A Short Lived Protein Involved in the Heat Shock Sensing Mechanism Responsible for Stress-activated Protein Kinase 2 (SAPK2/p38) Activation*

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The stress-activated protein kinase 2 (SAPK2/p38) is activated by various environmental stresses and also by a vast array of agonists including growth factors and cytokines. This implies the existence of multiple proximal signaling pathways converging to the SAPK2/p38 activation cascade. Here, we show that there is a sensing mechanism highly specific to heat shock for activation of SAPK2/p38. After mild heat shock, cells became refractory to reinduction of the SAPK2/p38 pathway by a second heat shock. This was not the result of a toxic effect because the cells remained fully responsive to reinduction by other stresses, cytokines, or growth factors. Neither the activity of SAPK2/p38 itself nor the accumulation of the heat shock proteins was essential in the desensitization process. The cells were not desensitized to heat shock by other treatments that activated SAPK2/p38. Moreover, inhibiting SAPK2/p38 activity during heat shock did not block desensitization. Also, overexpression of HSP70, HSP27, or HSP90 by gene transfection did not cause desensitization, and inhibiting their synthesis after heat shock did not prevent desensitization. Desensitization rather appeared to be linked closely to the turnover of a putative upstream activator of SAPK2/p38. Cycloheximide induced a progressive and eventually complete desensitization. The effect was specific to heat shock and minimally affected activation by other stress inducers. Inhibiting protein degradation with MG132 caused the constitutive activation of SAPK2/p38, which was blocked by a pretreatment with either cycloheximide or heat shock. The results thus indicate that there is a sensing pathway highly specific to heat shock upstream of SAPK2/p38 activation. The pathway appears to involve a short lived protein that is the target of rapid successive up- and down-regulation by heat shock.

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1 The abbreviations used are: HSP(s), heat shock protein(s); MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; JNK, Jun N-terminal kinase; EGF, epidermal growth factor; TNF, tumor necrosis factor; GST, glutathione S-transferase; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; MAPKAP, MAP-activated protein; ATF2, activating transcription factor-2.
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the cells to resist HSP27 phosphorylation and the thermotolerant state. This has led to the suggestion that some factors induced by the priming heat shock and generated along with the development of thermotolerance block the signaling pathway leading to HSP27 phosphorylation (10).

In the present study, we investigated the mechanisms responsible for inhibiting HSP27 phosphorylation in heat-induced thermotolerant cells. The results showed that the desensitization process occurs at a proximal step in the signaling pathway upstream of SAPK2/p38 phosphorylation and activation. Desensitization was strictly homologous affecting exclusively heat shock induction of the SAPK2/p38 pathway. We infer that there is a sensing mechanism highly specific to heat shock in the activation pathway of SAPK2/p38, and we showed that it involves a short lived protein whose activity depends on a tight regulation between synthesis and degradation.

**EXPERIMENTAL PROCEDURES**

**Materials—**\( \gamma^3P\)-ATP (3,000 Ci/mmol) was purchased from NEN Life Sciences Products, H$_2$O$_2$, MgSO$_4$, (N-carboxyanilide-Leu-Leu-norleucine, emetine, phenylmethylsulfonyl fluoride, and EGFP were from Sigma Chemical Co. Thrombin was from Life Technologies, Inc. TNF-\(\alpha\) and SB203580 were from Calbiochem. Recombinant HSP27 and ATF2-GST were purified from Escherichia coli transformed with appropriate plasmids (37, 38). Chemicals for electrophoresis were obtained from Bio-Rad and Fisher Scientific.

**Antibodies—**Anti-\(\alpha\) is a mouse monoclonal antibody recognizing the YPDVDYDA peptide sequence from human influenza hemagglutinin protein (Roche Molecular Biochemicals Corporation). All other antibodies used are polyclonal antibodies raised in rabbit. Anti-GST-MAPKAP kinase-2 recognizes the p45 and p54 isoforms of MAPKAP kinase-2 (30); anti-p38, SAPK2/p38 (34); LzR3, the Chinese hamster HSP27 (33); anti-HSP70 (no. 799), the inducible form of HSP70 (39); and phospho-p38 MAPK, the phosphorylated and activated form of SAPK2/p38 (New England BioLabs).

