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Proteasome Inhibitors MG132 and Lactacystin Hyperphosphorylate HSF1 and Induce hsp70 and hsp27 Expression

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MG132 and lactacystin, two 26S proteasome-specific protease inhibitors, can upregulate heat-shock gene transcription without heat shock. In this study, we showed that both of these inhibitors induce hyperphosphorylation and DNA-binding activity of HSF1 in the absence of heat shock (at 37°C). Since trimerization of HSF1 is known to precede the acquisition of HSF1–DNA binding activity, it seems that MG132- and lactacystin-induced hyperphosphorylation of HSF1 causes conformational changes of HSF1 molecules at 37°C and subsequently triggers its trimerization. Inhibition of protein synthesis by cycloheximide abolished the MG132- or lactacystin-induced hyperphosphorylation and DNA-binding activity of HSF1. These data suggest that the activity of a putative kinase(s) targeting HSF1 is upregulated in the presence of MG132 or lactacystin. The upregulation of the kinase activity requires *de novo* protein synthesis and is likely due to the inhibition of protein degradation of a short-lived, kinase(s) targeting HSF1 and/or the cofactor(s) for the kinases, through the ubiquitin–proteasome pathway.

Common to all cells and organisms, there is a highly conserved and exquisitely regulated cellular response to suboptimal physiological environments, so called heat shock or stress response. The activation of heat shock genes (or stress genes) results in the elevated synthesis of heat shock proteins (hsp), which protects cells from thermal or other environmental insults. These heat shock proteins, also known as molecular chaperones, have essential roles in protein biosynthesis, protein transport, translocation, and folding of proteins. Heat shock transcription factor HSF1 is the key factor in the regulation of hsp gene expression (1–3). In recent years, the molecular events underlying the transcriptional activation of HSF1 have been extensively studied, specifically in terms of their hyperphosphorylation (4), trimerization (5), and acquisition of heat shock element (HSE)-binding activity (6, 7). In the non-stressed cells HSF1 is maintained in monomeric, non-DNA binding and partially phosphorylated state. Upon stress, HSF1 is trimerized, and acquires DNA-binding ability (8). It is evident that trimerization precedes acquisition of DNA-binding activity; the sequential order of phosphorylation, however, is much less clearly defined. The trimerization of HSF1 accompanies conversion of the intramolecular to an intermolecular leucine zipper interaction between the N-terminal leucine zipper domains and that of C-terminus of HSF1 (9). Phosphorylation on multiple Ser/Thr sites in the central regulatory domain of HSF1, but not in the C-terminal activation domain, has been shown to play a major role in the regulation of transcriptional activation; on the other hand, these phosphorylation events are not necessary for the acquisition of HSF1-DNA-binding activity (10–12). Several attempts were made to investigate the signal transduction pathways for heat shock response and to search for protein kinases targeting HSF1. The Erk MAP kinase (11, 13–15), glycogen synthase kinase-3 (11, 15, 16), protein kinase C (16), SAPK/JNK (13) and p38 MAPK (13) have been shown to play a role in the phosphorylation of HSF1 and regulation of heat shock gene expression.

Recently, two proteasome inhibitors MG132 and lactacystin were shown to induce both the HSF1 trimerization (17) and the transcriptional activation of heat shock genes at 37°C (18–20). However, the molecular mechanism for these observations and specifically the role of phosphorylation in HSF1 trimerization is not clear. Here, we extended the earlier studies to examine the effect of lactacystin and MG132 on the state of

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phosphorylation and the HSE-binding activity of HSF1. We found that, in addition to its trimerization, HSF1 from MG132- or lactacystin-treated cells was hyperphosphorylated and acquired DNA-binding activity. Our data suggest that at 37°C, the normal physiological temperature, changes in the phosphorylation state of mammalian HSF1 have distinct effects on its DNA-binding activity. It is plausible that the trimerization of HSF1 is triggered by the conformational changes of HSF1 molecule caused by the lactacystin- or MG132-induced hyperphosphorylation. Analysis using cycloheximide and various protein kinase inhibitors, suggests that in the presence of MG132 or lactacystin the activity of some kinase(s) targeting HSF1 is upregulated. The upregulation of the kinase activity requires *de novo* protein synthesis and is likely the result of inhibition of protein degradation of a kinase targeting HSF1 and/or the cofactor for the kinase, through the ubiquitin-proteasome dependent pathway. Furthermore, kinases targeting HSF1 upon heat shock and kinases targeting HSF1 during MG132 or lactacystin treatment appear to be distinct, reconfirming that activation of heat shock response is under the control of multiple complex signaling pathways.

