Journal of Cellular Biochemistry 57:321–330 (1995)

MAPKAP Kinase 2 Is Activated by Heat Shock and TNF-α: In Vivo Phosphorylation of Small Heat Shock Protein Results From Stimulation of the MAP Kinase Cascade

Katrin Engel, Annette Ahlers, Marion A. Brach, Friedhelm Herrmann, and Matthias Gaestel

Max-Delbrück-Center for Molecular Medicine, 13122 Berlin, Germany

Abstract The activation of MAPKAP kinase 2 was investigated under heat-shock conditions in mouse Ehrlich ascites tumor cells and after treatment of human MO7 cells with tumor necrosis factor-α (TNF-α). MAPKAP kinase 2 activity was determined using the small heat-shock proteins (sHsps) Hsp25 and Hsp27 as substrates. In both cell types, about a threefold increase in MAPKAP kinase 2 activity could be detected in a time interval of about 10–15 min after stimulation either by heat shock or TNF-α. Phosphorylation of MAPKAP kinase 2, but not the level of MAPKAP kinase 2 mRNA, was increased after heat shock in EAT cells. It is further shown that activation of MAPKAP kinase 2 in MO7 cells is accompanied by increased MAP kinase activity. These data strongly suggest that increased phosphorylation of the sHsps after heat shock or TNF-α treatment results from phosphorylation by MAPKAP kinase 2, which itself is activated by phosphorylation through MAP kinases. Hence, we demonstrate that MAPKAP kinase 2 is responsible not only for phosphorylation of sHsps in vitro but also in vivo. The findings link sHsp phosphorylation to the MAP kinase cascade, explaining the early phosphorylation of sHsp that is stimulated by a variety of inducers such as mitogens, phorbol esters, thrombin, calcium ionophores, and heat shock.

Key words: mitogen activated protein kinases, heat shock, TNF-α, small heat-shock proteins

The small heat-shock proteins (sHsps) are ubiquitous, conserved, and evolutionary related to the lens α-crystallins [for recent review, see de Jong et al., 1993]. Their expression can be induced by heat shock and, in different organisms, they are also regulated during development [Arrigo and Pauli, 1988] or by hormones, i.e., by ecdisone in *Drosophila* [Rollet and Best-Belpomme, 1986] and by estradiol in human [Fuqua et al., 1989]. The sHsps form intracellular aggregates of about 400–800 kd, which are localised in the cytoplasm, but can also be detected in the nuclear region after heat shock [Arrigo et al., 1988]. Until now, the function of the sHsp is not well understood. Although an increase in thermoresistance of cells is achieved by overexpression of human and *Drosophila* Hsp27 [Landry et al., 1989; Rollet et al., 1992] as well as mouse Hsp25 [Knauf et al., 1992], it is not clear which mechanisms underlie this effect. The involvement in processes influencing the structure of the cytoskeleton has been supposed, since turkey Hsp27 inhibits actin polymerization in vitro [Miron et al., 1991] and a modulation of the stability of stress fibers in hamster Hsp27 overexpressing cells could be observed [Lavoie et al., 1993]. Recently, sHsps and the related αB-crystallins have been also demonstrated to exhibit chaperone-like properties in vitro by preventing thermal aggregation and assisting in refolding of proteins [Horwitz, 1992; Jakob et al., 1993; Merck et al., 1993]. To make the picture more complex, in many cases the sHsps represent a major phosphoprotein of the cell. Their phosphorylation occurs very rapidly in a variety of species in response to heat shock, growth factors, phorbol esters, calcium ionophores, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [Welch, 1985; Hepburn et al., 1988; Kaur et al., 1988]. It has been speculated that the phosphorylation of the sHsp which proceeds at serine residues in the more hydro-

Received December 27, 1993; revised June 30, 1994; accepted July 26, 1994.

Address reprint requests to Dr. Matthias Gaestel, MDC Berlin-Buch, R.-Rössle-Strasse 10, D-13122 Berlin, Germany.

phobic domain of the molecule can influence the high molecular weight aggregate structure of the sHsps and may regulate sHsp-mediated thermoresistance, their chaperone-like properties or other unknown functions of the sHsps. Surprisingly, at least the thermoresistance-mediating properties of Hsp25 in NIH 3T3 cells and its in vitro-chaperone properties seem to be phosphorylation-independent [Knauf et al., 1994]. The sHsp phosphorylation sites identified so far are localized within a protein kinase recognition motif R X X S, which is not recognized by S6 kinase II and only poorly phosphorylated in vitro by excess of PKC and PKA [Gaestel et al., 1991; Landry et al., 1992; Benndorf et al., 1992]. Recently, MAPKAP kinase 2, a protein kinase that could be activated via threonine phosphorylation by MAP kinases, has been described as the first enzyme that phosphorylates Hsp25 and Hsp27 in vitro, at similar rates to its standard substrate, a peptide similar to the N-terminus of glycogen synthase, and at the same phosphorylation sites that are phosphorylated after heat shock in vivo [Stokoe et al., 1992b]. Furthermore, the copurification of MAPKAP kinase 2 activity and Hsp25 kinase activity from rabbit skeletal muscle extracts as well as coexpression of Hsp25 and MAPKAP kinase 2 within rabbit muscle have been demonstrated, suggesting that MAPKAP kinase 2 is the enzyme responsible for phosphorylation of sHsp in vivo [Stokoe et al., 1992b], although no activation of MAPKAP kinase 2 (e.g., by heat shock) has been described so far.

This paper investigates the activation of MAPKAP kinase 2 under two different conditions known to lead to a significant increase in sHsp phosphorylation. We provide experimental data that MAPKAP kinase 2 activity is increased as a result of heat shock and TNF-α treatment, giving evidence for in vivo phosphorylation of the sHsps by MAPKAP kinase 2 under these conditions. The mechanism of MAPKAP kinase 2 activation is examined. It is shown that MAP kinase activity is increased upon exposure to TNF-α treatment and that the degree of phosphorylation of MAPKAP kinase 2 is elevated upon heat shock. These data indicate that MAPKAP kinase 2 activation by MAP kinases is probably a general mechanism for sHsp phosphorylation.

METHODS AND MATERIALS

Cell Culture

Ehrlich ascites tumor (EAT) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol. Cells were grown to near confluence and shifted to serum-free medium 16 h prior to exposure to heat shock at 43.5°C for the indicated periods of time.