**Cell Culture and Treatments—**Chinese hamster CCL39 and human HeLa cells were cultivated in Dulbecco’s modified Eagle’s medium containing 2.25 g/liter NaHCO$_3$ and 4.5 g/liter glucose, and supplemented with 5% or 10% fetal bovine serum, respectively. Cultures were maintained at 37 °C in a 5% CO$_2$ humidified atmosphere. Exponentially growing cells (10$^6$ cells/60 cm$^2$ culture flask) were either used directly in culture medium, and cells were maintained at 37 °C for the duration of treatments.

**Transfection—**The plasmids pSVHa27WT (31), bAPtrp70 (40), and pcDNA3-\(\alpha\)-p38 (41) were used for expression of Chinese hamster HSP27, inducible human HSP70, and HA-tagged SAPK2/p38, respectively. pAM-HSP90 contains the human HSP90\(\alpha\) cDNA (42) inserted at the SalI site of the expression vector pALTER-MAX (Promega). CCL39 cells were plated 24 h before transfection at a concentration of 0.75 $\times$ 10$^5$ cells/75 cm$^2$ culture flask. Transfection by calcium phosphate precipitation was done as described before using 21 $\mu$g of plasmid (7 $\mu$g of pcDNA3-\(\alpha\)-p38 and 14 $\mu$g of pSVHa27WT, bAPtrp70, PAM-HSP90, or carrier DNA) per flask (11). The cells were replated 24 h later and used 48 h after transfection.

**Immunoprecipitation—**After treatments, cells were scraped and extracted in lysis buffer containing 20 mM MOPS, pH 7.0, 10% glycerol, 80 mM $\beta$-glycerophosphate, 5 mM EGTA, 0.5 mM EDTA, 1 mM Na$_2$VO$_4$, 5 mM Na$_2$PO$_4$, 50 mM NaF, 1% Triton X-100, 1 mM benzamidine, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The extracts were vortexed and centrifuged at 17,000 $\times$ g for 12 min at 4 °C. The clarified supernatants were either used immediately for immunoprecipitation or stored at –80 °C. The further steps were carried out at 4 °C. The supernatant was diluted four times in buffer I (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM MgCl$_2$, 1 mM Na$_2$VO$_4$, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). Antibodies were added in limiting concentrations, and the mixtures were incubated for 1 h. 10–15 $\mu$l of protein A-Sepharose (Amersham Pharmacia Biotech) 50% v/v in buffer I were added, and the mixtures were incubated for 30 min. Samples were centrifuged for 15 s and washed three times with 300 $\mu$l of buffer I. Immunoprecipitates were used directly for the kinase assays.

**Kinase Assays—**Kinase assays were assayed in immune complexes. MAPKAP kinase-2 activity was measured using 1 $\mu$g of recombinant HSP27 as substrate (30). The assays were carried out in 25 $\mu$l of kinase buffer K (100 mM ATP, 3 $\mu$Ci of [\(\gamma^3P\)]-ATP, 40 mM p-nitrophenyl phosphate, 20 mM MOPS, pH 7.0, 10% glycerol, 15 mM MgCl$_2$, 0.05% Triton X-100, 1 mM diithiothreitol, 1 mM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.3 $\mu$g of protein kinase A inhibitor). The kinase activity was assayed for 30 min at 30 °C and was stopped by the addition of 10 $\mu$M SDS-polyacrylamide gel electrophoresis loading buffer. Immunoprecipitated SAPK2/p38 was assayed analogously using ATF2-GST as substrate (34). The kinase assay buffer for SAPK2/p38 contained 50 mM HEPES, pH 7.4, 50 mM $\beta$-glycerophosphate, 50 mM MgCl$_2$, 0.2 mM Na$_3$VO$_4$, 2 $\mu$g ATP-GST, and 4 $\mu$Ci of [\(\gamma^3P\)]-ATP. Kinase activities were evaluated by measuring incorporation of the radioactivity into the specific substrates after resolution by SDS-polyacrylamide gel electrophoresis and quantification using PhosphorImager (Molecular Dynamics). To ensure equal loading of the kinases on different lanes, immune complexes were analyzed by Western blotting using specific antibodies.

