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Inhibitors of Tyrosine and Ser/Thr Phosphatases Modulate the Heat Shock Response

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Abstract Following heat shock the expression of heat shock genes is regulated by the heat shock transcription factor, HSF, known to bind to arrays of the heat shock element, NGAAN, upstream of the heat shock genes. Phosphorylation of HSF is necessary for its activation. We report that the treatment of Chinese hamster HA-1 cells with 250 nM of okadaic acid (OA), a ser/thr phosphatase inhibitor, leads to an increase in activated HSF after heat shock. This is followed by the activation of the transcription of heat shock genes as assayed by the increase in the synthesis of β-galactosidase in an HA-1 cell line containing the heat shock promoter ligated to the β-galactosidase gene. To investigate the specificity of OA, we used other phosphatase inhibitors. We found that treatment of HA-1 cells with 500 μM of sodium vanadate, an inhibitor of tyr/phosphatases, resulted in a three to fivefold reduction in HSF activation and binding to the heat shock element following heat shock. Such reduction in HSF activation virtually abolished β-galactosidase induction. Reduced HSP synthesis was further confirmed by SDS-PAGE and Western blot analysis using anti–HSP-70 and 28 antibodies. Sodium vanadate treatment of heat shocked cells greatly reduced levels of thermotolerance. These results show that ser/thr and specifically tyr/phosphatase inhibitors modulate the signal transduction pathway of HSF activation.

Key words: heat shock factor, heat shock protein, phosphorylation, sodium vanadate

In higher eukaryotes, the expression of heat shock genes is known to be regulated by the heat shock transcription factor, HSF. Several HSF (HSF-1 and HSF-2) genes have been cloned and sequenced in human and mouse. Although they do contain some similarities in their DNA sequence, their regulation of heat shock response differs [Rabindran et al., 1991; Schuetz et al., 1991; Sarge et al., 1991]. The mRNA coding for the heat shock factor is constitutively expressed and translated in non–heat shocked cells, but after heat shock HSFs acquire the ability to bind the heat shock element (HSE). This consists of three repeats of NGAAN arranged in an inverted orientation upstream of the heat shock genes [Pelham, 1982; Amin et al., 1988; Xiao and Lis, 1988]. The steps involved in heat shock factor activation have been suggested to include an ATP-independent, heat-induced conformational change in the HSF that allows HSF-HSE binding [Rabindran et al., 1993]. A second step is the phosphorylation of HSF [Sorger and Pelham, 1988; Sorger et al., 1987; Larson et al., 1988; Sarge et al., 1993]. In mammalian cells HSF has not been detected to be bound to DNA under normal growth conditions. Heat shock or other stresses are required to cause binding competence and transcriptional activation of HSF. The mammalian HSF genes contain both DNA binding and multimerization domains [Rabindran et al., 1991, 1993; Schuetz et al., 1991; Sarge et al., 1993]. Multimers of heat shock factor proteins bind to the HSE [Clos et al., 1990; Rabindran et al., 1993; Baler et al., 1993]. Heat shock factor protein is folded under normal growth conditions, preventing it from forming a multimer [Rabindran et al., 1993]. In mammalian cells, phosphorylation seems to alter the binding ability or the activity of the heat

Abbreviations: HSF, heat shock factor; HSE, heat shock element; HSP, heat shock protein; SV, sodium vanadate; OA, okadaic acid.

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shock transcription factor [Larson et al., 1988; Sarge et al., 1993].

Very little is known about the signal transduction pathway leading to HSF activation, although evidence concerning HSF multimerization and its subsequent phosphorylation is accumulating [Rabindran et al., 1993; Baler et al., 1993; Sarge et al., 1993]. In yeast and mammalian cells, SDS gel electrophoresis and Western blot analysis has shown several modified species of HSF. The activated HSF is sensitive to phosphatase treatment. Such evidence indicates that most of the modifications are associated with phosphorylation [Sorger, 1991; Sarge et al., 1993; Morimoto, 1993]. Other evidence that phosphorylation is involved in HSF activation comes from experiments on murine erythroleukemia (MEL) cells. HSF in MEL cells does not readily bind to DNA after heat shock, and there is no detectable transcription of any of the heat inducible heat shock genes. MEL cells are apparently deficient in their ability to phosphorylate HSF [Hensold et al., 1990].

Negative regulatory effects of HSPs on activated HSF have been postulated [Sorger, 1991; Morimoto, 1993]. HSPs may bind to HSF in the absence of stress. Following heat shock and denaturation of proteins, HSPs are postulated to dissociate from HSF monomers. HSF may then form multimers and acquire DNA binding capability. During recovery from stress, newly produced HSPs may regulate phosphatases and/or kinases that normally dephosphorylate or phosphorylate HSF molecules [Sorger, 1991; Morimoto, 1993].

We have found that addition of purified HSP-72/73 kDa to a cell-free extract increases the activity of ser/thr phosphatases, specifically that of protein phosphatase 1 (PP1), resulting in dephosphorylation of several proteins [Mivechi et al., 1993]. We have now investigated the possibility of negative regulation of HSPs in vivo by using okadaic acid, a potent inhibitor of ser/thr phosphatases [Grahame et al., 1991]. We hypothesized that HSF is normally dephosphorylated by ser/thr phosphatases. Its activation state should then be altered by phosphatase inhibitors. We show that okadaic acid increases the levels of activated HSF by about 20% following heat shock. Further, pretreatment of cells with okadaic acid increases the levels of HSP-70 and HSP-28 proteins. Sodium vanadate, an inhibitor of tyr/phosphatases [Gordon, 1991], substantially reduced HSF activation after heat shock. We also show that sodium vanadate treatment of HA-1 cells results in inhibition of thermotolerance.

MATERIALS AND METHODS

Cell Culture and Cell Survival Assay

HA-1 (Chinese hamster ovary) cells [Yang et al., 1966] were maintained in Dulbecco's minimal essential medium (DMEM) plus 10% fetal calf serum (FCS) in a humidified CO_2 regulated incubator. HA-1 cells transfected with p1730R plasmids [Voellmy et al., 1985] were maintained in DMEM containing 100 μg/ml of Geneticin. Cell survival assays were performed by growing the cells in 60 mm tissue culture dishes. At 80% confluency, cultures were treated appropriately, trypsinized, and plated for colony formation and incubation at 37°C for 10 days. Colonies of 50 or more cells were counted.