MATERIALS AND METHODS

Chemicals and reagents. Lactacystin, *N*-carbobenzoxyl-Leu-Leu-leucinal (MG132), *N*-Ac-Leu-Leu-norleucinal (ALLN), *N*-Ac-Leu-Leu-normethional (ALLM), 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), SB203580, staurosporine, ML-7, H-89, KN-62, phorbol-12-myristate-13-acetate (PMA), and wortmannin were purchased from Calbiochem. Calyculin A was purchased from Gibco BRL. Polyclonal antibody against murine hsp25 (SPA-801) and monoclonal antibody against human hsp70 (SPA-810) were purchased from StressGen. Polyclonal antibody against human HSF1 was produced in our laboratory using the recombinant human HSF1 as an antigen, and were affinity purified with an HSF1-agarose column.

Cells. Mouse embryonic fibroblast (MEF) cells were immortalized by SV-40 transfection, and maintained in DMEM medium with 10% fetal calf serum in a humidified CO_2 incubator. Monolayers of exponentially growing cells (in 60-mm dishes) were exposed to lactacystin or MG132 in DMEM without serum. For heat shock experiments, cells were heated in a specially designed heat box equilibrated with 95% air and 5% CO_2.

Immunoblotting and electrophoretic mobility shift assay. Preparation of whole cell lysate or whole cell extract and procedure for Western blot was described previously (21). Protein content was quantified using BCA method for whole cell lysate (Pierce) or Bradford method (Bio-Rad) for whole cell extract. Equal amount of proteins was analyzed using 8 or 10% polyacrylamide SDS–PAGE and followed by Western blotting. Affinity purified polyclonal antibody for HSF1 was used for HSF1 detection. Monoclonal antibodies (SPA-810, StressGen) were used to detect hsp70 and hsc70. A rabbit polyclonal antibody (SPA-801, StressGen) was used to detect the murine hsp25. ECL system from Amersham was employed for Western blot.

Preparation of whole cell extract and electrophoretic mobility shift assay (EMSA) were performed as described previously (22). Equal amount of protein (50 μg) was subjected to electrophoretic mobility shift assay (EMSA) using a ^{32}P-labeled oligonucleotide probe.

RESULTS AND DISCUSSION

Lactacystin and MG132 induce hyperphosphorylation and DNA-binding activity of HSF1 at 37°C, and enhance hsp25 and hsp70 expression without heat shock. It has been shown that lactacystin and MG132, two proteasome inhibitors, induce enhanced hsps synthesis in the absence of heat shock (17). Here, we extended this study further to examine the mechanism of lactacystin- and MG132-induced hsps induction, specifically to determine the state of phosphorylation of HSF1, and to evaluate the effects of changes in the phosphorylation state of HSF1 on its activity at 37°C. As shown in Fig. 1A, HSF1 from cells exposed to lactacystin for 6 to 8 h was hyperphosphorylated. The extent of phosphorylation was comparable to that of heat-shocked cells (compare lane 1 and lane 3, Fig. 1A). The hyperphosphorylated HSF1 in lactacystin-treated cells also acquired HSE-binding activity as shown by the electrophoretic mobility shift assay (Fig. 1B), indicating that HSF1 is trimerized. Similar results were obtained from MG132-treated cells (Fig. 2). These data imply that at 37°C, the hyperphosphorylation of HSF1 induces conformation changes to the HSF1 molecules, triggers HSF1 trimerization and acquisition of HSE-binding activity. It is generally believed that trimerization of HSF1 in heat-shocked cells is triggered by the heat-induced conformation changes of HSF1 (23). Consistent with this notion, *in vitro* studies using purified HSF1 have shown that conformation change of HSF1 triggers HSF1 trimerization and acquisition of DNA-binding activity without phosphorylation, indicating that trimerization precedes phosphorylation (24, 25). On the other hand, in the case of lactacystin or MG132 treatment, the cause of HSF1 activation without heat shock is most likely due to conformation changes caused by protein modification, i.e., phosphorylation. The electrostatic charge on the phosphate groups may modify the tertiary structure of HSF1 and result in conformation changes of HSF1. It is less likely that lactacystin causes conformation change of HSF1 prior to its hyperphosphorylation, since lactacystin is known to specifically bind to proteasomes (26). As shown in Fig. 1A and by others (4), hyperphosphorylated HSF1 migrated slower in SDS–PAGE when compared to the HSF1 in control unstressed cells. Consistent with these observations, HSF1 from MG132-treated, or heat-shocked cells clearly showed an increase in mobility after treatment of alkaline phosphatase (lanes 4 and 6 in Fig. 1C). HSF1 from control unstressed cells also showed a similar effect, demonstrating that HSF1 is partially phosphorylated before heat shock (lane 2 in Fig. 1C).