**Western Blot Analysis—**Proteins were separated through 10% SDS/polyacrylamide gels and transferred onto nitrocellulose as described previously (33). After reacting the membranes with the specific antibodies, proteins were detected using an ECL detection kit (Amersham Pharmacia Biotech) or by iodinated secondary antibodies and quantified using PhosphorImager analysis.

**RESULTS**

**Desensitization of MAPKAP Kinase-2 and SAPK2/p38 Activation by Heat Shock—**Cells that had become thermotolerant after a mild heat shock are refractory to heat-induced phosphorylation of HSP27 (10). We investigated whether desensitization could be correlated with the loss of responsiveness of MAPKAP kinase-2 and SAPK2/p38, the two upstream activators of HSP27 phosphorylation. In CCL39 cells, MAPKAP kinase-2 and SAPK2/p38 are activated maximally right after a 20-min heat shock (Fig. 1, A and B). The activities of both kinases rapidly returned to basal level within the next 3–5 h. This was in perfect agreement with the previously published kinetics of HSP27 phosphorylation, which is maximal within 20 min and is back to normal basal levels within 3–5 h after the priming treatment (10). We examined the reactivation of these two kinases by heat shock at various times after a priming heat shock treatment. Neither kinase was reactivated when the cells were restimulated at any time between 5 and 10 h postpriming although both were present at normal levels as determined by immunoblot using anti MAPKAP kinase-2 and SAPK2/p38 antibodies (Fig. 1C). At later times (24 h), both kinases were activated normally as shown previously for HSP27 phosphorylation (10). Similar kinetics of desensitization was observed in HeLa cells (data not shown). In the case of SAPK2/p38, the results were confirmed using a phospho-specific antibody, which indicated that the failure to activate SAPK2/p38 correlated with lack of phosphorylation by the upstream kinases (data not shown).

The desensitization appeared to be total and not just a mere displacement in the dose-response curve for heat shock. Whereas a 20-min heat shock induced a maximal MAPKAP kinase-2 activation in naive cells, treatment for 1 h did not induce MAPKAP kinase-2 activity in fully desensitized cells (Fig. 1D).

**Activation of SAPK2/p38 and MAPKAP Kinase-2 by Heterologous Stimuli in Heat-desensitized Cells—**The Western blot analysis shown in Fig. 1C indicated that heat-induced desensitization of MAPKAP kinase-2 and SAPK2/p38 activation were not caused by a down-regulated expression of MAPKAP kinase-2 or SAPK2/p38 in thermotolerant cells. To determine whether these enzymes were physically available and func-
Exponentially growing CCL39 cells were heat shocked for varying lengths of times at 44 °C (HS2, second heat shock of 0 or 20 min at 44 °C (second heat shock, HS1) or left untreated (NT)). Proteins were then separated by SDS-polyacrylamide gel electrophoresis, and kinase activities were assayed using recombinant HSP27 and ATF2-GST as substrates, respectively. Proteins were then separated by SDS-polyacrylamide gel electrophoresis, and kinase activities were visualized by autoradiography of the 32P-labeled substrates. Panel C, at varying time points after heat shock, 20 μg of total cell extract proteins was also analyzed by Western blot using anti-GST MAPKAP kinase-2 (to detect both p45 and p54 MAPKAP kinase-2 isoforms) and anti-p38 antibodies. Panel D, cells were submitted to a 20-min heat shock at 44 °C (●), left untreated (●) and 5 h later exposed to a second heat shock for varying lengths of times at 44 °C (●, □) or 37 °C (□). After treatments, MAPKAP kinase-2 activity was determined. Results are expressed as the ratio of the kinase activities of stimulated cells over the activity of unstimulated cells.