Cells from the human megacaryocyte leukemia cell line MO7 (Avanzi et al., 1988) were cultured in RPMI medium with 10% fetal calf serum, 2 mM L-glutamine, 5 ng/ml recombinant human IL-3 (S. Gillis, Immunex, Seattle, WA), and 5 ng/ml recombinant human GM-CSF (S. Gillis, Immunex, Seattle, WA). Prior to treatment with TNF-α, IL-3 and recombinant human GM-CSF were withdrawn for 24 h to obtain quiescent cells. Cells were stimulated by TNF-α (G. Adolf, Bender-KG, Vienna, Austria) at a final concentrations of 100 or 250 U/ml for the times indicated.

Preparation and Fractionation of Cell Lysates

Cell cultures were scraped in ice-cold PBS buffer, washed twice, and the cell pellet was lysed by addition of buffer L (20 mM Tris-Acetate pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM α-glycerophosphate, 50 mM NaF, 5 mM pyrophosphate, 1% Triton X-100, 1 mM benzamidine, 2 μg/ml leupeptin, 0.1% β-mercaptoethanol, 0.27 M sucrose, 0.2 mM PMSF; 3.3 ml for 5×10^7 cells), vortexed for 30 sec, and clarified by centrifugation at 13,000*g* for 10 min at 4°C. Cell lysates analysed for MAPKAP kinase 2 activity were diluted 10-fold in buffer A (20 mM MOPS pH 7.0, 1 mM EDTA, 5% (v/v) glycerol, 0.01% (w/v) Brij 35, 0.2 mM PMSF, 1 mM benzamidine, 0.1% (v/v) β-mercaptoethanol) and applied to a Mono S column (Pharmacia, column dimensions 5 × 0.5 cm) equilibrated in buffer A. After washing with buffer until the absorbance at 280 nm was < 0.02, the column was developed with a 40-ml linear salt gradient from 0 to 500 mM NaCl in buffer A. The flow rate was 0.5 ml/min, and fractions of 1 ml were collected. For analysis of MAP kinase activity, cell lysates were diluted 1:10 in buffer B (50 mM Tris-HCl pH 7.3, 1.5 mM EGTA, 0.15 mM Na_3VO_4, 5% (v/v) glycerol, 0.03% (w/v) Brij 35, 0.2 mM PMSF, 1 mM benzamidine, 0.1% (v/v) β-mercaptoethanol) and applied to a Mono Q column (Pharmacia, column dimensions 5 × 0.5 cm) equilibrated in buffer B. After washing with buffer the column was developed with a 40 ml linear salt gradient from 0 to 600 mM NaCl in buffer B, a flow rate of 0.5 ml/min and a fraction size of 1 ml. Size exclusion liquid chromatography was carried out

on a Superose 12 HR 30/10 column (Pharmacia) equilibrated with buffer A supplemented with 50 mM NaCl, 5 mM pyrophosphate and 0.5 mM Na_3VO_4 at a flow rate of 0.4 ml/min and a fraction size of 0.33 ml. Phosphorylase b (94 kd), bovine serum albumin (67 kd), ovalbumin (44 kd), carbonic anhydrase (30 kd) and cytocrome C (dimeric, 24 kd) (Pharmacia) were used as standards for estimation of the molecular mass.

Protein Kinase Assays

The assays for sHsp kinase/MAPKAP kinase 2 or MAP kinase activity of unfractionated cell lysates were always performed in a final reaction volume of 25 μl containing 5 μl of a 1:20 dilution of the cell lysate in buffer A or B, respectively. This dilution ensures a linear dependence of protein kinase activity on protein concentration. Assay conditions were also tested to guarantee a linear dependence of kinase activity determined on the assay time chosen. For protein precipitation assay 5 μl of cell lysate diluted as described above or undiluted fractions of Mono S chromatography were incubated in a kinase reaction mixture of a final volume of 25 μl, containing 50 mM α-glycerophosphate, 0.1 mM EDTA, 2.5 μM PKI (the specific peptide inhibitor of PKA, GIBCO, Gaithersburg, MD), 4 mM $(CH_3COO)_2Mg$, 0.1 mM ATP, 1–3 μCi [γ-^{32}P]ATP and protein substrate (10 μg recombinant Hsp25 or Hsp27 purified from *Escherichia coli* [Gaestel et al., 1989; Jakob et al., 1993]. After 15 min at 30°C, reactions were stopped by adding 1 ml 5% (w/v) tricloroacetic acid. The precipitate obtained by centrifugation at 13,000*g* was washed three times with 20% (w/v) trichloroacetic acid (TCA) and analysed by Cerenkov counting.

In the case of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) assay, the above phosphorylation reaction was terminated by adding 8 μl 4 × SDS sample buffer. Proteins were separated by SDS–PAGE, ^{32}P-labeled proteins were detected using a Bio Imaging Analyser BAS 2000 (Fuji), and labeling was quantified by photo-stimulated luminescence (PSL). Where indicated, the inhibitors H7 and HA1077 were added to a final concentration of 20 μM.

MAP kinase activity was determined by incubation of 5 μl diluted cell lysate or undiluted fractions of Mono Q chromatography in 25 μl final volume containing 25 mM Tris–HCl, pH 7.0, 0.1 mM EGTA, 0.1 mM Na_3VO_4, 1 μM PKI, 4 mM $(CH_3COO)_2Mg$, 20 μM ATP, 2 μCi [γ-^{32}P]ATP and 0.33 mg/ml myelin basic protein (MBP; Sigma, St. Louis, MO). Phosphorylation of MBP was analysed by combining SDS–PAGE and phospho-imaging as described above.

Northern Blot Detection of MAPKAP Kinase 2

Degenerate oligonucleotides derived from the sequence of tryptic peptides of domains VIb and XI [Hanks et al., 1991; Stokoe et al., 1992a] were used as primers in a PCR reaction with mouse cDNA as template. The fragment amplified was cloned into pUC18 (Sure Clone Ligation Kit, Pharmacia). DNA sequencing was carried out in both directions using double-stranded plasmid and Sequenase 2.0 (U.S. Biochemical Corp.). The deduced amino acid sequence of the fragment was verified to be a part of the MAPKAP kinase 2 sequence [Engel et al., 1993]. A hybridization probe was generated from the cloned PCR fragments by random priming and labeling with [α-^{32}P]ATP (3,000 Ci/mmol) (Megaprime labeling kit, Amersham). RNA was prepared from EAT cells [Chomczynski and Sacchi, 1987], cultured under normal conditions or exposed to a 15-min heat shock of 43.5°C. 20 μg of RNA was separated under denaturing conditions in 1% agarose gel, visualized by ethidium bromide staining to ensure loading of equal amounts and transferred to nitrocellulose. Hybridization was carried out in 5 × SSPE, 10× Denhardt's reagent, 2% SDS, 50 μg/ml fragmented salmon sperm DNA, 50% formamide at 42°C overnight. Filters were washed three times for 10 min in 2× SSC, 0.05% SDS at room temperature, and twice for 15 min in 2× SSC, 0.1% SDS at 50°C. Northern blot was analyzed quantitatively using the Bio Imaging analyzer BAS 2000 (Fuji).