As shown in Fig. 1D, lactacystin and MG132 induced hyperphosphorylation of HSF1 (upper panel) and enhanced the expression of hsp25 and hsp70 (middle and lower panels in Fig. 1D). The effect of lactacystin and

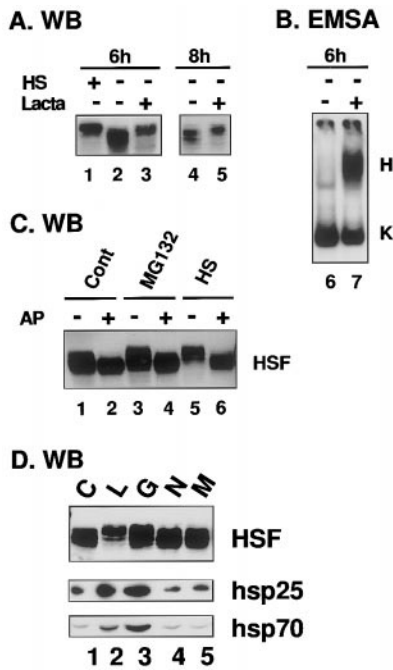

FIG. 1. Lactacystin induces the DNA-binding activity and hyperphosphorylation of HSF1 at 37°C, and hsp25 and hsp70 expression without heat shock. (A) Cells were exposed to 20 μM of lactacystin for up to 8 h and the whole cell lysates were analyzed by Western blot following SDS–PAGE using an anti-HSF1 antibody. HSF1 from cells exposed to lactacystin for 6 or 8 h were hyperphosphorylated (lanes 3 and 5). Hyperphosphorylated HSF1 from heat-shocked cells was shown for comparison (lane 1); (B) electrophoretic mobility shift analysis showing that lactacystin induces HSF1-DNA binding activity. Cell extract from cells described in A without (lane 6) or with lactacystin treatment (lane 7) were analyzed; (C) whole cell extract of control cells, MG132-treated cells (20 μM/6 h), and heat shocked cells (45°C/15 min) were treated with alkaline phosphatase, subjected to SDS–PAGE, and analyzed by Western blot using anti-HSF1 antibody. The phosphatase-treated HSF1 showed a faster mobility in SDS–PAGE, indicating that molecular size was reduced by dephosphorylation (lanes 2, 4, and 6). Data as shown above in A, B, and C suggest that kinase(s) activity for HSF1 is elevated by lactacystin or MG132; (D) cells were exposed to 10 μM of lactacystin (L), MG132 (G), ALLN (N), and ALLM (M) for 6 h and the whole cell lysates were analyzed by Western blot using anti-HSF1 or anti-hsp25 or anti-hsp70 antibody following SDS–PAGE. HSF1 from lactacystin- (lane 2) or MG132- (lane 3) treated cells showed slower mobility than that from the control cells, indicating hyperphosphorylation of HSF1. In parallel, hsp25 and hsp70 expression was induced in lactacystin- or MG132-treated cells, but not in cells treated with proteasome nonspecific protease inhibitors such as ALLN or ALLM (lanes 4 and 5, respectively) or in control, untreated cells (lane 1).

MG132 appears to be specific, since ALLN and ALLM, (calpain inhibitors I and II, respectively) did not induce hyperphosphorylation of HSF1 nor enhanced synthesis of hsp25 and hsp70 (middle and lower panels in Fig. 1D).