Following, we evaluated if the desensitization process also affected MAPKAP kinase-2 activation by other known activators of the pathway. Cells were first exposed to heat shock and then re-stimulated with various growth factors, cytokines, or stress agents. In control cells, thrombin and TNF-α elicited a strong activation of MAPKAP kinase-2 (Fig. 2A and B). The ability of thrombin and TNF-α to activate MAPKAP kinase-2 was unaffected in heat-desensitized cells. Similarly, heat-desensitized cells remained fully responsive to restimulation with all other agonists tested at 5 h after heat shock, namely EGF, platelet-derived growth factor, readdition of serum to serum-deprived cells, sphingomyelinase, and phorbol ester (data not shown). The ability of physical or chemical stresses to activate the SAPK2/p38 pathway in heat-desensitized cells was also tested by looking either directly at the activity of SAPK2/p38 or at the activity of MAPKAP kinase-2. Both H2O2 and sodium arsenite induced a normal activation of the pathway at all times after the desensitizing heat shock (Fig. 2, C and D). Similarly, sorbitol induction of the pathway was about 75% of the control response at 5 h after the priming heat shock (data not shown). Hence, heat shock-induced desensitization of the SAPK2/p38 pathway was not caused by a general toxic response and affected specifically the heat shock-sensitive pathway. Thus, the failure to induce phosphorylation of HSP27 in heat-induced thermotolerant cells was the result of a total inhibition of some proximal elements of the heat shock signaling pathway preventing phosphorylation and activation of SAPK2/p38 and activation of the HSP27 kinase MAPKAP kinase-2.

**Desensitization Does Not Require SAPK2/p38 Activity**—Having shown clear homologous desensitization of the SAPK2/p38 pathway activation by heat shock, we next tested if other agonists were also capable of eliciting a desensitization to heat shock. Cells pretreated with EGF showed virtually total desensitization of MAPKAP kinase-2 activation to restimulation with EGF (Fig. 3A). MAPKAP kinase-2 activation was not induced above the residual level remaining after the first stimulation. In contrast, heat shock induction was unaffected in EGF-desensitized cells. Similarly, thrombin treatment induced homologous desensitization of MAPKAP kinase-2 activation but did not desensitize to heat shock (Fig. 3B). One conclusion of these results is that activation of SAPK2/p38 is not a sufficient condition for heat desensitization. The possibility that it could be required during heat-induced homologous desensitization was tested by determining the effect on heat-induced desensitization of SB203580, a pyridinil imidazol derivative that efficiently inhibits SAPK2/p38 activity (43). As reported previously, SB203580 did not prevent SAPK2/p38 activation but led to a total inhibition of MAPKAP kinase-2 activation (Fig. 4) and HSP27 phosphorylation. Inhibition of SAPK2/p38 activity during the priming heat shock treatment had no effect on induction of desensitization, indicating that events occurring downstream of SAPK2/p38 were not required for inducing homologous heat desensitization.

**HSP90, HSP70, or HSP27 Accumulated during Development of Thermotolerance Is Not Implicated in Heat-induced Homologous Desensitization**—Another possibility for explaining the heat-induced desensitization is that one of the heat shock proteins that accumulate after the first heat shock acts as a repressor of one of the elements in the heat-specific sensing pathway that triggers SAPK2/p38 activation. This appeared unlikely because the kinetics of accumulation of the HSP after heat shock does not match the kinetics of desensitization. For example, HSP27 and HSP70 concentrations peak at 10 and 14 h after heat shock, whereas desensitization is maximal at 5 h (10). Nevertheless, we tested directly the effect of overexpressing HSP70, HSP90, or HSP27 on heat activation of SAPK2/p38. HSP70, HSP90, or HSP27 was cotransfected with HA-tagged SAPK2/p38 in CCL39 cells. Under the conditions of transfection used, we calculated, after correcting for the transfection efficiency (about 20%), that individual transfected cells expressed amounts of HSP equivalent (HSP70 and HSP90) or 2-fold higher (HSP27) than what control cells express 5 h after heat shock. Epitope-tagged SAPK2/p38 was immunoprecipitated from the transfected cells at various times after shifting the temperature to 44 °C, and p38 activity was determined.

