Differential Activation of p38 Mitogen-activated Protein Kinase and Extracellular Signal-regulated Protein Kinases Confers Cadmium-induced HSP70 Expression in 9L Rat Brain Tumor Cells*

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We have reported that treatment with CdCl₂ at 40–100 μM induces the heat shock proteins (HSPs) in nine rat brain tumor cells, during which the activation of heat shock factor (HSF) is essentially involved. By exploiting protein kinase inhibitors, we further analyzed the possible participation of specific protein kinases in the above processes. It was found that induction of HSP70 in cells treated with a high concentration of cadmium (i.e., 100 μM) is preceded by the phosphorylation and activation of p38 mitogen-activated protein kinase (p38 MAPK), while that in cells treated with low concentrations (60 μM) is accompanied by the phosphorylation and activation of extracellular-regulated protein kinases 1 and 2 (ERK1/2). In 100 μM cadmium-treated cells, both HSP70 induction and HSF1 activation are eliminated in the presence of SB203580, a specific inhibitor of p38 MAPK. By contrast, in 60 μM cadmium-treated cells, the processes are not affected by SB203580 but are significantly suppressed by PD98059, which indirectly inhibits ERK1/2 by acting on MAPK-ERK kinase. Taken together, we demonstrate that p38 MAPK and ERK1/2 can be simultaneously or independently activated under different concentrations of cadmium and that the signaling pathways participate in the induction of HSP70 by acting on the inducible phosphorylation of HSF1. We thus provide the first evidence that both p38 MAPK and ERK signaling pathways can differentially participate in the activation of HSF1, which leads to the induction of HSP70 by cadmium.

Heat shock proteins (HSPs)† are induced in cells responding

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The abbreviations and trivial names used are: RBT cells, rat brain tumor cells; HSP, heat shock protein; HSP70, 70-kDa inducible HSP; HSF, heat shock factor; HSE, heat shock element; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; SAPK, stress-activated protein kinase; SB203580, 4-(fluorophenyl)-2-(4-methylsulfanyl-phenyl)-5-(4-pyridyl)imidazole; PD98059, 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalene-4-one; BIM, bisindolylmaleimide; H89, N-[2-(3-fluoropropyl)cinnamyl]aminoethyl]-5′-isoquinoline-sulfonamide, HCl; MBP, myelin basic protein; MAPKAPK-2, MAPK-activated protein kinase-2; [32P]orthophosphate; PAGE, polyacrylamide gel electrophoresis; PI, pro-pidium iodide.
and that HSF-HSE interaction is involved in this process (33). In this study, we have employed SB203580 and PD98059 to investigate whether activation of p38 MAPK and ERK1/2 are necessary for cadmium-induced HSP70 expression. We have focused on the effects of the protein kinase inhibitors on the induction of HSP70 as well as the activation of HSF1 in cadmium-treated 9L RBT cells. Herein we demonstrate that p38 MAPK and ERKs can be simultaneously or distinctly activated under different concentrations of cadmium and that the signaling pathways may participate in the induction of HSP70 by acting on the inducible phosphorylation of HSF1. We thus provide the first evidence that both p38MAPK and ERKs may differentially participate in the activation of HSF1, which leads to the induction of the heat shock genes. The differential role of each of these two signaling pathways under stress is also discussed.

EXPERIMENTAL PROCEDURES

Materials—Cultureware was obtained from Corning, and culture medium components were purchased from Life Technologies, Inc. [35S]methionine (specific activity >800 Ci/mmol), [γ-32P]ATP (5,000 Ci/mmol), [γ-32P]ATP (5,000 Ci/mmol), orthophosphoric (5,000 Ci/mmol), antibodies against HSP70, horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence (ECL) Western blotting detection kits were purchased from Amersham Pharmacia Biotech. Antibodies against p38 MAPK, phospho-p38 MAPK (Tyr182), ERK1/2, phospho-ERK1/2, c-Jun, and phospho-c-Jun (Ser63, Ser73) were from New England Bio-labs. Synthetic oligonucleotides were ordered from Life Technologies, Inc. Chemical reagents for electrophoresis were from Bio-Rad. Other chemicals were obtained from Merck or Sigma.