One-Dimensional Gel Electrophoresis and Western Blot Analysis

Approximately $1–2 \times 10^6$ cells were labeled with ^{35}S-met (40 μCi/ml, sp act > 6,000 Ci/mM; Amersham) in methionine-free MEM for 3 hr, following appropriate treatment. At the end of the labeling period, cells were washed with PBS and lyzed in sodium dodecyl sulfate (SDS) sample buffer and analyzed by PAGE as described previously [Laemmli, 1975]. Equal cell numbers were loaded in each lane. For Western blot analysis equal number of cells were loaded, and following electrophoresis proteins were transferred to the nitrocellulose membrane and exposed to appropriate dilutions of N27 (anti-HSP72/73) antibodies (gift of Dr. W. Welch); anti-HSP72 antibody (C92) (Amersham); anti-HSP 28 antibody (gift of Dr. J. Landry); or anti-mouse HSF-1 antibody (gift of Dr. R. Morimoto). Membranes were then exposed to alkaline phosphatase goat anti-mouse IgG (or goat anti-rabbit IgG in the case of anti-HSP 28 antibody) and subsequently stained. When anti-murine HSF-1 antibody was used, following the incubation in the presence of primary antibody, the blots were incubated with Streptavidin/Biotin IgG-HRP and subsequently developed with the ECL system (Amersham).

Gel Retardation Assays

For quantifying the binding of activated HSF to HSE, the procedure of Zimarino and Wu [1987] was used. Ten to twenty microliters of

the cell extract (equivalent to 2×10^6 cells) in extraction buffer (10 mM HEPES, pH 7.9; 0.4 M NaCl; 0.1 mM EGTA; 0.5 mM DTT; 5% glycerol; 0.5 mM PMSF) was added to 10 μl of binding buffer (37.5 mM NaCl; 15 mM Tris HCl, pH 7.4; 0.1 mM EGTA; 0.5 mM DTT; 5% glycerol) which also contained 4 μl of yeast tRNA (10 μg); poly dIdC (10 μg); 1 μg of sheared *E. coli* DNA; and 1 ng of ^{32}P-labeled HSE. The nucleotide sequence for HSE was the same as described by Zimarino and Wu [1987]. The mixture was incubated at 25°C for 15 min and electrophoresed on a nondenaturing polyacrylamide gel [Mivechi et al., 1992].

Gel retardation assay of cytoplasmic fraction: $1 \times 10^8 - 2 \times 10^8$ HA-1 cells were grown and scraped from tissue culture dishes, washed with PBS, and resuspended in two packed cell volumes of buffer A (10 mM HEPES, pH 7.9; 1.5 mM Mg_2Cl; 10 mM KCl; and 0.5 mM DTT). Cells were lysed by dounce homogenizer and nuclei were spun down. The supernatant was mixed with 0.11 volumes of buffer B (0.3 M HEPES, pH 7.9; 1.4 M KCl; 0.3 M $MgCl_2$). The mixture was centrifuged at 100,000 *g* for 60 min. The supernatant was dialysed against buffer D [20 mM HEPES, pH 7.9; 20% (v/v) glycerol; 0.1 M KCl; 0.2 mM EDTA; 0.5 mM PMSF; 0.5 mM DTT] for 8 hr and used immediately [Mosser et al., 1990].

β-galactosidase Assay

Cells were rinsed with PBS and lysed with lysis buffer 0.5% (v/v) NP40 in the reaction buffer (60 mM sodium phosphate, pH 7.0; 10 mM KCl; 1 mM $MgSO_4$). The lysed cells were microfuged for 10 min and O-nitrophenyl-b-D-galactosidase solution (4 mg/ml in reaction buffer) was added to the appropriate amounts of cell lysate. The mixture was vortexed and incubated for 30 min at 37°C. The reaction was stopped with 1 M sodium bicarbonate and absorbance was measured at 420 nm. The protein concentration was measured for each sample and data are reported per milligram of protein [Voellmy et al., 1985].

RESULTS

Okadaic Acid Increases the Binding of Activated HSF to the HSE in Heat Shocked HA-1 Cells

The addition of purified HSP-72/73 kDa to a reticulocyte lysate increases the activity of ser/thr phosphatases (as measured by ^{32}P release from ^{32}P-labeled phosphorylase-β as a substrate) as shown by reduced phosphorylation of several proteins [Mivechi et al., 1993]. We therefore investigated the possibility that OA, a potent and specific inhibitor of ser/thr phosphatases, affects the regulation of HSP synthesis in vivo. Exposure of HA-1 cells to a non-toxic dose of OA (250 nM, 45 min), followed by heat shock, caused an increase in binding of the activated HSF to HSE (data not shown). This increase was apparent immediately following heat shock. The increase in bound HSF activated transcription, which was measured by an increase in β-galactosidase activity (by approximately 20%) in HA-1 cells that contain the plasmid p1730R [Schiller et al., 1988] with the heat shock promoter [Voellmy et al., 1985] ligated to the β-galactosidase gene (data not shown).

Reduction of the Binding of Activated HSF to the HSE by Sodium Vanadate, an Inhibitor of Tyr/Phosphatases

We tested several phosphoserine or phosphotyrosine phosphatase inhibitors for their ability to affect HSF activation and binding. Sodium vanadate (SV) is an inhibitor of phosphotyrosine phosphatases [Klarlund, 1985]. Cells exposed to 500 μM of SV for 2.5 hr at 37°C showed a substantial decrease in the binding of HSF to the HSE following heat shock (Fig. 1). Sodium vanadate did not abolish HSF-HSE binding entirely, but its presence caused a more rapid decline of the residual activated HSF-HSE binding during the 3 hr recovery at 37°C following heat shock when compared to the HA-1 cells that received heat shock only. In parallel, SV abolished β-galactosidase activity in transfected HA-1 cells (Fig. 2A). The effect was concentration dependent; 63 μM of sodium vanadate inhibited β-galactosidase induction by more than 50% (Fig. 2B). Only 30 min of preincubation with 500 μM of SV was required to inhibit β-galactosidase activity by more than 50% following heat shock (Fig. 2C).

SV caused reduction of synthesis of HSP-70, 90, 110 (Fig. 3A, lanes 1–9, and B), and 28 (Fig. 3B) with little inhibition of general protein synthesis (note that ^{35}S-methionine labeling was for 3 hr) (compare lanes 1 and 10, Fig. 3A). As Fig. 3B (lanes 1 and 6–9) shows, C92 detected no inducible HSP-70 in sodium vanadate–treated cells, even 24 hr following heat shock. The levels of HSP-28 were also sharply reduced as measured by Western blot analysis (Fig. 3B, lanes 1 and 6–9).

