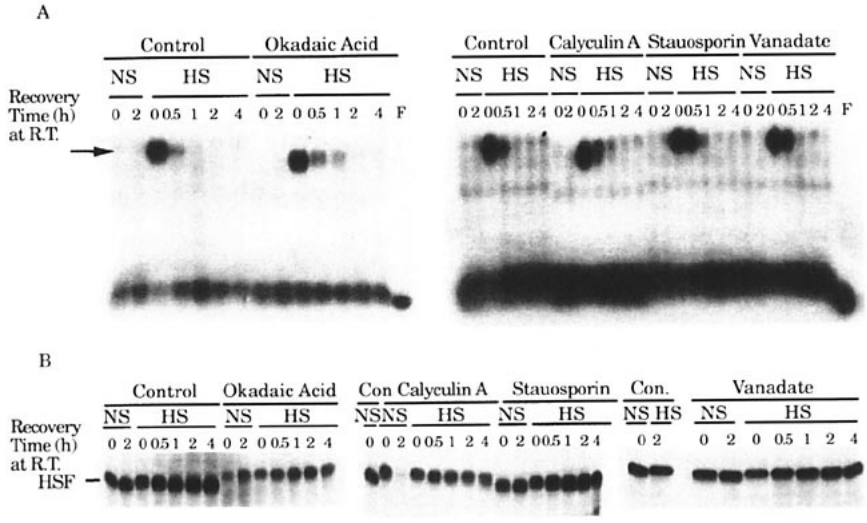

Fig. 4 Commonly used phosphatase and kinase inhibitors have little effect on the ability of DmHSF to bind DNA upon heat-shock or to recover to the non-DNA binding state. (A) Gel mobility shift assay of WCEs from SL2 cells grown in suspension and treated with DMSO (control), okadaic acid (200 nM), calyculin A (100 nM), staurosporine (100 nM), or sodium orthovanadate (10 mM) for 1 h. The cells were then either left at non-shocked (NS) temperature (room temperature, RT) or heat shocked for 30 min at 37°C. Heat shocked cells were either processed immediately (time 0) or placed at non-shocked temperatures for varying amounts of time in order for HSF to recover to the non-shocked, non-DNA binding state. Similar results were seen at concentrations up to 20 μM for okadaic acid, calyculin A, and staurosporine (data not shown). The free ^{32}P labeled oligonucleotide without extract is shown in the lane marked F. (B) Whole cell extracts from A were used for Western blot analysis for HSF and show approximately equal recovery of HSF protein. Con represents control samples. The HSF protein from calyculin A treated, non-shocked cells maintained at RT for 2 h was degraded.

shown to decrease heat induced hsp70 transcription (Yamamoto et al. 1994) and we observed that H7 prevents heat stress induced production of hsp70 protein (data not shown). The PKC activator PMA/TPA has no effect on induction or recovery of DNA binding activity by HSF. The recovery of HSF protein (Fig. 7D) with all of these compounds is approximately equivalent. Of the four PKC inhibitors tested, only iso-H7 showed some effects on constitutive induction and retarded recovery of the DNA binding activity of HSF. The effects of a PKC activator were minimal. These results are inconsistent with involvement of PKC as the primary pathway in heat shock signaling and suggest that iso-H7 may exert its effects on HSF by secondary mechanisms.

DISCUSSION

Under steady state conditions of ^{32}P labeling we found no difference in the total amount of phosphorylation between the non-stressed and heat-stressed DmHSF molecules (Fig. 1). This observation is in contrast to yeast and human HSFs which even under steady-state ^{32}P labeling conditions show strong heat shock induced hyperphosphorylation (Sorger and Pelham 1988; Nieto-Sotelo et al. 1990; Hoj and Jakobsen 1994; Cotto et al. 1996; Knauf et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997; Xia et al. 1998). As reported for other HSF molecules, the phosphorylation pattern of DmHSF is predominantly on serine residues with only minimal threonine phosphorylation and no tyrosine phosphorylation (Hoj and Jakobsen 1994; Cotto et al. 1996; Knauf et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997). Phosphoamino-acids that are acid labile, such as phosphohistidine (P–N), would not be detected by the methods used in this paper (Boyle et al. 1991). The role for P–N phosphorylations (base stable) in modulating HSF activity has not yet been reported.