In Vitro Activation of MAPKAP Kinase 2 by MAP Kinase (erk 2) and Inactivation by PP2A

Five μl of pooled MAPKAP kinase 2 peak fractions from Mono S chromatography (equal amounts of protein were applied to the column) of control and heat shocked (43.5°C, 15 min) EAT cells were incubated at 30°C for 30 min with 7.5 μl activating mix (41.6 mM Tris–HCl, pH 7.4, 0.16 mM EGTA, 4.2 μM PKI, 16.6 mM $(CH_3COO)_2Mg$, 0.16 mM ATP, 0.36 U/ml of p42 MAP kinase) and diluted 1:1 with buffer A. Five-μl aliquots of this dilution were assayed for MAPKAP kinase 2 activity. Reaction conditions were tested to provide complete phosphorylation of MAPKAP kinase 2. Control incubation without MAP kinase and with fractions that do not contain MAPKAP kinase 2 were performed.

In order to inactivate MAPKAP kinase 2, a 5-μl sample of partial purified MAPKAP kinase 2 fractions as described above was incubated for 30 min at 30°C with 15 μl 10 mU/ml PP2A in 20 mM Tris–HCl pH 7.4, 0.1 mM EGTA, 0.6 mg/ml bovine serum albumin (BSA), 0.1 (v/v)% β-mercaptoethanol. Five-μl aliquots of a 1:1 dilution with buffer A were assayed for MAPKAP kinase 2 activity. Control incubation were carried out with buffer alone and with 0.5 μM *okadaic acid*.

RESULTS

Detection of sHsp Kinase Activity Indistinguisable From MAPKAP Kinase 2 in Ehrlich Ascites Tumor Cells Before and After Heat Shock

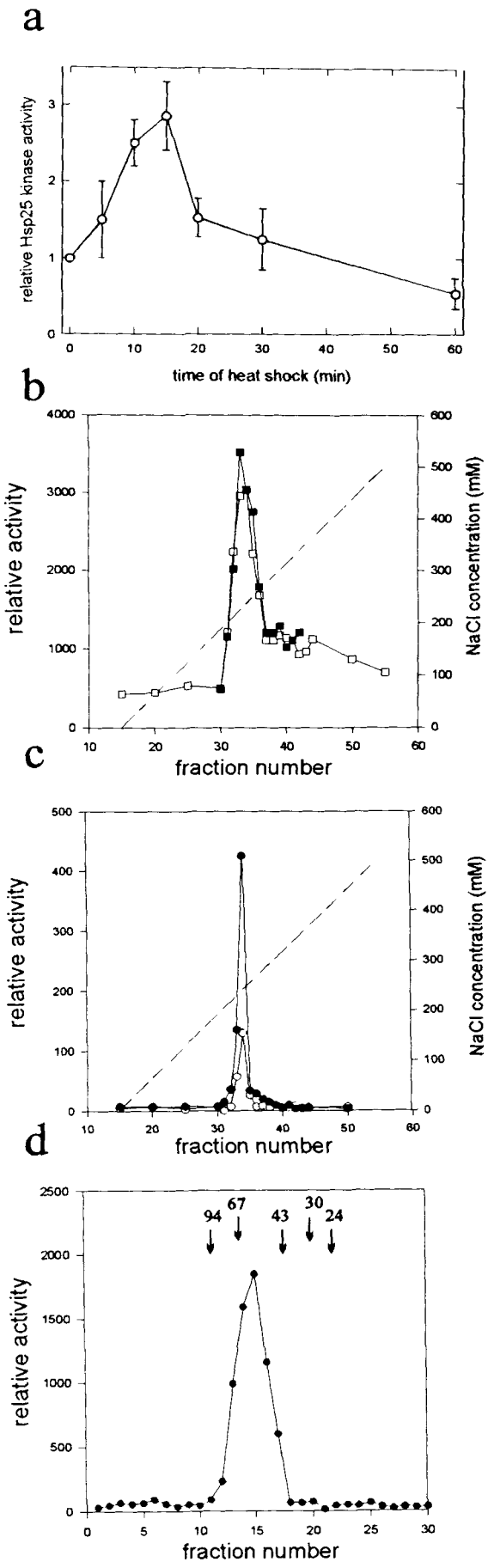
EAT cells were subjected to a heat shock of 43.5°C for 5, 10, 15, 20, 30, and 60 min. After various times of heat shock, sHsp kinase activity was determined in the EAT cell lysate in the presence of the protein kinase A inhibitor PKI [Glass et al., 1989]. As substrate we used recombinant mouse Hsp25 purified from *E. coli* BL21 [Gaestel et al., 1989] containing the MAPKAP kinase 2 recognition sites at serine 15 ... SLLRSPS*WEP ... and serine 86 ... ALNRQLS*SGV ... [Stokoe et al., 1992b]. The assay combining SDS–PAGE and phosphoimaging analysis for monitoring the phosphorylation reaction was applied. In this assay, the protein kinase inhibitors H7 [Kawamoto and Hidaka, 1984] and HA1077 [Asano et al., 1989], which do not inhibit MAPKAP kinase 2, were included at concentrations that inhibit cGMP-dependent protein kinase, smooth muscle myosin light chain kinase and protein kinase C, as well as insulin-stimulated protein kinase 1. The analysis of the time course of sHsp kinase activity after heat shock is depicted in Figure 1a. Activity peaks after about 15 min at a level threefold higher than the value before heat shock, followed by a decline of sHsp kinase activity that reaches a level below the pre-heat shock value after a heat shock of 60 min.