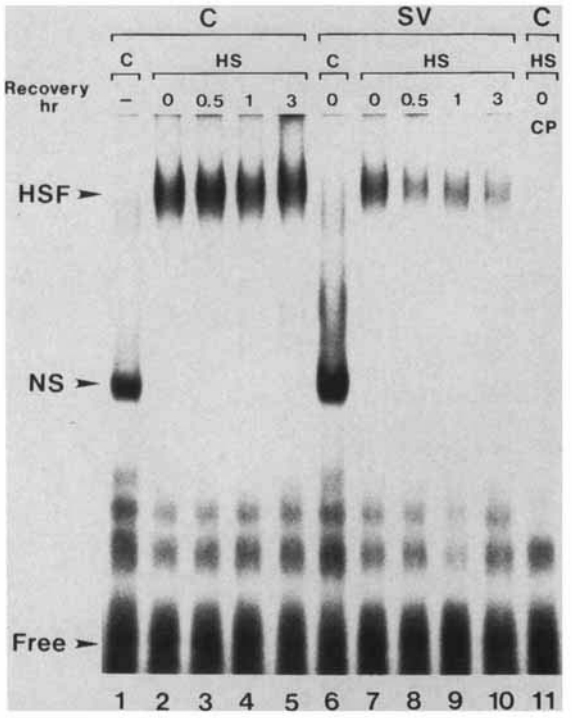

Fig. 1. Effect of sodium vanadate on HSF activation in HA-1 cells. HA-1 cells were treated with 500 μM of SV for 2.5 hr at 37°C. Cells were then treated at 45°C for 15 min and analyzed by gel retardation assays. Control groups received 45°C, 15 min heat shock. (–) indicates control group that received no heat shock; 0, 0.5, 1, and 3 are recovery hr at 37°C after heat treatment. Cp (lane 11) is the same group as lane 2 supplemented with 200-fold excess unlabeled HSE showing specific binding of HSF to HSE. NS, constitutive HSE binding activity. Free, unbound ^{32}P-HSE.

The sodium vanadate exposure did not kill any cells. It did, however, reduce the development of thermotolerance (Fig. 4). Thermotolerance results at least in part from accumulation of HSP-70 [Li and Werb, 1982; Hahn and Li, 1990; Mivechi et al., 1991]. We determined if the inhibition of HSP synthesis simply reflected reduction in protein synthesis as a whole. Treatment of cells by SV for 0.5 hr or 2.5 hr resulted in inhibition by 25 or 65%, respectively, of protein synthesis. Following removal of the drug, protein synthesis rapidly recovered (data not shown). The amount of protein synthesis and the kinetics of recovery of protein synthesis in both vanadate-treated and control cells following heat shock were similar. Therefore, the effects on thermotolerance resulted from specific inhibition of HSP synthesis.

Although preincubation of cells with 500 μM of SV inhibited HSF activation and binding to the HSE, SV concentrations as high as 1.5 mM did not totally abolish activated HSF-HSE binding in vivo (Fig. 5A). In vitro, however, 500 μM of SV added to the cytoplasmic fraction of HA-1 cells before heat shock completely inhibited HSF activation and binding to the HSE (Fig. 5B). For these experiments, the cytoplasmic fraction of unheated HA-1 cells was isolated. SV was added to the lysate either before or immediately after heating. As seen in Figure 5B, SV interfered with binding of the HSF only if it was present before heating. Therefore, sodium vanadate blocked steps prior to the HSF activation. Similarly, there was no effect on activated HSF-HSE binding when intact HA-1 cells were heat shocked and then treated with SV (data not shown).

The reason why in vivo (Fig. 5A), the highest concentrations of SV (1.5 mM) could not totally abolish HSF-HSE binding may be due to the levels of SV concentrations that can actually be achieved in vivo.

We measured the tyrosine phosphatase activity in HA-1 cells or in cell lysates treated with SV using ^{32}P-labeled Raytide as a substrate. Raytide, a common substrate for tyrosine phosphatase assay system, contains one tyrosine residue that labeled with γ-^{32}P-ATP in the presence of Src kinase as previously described [Krueger et al., 1990]. When HA-1 cells were treated with 500 μM of SV in vivo, over 70% inhibition of tyrosine phosphatase activity was observed within 30 min. Similar results were obtained in vitro, when SV was added to the cytoplasmic fraction of HA-1 cells and a fraction of the lysate was used to measure ^{32}P release from ^{32}P-labeled Raytide (data not shown). As shown in Figure 2C, 30 min preincubation of HA-1 cells with SV resulted in over 50% inhibition of β-galactosidase activity with little or no inhibition of synthesis of most other proteins (Fig. 3, and data not shown). Further, inhibition of tyrosine phosphatases were over 70% after 30 min. These results suggest that inhibition of tyrosine phosphatases may be a likely cause of inhibition of HSF activation.

We asked if SV inhibited the heat shock response by interfering with phosphorylation. An alternative possibility was that SV interfered directly with the binding of HSF to the HSE. We took advantage of the inability of MEL cells to achieve phosphorylation of the HSF [Hensold et al., 1990]. Preincubation with SV for 2.5 hr before heating at 43°C did not interfere with