Although there was no overall change in the level of steady-state phosphorylation of DmHSF, there were numerous differences between the phosphopeptide maps generated for the non-shocked and heat-shocked HSF molecules. Our two-dimensional phosphopeptide maps show many more phosphorylated peptides than reported for human HSF1 (Cotto et al. 1996; Knauf et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997; Xia et al. 1998). It is unlikely that this difference can be attributed to incomplete tryptic digestion of HSF, as complete cleavage was confirmed by mass spectrometry (our unpublished observations). We treated purified DmHSF overexpressed in *E. coli* or Sf9 cells (using a baculovirus expression system) with trypsin at the same ratio of HSF:trypsin as for immunopurified SL2 HSF used for the 2-dimensional electrophoresis experiments. The resulting fragments were separated by HPLC, and mass

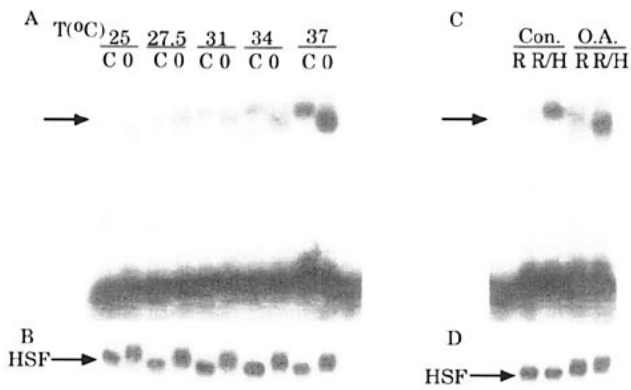

Fig. 5 Okadaic acid does not facilitate the heat-shock response at a lower temperature. (A) Gel mobility shift assay using WCEs from SL2 cells grown in suspension and treated with okadaic acid at 200 nM (O) or with DMSO (C) for 1 h prior to exposure to the indicated temperatures for 30 min. The final unlabeled lane represents free ^{32}P labeled oligonucleotide without extract. (B) Western blot analysis for HSF of the same WCEs as in A showing approximately equal recovery of HSF protein. (C) Gel mobility shift assay using WCEs from SL2 cells grown in suspension that were treated with okadaic acid (OA) or with DMSO (Con) for 1 h before heat shock at 37°C for 30 min with recovery at non-shocked temperature for 1.5 h (R) or recovery for 1.5 h followed by a second heat-shock at 37°C for 30 min (R/H). The arrow indicates the HSF:HSE complex. The first unlabeled lane represents free ^{32}P labeled oligonucleotide without extract. (D) Western blot analysis of the same samples as in C shows approximately equal recovery of full length HSF under all conditions.