In order to demonstrate the identity of the heat inducible sHsp kinase activity in EAT cells with MAPKAP kinase 2, cell lysates were prepared from EAT cells before and after heat shock and were fractionated by ion-exchange chromatography on a Mono S column. The kinase activity was determined in the Mono S fractions using the substrate Hsp25 in the presence of PKI. Figure 1b shows the sHsp kinase activity from EAT cells prior to heat shock, as determined in the different fractions of the Mono S chromatography, using recombinant Hsp25 and the TCA precipitation assay in the presence and absence of the inhibitors H7 and HA1077. sHsp kinase activity is eluting at about 220 mM NaCl in Mono S buffer. The elution characteristics of the kinase from Ehrlich ascites tumor cells is very similar to that of the rabbit muscle MAPKAP kinase 2, which also elutes in the gradient at 200–250 mM NaCl [Stokoe et al., 1992a]. As expected for MAPKAP kinase 2, addition of the inhibitors H7 and HA1077 does not influence the activity of the kinase from EAT cells. No sHsp kinase activity was found in the flowthrough of the Mono S column and no activity was eluted in the final wash of the column with 1 M NaCl. Similar results were obtained using another known substrate for MAPKAP kinase 2, the peptide KKPLNRTLS*VASLPGLamide derived from the N terminus of glycogen synthase [Stokoe et al., 1992a; data not shown].

To demonstrate that heat shock induces the same kinase activity, sHsp kinase activity in EAT cells after a 15-min heat shock was analysed by Mono S chromatography. As shown in Figure 1c, sHsp kinase activity after 15-min heat shock assayed as above in the presence of PKI, H7, and HA1077 elutes as a single peak at the same position in the Mono S gradient as before heat shock and is increased about three- to fourfold, which corresponds to the increase of activity measured in the EAT lysate (Fig. 1a).

For estimation of the molecular mass of the sHsp kinase from EAT cells, the Mono S peak fraction was concentrated and applied to Superose 12 (Pharmacia) size exclusion liquid chromatography. The eluting fractions were assayed for kinase activity as described above. As shown in Figure 1d, sHsp kinase activity elutes as a single peak from the column. Using the marker proteins indicated in Figure 1d and a half-logarithmic regression of retention volume versus the molecular mass of the marker proteins (not shown), a molecular mass of 58 kd could calculated for the kinase. This corresponds to the elution behavior of rabbit muscle MAPKAP kinase 2 in size exclusion liquid chromatography and is in agreement with its appearent molecular mass of 53 kd and 60 kd, as determined in SDS–PAGE [Stokoe et al., 1992a].

Taken together, the biochemical properties of the sHsp kinase of EAT and the rabbit skeletal muscle MAPKAP kinase 2 show broad similarities that strongly suggest the identity of the



sHsp kinase from EAT cells to mouse MAPKAP kinase 2.

Mechanism of MAPKAP Kinase 2 Activation in EAT Cells

It is known that rabbit MAPKAP kinase 2 can be phosphorylated at a specified threonine residue by p42 and p44 MAP kinases and that this phosphorylation leads to an activation of the enzyme [Stokoe et al., 1992a]. This led us to ask, whether heat shock-induced activation of the sHsp phosphorylating activity is also the result of increased phosphorylation of mouse MAPKAP kinase 2. As a first attempt to answer this question the expression level of the MAPKAP kinase 2 mRNA was investigated by Northern blot hybridization. If activation of the enzyme would be achieved via phosphorylation, a constant transcript level of the enzyme before and after heat shock should be expected. Using a mouse MAPKAP kinase 2 specific cDNA probe [Engel et al., 1993], which was derived from a PCR-fragment spanning the catalytic domain of the enzyme from subdomains VIb to XI [Hanks et al., 1991], the amount of mouse MAPKAP kinase 2 mRNA was determined by Northern blot hybridization (Fig. 2). Further quantitative analysis of the photo-stimulated luminescence of the Northern blot demonstrates that after a heat shock of 15 min there is no significant increase in MAPKAP kinase 2 mRNA expres-

Fig. 1. Detection of a heat-inducible sHsp kinase activity in EAT cells that is biochemically indistinguishable from MAPKAPK 2. **a:** Time course of sHsp kinase activation by heat-shock. sHsp kinase activity of EAT cell lysates from cells after different times of heat shock was analysed by phosphorylation of recombinant mouse Hsp25. The data represent the results of three independent experiments. Relative sHsp kinase activity is shown as the ratio of units per mg protein after and before heat shock. **b:** Ion-exchange chromatography of EAT cell lysates and sHsp kinase assay in the presence and absence of the inhibitors H7 and HA1077. EAT cell lysates were fractionated by chromatography on Mono S using a linear gradient of NaCl from 0 to 500 mM (dashed line). sHsp kinase activity in the different fractions was determined using the recombinant nonphosphorylated mouse Hsp25 in the presence of the inhibitor PKI (■). The kinase activity peak fractions were also tested after addition of the inhibitors H7 and HA1077 to a final concentration of 20 μM (□). **c:** Ion-exchange chromatography of EAT cell lysates before (○) and after heat shock (●). sHsp kinase activity was assayed as in b in the presence of PK1, H7, and HA1077. **d:** Size exclusion liquid chromatography of the Mono S peak fraction from non-heat-shocked EAT cells (cf. b) assayed as described in c. Standards for estimation of the molecular mass were phosphorylase b (94 kd), bovine serum albumin (67 kd), ovalbumin (44 kd), carbonic anhydrase (30 kd) (Pharmacia), and cytochrome c (dimeric, 24 kd).

sion (data not shown). This excludes the possibility that transcriptional regulation accounts for the two- to threefold increase in mouse MAPKAP kinase 2 activity.

Further evidence for the notion that heat shock-induced activation of mouse MAPKAP kinase 2 is the result of phosphorylation of the enzyme can be obtained by in vitro phosphorylation and activation of MAPKAP kinase 2. For this reason, the MAPKAP kinase 2 activity containing peak fractions from Mono S chromatography of lysates of control and heat shocked EAT cells were pooled, subjected to in vitro phosphorylation by recombinant p42 MAP kinase (erk2), and assayed for MAPKAP kinase 2 activity by analyzing ^{32}P incorporation into Hsp25 using SDS–PAGE as described above. Both nonactivated MAPKAP kinase 2 obtained from control cells and MAPKAP kinase 2 obtained from cells subjected to a 15-min heat shock could be stimulated by MAP kinase. Interestingly, both MAPKAP kinase 2 fractions could be stimulated to the same final activity (Fig. 3). The increased MAPKAP kinase 2 activity from heat shocked cells could not be stimulated to a higher final activity by phosphorylation with p42 MAP kinase in vitro compared to the activity of stimulated fractions from non-heat-shocked cells. Thus it can be concluded that the increased activity after heat shock is almost certainly caused by a similar activation process, i.e., by phosphorylation.