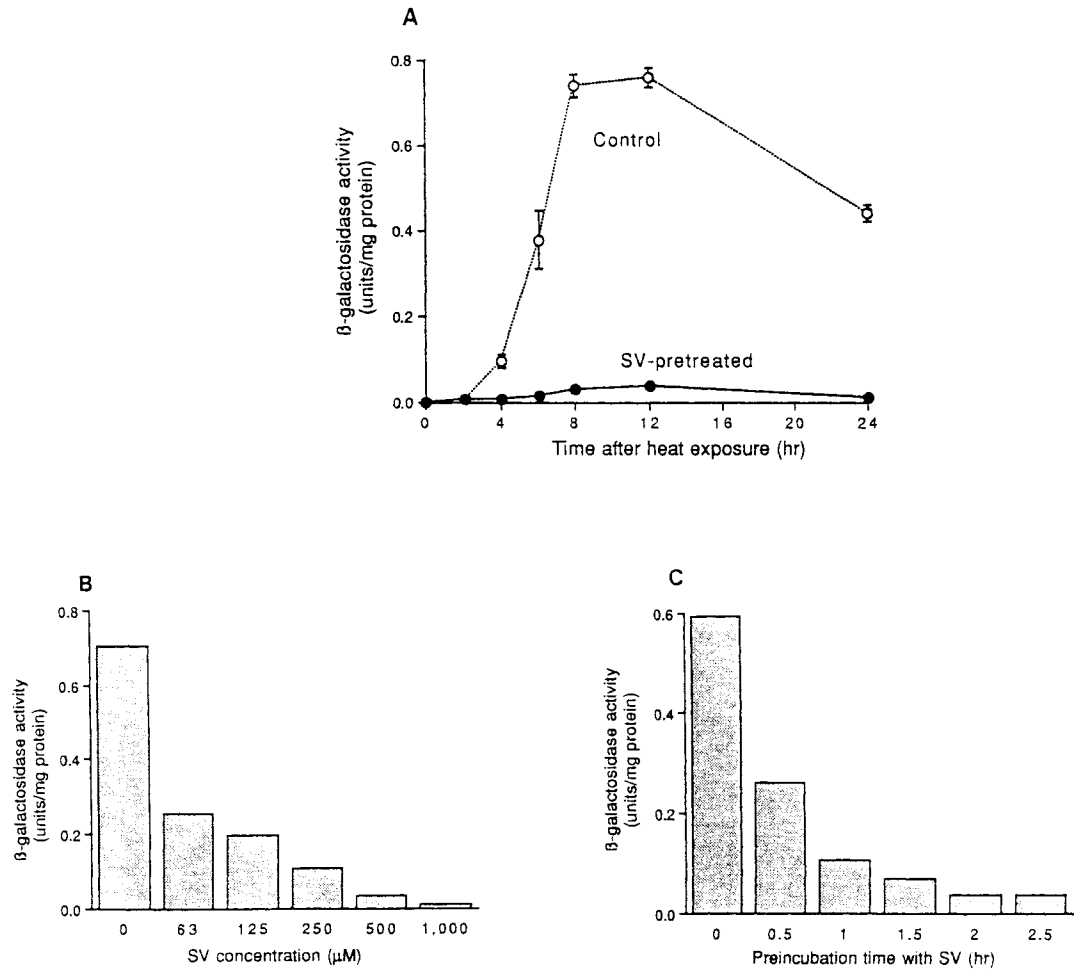

Fig. 2. Sodium vanadate inhibits β-galactosidase activity following heat shock in transfected HA-1 cells. **A:** Cells received 2.5 hr of 500 μM of SV treatment. Cells were then rinsed with PBS and challenged with 45°C, 15 min, and then incubated at 37°C. At indicated times, the β-galactosidase activities were measured. Control cells did not receive any SV pretreatment but received 45°C, 15 min heat shock. **B:** Cells were treated with increasing concentrations of SV for 2.5 hr. Cells were then rinsed with PBS and received 45°C, 15 min heat shock, and then were incubated at 37°C for 8 hr before β-galactosidase activities were measured. **C:** Cells were treated with 500 μM of SV for various times before cells were rinsed with PBS and heat shocked at 45°C, 15 min. The activity of β-galactosidase was determined 8 hr post–heat treatment.

residual binding of the HSF in heat shocked MEL cells (data not shown).

To investigate if other tyrosine phosphatase inhibitors also affect HSF-HSE binding activity, the following experiments were performed. Cytoplasmic fraction of HA-1 cells were heat shocked at 43°C for 1 hr either alone or in the presence of 500 μM of SV, 100 μM of Zn^{2+}, 500 μM of NaF, and 100 or 500 μM of ammonium molybdate. As Figure 6 shows, as with SV, the presence of both Zn^{2+} and ammonium molybdate before heat shock interfered with HSF-HSE binding activity. NaF, although not effective in this experiment in reducing HSF-HSE binding, did reduce HSF-HSE binding by more than 50% at a 500 μM concentration in other experiments (data not shown). The variations in the effectiveness of various tyrosine phosphatases are most likely due to the fact HSF can be partially activated during preparation of cytoplasmic fractions [Mosser et al., 1990], in which case the inhibitors may not be as effective.

In the order of effectiveness of inhibition of tyrosine phosphatases, Zn^{2+} was more effective than either SV or ammonium molybdate. In Western blot analysis using anti-phosphotyrosine antibody to measure increases in tyrosine phosphorylation after treatment with tyr/phosphatase inhibitors, we found that tyrosine phosphorylated proteins increased by 30–50% follow-

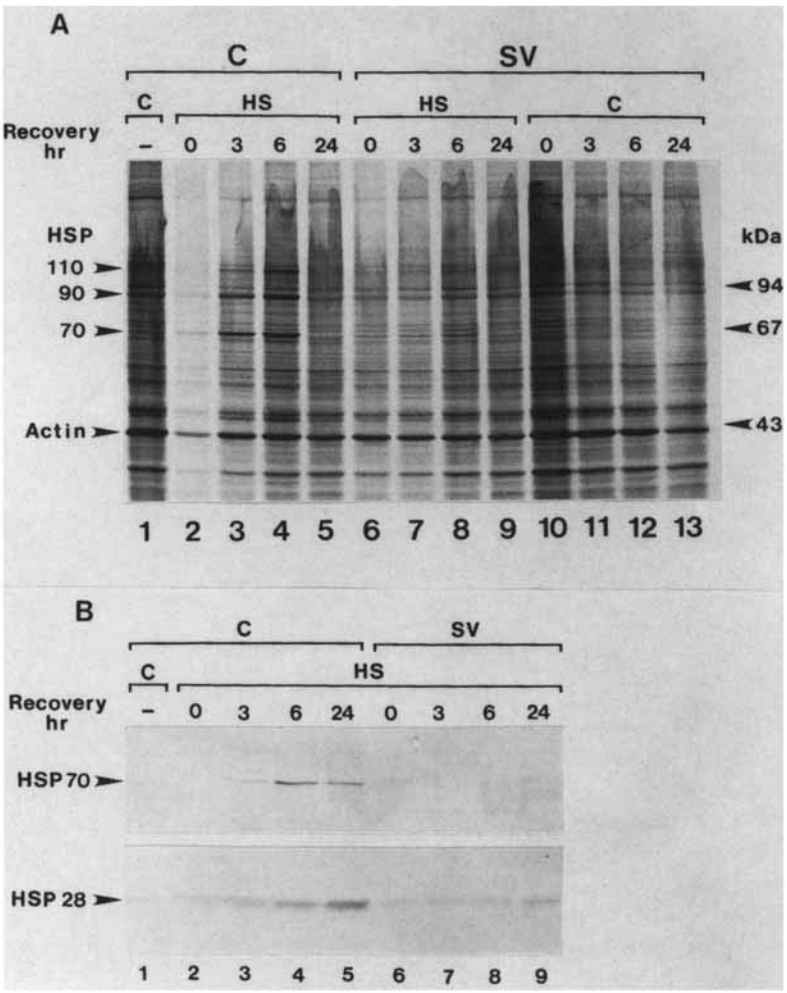

Fig. 3. Heat shock protein synthesis in HA-1 cells following treatment with sodium vanadate and heat shock. **A:** Cells were pretreated with 500 μM of SV for 2.5 hr at 37°C. Cells were then rinsed with PBS, heat shocked at 45°C for 15 min, and then labeled with ^{35}S-met for 3 hr at 37°C. Control cells did not receive any SV treatment prior to heat shock (lanes 1–5). SV control group (lanes 1 and 10–13) did not receive any heat treatment after SV treatment. Equal cell numbers were loaded in each lane and analyzed by SDS-PAGE. (–) indicates control cells labeled with ^{35}S-met for 3 hr; 0, 3, 6, and 24 are hr at 37°C following heat shock before labeling. The positions of HSPs 110, 90, and 70 have been shown on the left. C, control; HS, heat shock, 45°C, 15 min; SV, sodium vanadate–treated groups. **B:** Western blot analysis using anti–HSP-70 and anti–HSP-28 antibodies. In lanes 2–5 cells received heat shock. In lanes 6–9, cells were heat shocked after SV treatment. C, control; HS, 45°C, 15 min heat shock. (–) indicates control cells, no treatment; 0, 3, 6, and 24 are hr at 37°C after SV and/or heat treatment.