Cells and Drug Treatment—The 9L RBT cells (34) were maintained in Eagle’s minimal essential medium plus 10% fetal calf serum supplemented with 100 units/ml penicillin G and 100 μg/ml streptomycin in a 37 °C incubator under 5% CO2 and 95% air. Prior to each experiment, stock cells were plated in 25-cm2 flasks or six-well plates at a density of 4–6 × 104 cells/cm2. Exponentially growing cells at 80–90% confluency were used. Cadmium chloride was dissolved in water at a concentration of 100 mM and added to the culture medium to the desired concentrations for treatment. The cells were treated at 37 °C for 2 h and allowed to recover for various durations as specified. For the studies concerning the effects of protein kinase inhibitors, the cells were pre-incubated with respective inhibitors, at various concentrations for 1 h, followed by treatment with cadmium in the presence of the inhibitors.

Metabolic Labeling and SDS-PAGE—De novo protein synthesis was revealed by [35S]methionine labeling at a concentration of 20 μCi/ml. After various treatments, the cells were labeled for 1 h, washed in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO4, 1.4 mM KH2PO4, pH 7.4), and lysed in sample buffer (35). Equal amounts of cell lysates were resolved in 10% SDS-PAGE gels and transferred to membrane filters, which were incubated with a 1:5,000 dilution of respective antibodies against phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2, ERK1/2, c-Jun, and phospho-c-Jun (Ser63, Ser73) for 12 h. Immunoreactive bands were visualized by autoradiography.

Activity Assays for p38 MAPK and ERK1/2—Cells were stressed by 0, 10, 50, 100 μM CdCl2, for 2 h and then lysed in kinase lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, 10 μM β-glycerophosphate, 50 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM benzamidine, 0.1% β-mercaptoethanol) for 10 min on ice. Insoluble material was removed by centrifugation (10,000 × g, 20 min, 4 °C). The cell lysate was mixed with 5 μl (200 ng) of glutathione S-transferase-MAPKAPK-2 or MBP, 5 μl of [γ-32P]ATP, and 2 μl of magnesium/ATP mixture and then incubated for 15 min at 30 °C with agitation. The solution was finally mixed with an equal volume of 2× sample buffer for SDS-PAGE. After electrophoresis, the gels were dried and processed for autoradiography.

Differential Activation of p38 MAPK and ERK1/2 by Cadmium—Differential activation of each of these two signaling pathways under stress is also examined by three strokes with a Dounce grinder. The samples were lysed in sample buffer (35). Equal amounts of cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and then incubated for 15 min at 30 °C with agitation. The solution was finally mixed with an equal volume of 2× sample buffer for SDS-PAGE. After electrophoresis, the gels were dried and processed for autoradiography.

Flow Cytometry—Flow cytometry was performed on a fluorescence-activated cell sorter, FACScan (Becton-Dickinson, San Jose, CA), using CellQuest and Modfit LT software. For cell cycle analysis, aliquots of 1 × 106 cells were fixed in 70% ethanol on ice for 2 h and collected by centrifugation (1,000 × g, 10 min, 4 °C). The pellets were resuspended in 1 ml of PBS buffer containing 20 μg/ml RNase A and incubated at room temperature for 30 min. RNase was removed by centrifugation (1,000 × g, 10 min, 4 °C), and the fixed cells were resuspended in 1 ml of PBS followed by incubation with 100 μl of 10 μg/ml propidium iodide (PI) for 30 min. The labeled cells were analyzed with FACScan according to the manufacturer’s instructions.