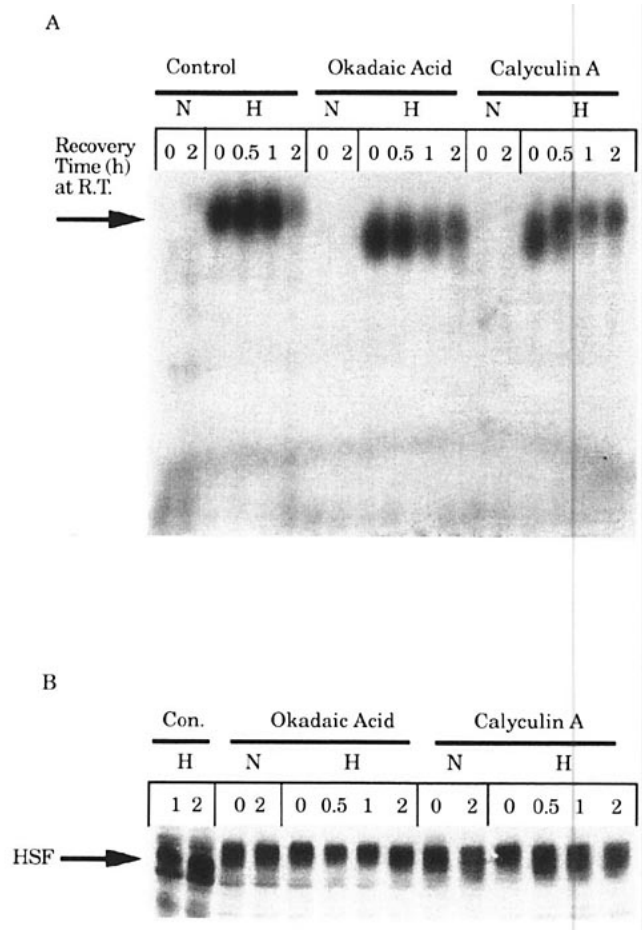

Fig. 6 SL2 cells grown adherent in flasks show a similar response to okadaic acid and calyculin A as SL2 cells grown in suspension. (A) Gel mobility shift assay of WCEs from SL2 cells grown adherent to T25 flasks for 5 days, fed fresh media 24 h prior to harvest and on the day of harvest treated with DMSO (control), okadaic acid (200 nM), or calyculin A (100 nM) for 1 h, left at non-shocked temperature, RT, (N) or exposed to heat shock for 30 min at 37°C (H) followed by recovery at the non-shocked temperature for 0–2 h. The arrow indicates the HSF:HSE complex. The kinetics of the return of HSF to the non-shocked state were much slower than for SL2 cells grown in suspension even for the controls (Fig. 4). (B) Western blot analysis for HSF of the same samples as in A shows approximately equal recovery of HSF. Con. represents control samples.

spectrometry of each HPLC peak was performed. The resulting profile demonstrated numerous peptides, many as small as 700 Da (the limit of resolution), and none larger in molecular weight than predicted if complete trypsinization of HSF had been obtained. It is unlikely that the difference in the number of phosphorylated peptides observed for DmHSF compared to human HSF1 is due to a difference in the number of available serines because DmHSF possesses 73 serine residues out of a total of 691 amino acids (Clos et al. 1990), whereas human HSF1 possesses 70 serine residues out of a total of 529 amino acids (Rabindran et al. 1991). A possible explanation is that we added to our modified RIPA extraction buffer a relatively high concentration of microcystin, a potent in vitro inhibitor of protein phosphatases 1 and 2a, in addition to the other phosphatase inhibitors (NaF, pyrophosphate, and sodium orthovanadate) reported by others as used in their extraction buffers (Cotto et al. 1996; Kanuf et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997; Xia et al. 1998). The addition of microcystin may have allowed the true baseline level of phosphorylation of DmHSF to be preserved throughout purification. We observed the presence of specific phosphopeptides in the non-shocked state and the appearance of new phosphopeptides along with dephosphorylation of other peptides upon heat-shock. The changes in phosphorylation identified by HPLC and two-dimensional phosphopeptide mapping may be directly related to the regulation and function of HSF or could simply represent changes in the equilibrium activity of cellular kinases and phosphatases that occur during heat stress.

Accordingly, we studied the effects of several phosphatase inhibitors, protein kinase inhibitors, and a protein kinase activator on the DNA binding activity of HSF. All compounds tested in SL2 cells have been shown to function in higher eukaryotic cells at Kis reported in Table 1.