ing the addition of the inhibitors to the cell lysate (or when HA-1 cells were treated with 500 μM of SV in vivo). The increase in tyrosine phosphorylated proteins correlated with the amount of inhibition of tyr/phosphatase activities measured. There was a positive correlation between the increase in tyr/phosphorylated proteins and the degree by which different cell lines react to tyr/phosphatase inhibitors in terms of inhibition of HSF-HSE binding activity (data not shown). These results suggest that tyr/phosphatase inhibition is the most likely mechanism of inhibition of HSF activation.

Modulation of HSF Phosphorylation by Heat Shock in the Presence of Phosphatases or Phosphatase Inhibitors

Several investigators have shown that HSF is phosphorylated after heat shock [Larson et al., 1988; Sarge et al., 1993]. HSF-HSE binding can be inhibited when the lysate is treated with potato acid phosphatase before heat shock. Fig-

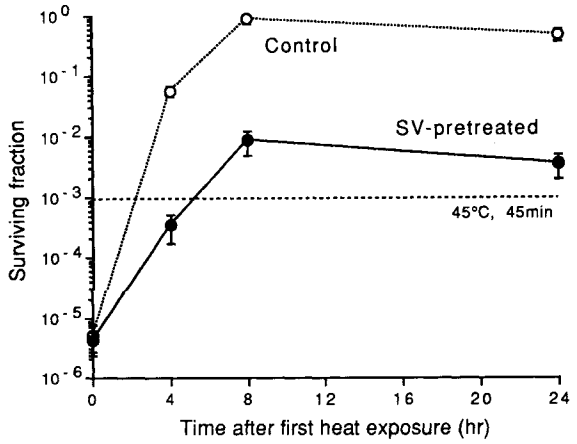

Fig. 4. Effect of sodium vanadate and heat shock on development of thermotolerance in HA-1 cells. Cells were treated with 45°C, 15 min (control) or treated with 500 μM of SV before receiving 45°C, 15 min heat shock (SV, pretreated). Cells were then incubated at 37°C for increasing times before they were challenged with a heat dose of 45°C for 45 min. Dashed line shows the survival level for HA-1 cells receiving a heat dose of 45°C for 45 min only, without any pretreatment.

ure 7A shows the result of such an experiment. HA-1 cells were heat shocked to activate HSF, the whole cell extracts were prepared and 20 μg of lysate was incubated in the presence of five units of acid phosphatase for 1 hr at 37°C. The phosphatase treatment inhibited HSF-HSE binding activity (Fig. 7A, lane 2 in comparison with lane 3). The slight reduction in HSF-HSE binding in lane 3 compared to lane 1 could be due to the in vitro incubation of cell lysates at 37°C for 1 hr, which may effect the HSF-HSE binding ability. These results indicate that phosphorylation of at least some residues in the HSF protein are needed for maximal HSF-HSE binding. Phosphatase activity can also be abolished by the addition of ammonium molybdate and HSF-HSE binding can thus be restored [Larson et al., 1988; Sarge et al., 1993].

Sarge et al. [1993] observed an increase of 11 kDa in the molecular weight of HSF protein upon heat shock. This increase was reduced or abolished by phosphatase treatment of the heat shocked cell extracts, suggesting strongly that the HSF had been phosphorylated. Figure 7B shows the results of an experiment in which we heat shocked HA-1 cells, and prepared whole cell extracts and then treated these with 2.5 units of acid phosphatase for 1 hr at 37°C. Lane 1 shows that after heat shock (45°C, 15 min) HSF proteins have molecular weights ranging between 70 kDa and 80 kDa. Phosphatase treatment of