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**Fig. 3.** Activation of MAPKAP kinase-2 by heat shock is not affected in EGF- or thrombin-desensitized cells. Cultures of CCL39 cells were serum starved for 24 h and then exposed to a 5-min EGF treatment (2.5 ng/ml) (panel A) or a 5-min thrombin treatment (1 unit/ml, THR) (panel B). Some cells were left untreated (NT). At varying times (Δt) thereafter, the cells were left untreated or submitted to a second identical treatment with EGF or thrombin or submitted to a 20-min heat shock at 44 °C (HS). Cells extracts were prepared at the end of the treatments, and MAPKAP kinase-2 activity was assayed using recombinant HSP27 as substrate. 

**Fig. 4.** Heat desensitization does not require SAPK2/p38 activity. Panel A, exponentially growing CCL39 cells were pretreated (+ SB) or not (− SB) for 1 h with the SAPK2/p38 inhibitor SB203580 (5 μM) and then exposed (HS) or not (Ctl) to a 20-min heat shock at 44 °C before being extracted. Panel B, cells were pretreated (+ SB) or not (− SB) for 1 h with SB203580 (5 μM), exposed to a 20-min heat shock at 44 °C, and returned at 37 °C. 5 h later, the cells were exposed (HS) or not (Ctl) to a second identical heat shock before being extracted. Extracts were assayed for MAPKAP kinase-2 (black bars) or SAPK2/p38 (white bars) activities using recombinant HSP27 or ATF2-GST as substrates, respectively. Results are expressed as the ratio of the kinase activities of stimulated cells to the activity of unstimulated cells.

**Fig. 5.** Overexpression of HSP90, HSP70, or HSP27 is not involved in heat-induced homologous desensitization. CCL39 cells were transfected with the HA-tagged p38 vector together with HSP90, HSP70, or empty pSV7 vectors. Panel A, epitope-tagged SAPK2/p38 was immunoprecipitated from the transfected cells at various times after shifting the temperature from 37 to 44 °C, and SAPK2/p38 activity was determined using ATF2-GST as substrate. Panel B, the transfected cells were exposed (+) or not (−) to a priming heat shock (HS1) and 5 h later exposed (+) or not (−) to a second heat shock (HS2). Both heat shock treatments were for 20 min at 44 °C. Epitope-tagged SAPK2/p38 was immunoprecipitated, and SAPK2/p38 activity was determined using ATF2-GST as substrate.

A Short Lived Protein Regulates SAPK2/p38 Activation by Heat Shock—Cycloheximide desensitized MAPKAP kinase-2 to activation by heat shock (Fig. 6A). The capacity to activate MAPKAP kinase-2 with heat shock decreased progressively upon exposure to cycloheximide and was totally inhibited after 5 h, suggesting the existence of an essential element with a half-life in the order of 2–3 h in the SAPK2/p38 activation pathway. To determine whether this element was specific to heat shock, cells were exposed to cycloheximide for 5 h and then treated with H2O2, sodium arsenite, or sorbitol. In contrast to heat shock, activation of MAPKAP kinase-2 by these agents was not or only slightly affected by the cycloheximide pretreatment (Fig. 6B). Similar results were obtained using two other structurally and mechanistically unrelated inhibitors of protein synthesis, puromycin and emetine. Preincubation with these agents also blocked activation of the p38 pathway by heat shock but not by hyperosmotic shock (Fig. 6C). This indicated that the putative short lived protein was a proximal element of the heat shock response pathway and was not required for these inducers. To investigate further the hypothesis of a short lived regulator, we looked at the effects of the proteasome inhibitor MG132 on SAPK2/p38 activation. As reported before (44), MG132 induced a robust activation of SAPK2/p38 which developed to maximal level within 5 h. In contrast to SAPK2/p38 activation by other inducers, activation by MG132 was not transient and was maintained for as long as MG132 was present in the medium for up to 24 h (Fig. 7, top panel). At longer times, MG132 was toxic. Pretreating cells with cycloheximide or heat shock prevented MG132 activation of SAPK2/p38 (Fig. 7, lower panels), suggesting that a protein that was up-regulated by MG132 and responsible for SAPK2/p38 activation was degraded during prolonged treatment with cycloheximide or after heat shock.