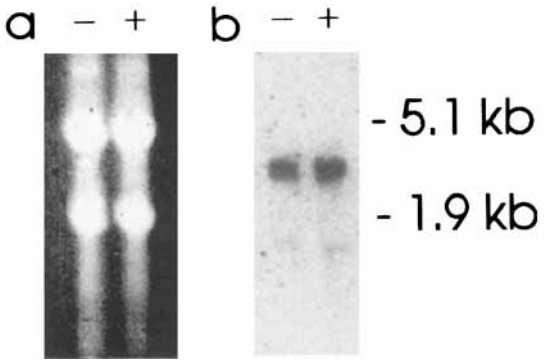


Fig. 2. Northern blot analysis of MAPKAP kinase 2 expression before and after heat shock. EAT cells were subjected to a heat shock of 43.5°C for 15 min. RNA of non-heat-shocked (−) and heat-shocked (+) cells was prepared according to Chomczynski and Sacchi (1987). Twenty μg of total RNA was separated in a 1% denaturing agarose gel and blotted onto nitrocellulose. MAPKAP kinase 2 mRNA was detected using a specific PCR fragment of the MAPKAP kinase 2 cDNA that was labeled by random priming and [^{32}P-α]dATP. **a:** Ethidium bromide staining of electrophoretically separated RNA. **b:** Phospho-image of hybridization signals.

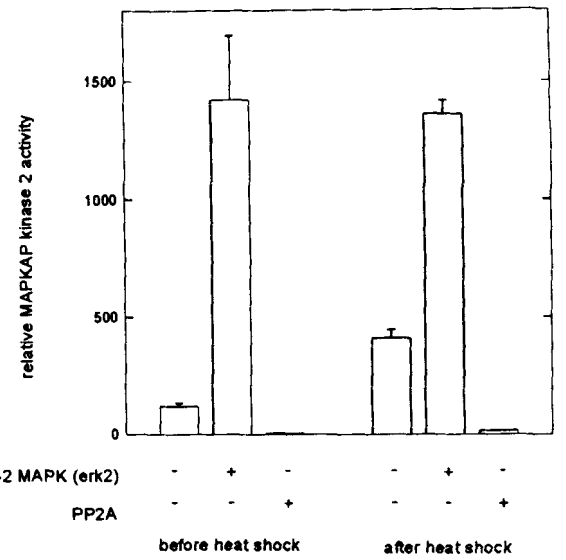


Fig. 3. Characterisation of MAPKAP kinase 2 activity from control and heat-shocked EAT cells by in vitro phosphorylation and dephosphorylation. MAPKAP kinase 2 containing fractions from Mono S chromatography of cells lysates prepared before and after a 15-min heat shock (43.5°C) were pooled and subjected to in vitro phosphorylation by recombinant p42 MAP kinase (erk2) and to dephosphorylation by protein phosphatase 2A. MAPKAP kinase 2 activity (in relative units) was determined before treatment, after phosphorylation, and after dephosphorylation with 2.7 mU p42 MAP kinase and 0.15 mU PP2A [Cohen et al., 1988], respectively, as described under Experimental procedures. Relative MAPKAP kinase 2 activity was normalized per amount of protein applied to Mono S chromatography.

Further evidence for activation of mouse MAPKAP kinase 2 by in vivo phosphorylation during heat shock comes from dephosphorylation studies. The pooled peak fractions of mouse MAPKAP kinase 2 activity were subjected to dephosphorylation by the catalytic subunit of protein phosphatase 2A (PP2A), which dephosphorylates rabbit MAPKAP kinase 2 leading to inactivation of the enzyme [Stokoe et al., 1992a]. PP2A treatment greatly diminished the activity of MAPKAP kinase 2 preparations from heat shocked and control EAT cells (Fig. 3), indicating that the only difference between both MAPKAP kinase 2 preparations is phosphorylation, probably at the threonine residue phosphorylated by p42 and p44 MAP kinases. Interestingly, treatment of EAT cells under control and heat-shock conditions with the serine/threonine protein phosphatase inhibitor ocadaic acid at concentrations up to 500 nM does not significantly increase neither MAPKAP kinase 2 activity nor Hsp25 phosphorylation (data not shown), indicating that protein dephosphorylation is not involved in the rapid activation of

MAPKAP kinase 2 upon 15-min heat shock. However, a downregulation of MAPKAP kinase 2 activity by protein phosphatase PP2A in vivo as proposed by Cairns et al. (1994) could not be excluded.

TNF-α Induction of MAPKAP Kinase 2 in Human MO7 Cells

As a second system for the analysis of the mechanism of rapid sHsp phosphorylation, we studied the induction of Hsp27-phosphorylation by TNF-α, which proceeds in a time period comparable to the heat shock induced phosphorylation of the sHsps [Kaur et al., 1988]. Human hematopoietic MO7 cells [Avanzi et al., 1988] were used for these experiments and the MAPKAP kinase 2 activity was monitored in the cell lysate, using the inhibitors PKI, H7 and HA1077 and the homologous recombinant human Hsp27, containing three MAPKAP kinase 2 phosphorylation sites at serines 15, 78, and 82 [Stokoe et al., 1992b]. As shown in Figure 4A, sHsp kinase activity in MO7 cells is induced about threefold within 15 min following treatment with 100 U/ml of recombinant TNF-α and returns to starting levels 2 h after induction. A dose dependence of the induction can be demonstrated, leading to an about fourfold induction when the cells were treated with 250 U/ml of recombinant TNF-α (cf. Fig. 4a). To demonstrate the identity of the ion-exchange elution properties and inhibition characteristics of the TNF-α-induced sHsp kinase from MO7 cells to that of rabbit and mouse MAPKAP kinase 2, we fractionated MO7 cell lysates before and after 15 min of treatment with 100 U/ml TNF-α, using Mono S chromatography and kinase assay in the presence of PKI, H7, and HA1077, as described above. As shown in Figure 4b, the TNF-α-induced kinase elutes at the same position in the gradient and is about threefold increased compared to the control indicating that human MAPKAP kinase 2 is induced by TNF-α.

In order to understand the mechanism of activation of human MAPKAP kinase 2 by TNF-α, we also monitored MAP kinase activity from MO7 cell lysates before and different times after TNF-α treatment. It can be seen that induction of MAP kinase activity occurs with similar kinetics to MAPKAP kinase 2 (Fig. 4a). To characterize further the TNF-α-induced MAP kinase activity, cell lysates of untreated MO7 cells and MO7 cells stimulated for 10 min with 100 U/ml recombinant TNF-α were fractionated by Mono Q chromatography and MAP kinase activity in the eluted fractions was measured. An about two- to threefold increased MAP kinase activity in stimulated cells was detected (Fig. 4c). The inducible activity elutes in a position of the gradient, which is characteristic for p42 MAP kinase [Stokoe et al., 1992a], which was recently shown to be preferentially phosphorylated and activated by TNF-α treatment [Raines et al., 1993]. These findings support the notion that MAPKAP kinase 2 is activated by MAP kinases in vivo.