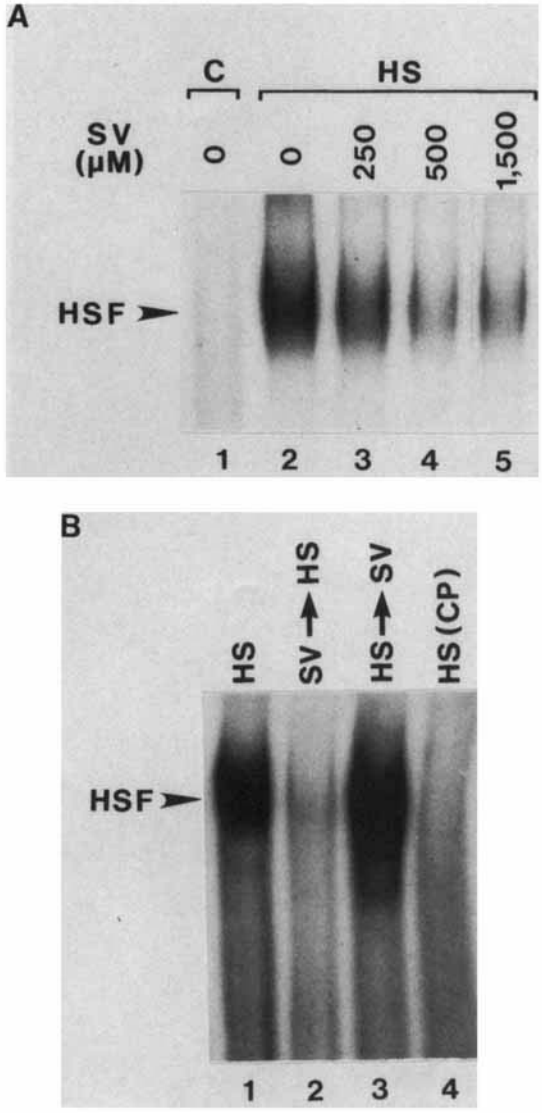

Fig. 5. **A:** Effect of increasing concentrations of sodium vanadate on HSF-HSE binding in vivo. HA-1 cells were treated with 0, 250, 500, or 1,500 μM of sodium vanadate for 2.5 hr at 37°C. After rinsing with PBS, cells were heat shocked at 45°C for 15 min. Immediately after heat shock, HSF-HSE binding was measured using gel retardation assays. Lane 1, control, no treatment; lane 2, cells received 45°C, 15 min heat shock only. Lanes 3–5, cells treated with SV and then heat shocked. **B:** Effect of sodium vanadate added to the cytoplasmic fraction before or after heat shock on HSF-HSE binding. Cytoplasmic fractions of HA-1 cells were prepared as described in Materials and Methods. The cytoplasmic fractions were then treated at 43°C for 1 hr and 500 μM of SV was added either immediately before or after heat shock. HSF-HSE activation and binding were then measured by gel retardation assays. **Lane 1,** HA-1 cytoplasmic fraction received 43°C, 1 hr; **lane 2,** 500 μM SV was added to the cytoplasmic fraction immediately before heat shock of 43°C, 1 hr. **Lane 3,** cytoplasmic fraction of HA-1 cells were heat shocked at 43°C for 1 hr and 500 μM SV was added. **Lane 4,** lane 1 plus 200-fold excess unlabeled HSE used as a competitor indicating specific HSF-HSE binding. HS, heat shock; SV, sodium vanadate; CP, competitor.

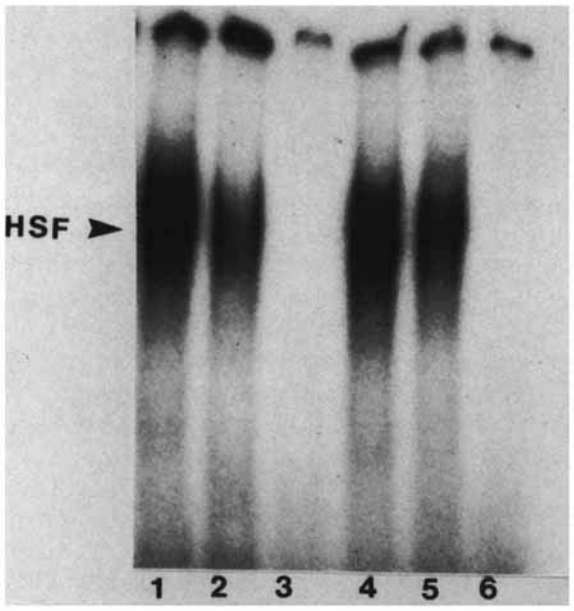

Fig. 6. Effect of SV, Zn^{2+}, ammonium molybdate, and NaF on HSF-HSE binding activity. The cytoplasmic fraction of control HA-1 cells was incubated with a variety of tyr/phosphatase inhibitors. The fractions were then incubated at 43°C for 1 hr and HSF-HSE binding activity was measured. **Lane 1,** 43°C, 1 hr. **Lane 2,** 500 μM of SV plus 43°C, 1 hr. **Lane 3,** 100 μM Zn^{2+} plus 43°C, 1 hr. **Lane 4,** 500 μM of NaF plus 43°C, 1 hr. **Lanes 5 and 6,** 100 or 500 μM of ammonium molybdate plus 43°C, 1 hr, respectively.

the heat shocked cell lysate for 1 hr at 37°C brings the molecular weight back to that of the original unheated HSF, that is, approximately 70 kDa (lane 2). Addition of 100 μM of Zn^{2+} to the heat shocked cell lysate containing 2.5 units of acid phosphatase and its further incubation for 1 hr at 37°C counteracts the phosphatase effect (lane 3). Zn^{2+} by itself has no effect on the HSF protein when added to the heat shocked cell lysate and incubated at 37°C for 1 hr (lane 4). From these results we conclude that the 70 kDa HSF is in an uphosphorylated state, the 80 kDa phosphorylated, consistant with the data of Sarge et al. [1993].

To investigate whether the treatment of HA-1 cells with sodium vanadate before heat shock changes the molecular weight of HSF, cells were heat shocked with or without pretreatment with 500 μM of sodium vanadate for 2½ hr. As Figure 7C shows, pretreatment with SV does not inhibit the increase in the molecular weight of HSF after heat shock. These results suggest that SV does not interfere with phosphorylation of HSF after heat shock, although we cannot rule out partial reduction in phosphorylation that cannot be detected by gel electrophoresis.

Sodium Vanadate Does Not Affect Nuclear Localization of HSF

HSF monomer normally is translocated into the nuclei and forms a trimer after heat shock. To investigate the possibility that SV interferes with either process, that is, nuclear translocation and trimerization, the following experiments were performed. Immunofluorescence studies were performed to find out whether sodium vanadate prevents nuclear translocation of HSF, that is, if HSF is translocated into the nuclei in sodium vanadate–treated, heat shocked HA-1 cells (Fig. 8B and D). The only apparent difference observed between control and SV-treated heat shocked cells is that the number of foci observed in the nuclei in SV-treated cells is consistently higher than in control heat shocked cells (compare Fig. 8A and B). As has been previously reported [Sarge et al., 1993], there are four to six foci of HSF proteins in heat shocked human cells (these foci are not present in murine cells; these show uniform nuclear staining after heat shock, same for HA-1 cells, see Fig. 8C and D). We also observed four to six foci of HSF protein after heat shock in control human HT1080 cells. In SV-treated, heat shocked cells, the number of these foci is increased consistently to 10 or more.

These results prompted us to measure the trimer formation of HSF in SV-treated, heat shocked (45°C, 15 min), or heat shocked HA-1 cells. For such experiments, HA-1 cells were either heat shocked or treated with SV for 2½ hr, rinsed with PBS, and immediately heat shocked at 45°C for 15 min. The cell lysates were prepared and incubated in the presence of 0, 0.2, or 1 mM of cross-linking reagent EGS [Sarge et al., 1993] for 20 min at 25°C. Cell lysates were then analyzed by gel electrophoresis and Western blotting. Our data indicated that there was no difference between heat shocked and SV-treated, heat shocked cells in terms of HSF trimerization (data not shown). HSF formed complexes of approximately 230 kDa at 1 mM EGS concentration in both groups.

DISCUSSION

Regulation of transcription factors by phosphorylation has been suggested to occur at several levels [Hunter and Karin, 1992]. Phosphorylation/dephosphoylation of transcription factors

regulates nuclear translocation, DNA binding, regulation of transactivation, or stimulation of transrepression. Most often phosphorylation of one or two residues has been shown to regulate such activities. In yeast, where HSF is constitutively bound to DNA, phosphorylation has been suggested to stimulate transactivation [Sorger and Pelham, 1988]. In higher eukaryotes, where HSF is not bound to the HSE under normal growth conditions, the precise regulation of HSF activation by phosphorylation is currently not understood. Recently it was shown that there is as much as an 11 kDa increase in the molecular weight of HSF protein upon heat shock. Phosphatase treatment returns HSF to its original molecular weight [Sarge et al., 1993, and data presented here]. Phosphatase treatment of unheated HSF decreases its molecular weight by 4 kDa, indicating HSF is partially phosphorylated under normal growth conditions [Sarge et al., 1993]. It is very likely, therefore, that HSF is regulated at multiple levels by phosphorylation.