**DISCUSSION**

SAPK2/p38 is activated in mammalian cells by diverse agents including chemical and physical stresses such as heat shock, oxidants, hyperosmolarity, and also numerous cytokines and growth factor agonists (for review, see Ref. 45). Hence multiple sensing pathways exist which must eventually converge on the main signaling elements of SAPK2/p38. Two MAP kinase kinases, MKK3 and MKK6, have been shown to phosphorylate and activate SAPK2/p38 selectively (38, 46–48), and several different MAP kinase kinase kinases including MLK-2 using recombinant HSP27 as substrate.
and -3, MEKK1, ASK1, and TAK1 (48–51) can potentially activate MKK3 or MKK6. The MAP kinase kinase kinases are themselves activated either by kinases of the ste-20-like family of protein kinases or more directly by interacting with adaptors of specific receptors (for review, see Ref. 45). Ligation of Fas to its ligand, for example, recruits the adaptor Daxx yielding to its ligand, for example, recruits the adaptor Daxx yielding to

This work stemmed from a previous observation that cells that have developed thermotolerance as a result of a short exposure to mild heat shock are refractory to induction of HSP27 phosphorylation by a second triggering heat shock (10). Here we showed that the failure to reinduce phosphorylation of HSP27 is accompanied by a failure to activate the SAPK2/p38 kinase, MAPKAP kinase-2, and to phosphorylate and activate the MAPKAP kinase-2 activator, SAPK2/p38. This temporary refractoriness to restimulation was specific to heat shock and did not interfere with activation by all of the growth factors, cytokines, and stressing agents tested. This implies that heat desensitization is not the result of heat-induced alterations in MAPKAP kinase-2, SAPK2/p38, or any upstream signaling molecules that are also used by growth factors, cytokines, or other stress. Pathways connecting receptors/sensors for growth factors, cytokines, or other stress to the common signaling components essential for MAPKAP kinase-2 activation were not desensitized and were still functional in heat-desensitized cells. Only components specific to heat shock signaling were altered, implying that such components do exist and play the role of a heat shock sensor. The heat-desensitized element must lie upstream of the various pathways that lead to activation of SAPK2/p38 and upstream of the converging point of the various pathways that lead to activation of SAPK2/p38. It also must lie between the heat shock "sensor" and the diverging point leading to activation of SAPK2/p38 because SAPK1/JNK activation is also homologously desensitized by heat shock in the CCL39 cell line used in this study (data not shown) and in other cell lines (25, 58, 59).

Very little is known concerning the mechanisms by which stress activates signaling pathways in mammalian cells. In the case of oxidative stress, oxidation of thioredoxin, a direct inhibitor of ASK1, and dimerization of ASK1 lead to activation of kinases upstream of SAPK2/p38 (53, 60). In the case of UV light and hyperosmotic shock, activation of the SAPK1/JNK signal transduction pathway and activation of new gene activity result from the perturbation of the cell membrane inducing conformation changes in receptors. These agents induce activation, clustering, and internalization of the receptors for EGF, TNF-α, and interleukin-1 and the assembly of signaling molecules at these receptors (61). Agonist-independent phosphorylation and activation of the EGF receptor also seem to be involved in the activation of the MAP kinase ERK by heat shock.