DISCUSSION

We have demonstrated protein kinase activity in EAT and MO7 cells, which is indistinguishable from MAPKAP kinase 2 and is inducible by heat shock and TNF-α. This result supports the notion that MAPKAP kinase 2 is the enzyme responsible for sHsp phosphorylation also in vivo. The identity of the kinase activity described and MAPKAP kinase 2 is established by (1) the ability to phosphorylate the sHsps; (2) the elution characteristics in Mono S chromatography; (3) the nonresponsiveness to the inhibitors PKI, H7, and HA1077; (4) the elution characteristics in size-exclusion chromatography; (5) activation by p42 MAP kinase phosphorylation; and (6) inactivation by PP2A dephosphorylation. The inducibility of MAPKAP kinase 2 activity by heat shock and TNF-α is shown to be about threefold and proceeds in a time span of 10–15 min. The data presented strongly suggest that MAPKAP kinase 2 activation under heat shock and TNF-α treatment is the result of phosphorylation by MAP kinases. First, it is demonstrated that increased MAPKAP kinase 2 activity under heat-shock conditions is not regulated by the amount of mRNA. Furthermore, a translational up-regulation of MAPKAP kinase protein level during exposure to a short heat shock (10–15 min) can be almost excluded, since it is known that heat shock causes a transient halt to general protein synthesis connected with the breakdown of polysomes in the same time span over which the activation of MAPKAP kinase 2 proceeds [Nover, 1991]. Second, the activities of MAPKAP kinase 2 from control and heat-shocked EAT cells are obviously different, but can be stimulated by MAP kinase phosphorylation to equal maximal values. This also implies comparable amounts of MAPKAP kinase 2 protein in control and heat shocked cells, which may be distinguished probably only in the phos-

phorylation status. Both control and heat-shock-activated MAPKAP kinase 2 activity could be inactivated by dephosphorylation with PP2A. Third, TNF-α treatment induces stimulation of MAPKAP kinase 2 activity accompanied by increased MAP kinase activity.

Although the function of sHsp and the modulation of their function by phosphorylation is unclear, the findings that MAPKAP kinase 2 is responsible for sHsp phosphorylation in vivo and that MAPKAP kinase 2 could be activated by MAP kinases in response to heat shock and TNF-α provides a comprehensive explanation for the various data concerning sHsp phosphorylation obtained so far. It is known that MAP kinase activation can be triggered by many different kinds of signals, including heat shock [Chung et al., 1992] as well as the variety of other inducers known to be responsible for sHsp phosphorylation, like growth factors linked to receptor tyrosine kinases [Saklatvala et al., 1991], agonists for protein kinase C, e.g., phorbol esters such as PMA [Welch, 1985], G protein agonists such as thrombin [Santell et al., 1992], calcium ionophores [Welch, 1985], TNF-α and IL-1 [Kaur et al., 1988]. After being activated by phosphorylation, MAP kinases phosphorylate protein kinases such as S6 kinase II (pp90rsk) [Chen et al., 1992], which can also be designated MAPKAP kinase 1 (MAP kinase-activated protein kinase 1), and, as suggested in this paper, MAPKAP kinase 2, which is responsible for sHsp phosphorylation. Hence, sHsp phosphorylation appears to be linked to the MAP kinase cascade. This explains that a variety of different signals induce sHsp phosphorylation as an early event of the cellular response.