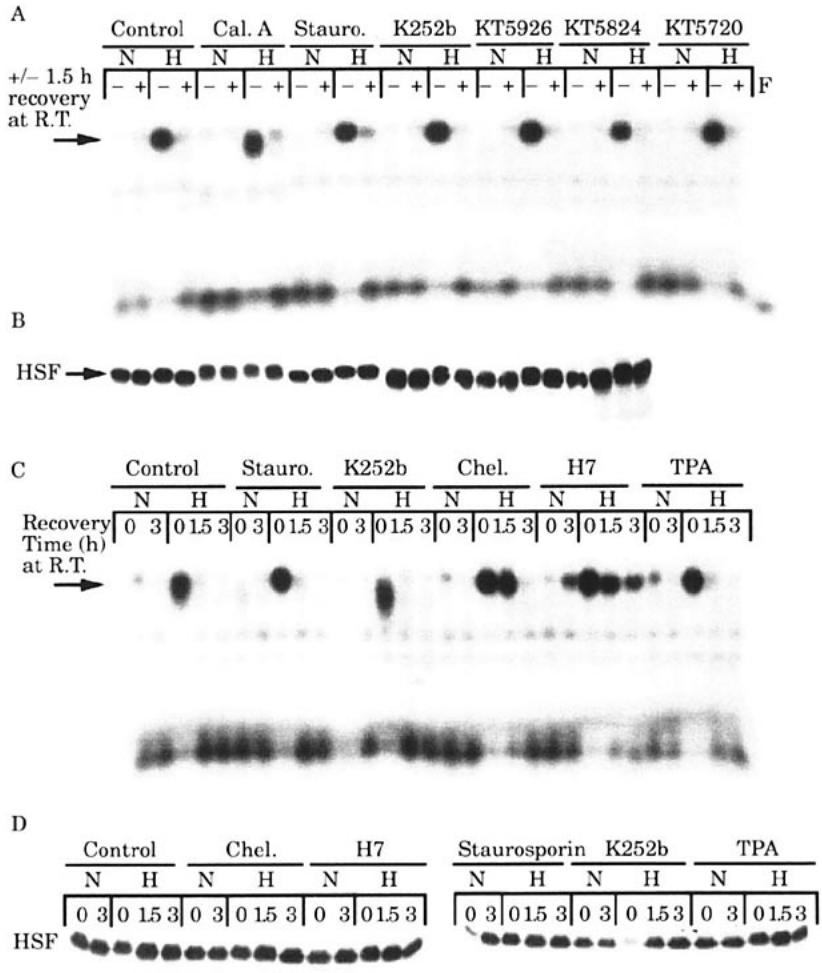

Fig. 7 The protein kinase C pathway does not appear to regulate heat induced DNA binding activity of DmHSF. (A) Gel mobility shift assay using WCEs from SL2 cells grown in suspension and treated with a variety of phosphatase and kinase inhibitors (calyculin A (Cal A) at 100 nM, staurosporine (Stauro.) at 100 nM, K252b at 0.5 μM, KT5926 at 0.5 μM, KT5823 at 2.5 μM, and KT5720 at 1 μM) for 1 h followed by heat shock (H) for 30 min at 37°C or no heat shock (N). Cells underwent no recovery (–) or 1.5 h of recovery at RT (+) prior to harvesting. The free ^{32}P labeled oligonucleotide without extract is shown in the last lane. (B) Western blots for HSF of the same samples as in A. No Western blot data on KT5720 were obtained. (C) Gel mobility shift assay using WCEs from SL2 cells grown in suspension and treated with a variety of PKC inhibitors (staurosporine (Stauro.) at 10 nM, K252b at 0.1 μM, chelerythrine chloride (Chel.) at 10 μM, and iso-H7 at 200 μM) or a PKC activator (TPA at 60 nM), for 1 h followed by heat-shock (H) for 30 min at 37°C or no heat-shock (N). Cells then underwent recovery at RT for times ranging from 0 to 3 h as indicated. (D) Western blots for HSF of the same samples as in C and as labeled. The HSF protein from the heat shocked (H) zero time point (0) sample for K252b was partially degraded and therefore migrated as a smear in C.

In all of our studies with Drosophila SL2 cells we made the assumption that absorption, metabolism, and excretion of these compounds by SL2 cells is similar to that in higher eukaryotes. Clearly, okadaic acid and calyculin A entered SL2 cells and led to hyperphosphorylation of HSF as demonstrated by Western blotting (Fig. 4B). We also demonstrated that even at very high concentrations of some of these compounds (okadaic acid, calyculin A, and staurosporine) there was still no effect on HSF DNA binding activity (data not shown). Two serine/threonine phosphatase inhibitors, okadaic acid and calyculin A, led to hyperphosphorylation but did not induce DNA binding of HSF at non-shocked temperature, nor did they prevent induction of DNA binding by heat stress. Neither compound prevented recovery of HSF back to the non-shocked form, although both did slightly delay the time course of recovery compared to controls. Our data suggest that activation and inactivation of DNA binding of DmHSF are independent of phosphatase 1 and 2A activities in SL2 cells. The single tyrosine phosphatase inhibitor tested (vanadate) had no effect on DNA binding of HSF, which is consistent with the observation that there was no tyrosine phosphorylation of HSF.

However, both okadaic acid (Chang et al. 1993) and calyculin A (Xia and Voellmy 1997) have previously been shown to lead to hyperphosphorylation of HSF and

enhance heat-induced hsp70 transcription, but transcription was not induced without heat shock. Furthermore, calyculin A has been shown to retard dissociation of human HSF1 trimers (Xia and Voellmy 1997), and was also found to activate transcription of hsp70 genes at non-stress temperatures if HSF1 trimers were preformed by salicylate treatment of the cells (Xia and Voellmy 1997). These reports suggest that hyperphosphorylation of HSF is involved in some way with its transcriptional competence.

We also tested the PKC activator (PMA/TPA) and found no effect on DNA binding activity of HSF or recovery of HSF from heat induced DNA binding. Holmberg et al. (1997), showed that TPA did not induce the heat shock response, but it did markedly enhance the response, whereas Ding et al. (1997) showed that PMA increased hsp72 mRNA levels in cells containing overexpressed HSF1 and this increase in hsp72 was blocked by staurosporine.

Numerous protein kinase inhibitors were tested for effects on DNA binding. Staurosporine is a commonly used general protein kinase inhibitor with potent PKC inhibitory activity. Previous studies (Erdos and Lee 1994; Yamamoto et al. 1994) showed that staurosporine inhibited heat shock induced hsp70 mRNA levels, but did not affect heat shock-induced DNA binding of HSF1 (Erdos and Lee 1994). In general agreement with those results, staurosporine was found to have no effect on DNA binding of DmHSF, and recovery was slightly delayed but not prevented.