**Fig. 6.** Protein synthesis inhibitors desensitize the activation of the SAPK2/p38 pathway by heat shock but not by other stresses. Panel A, exponentially growing CCL39 cells were incubated in the presence of 100 μg/ml cycloheximide for different periods of time (1–5 h) before being submitted (+, HS) or not (−, C) to a 20-min heat shock at 44 °C. Panel B, cells were preincubated (+) or not (−) with 100 μg/ml cycloheximide for 5 h (CHX) prior to being exposed to H2O2 (5 mM, 15 min), sorbitol (0.3 M, 15 min), or sodium arsenite (Ars, 200 μM, 60 min). Panel C, cells were preincubated with puromycin (50 μg/ml), emetine (5 μM), or left untreated (−) for 5 h prior to being exposed to heat shock or sorbitol. At the end of treatments, MAPKAP kinase-2 (panels A and B) or SAPK2/p38 (panel C) activities were determined using recombinant HSP27 or ATF2-GST as substrate. In panel A, the results are presented as the ratio of the kinase activities of stimulated cells over the activity of unstimulated cells.

**Fig. 7.** SAPK2/p38 activation by MG132 is blocked by cycloheximide and heat shock. Control (○), cycloheximide-pretreated (■), or heat shock-pretreated (○) CCL39 cells were incubated with 5 μM MG132 for the indicated time periods. At the end of the treatments, SAPK2/p38 activity was determined using ATF2-GST as substrate. Pretreatment with cycloheximide was for 5 h at a concentration of 100 μg/ml (cycloheximide was left in the medium during exposure to MG132). Preheat shock consisted of a 20-min exposure at 44 °C administered 4 h before MG132. Results are expressed as the ratio of the kinase activities of stimulated cells over the activity of unstimulated control cells.
However, activation of MAPKAP kinase-2 by heat shock is not blocked by tryptophan AG1478, a selective EGFr receptor inhibitor, nor by a dominant negative mutant of EGFr receptor (62), and there is no cross-desensitization of the SAPK2/p38 pathway between heat shock and EGFr (this study), suggesting that activation of SAPK2/p38 uses a sensing pathway different from ERK. There is also no cross-desensitization between heat shock and thrombin, and we found that the heat-desensitized component is not required for transmitting signal from growth factor and cytokine receptors to MAPKAP kinase-2, strongly suggesting that heat shock does not use proximal elements of these pathways for triggering SAPK2/p38. All of these results contribute to the conclusion that a specific receptor/sensor pathway is used by heat shock to activate the SAPK2/p38 pathway. It should also be added that the pathway is unlikely to use a membrane-bound molecule because suramin, an extracellular antagonist of several membrane receptors, did not block heat shock activation of p38 but it completely blocked activation of ERK by heat shock and also induction of ERK and SAPK1/JNK by UV light and hyperosmotic shock (61–63). Moreover, it was also reported that a low concentration of the anionic detergent Triton X-100, which totally inhibited activation of SAPK1/JNK by UV light, did not prevent activation of p38 by heat shock (5).

Several mechanisms have been demonstrated for homologous desensitization of receptor-mediated responses to agonists including direct down-regulation of the receptor expression and feedback inhibition of essential signaling components by phosphorylation or other mechanisms. We clearly showed that heat-induced homologous desensitization of HSP27 phosphorylation is not caused by down-regulation of MAPKAP kinase-2 or SAPK2/p38 expression. Both upstream regulators of HSP27 phosphorylation were expressed at normal levels in heat-desensitized cells and could be activated by other stress, growth factors, and cytokines. We also showed that inhibition of SAPK2/p38 during the priming treatment had no effect on the heat desensitization process, indicating that heat-induced homologous desensitization is not the result of a negative feedback loop involving activation of SAPK2/p38. This is consistent with the results that EGFr and thrombin also induced SAPK2/p38 without desensitizing the cells to heat shock.