Preparation of Genomic DNA from 9L RBT Cells—The DNA extraction procedure was carried out at 4 °C unless otherwise specified. Approximately 106 cells treated with or without CdCl2 were collected by centrifugation (1,000 × g, 5 min) and resuspended in 10 ml of PBS buffer, and the pellet was collected by centrifugation (1,000 × g, 5 min). The cells were resuspended in 3.6 ml of proteinase K buffer (10 ml Tris-CI, pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.4% SDS), and 0.4 ml of 10 mg/ml proteinase K was added. The mixture was incubated at 65 °C
for 15 min and 37 °C overnight. The solution was extracted three times with an equal volume of phenol/chloroform (1:1) and finally one time with an equal volume of chloroform followed by centrifugation (2,500 × g, 10 min). One-tenth volume of 3 M sodium acetate, pH 7.0, and 2.5 volumes of ethanol were both added to the aqueous layer with gentle mix to precipitate the genomic DNA. The DNA pellet was rinsed with 5 ml of 80% ethanol and recollected by centrifugation (2,500 × g, 5 min). To dissolve the precipitated DNA, 4.5 ml of proteinase K buffer without SDS was added, and the solution was incubated at room temperature overnight. Subsequently, 25 μl of 10 mg/ml RNase A was added, and the solution was further incubated at 37 °C for 30 min, extracted with 5 ml of phenol/chloroform (1:1), and separated by centrifugation (2,500 × g, 10 min). The genomic DNA was precipitated by the addition of 9 ml of 80% ethanol and recollected by centrifugation (2,500 × g, 10 min). The isolated DNAs were loaded and separated on a 1% agarose gel in 1× TAE buffer and stained with ethidium bromide for examination with a UV transilluminator.

RESULTS
Effects of Protein Kinase Inhibitors on Cadmium-induced HSP70 Expression in 9L RBT Cells—As a first step to elucidate the possible involvement of protein kinases in the induction of HSP70 under cadmium treatment, several protein kinase inhibitors, including staurosporine, SB203580, bisindolylmaleimide (BIM), and H89, which inhibit general kinases, p38 MAPK, protein kinase C, and protein kinase A, respectively, were employed. Cells were respectively incubated with the above inhibitors for 1 h, treated with 100 μM CdCl₂ for 2 h along with each inhibitor, and metabolically labeled with [35S]methionine for 1 h. De novo protein synthesis was monitored by autoradiography (Fig. 1A). Staurosporine appeared to inhibit the induction of HSP70, indicating that certain protein kinase(s) are involved in the process (Fig. 1, lane 3). Furthermore, SB203580, but not BIM or H89, was found to be able to significantly suppress the induction of HSP70 in cadmium-treated cells (Fig. 1, lane 4). The results indicated that the p38MAPK, but not the protein kinase A or protein kinase C, signaling pathway is involved in this process.

Effects of Protein Kinase Inhibitors on the HSE Binding Activities of Nuclear Proteins Extracted from Cadmium-treated 9L RBT Cells—We have previously demonstrated the formation of HSE-HSF complexes during the induction of HSPs in cadmium-treated 9L RBT cells (33). Herein we further analyzed the effects of the protein kinase inhibitors on the HSE binding activities of the nuclear proteins extracted from both the treated and untreated cells. As shown in Fig. 2, two slowly migrating complexes, designated as complexes I and II, were detected (Fig. 2, lane 2). In the untreated cells, staurosporine could completely abolish the formation of complex I (Fig. 2, lane 3), while SB203580, BIM, and H89 could only reduce the binding intensity but did not affect the binding patterns (Fig. 2, lanes 4–6). Enhanced binding activity toward HSE was found in the cadmium-treated cells (Fig. 2, lane 7); however, both staurosporine and SB203580 eliminated the formation of complex I (Fig. 2, lanes 8 and 9), which could still be detected in cells preincubated with BIM or H89 (Fig. 2, lanes 10 and 11). These observations supported the notion that there are constitutive as well as inducible phosphorylation processes of HSFs and indicated that complex I may represent the presence of the inducible phosphorylation form. Moreover, the effects of the protein kinase inhibitors on the formation of complex I (inducible phosphorylation form) closely correspond to those on the induction of HSP70 shown in Fig. 1. Taken together, the data indicated that in cells treated with 100 μM CdCl₂ for 2 h, the p38 MAPK signaling pathway is involved in the cadmium-induced activation of HSF, which in turn confers the induction of HSP70.