Kinetics of lactacystin- and MG132-induced hyperphosphorylation and acquisition of DNA-binding activity of HSF1. To define the sequential order of hyperphosphorylation and trimerization of HSF1, the kinetics of phosphorylation and acquisition of DNA-binding activity of HSF1 induced by MG132 and lactacystin were determined in the next set of experiments. Monolayers of cells were exposed to 10μM of MG132 and lactacystin for 0, 0.5, 1, 2, 3, and 4 h. Cell extracts were prepared and subjected to immunoblot and gel mobility shift analysis (Figs. 2A and 2B). Our data clearly showed that HSF1 is hyperphosphorylated after 2–4 h of MG132-treatment (lanes 4–6, Fig. 2A), or lactacystin-treatment (lanes 10–12, Fig. 2A). In parallel, HSF1 acquired DNA-binding activity (Fig. 2B). The kinetics of HSF1 hyperphosphorylation and acquisition of HSF1–DNA-binding activity was nearly identical. It is plausible that the trimerization induced by conformation change of HSF1 is very fast and the sequential order of the events may not be easily separable using the conventional techniques.

Cycloheximide abolishes lactacystin- and MG132-induced hyperphosphorylation and DNA-binding activity of HSF1. Since HSF1 is hyperphosphorylated in cells treated with lactacystin (or MG132), it seems reasonable to assume that lactacystin (or MG132) may upregulate the activity of some putative HSF1 kinase(s) by inhibiting the proteasome-dependent protein degradation of the kinase(s) and/or cofactor(s) for the kinases. In other words, if hyperphosphorylation of HSF1 is mediated by a putative HSF1 kinase(s), whose activity is upregulated in MG132- or lactacystin-treated cells, one would expect that the kinase(s) activity would be affected by inhibition of *de novo* protein synthesis. To test this hypothesis, a protein synthesis inhibitor, cycloheximide (CHM), was used in combination with lactacystin or MG132. As shown in Fig. 3A,

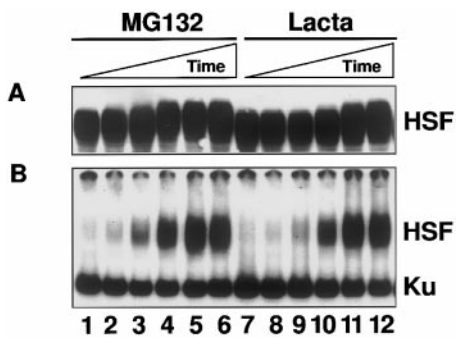

FIG. 2. Time course of HSF1 phosphorylation and acquisition of DNA-binding activity induced by MG132 or lactacystin at 37°C. (A) Cells were exposed to 10 μM of MG132 or lactacystin for 0 h (lane 1, 7), 0.5 h (lanes 2 and 8), 1 h (lanes 3 and 9), 2 h (lanes 4 and 10), 3 h (lane 5, 11), 4 h (lanes 6 and 12) in DMEM (without serum), and followed by Western blot to determine the phosphorylation status of HSF1 using antibody specific to HSF1. Samples from 1 to 4 h treatment of either MG132 or lactacystin showed that HSF1 migrates with a slow mobility, indicating that HSF1 is hyperphosphorylated. The kinetics of hyperphosphorylation and acquisition of DNA-binding activity was nearly identical; (B) the same cell extracts as used in A were analyzed with EMSA. The DNA-binding activity reached a plateau after 3–4 h of incubation with MG132 or lactacystin (lanes 5 and 6 and lanes 11 and 12, respectively).

simultaneous treatments of CHM and lactacystin abolished the hyperphosphorylation of HSF1 (Fig. 3A, lane 6); whereas in the absence of cycloheximide (CHM), there was significant hyperphosphorylation of HSF1 in the lactacystin-treated cells (Fig. 3A, lane 2). Cells pre-treated with CHM for 3 to 6 hours before lactacystin treatment also showed an inhibition of hyperphosphorylation of HSF1 (Fig. 3B), suggesting that the putative HSF1 kinase(s) and/or cofactor(s) is short-lived and that they are probably degraded through the ubiquitin-proteasome pathway. Our data further suggest that hyperphosphorylation of HSF1 in control cells may require the accumulation of the putative HSF1 kinase(s) synthesized for approximately 2–4 h and this protein turned over rapidly within 3 h. In other words, our results suggest that the lactacystin-induced hyperphosphorylation of HSF1 may be caused by an enhanced level of kinase(s) activity by blocking proteasome-dependent protein degradation. Interestingly, in contrast to lactacystin treatment alone, hyperphosphorylation of HSF1 induced by heat-shock plus lactacystin was not affected by CHM (compare lane 7 to lane 3 in Fig. 3A). The difference in response to cycloheximide between lactacystin-treated and heat-shocked cells suggests that protein kinase(s) targeting HSF1 during heat shock and during lactacystin treatment may be different. From the fact that HSF1 is hyperphosphorylated by heat shock or by MG132/lactacystin and HSF1 is dephosphorylated during post-heat-shock-recovery or upon the removal of MG132/lactacystin, it is apparent that the phosphorylation status of HSF1 is tightly regulated by the activity of protein kinase and protein phosphatase. Thus, either by increasing the HSF1 kinase activity or by blocking the HSF1 phosphatase activity, one can shift the equilibrium favoring phosphorylation of HSF1.