Homologous heat-induced desensitization of HSP27 phosphorylation occurs coincidentally with HSP accumulation (10), but none of the HSP tested inhibited the activation of p38 nor the process of heat-induced homologous desensitization in CCL39 cells. Our results contrast with those of Mosser et al. (58) and Gabai et al. (29), who found that extremely high (100-fold) overexpression of HSP70 blocked activation of SAPK1/JNK and SAPK2/p38 by a variety of stressful conditions in PEER cells. They are however consistent with other observations by the same (58) and other authors (10, 18, 23, 24), indicating that permanent cell lines constitutively expressing either HSP27 or HSP70 genes respond normally or even slightly better to heat induction of HSP27 phosphorylation or activation of SAPK1/JNK than control cells. In WEHI and RIN cells, we also found that overexpression of HSP70 either in a permanent cell line or in transient transfection assays enhanced (2-fold) induction of SAPK2/p38 by cytokines and had only a slight inhibitory effect on heat shock. The reason for the variable results obtained with HSP70 is not clear but may reflect the existence of distinct pathways for induction of SAPK1/JNK and SAPK2/p38 by stress. One pathway may be triggered as a consequence of damages caused by heat shock. During apoptosis, for example, damages can activate caspases, which in turn can activate SAPK1/JNK and SAPK2/p38 thereby activating even more caspase activities in a loop of amplification (64–66). In that situation, the capacity of HSP70 to block apoptosis downstream of caspase activation (24) would also result in blocking SAPK activation. A second pathway involving a more specific heat shock sensing mechanism independent of HSP70 may prevail during nontoxic heat shock treatment.

Results obtained with cycloheximide and MG132 suggest a possible mechanism for activation of SAPK2/p38 by heat shock and for homologous heat desensitization. We found that pretreatment with cycloheximide progressively blocks SAPK2/p38 activation by heat shock. This finding suggests that an essential component of the SAPK2/p38 activation pathway is short lived and rapidly eliminated during pretreatment with cycloheximide. This component is, however, essential only in the heat shock activation pathway because cycloheximide treatments do not interfere or interfere only slightly with activation by hydrogen peroxide, sorbitol, or sodium arsenite. We also found that MG132, an inhibitor of proteasome function, caused a sustained activation of SAPK2/p38. This suggests that accumulation of a normally rapidly degraded protein acts as a positive regulator of the SAPK2/p38 pathway. The finding that cycloheximide-pretreated cells do not respond to MG132 supports the view that this regulator is rapidly turning over under normal conditions and thus in tight equilibrium between rapid synthesis and rapid degradation. Heat shock-desensitized cells failed to respond to MG132, indicating that this regulator is also eliminated after heat shock. In mammalian cells, 80–90% of the protein breakdown including short and long lived proteins occurs by the ubiquitin-proteasome pathway (67). Upon heat shock, accumulation of unfolded proteins causes an initial overload of the ubiquitin-proteasomal system, a decrease in the free-ubiquitin pool, and a transient accumulation of short half-life protein normally degraded by this system (68, 69). Thus, we hypothesize that like MG132, heat shock causes an accumulation of this upstream activator of SAPK2/p38. In contrast to MG132, which maintains this factor at a high concentration and thus keeps SAPK2/p38 activated, the accumulation of the factor is only transient in the case of heat shock. Several mechanisms can contribute to the rapid disappearance of the factor after returning cells to normal temperature. The general protein synthesis rate is decreased after heat shock in mammalian cells (70); ubiquitin is itself a heat shock protein, and its concentration is increased after heat shock (71); an increased concentration of HSP70 and/or HSP90, which under some conditions may facilitate protein degradation by proteasomes, can contribute to accelerate protein degradation transiently (72, 73).

In conclusion, we have shown that heat shock-induced desensitization of the SAPK2/p38 pathways occurs through a specific and regulated manner involving a rapidly turning-over activator located at a proximal step of the activation cascade. Desensitization affected only the heat shock activation pathway pointing out at the existence of a specific sensing pathway for heat shock, distinct from pathways used by other stresses, growth factors and cytokines.

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