Correlation of HSP70 Induction and p38 MAPK Activation in Cadmium-treated 9L RBT Cells—To study the relationship between HSP70 induction and p38 MAPK activation, the levels of p38 MAPK, tyrosine phosphorylation of p38 MAPK, and HSP70 were determined by immunoblotting analysis. The anisomycin-treated cell lysates, supplied by the manufacturer, were also used as additional controls to assure the authentic detection of p38 MAPK and phospho-p38 MAPK. The results clearly demonstrated that Tyr^{182} of p38 MAPK underwent phosphorylation in cells treated with 100 μM CdCl₂ (Fig. 3, lane 5) despite no observable changes in the levels of p38 MAPK with or without cadmium treatment. Furthermore, it was found that phosphorylation of p38 MAPK was completely abolished and inductive accumulation of HSP70 was significantly suppressed in cells preincubated with SB203580 (Fig. 3, lane 6).

The correlation between p38 MAPK activation and HSP70 induction was further examined in cells treated with 100 μM
CdCl₂ for 2 h followed by different recovery durations (Fig. 4). The synthesis of HSP70 was evaluated by metabolic labeling followed by autoradiography, while the alterations in the phosphorylation of p38MAPK and total expression of p38 MAPK were determined by immunoblotting using respective antibodies. Fig. 4A showed that synthesis of HSP70 was induced immediately after treatment, reached its maximum after 2 h, and diminished after 8 h of recovery. Consistently, p38MAPK remained unphosphorylated in the untreated cells, but the phosphorylated form of p38MAPK appeared immediately after treatment. The protein kinase was found to be phosphorylated until 4 h but completely dephosphorylated after 8 h of recovery (Fig. 4B). Quantitative analysis of the data showed that phosphorylation of p38MAPK appeared to precede the induction HSP70.

Concentration-dependent Activation of p38MAPK and ERK1/2 by Cadmium—The correlation between the HSP70 induction and MAPK activation was further investigated in cells treated with different concentrations of cadmium for 2 h and allowed to recover for up to 8 h. At different intervals as indicated, the cells were labeled with [³⁵S]methionine for 1 h and then lysed. The cell lysates were resolved by SDS-PAGE followed by autoradiography (A). Additionally, the cell lysates were analyzed by immunoblotting using anti-phospho-p38MAPK and anti-p38MAPK as the primary antibodies (B). The relative synthesis rate of HSP70 was determined as described in the legend of Fig. 1, while the relative levels of p38MAPK phosphorylation were presented as the sum of pixel values of each phospho-p38MAPK band divided by that of p38MAPK in the same lane (C). Data represent the means ± S.D. of three independent experiments.

**Fig. 3.** SB203580 prevents phosphorylation of p38MAPK and expression of HSP70 in the cadmium-treated 9L RBT cells. Cells were treated with 100 μM CdCl₂ with or without a 1-h preincubation with 20 μM SB203580. After 2 h of recovery, the cells were lysed, and the cell lysates were analyzed by immunoblotting using antibodies against p38MAPK (A), phospho-p38MAPK (specific for Tyr182 in p38MAPK) (B), and HSP70 (C), respectively. The immunocomplexes were visualized by ECL. NC and PC represent negative and positive controls that, respectively, consist of untreated and anisomycin-treated C-6 glioma cell lysates supplied by the manufacturer. Similar results were obtained in three independent experiments.