To examine the effect of cycloheximide on MG132-induced activation of HSF1, cells were exposed simultaneously with CHM and MG132 for 1–3 h and the DNA-binding activity was determined. Figure 3C shows that in the presence of CHM, MG132-induced activation of HSF1-DNA-binding was completely abolished (compare lanes 2–4 to lanes 5–7, Fig. 3C). In contrast, CHM did not affect the heat-shock-induced activation of HSF1 (Fig. 3D). Taken together, these data demonstrate that the kinase(s)/cofactor(s) phosphorylating HSF1 in MG132-and lactacystin-treated cells may be distinct, at least in part, from that in heat-shocked cells. Furthermore, *de novo* protein synthesis appears to be required for the enhanced kinase activity in MG132- and lactacystin-treated cells. However, it is not clear at present whether the candidates modulated by proteasome inhibitors and/or cycloheximide are kinase(s) per se and/or the cofactor(s) for the kinase(s).

To search for the putative HSF1 kinase(s) which was upregulated by proteasome inhibitors, cells were si-

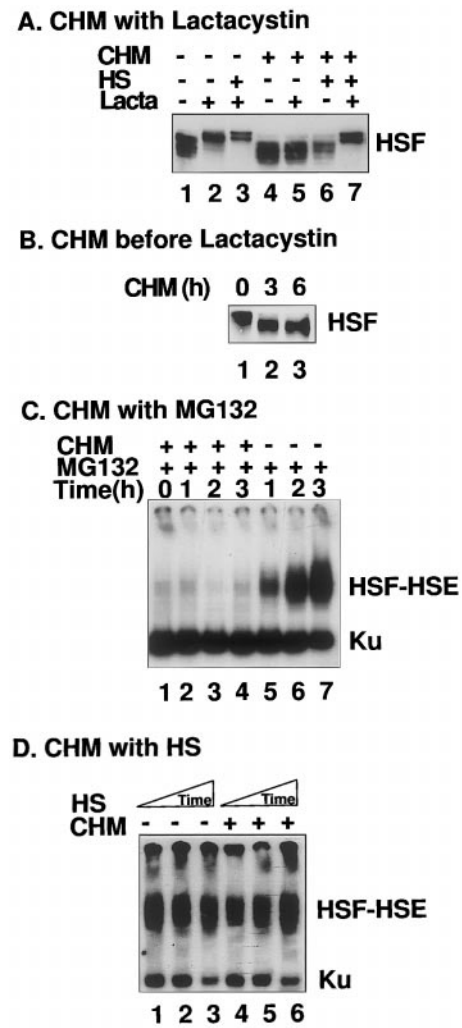

FIG. 3. Inhibition of protein synthesis by cycloheximide abolishes lactacystin- or MG132-induced hyperphosphorylation of HSF1. (A) Cells were exposed to 10 μM of lactacystin for 6 h with or without cycloheximide (CHM, 10 μg/ml). The whole cell lysates were then subjected to SDS–PAGE and Western blot using an anti-HSF1 antibody. HSF1 from cells treated with lactacystin and CHM simultaneously were not hyperphosphorylated (compare lane 2 to lane 5). HSF1 from cells heat-shocked (45°C/10 min) and subsequently treated with 10 μM of lactacystin for 6 h remained hyperphosphorylated regardless of the presence or absence of CHM (compare lane 3 to lane 7); (B) cells were treated up to 6 h with 10 μg/ml of CHM and followed by 2 h treatment of 10 μM MG132 in the presence of CHM. The whole cell lysates were prepared and subjected to SDS–PAGE and Western blot using an anti-HSF1 antibody. The hyperphosphorylation of HSF1 was abolished by CHM pretreatment, suggesting that the kinase(s) activity for HSF1 is short-lived (less than 3 h); (C) cells were treated for up to 3 h with MG132 in the presence (lanes 1–4) or absence (lanes 5–7) of CHM (5 μg/ml), and whole cell extracts were analyzed by EMSA. The acquisition of DNA-binding activity of HSF1 was abolished in the presence of CHM, indicating that either the kinase(s) for HSF1 is short-lived or only newly synthesized kinase(s)/cofactors is required for activity; (D) cells were heat shocked for 5 (lanes 1 and 4), 10 (lanes 2 and 5), and 20 min (lanes 3 and 6) at 42°C without (lanes 1–3) or with CHM (5 μg/ml) pretreatment for 30 min (lanes 4–6). In contrast to MG132-treated cells as in C, CHM did not affect acquisition of HSF1 DNA-binding activity during heat shock.