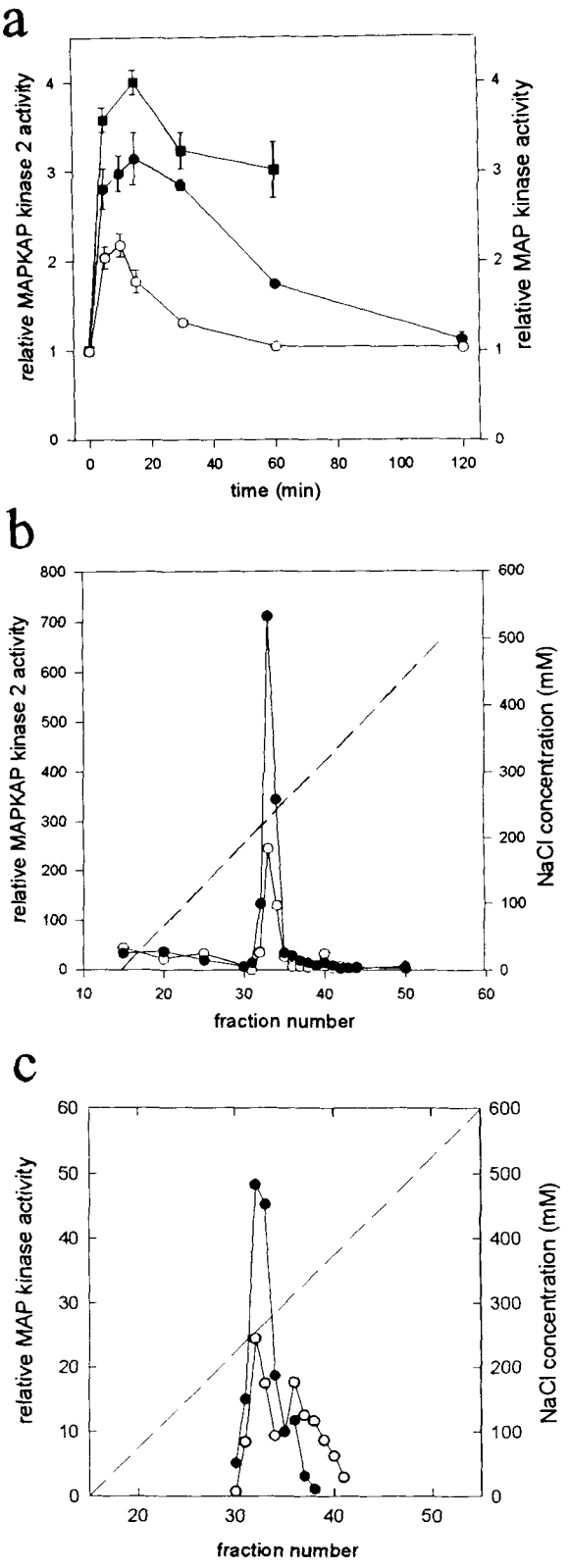


Fig. 4. TNF-α induction of MAPKAP kinase 2 and MAP kinase activity in MO7 cells. **a:** MO7 cells were treated with TNF-α and MAPKAP kinase 2 activity (●, after treatment with 100 U/ml TNF-α, ■, after treatment with 250 U/ml TNF-α) and MAP kinase activity (○, after treatment with 100 U/ml TNF-α) were determined using recombinant Hsp27 and MBP as substrate at different times after treatment. Relative activity is normalized by the amount of lysate protein. **b:** Mono S ion-exchange chromatography of MO7 lysates from cells before (○) and after 15 min of treatment with 100 U/ml TNF-α (●) (cf. a). The kinase activity elutes at the gradient position characteristic for MAPKAP kinase 2 and is about threefold increased after TNF-α treatment. **c:** Control MO7 cells (○) and cells stimulated for 10 min with 100 U/ml of TNF-α (●) were lysed and fractionated by Mono Q ion-exchange chromatography developed with a linear NaCl gradient (0–600 mM). MAP kinase activity was monitored by ^{32}P incorporation into MBP and is given normalized per amount of protein loaded onto the column.

Recently, sHsp kinase activities have been analysed from EAT cells [Benndorf et al., 1992], MRC-5 fibroblasts [Guesdon et al., 1993] and Chinese hamster cells [Zhou et al., 1993]. The sHsp kinase activity in EAT cell lysates was shown to be independent of calcium ions, phosphatidylserine, cAMP and cGMP and not to be inhibited by trifluoperazine, staurosporine and PKI [Benndorf et al., 1992], consistent with the properties of MAPKAP kinase 2. The sHsp-kinase from Chinese hamster cells has been demonstrated to be activated by heat shock, serum, FGF and thrombin and to be inactivated by acid phosphatase treatment [Zhou et al., 1993]. These data are also congruent with the mechanism of activation of MAPKAP kinase 2 demonstrated in this paper. In MRC-5 fibroblasts an Hsp27 kinase was described that had similar physicochemical properties as MAPKAP kinase 2 but lacked the ability to be activated by the phorbol ester PMA, which increased the myelin basic protein (MBP) kinase activity of these cells which is probably MAP kinase [Guesdon et al., 1993]. Whether this finding means that another sHsp kinase or another mechanism of MAPKAP kinase 2 activation exists, at least in MRC-5 cells, or whether the major MBP-kinases in these cells are distinct from MAP kinase, remains to be clarified. However, in EAT no further MAPKAP kinase 2 activity was detectable in the flowthrough and wash fractions of the Mono S column, as monitored by substrate peptide and recombinant Hsp25 phosphorylation in an assay using the inhibitor PKI. Since sHsp kinases are not inhibited by PKI [Benndorf et al., 1992; Guesdon et al., 1993], this finding strongly suggests that MAPKAP kinase 2 is the only enzyme that can phosphorylate the sHsps in these cells.

The data presented provide compelling evidence that small Hsp phosphorylation after heat shock and mitogenic stimulation by MAPKAP kinase 2 might be the result of activation of the MAP kinase cascade. By contrast, a calcium/calmodulin-dependent dephosphorylation of the sHsps has been described [Gaestel et al., 1992], which could be activated by the increased intracellular free calcium concentration after heat shock [Stevenson et al., 1986], or as a result of the activation of hormone receptors [Carafoli, 1987]. Hence, both sHsp phosphorylation and dephosphorylation in response to extracellular signals takes place in vivo, making the phosphorylation status of the sHsps a sensitive signal dependent factor within the cell.

ACKNOWLEDGMENTS

We thank P. Cohen and D. Stokoe, MRC Protein Phosphorylation Unit, University of Dundee, Scotland, for introducing K.E. into the analysis of MAPKAP kinase 2. Furthermore, we thank P. Cohen for the generous support in supplying materials such as substrate peptide and PP2A from his lab and for critical reading of the manuscript. This work was supported by grants Ga 453-2/1 and SFB 344 YE1 from the Deutsche Forschungsgemeindschaft, by grant A10172 from the Bundesministerium für Forschung und Technologie to M.G., and by grant W 27/90 He1 from the Deutsche Krebshilfe to F.H.

REFERENCES

Arrigo AP, Pauli D (1988): Characterization of HSP27 and three immunologically related polypeptides during *Drosophila* development. Exp Cell Res 175:169–183.

Arrigo AP, Suhan JP, Welch WJ (1988): Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein. Mol Cell Biol 8:5059–5071.

Asano T, Suzuki T, Tsuchiya M, Satoh S, Ikegaki I, Shibuya M, Suzuki Y, Hidaka H (1989): Vasodilator actions of HA1077 in vitro and in vivo putatively mediated by the inhibition of protein kinase. Br J Pharmacol 98:1091–1100.

Avanzi GC, Lista P, Giovinazzo B, Miniero R, Saglio G, Benetton G, Coda R, Cattoretti G, Pegoraro L (1988): Selective growth-response to Il-3 of a human-leukemic cell-line with megakaryoblastic features. Br J Haematol 69:359–366.

Benndorf R, Hayess K, Stahl J, Bielka H (1992): Cell-free phosphorylation of the murine small heat-shock protein hsp25 by an endogenous kinase from Ehrlich ascites tumor cells. Biochim Biophys Acta 1136:203–207.

Cairns J, Qin SX, Philp R, Tan YH, Guy GR (1994): Dephosphorylation of the small heat shock protein Hsp27 in vivo by protein phosphatase 2A. J Biol Chem 269:9176–9183.

Carafoli E (1987): Intracellular calcium homeostasis. Annu Rev Biochem 56:395–433.

Chen RH, Sarnecki C, Blenis J (1992): Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Mol Cell Biol 12:915–927.

Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem 162:156–159.

Chung J, Kuo CJ, Crabtree GR, Blenis J (1992): Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell 69: 1227–1236.

Cohen P, Alemany S, Hemmings BA, Resink TJ, Stralfors P, Tung HYL (1988): Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal-muscle. Methods Enzymol 159:390–480.

de Jong WW, Leunissen JAM, Voorter CEM (1993): Evolution of the alpha-crystallin/small heat-shock protein family. Mol Biol Evol 10:103–126.

Engel K, Plath K, Gaestel M (1993): The MAP kinase activated protein kinase 2 contains a proline-rich SH3-binding domain. FEBS Lett 336:143–147.