**Fig. 4.** Induction of HSP70 in cadmium-treated 9L RBT cells is preceded by the activation of p38MAPK. Cells were treated with 100 μM cadmium for 2 h and allowed to recover for up to 8 h. At different intervals as indicated, the cells were labeled with [³⁵S]methionine for 1 h and then lysed. The cell lysates were resolved by SDS-PAGE followed by autoradiography (A). Additionally, the cell lysates were analyzed by immunoblotting using anti-phospho-p38MAPK and anti-p38MAPK as the primary antibodies (B). The relative synthesis rate of HSP70 was determined as described in the legend of Fig. 1, while the relative levels of p38MAPK phosphorylation were presented as the sum of pixel values of each phospho-p38MAPK band divided by that of p38MAPK in the same lane (C). Data represent the means ± S.D. of three independent experiments.
thus may be responsible for HSP70 induction under these conditions. In an attempt to investigate whether another class of MAPK pathways is involved in HSP70 induction upon cadmium treatment, we have examined the roles of ERKs, which have been reported to be involved in the in vitro phosphorylation of HSF1. By exploiting antibodies against ERK1/2 and phospho-ERK1/2, we found that phosphorylation of the ERKs was initially induced by 40 μM CdCl₂, peaked at 60 μM CdCl₂, and subsided at higher concentrations. Phosphorylation of ERK1/2 became barely detectable in cells treated with 100 μM CdCl₂ (Fig. 5C). On the other hand, antibodies against the substrate of SAPKs (c-Jun) and phospho-c-Jun were employed, no change in protein level and phosphorylation level was detected in cells treated with up to 100 μM CdCl₂ (data not shown). Taken together, our results showed that both p38MAPK- and ERK1/2-mediated pathways can be simultaneously or independently activated in cadmium-treated cells, depending on the concentration used, whereas the SAPK signaling pathway appeared not to be involved in this process. Concentration-dependent differential involvement of the p38MAPK and ERKs in cadmium-induced HSP70 was further substantiated by the employment of specific inhibitors of these kinases, PD98059 and SB203580. As shown in Fig. 6, preincubation with PD98059 abolished HSP70 induction in cells treated with 60 μM CdCl₂ but not in those treated with 100 μM CdCl₂ (Fig. 6A, lanes 3 and 6). Conversely, SB203580 effected abolishment of HSP70 induction upon CdCl₂ treatment at 100 μM but not at 60 μM (Fig. 6A, lanes 4 and 7). Furthermore, induction of HSP70 by 60 μM CdCl₂ was strictly associated with the activation of ERK1/2, since both processes in these cells were simultaneously inhibited by PD98059 but not by SB203580 (Fig. 6B, lanes 2–4). In contrast, the induction of HSP70 by 100 μM CdCl₂ was strictly confined to the activation of p38MAPK, since both processes in these cells were simultaneously inhibited by SB203580 but not by PD98059 (Fig. 6B, lanes 5–7). In addition to examining the phosphorylation of p38MAPK and ERK1/2 themselves, the activation of the protein kinases was assessed by the phosphorylation of their respective downstream effectors, MAPKAPK-2 and MBP. It was found that MAPKAPK-2 was phosphorylated in the presence of 100 μM CdCl₂ (Fig. 7A, lane 3), while MBP was phosphorylated in the presence of 60 μM CdCl₂ (Fig. 7B, lane 2). Furthermore, each phosphorylation process could be blocked by its specific inhibitor (Fig. 7A, lane 4, and B, lane 3). Altogether, we provided concrete evidence that there is differential involvement of p38MAPK and ERK1/2 in cadmium-induced synthesis of HSP70.
The cell lysate was mixed with 100 \( \mu \text{M} \) CdCl\(_2\), respectively, for 2 h with or without a 1-h pretreatment with 20 \( \mu \text{M} \) SB203580 (SB) or 10 \( \mu \text{M} \) PD98059 (PD). The cell lysate was mixed with \( \gamma^{32}\text{P}\)orthophosphate and MAPKAPK-2 (A) or MBP (B) and analyzed by SDS-PAGE followed by autoradiography. Similar results were obtained in three independent experiments.