Of the other compounds tested only two showed any effect on DNA binding. Two PKC inhibitors, chelerythrine and H7, led to delayed recovery of HSF back to the non-shocked state. H7 had the clearest effect on DNA binding of HSF. H7 induced some DNA binding activity of DmHSF without heat shock, although heat stress increased the amount of DNA binding. Although there was some recovery of HSF to the non-shocked form, there remained a significant amount of DNA binding activity compared to control values. Yamamoto et al. (1994) showed that H7 inhibits mammalian hsp70 RNA induction in response to heat stress; however, they did not assess DNA binding activity of HSF1. Our data with PKC inhibitors and the PKC activator (PMA/TPA) do not support a role for PKC alone in modulating HSF activity, since only one inhibitor of PKC had any significant effect on HSF activity (Fig. 7) out of the four PKC inhibitors tested. The effects of H7 on the DNA binding activity of HSF and hsp70 production may be through a mechanism secondary to phosphorylation. In summary, we showed that phosphorylation plays no substantial role in regulating DNA binding activity of DmHSF, in agreement with DNA binding studies of yeast and human HSFs (Sorger and Pelham 1988; Nieto-Sotelo et al. 1990; Cotto et al. 1996; Knauf et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997).

However, another phosphorylation pathway has been proposed to regulate Arabidopsis HSF DNA binding activity through a cyclin dependent kinase (Reindl et al. 1997). Phosphorylation of Arabidopsis HSF1 by CDC2a inhibits DNA binding. Other reports also suggest a role for phosphorylation in deactivation of transcriptional activity in HSF (Hoj and Jakobsen 1994; Cotto et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997; Xia et al. 1998). Several reports have limited this area of regulation within the HSF1 molecule to serines 303 and 307 for human HSF1 and implicated MAP kinases as the regulatory enzymes (Mivechi et al. 1994; Mivechi and Giaccia 1995; Chu et al. 1996; Knauf et al. 1996; Kline et al. 1997; Chu et al. 1998; Kim et al. 1998). With regards to regulation of transcription by phosphorylation of human HSF1, it has been demonstrated that ERK1/ERK2 can phosphorylate serines 303/307 in vitro. These serines are in the regulatory domain of human HSF1 and when they are phosphorylated at control temperatures hsp70 transcription is repressed (Knauf et al. 1996). However, dephosphorylation of these serine residues is not required for heat shock induction, suggesting that other mechanisms are brought into play for full regulation of HSF activity. A recent report indicates that dephosphorylation of serine 307 in human HSF1 may be an important step in transcriptional activation or serve to maintain HSF1 in the transcriptionally active conformation (Xia et al. 1998). Another report implicates alterations in phosphorylation of serines 303, 307, and 363 in modulating human HSF1 transcriptional activity and proposes that three protein kinase pathways are involved including MAP kinase, PKC, and glycogen synthase kinase (Chu et al. 1998).

Other mechanisms, including the interaction of HSF with hsps, have been proposed to influence the activation of DNA binding, trimerization, and transcriptional activity of HSF (Boorstein and Craig 1990; Abravaya et al. 1992; Baler et al. 1992; Mosser et al. 1993; Schlesinger and Ryan 1993; Rabindran et al. 1994; Kim et al. 1995; Shi et al. 1998; Zuo et al. 1998). In addition, a recent report using purified HSF suggests that the DmHSF molecule itself may be capable of direct sensing of heat and oxidative stress leading to conformative changes that result in altered DNA binding, although the transcriptional competence of these preparations of DmHSF was not investigated (Zhong et al. 1998).

In conclusion, we have shown that DmHSF, unlike other HSF1 molecules, does not show hyperphosphorylation upon heat shock when studied under steady state ^{32}P labeling conditions. However, like other HSF1 molecules, DmHSF shows predominantly serine phosphorylation with numerous phosphopeptides identified. Upon heat shock new phosphopeptides appear and others disappear,

suggesting both increased phosphorylation and dephosphorylation of DmHSF in response to heat stress. As with yeast and human HSF, phosphorylation/dephosphorylation of DmHSF appears to play no role in regulating DNA binding activity. It will be of interest to further explore the role of phosphorylation and other regulatory mechanisms in modulating the transcriptional potency of Drosophila HSF.

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