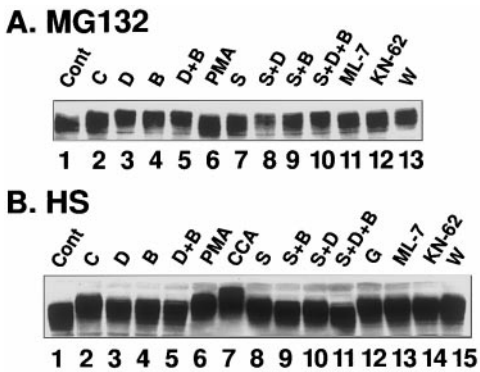

FIG. 4. Effect of various protein kinase inhibitors on MG132- or heat-shock-induced HSF1 phosphorylation. (A) Exponentially growing cells were treated with various kind of protein kinase inhibitors in the presence of MG132 for 6 h. Whole cell Extract were prepared, analyzed by SDS–PAGE and followed by Western blot using anti-HSF1 antibody; (B) cells were treated with various kind of protein kinase inhibitors in DMEM without serum for 1 h and then heat shocked for 15 min at 43°C. Whole cell extracts were prepared and HSF1 was detected as above. Cont and C indicate control cells and cells treated with MG132 only (in A) or heat shock only (in B), respectively. D, B, S, G, and W indicate DRB, SB203580, staurosporine, MG132, and wortmannin, respectively. CCA indicates calyculin A.

multaneously treated with various protein kinase inhibitors and MG132; specifically, the effect of protein kinase inhibitor DRB (for casein kinase II), SB203580 (for P38 MAPK), staurosporine (for protein kinase C), ML-7 (for myosin light chain kinase), KN62 (for calmodulin protein kinase), and wortmannin (for PI-3K and DNA-PK) were examined singly or in combination. As shown in Fig. 4A, MG132-induced hyperphosphorylation of HSF1 was not affected by any of these protein kinase inhibitors or combination of inhibitors. On the contrary, heat-shock-induced HSF1 hyperphosphorylation was partially inhibited by kinase inhibitors such as DRB, SB203580, and staurosporine if cells were pre-treated with these inhibitors before heat shock (Fig. 4B, lanes 3, 4, and 8). A more significant inhibition was observed if the treatment consisted of a combination of DRB and SB203580 (Fig. 4B, lane 5), or staurosporine and SB203580 (Fig. 4B, lane 9), staurosporine and DRB (Fig. 4B, lane 10), or a combination of staurosporine, DRB, and SB203580 (Fig. 4B, lane 11). Thus, the sensitivity of HSF1 phosphorylation to various protein kinase inhibitors was significantly different between MG132-treated cells and heat-shock-treated cells, again supporting the notion that the HSF1 kinase(s) upregulated by proteasome inhibitors and by heat shock is different. Furthermore, Fig. 4B demonstrates that multiple protein kinases are involved in heat-shock-induced HSF1 phosphorylation; protein kinase C, casein kinase II, and P38 MAPK seem to be the most likely candidates (lane 11 in Fig. 4B). The identity of the putative kinase(s) involved in lactacystin- or MG132-induced HSF1 hyperphosphorylation is not known at present. The putative HSF1 kinase(s) may be a novel protein kinase or one of the known protein kinases which cannot be deduced from our present study.

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