**Phosphorylation of HSF1 in Cadmium-treated 9L RBT Cells**—We subsequently measured the phosphorylation level of HSF1 in the cells under cadmium treatment to investigate whether the processes of differential activation of p38MAPK and ERK1/2 were authentically coupled to the inductive phosphorylation of HSF1. As in the previous experiments, cells were prelabeled by \( \gamma^{32}\text{P}\)orthophosphate and then treated with 60 or 100 \( \mu \text{M} \) CdCl\(_2\) with or without preincubations of the protein kinase inhibitors. After treatment, HSF1 was immunoprecipitated from the cell lysates, and the immunocomplexes were analyzed by SDS-PAGE followed by autoradiography. We found that HSF1 was phosphorylated in cells treated with both 60 and 100 \( \mu \text{M} \) CdCl\(_2\) (Fig. 8). In accordance with the results shown in Fig. 6, preincubation with PD98059 remarkably eliminated the HSF1 phosphorylation in cells treated with 60 but not 100 \( \mu \text{M} \) CdCl\(_2\). In contrast, the phosphorylation of HSF1 under 100 \( \mu \text{M} \) CdCl\(_2\) could only be abolished in cells preincubated with SB203580. These data further substantiated the notion that phosphorylation of HSFs and HSP70 induction in cells treated with different concentrations of cadmium are mediated through p38MAPK and ERK1/2 differentially.

**Mitogenic and Apoptotic Effects Induced in Cadmium-treated 9L RBT Cells**—In order to investigate whether 60 and 100 \( \mu \text{M} \) CdCl\(_2\), respectively, cause the mitogenic and apoptotic effect in 9L RBT cells, changes in DNA synthesis and fragmentation in each condition have been monitored by flow cytometry employing PI staining and agarose gel electrophoresis as shown in Fig. 9. In our experiments, CdCl\(_2\) at a concentration of 60 or 100 \( \mu \text{M} \) was used for induction, and the cells were allowed to execute their stress response in 6 or 12 h. As shown in Fig. 9A, the percentage of cells arrested in G2/M phase was 13.6%, which drastically increased to 30.8% once the cells were treated with 60 \( \mu \text{M} \) CdCl\(_2\) and 6 h of recovery. The increase indicated vigorous mitogenesis in the treated cells. On the other hand, for cells treated with 100 \( \mu \text{M} \) CdCl\(_2\) and 6 h of recovery, the percentage increased to only 21.1% accompanied by the appearance of some apoptotic cells. The apoptotic cells became the major component as the cells treated with 100 \( \mu \text{M} \) CdCl\(_2\) were allowed to recover for 12 h. Meanwhile, the fraction arrested in G2/M phase decreased to only 2.3%. The genomic DNAs derived from cells with or without CdCl\(_2\) treatment were further examined on a 1% agarose gel as shown in Fig. 9B. It was clear that no additional DNA fragmentation occurred in cells treated with 60 \( \mu \text{M} \) CdCl\(_2\) and 6 h of recovery as compared with the control, but severe DNA fragmentation was observed in cells treated with 100 \( \mu \text{M} \) CdCl\(_2\). It was also evident that as the post-treatment time extended, more smaller fragments could be detected. The results were in agreement with what was derived with the PI assay, i.e., low and high concentration of cadmium chloride applied in the system indeed induces different response in the treated cells. We have proved that 60 and 100 \( \mu \text{M} \) CdCl\(_2\) individually cause the mitogenic and apoptotic effect, respectively.