Treatment of cell extracts with sodium vanadate, Zn^{2+}, or ammonium molybdate to inhibit tyr/phosphatases resulted in substantial reduction in HSF-HSE binding. Therefore, phosphorylation may not only be required for maximal transcription, but also for efficient HSF binding to the HSE. Phosphorylation is known to increase binding to DNA of other factors such as

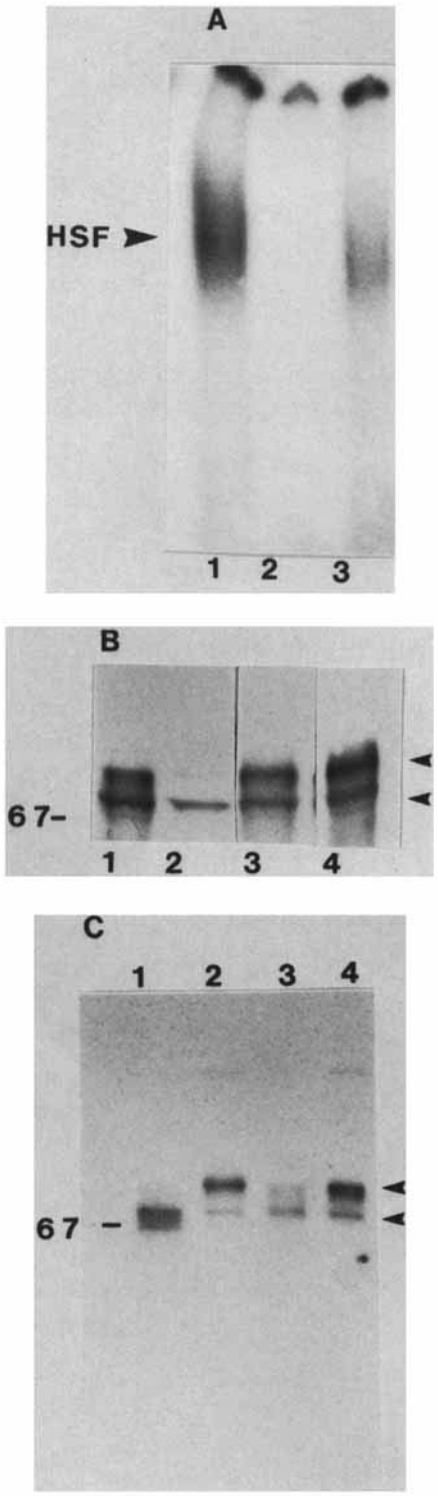

Fig. 7. Effect of phosphatase and phosphatase inhibitors on HSF-HSE binding ability and HSF phosphorylation. **A:** HA-1 cells were heat shocked in vivo at 45°C for 15 min. Whole cell extracts were prepared [Mivechi et al., 1992] and incubated at 37°C for 1 hr in the presence or absence of 5 units of potato acid phosphatase. HSF-HSE binding was then measured. **Lane 1,** heat shocked whole cell extracts without further incubation at 37°C in vitro. **Lane 2,** heat shocked whole cell extracts were incubated in the presence of 5 units of potato acid phosphatase for 1 hr at 37°C. **Lane 3,** heat shocked whole cell extracts were incubated at 37°C for 1 hr. HSF-HSE binding assay was then performed with treated extracts. **B:** Whole cell extracts were prepared from heat shocked cells (45°C, 15 min) and analyzed by SDS-PAGE and Western blot analysis using anti-murine HSF-1 antibody. **Lane 1,** heat shocked cell extract incubated at 37°C for 1 hr. **Lane 2,** heat shocked cell extracts incubated at 37°C for 1 hr in the presence of 2.5 units of potato acid phosphatase. **Lane 3,** heat shocked cell extracts incubated at 37°C for 1 hr in the presence of 2.5 units of potato acid phosphatase and 100 μM of Zn^{2+}. **Lane 4,** heat shocked cell extracts incubated at 37°C for 1 hr in the presence of 100 μM of Zn^{2+}. The arrowheads indicate the phosphorylated and unphosphorylated forms of HSF protein. Molecular weight marker is shown on the left. **C:** HA-1 cells were treated with 500 μM of SV for 2.5 hr. Cells were then heat shocked and analyzed by SDS-PAGE and Western blot analysis using anti-murine HSF antibody. **Lane 1,** control HA-1 cells. **Lane 2,** HA-1 cells heated at 45°C, 15 min. **Lane 3,** HA-1 cells were treated with SV for 2.5 hr. **Lane 4,** HA-1 cells were treated with SV for 2.5 hr and then heat shocked at 45°C, 15 min. The arrowheads show the phosphorylated and unphosphorylated forms of HSF. Molecular weight marker is shown on the left.