Fuqua SA, Blum Salingaros M, McGuire WL (1989): Induction of the estrogen-regulated "24K" protein by heat shock. Cancer Res 49:4126–4129.

Gaestel M, Gross B, Benndorf R, Strauss M, Schunk WH, Kraft R, Otto A, Böhm H, Stahl J, Drabsch H, et al. (1989): Molecular cloning, sequencing and expression in Escherichia coli of the 25-kDa growth-related protein of Ehrlich ascites tumor and its homology to mammalian stress proteins. Eur J Biochem 179:209–213.

Gaestel M, Schröder W, Benndorf R, Lippmann C, Buchner K, Hucho F, Erdmann VA, Bielka H (1991): Identification of the phosphorylation sites of the murine small heat shock protein hsp25. J Biol Chem 266:14721–14724.

Gaestel M, Benndorf R, Hayess K, Priemer E, Engel K (1992): Dephosphorylation of the small heat shock protein hsp25 by calcium calmodulin-dependent (Type-2B) protein phosphatase. J Biol Chem 267:21607–21611.

Glass DB, Cheng HC, Mende Mueller L, Reed J, Walsh DA (1989): Primary structural determinants essential for potent inhibition of cAMP-dependent protein kinase by inhibitory peptides corresponding to the active portion of the heat-stable inhibitor protein. J Biol Chem 264:8802–8810.

Guesdon F, Freshney N, Waller RJ, Rawlinson L, Saklatvala J (1993): Interleukin 1 and tumor necrosis factor stimulate two novel protein kinases that phosphorylate the heat shock protein hsp27 and beta-casein. J Biol Chem 268: 4236–4243.

Hanks SK, Quinn AM (1991): Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol 200:38–62.

Hepburn A, Demolle D, Boeynaems JM, Fiers W, Dumont JE (1988): Rapid phosphorylation of a 27 kDa protein induced by tumor necrosis factor. FEBS Lett 227:175–178.

Horwitz J (1992): Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad Sci USA 89:10449–10453.

Jakob U, Gaestel M, Engel K, Buchner J (1993): Small heat shock proteins are molecular chaperones. J Biol Chem 268:1517–1520.

Kaur P, Welch WJ, Saklatvala J (1989): Interleukin 1 and tumour necrosis factor increase phosphorylation of the small heat shock protein. Effects in fibroblasts, Hep G2 and U937 cells. FEBS Lett 258:269–273.

Kawamoto S, Hidaka H (1984): 1-(5-Isoquinolinesulfonyl)-2-Methylpiperazine (H-7) is a selective inhibitor of protein kinase-C in rabbit platelets. Biochem Biophys Res Commun 125:258–264.

Knauf U, Bielka H, Gaestel M (1992): Over-expression of the small heat-shock protein, hsp25, inhibits growth of Ehrlich ascites tumor cells. FEBS Lett 309:297–302.

Knauf U, Jakob U, Engel K, Buchner J, Gaestel M (1994): Stress- and mitogen-induced phosphorylation of the small heat shock protein Hsp25 by mapkap kinase 2 is not essential for chaperone properties and cellular thermoresistance. EMBO J 13:54–60.

Landry J, Chretien P, Lambert H, Hickey E, Weber LA (1989): Heat shock resistance conferred by expression of the human hsp27 gene in rodent cells. J Cell Biol 109:7–15.

Landry J, Lambert H, Zhou M, Lavoie JN, Hickey E, Weber LA, Anderson CW (1992): Human hsp27 is phosphorylated at serines-78 and serines-82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 Kinase-II. J Biol Chem 267:794–803.

Lavoie JN, Gingrasbreton G, Tanguay RM, Landry J (1993): Induction of chinese hamster hsp27 gene expression in mouse cells confers resistance to heat shock–hsp27 stabilization of the microfilament organization. J Biol Chem 268:3420–3429.

Merck KB, Groenen PJTA, Voorter CEM, Dehaardhoekman WA, Horwitz J, Bloemendal H, de Jong WW (1993): Structural and functional similarities of bovine alpha-crystallin and mouse small heat-shock protein—A family of chaperones. J Biol Chem 268:1046–1052.

Miron T, Vancompernolle K, Vandekerckhove J, Wilchek M, Geiger B (1991): A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J Cell Biol 114:255–261.

Nover L (1991) Translational control. In Nover L (ed): "Heat Shock Response." Boca Raton, FL: CRC Press, pp 299–317.

Raines MA, Kolesnick RN, Golde DW (1993): Sphingomyelinase and ceramide activate mitogen-activated protein kinase in myeloid HL-60 cells. J Biol Chem 268:14572–14575.

Rollet E, Best-Belpomme M (1986): Hsp26 and 27 are phosphorylated in response to heat shock and ecdysterone in *Drosophila melanogaster* cells. Biochem Biophys Res Commun 141:426–433.

Rollet E, Lavoie JN, Landry J, Tanguay RM (1992): Expression of *Drosophila*'s 27 kDa heat shock protein into rodent cells confers thermal resistance. Biochem Biophys Res Commun 185:116–120.

Saklatvala J, Kaur P, Guesdon F (1991): Phosphorylation of the small heat-shock protein is regulated by interleukin 1, tumour necrosis factor, growth factors, bradykinin and ATP. Biochem J 277:635–642.

Santell L, Bartfeld NS, Levin EG (1992): Identification of a protein transiently phosphorylated by activators of endothelial cell function as the heat-shock protein-hsp27—A possible role for protein kinase-C. Biochem J 284:705–710.

Stevenson MA, Calderwood SK, Hahn GM (1986): Rapid increases in inositol trisphosphate and intracellular Ca^{++} after heat shock. Biochem Biophys Res Commun 137:826–833.

Stokoe D, Campbell DG, Nakielny S, Hidaka H, Leevers SJ, Marshall C, Cohen P (1992a): MAPKAP kinase-2—A novel protein kinase activated by mitogen-activated protein kinase. EMBO J 11:3985–3994.

Stokoe D, Engel K, Campbell DG, Cohen P, Gaestel M (1992b): Identification of MAPKAP kinase-2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. FEBS Lett 313:307–313.

Welch WJ (1985): Phorbol ester, calcium ionophore, or serum added to quiescent rat embryo fibroblast cells all result in the elevated phosphorylation of two 28,000-dalton mammalian stress proteins. J Biol Chem 260:3058–3062.

Zhou M, Lambert H, Landry J (1993): Transient activation of a distinct serine protein kinase is responsible for 27-kDa heat shock protein phosphorylation in mitogen-stimulated and heat-shocked cells. J Biol Chem 268:35–43.