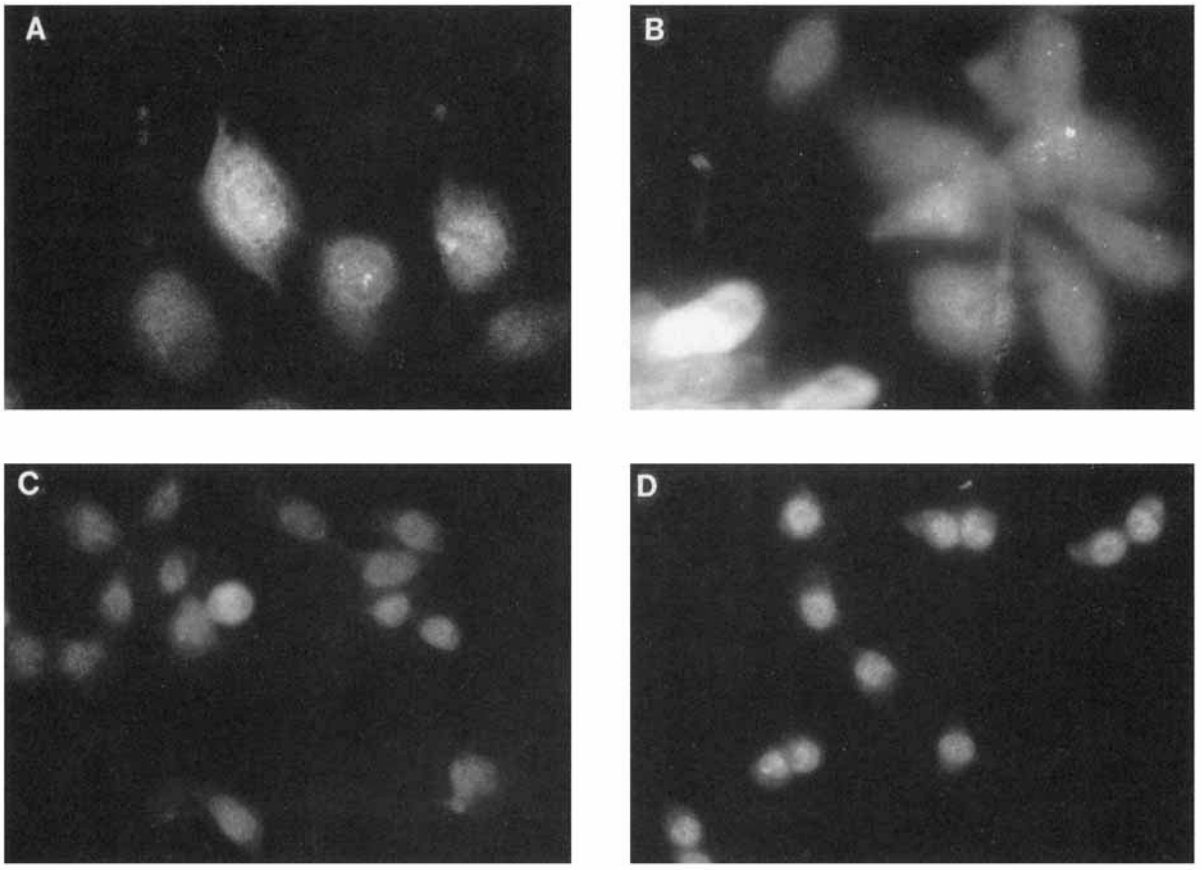

Fig. 8. Immunofluorescence analysis of control and SV-treated cells. **A** and **B:** Heat shocked (45°C, 20 min) or SV-treated (500 μM, 2.5 hr at 37°C) and then heat shocked HT1080, a human colon carcinoma cell. **C** and **D:** Heat shocked (45°C, 15 min) or SV-treated then heat shocked HA-1 cells. Immunofluorescence studies were performed as described by Sarge et al. [1993]. Control or SV-treated but not heat shocked cells do not show any nuclear staining. There are also no differences visually apparent between the control or SV-treated groups (data not shown). Anti-human HSF-1 (gift of Dr. C. Wu) and anti-murine HSF-1 (gift of Dr. R. Morimoto) antibody was used for HT1080 and HA-1 cells, respectively.

E2F, E4F, and serum response factor. Phosphorylation sites upstream of the DNA-binding domain control the binding on/off rate [Hunter and Karin, 1992].

The following conclusions can be made for the regulation of HSF activation by heat shock. HSF is known to be present mostly in the cytoplasm and some in the nuclei [Sarge et al., 1993; Baler et al., 1993]. Under normal growth conditions, HSF cannot bind to the DNA, perhaps because the HSF monomer is folded on itself, preventing it from forming a trimer and binding to the DNA [Rabindran et al., 1993]. Before heat shock, HSF is most likely under negative regulation, because HSF transiently transfected into mammalian cells constitutively binds to DNA [Rabindran et al., 1993; Sarge et al., 1993].

The signal transduction pathway leading to HSF activation, however, is not understood. We have previously shown [Mivechi et al., 1993] that purified HSP-70 kDa activated ser/thr phosphoprotein phosphatases [Lau et al., 1989] in vitro. Such results indicated to us that the negative regulatory effect of HSP-70 kDa on the heat shock cycle which has been proposed recently [Morimoto, 1993] may in fact be due to HSP-70 kDa activation of ser/thr phosphatases. Such an hypothesis would mean that OA, an inhibitor of phosphoprotein phosphatase type 1 and 2A, should inhibit dephosphorylation of phosphorylated HSF (HSF most likely is phosphorylated on ser/thr residues), therefore resulting in an increase in accumulation of HSPs.

Surprisingly, the OA effect on HSF phosphorylation and β-galactosidase was rather minor in HA-1 cells. It is possible that although phosphorylated HSF may be dephosphorylated by ser/thr phosphatases, there is a tight regulation in vivo by the kinase(s) that phosphorylates HSF. Alternately, the concentration of OA used

here to inhibit ser/thr phosphatases did not completely inhibit the enzymes. Using another HSP-70 promoter [Hunt and Morimoto, 1985] ligated to the CAT gene in cell lines other than HA-1, Chang et al. [1993] have recently shown that OA given before heat shock increases CAT activity in transient transfected cells by three to 10-fold. These data suggest that HSF may indeed be dephosphorylated by ser/thr phosphoprotein phosphatases type 1 or 2A.

Sodium vanadate and other tyrosine phosphatase inhibitors prevent or reduce DNA binding of HSF to the HSE. Sodium vanadate does not interfere with HSF's nuclear localization or its trimerization. Further, sodium vanadate does not appear to inhibit phosphorylation of HSF protein. There are several possibilities for these results. It has been recently suggested that HSF is phosphorylated at multiple residues and such phosphorylation may be differentially regulated [Sarge et al., 1993]. Sodium vanadate may affect phosphorylation of HSF at a few residues, and therefore, there is no measureable shift in the molecular weight. It is also possible that sodium vanadate affects the phosphorylation of some factor(s) that, in turn, affects DNA binding of HSF, consistent with the studies showing that sodium vanadate is effective in reducing DNA binding of HSF only when it is added before heat shock.

It is also possible that SV and other tyrosine phosphatase inhibitors affect reactions several steps prior to HSF activation, and inhibition of HSF-HSE binding is a consequence. It has recently been shown that tyrosine phosphorylation of several proteins increases upon heat shock [Maher and Pasquale, 1989]. Whether such proteins play a role in the activation of the heat shock response needs to be investigated. However, in those studies the heat shock used to increase tyr/phosphorylated proteins were extremely toxic heat doses.

Our data suggest that abolishment of activated HSF-HSE binding results in a substantial reduction of synthesis of various HSPs after heat shock. Such reduction of newly synthesized HSPs results in a clear reduction of thermotolerance. The only other agent known to reduce HSP synthesis following heat shock and also reduce thermotolerance is quercetin [Hosokawa et al., 1992]. However, quercetin affects many cellular processes. Which one (or more) of these is responsible for its inhibition of the heat shock response is not known.

In conclusion we have shown that mammalian heat shock factor activation and binding to the heat shock element is affected by tyrosine phosphatase inhibitors. Furthermore, tyrosine phosphatase inhibitors can effectively inhibit heat shock protein synthesis and reduce the development of thermotolerance.

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