**DISCUSSION**

We have demonstrated the differential activation of ERK1/2 and p38MAPK upon CdCl\(_2\) treatment in 9L RBT cells. Both of these two signal cascades lead to the phosphorylation and activation of HSF1, which in turn confers HSP70 induction upon treatment with CdCl\(_2\). Under low cadmium concentration (60 \( \mu \text{M} \)), ERK1/2 were activated and then HSF1 was phosphorylated. On the other hand, p38MAPK was activated and respon-
sible for phosphorylation of HSF1 in cells treated with a high concentration (100 μM) of cadmium. ERK1/2 and p38 MAPK are subgroups of the MAPK family that play key roles in transducing extracellular signals to the nucleus (25, 38). In general, ERK- and p38 MAPK-mediated signaling represent independent pathways, with distinct upstream activators and downstream targets. The ERK1/2 pathway consists of a protein kinase cascade linking growth and differentiation signals with transcription in the nucleus. Growth factor receptors and tyrosine kinases activate Ras, which in turn activates c-Raf, MAPK-ERK kinase 1/2, and thus ERK1/2. Activated ERK1/2 then translocates to the nucleus and activates transcription by phosphorylation of transcription factors such as Elk1 and Stat (14, 39). On the other hand, p38 MAPK has been shown to be activated by MAPK kinases 3 and 6 (40, 41). Subsequently, activated p38 MAPK phosphorylates and activates MAPKAPK-2 (20) and ATF-2 (21, 42). We have demonstrated that ERK1/2 are activated in cells treated with 60 μM CdCl2, while p38 MAPK is activated in cells treated with 100 μM CdCl2. Moreover, both ERK1/2 and p38 MAPK are found to be phosphorylated by 80 μM CdCl2, indicating that the kinase cascades can be simultaneously or separately activated in cadmium-treated cells. However, the SAPK signaling pathway appeared to be uninvolved in the cadmium-activated HSF1, since the processes could be completely abolished by the protein kinase inhibitors PD98059 and SB203580. Since the distinct ERK and p38 MAPK signaling pathways principally represent mitogenic response and stress response, respectively, it is conceivable that there are concentration-dependent responses to cadmium with different biological consequences. Our experimental data proved that activation of ERK1/2 in cells upon treatment with a low concentration of cadmium causes a mitogenic response representing an adaptation route for a future down-regulation stress response elicited by cadmium. By contrast, activation of p38 MAPK upon treatment with a high concentration of cadmium is associated with induction of an apoptotic response (43, 44). However, whether p38 MAPK and ERK1/2 are directly involved in the apoptotic and mitogenic processes still remains to be investigated.

As mentioned previously, it has been shown that HSF1 is a suitable in vitro substrate for proline-directed kinases including ERK1/2 (11, 12). However, two-dimensional PAGE analysis showed that the pattern of phosphorylation in vitro varies from that of in vivo with only one phosphopeptide in common (11). Moreover, phosphorylation of HSF1 by ERK1/2 is likely to result in a suppression of the transactivation activity (11, 13, 14). In vivo studies, both of the ERK and p38 MAPK signaling pathways have been linked to the regulation of HSF1. For instance, it has been shown that overexpression of c-Raf and thus up-regulation of the Raf/MAPK-ERK kinase/ERK pathway would lead to enhanced phosphorylation of HSF1 (10, 45) and that p38 MAPK is activated in heat-shocked and osmotically stressed cells in which heat shock genes are induced (18, 46). In the present studies, the respective activations of ERK1/2 and p38 MAPK by low and high concentrations of cadmium are strictly coupled to the inducible phosphorylation of HSF1, as revealed by exploiting PD98059 and SB203580 (Fig. 8). Our findings indicate that activation of ERK1/2 or p38 MAPK could be directly linked to phosphorylation and activation of HSF1, suggesting that HSF1 can serve as a downstream effector for both ERK1/2 and p38 MAPK signaling pathways.

Our electrophoretic mobility shift assay and 32P-labeling experiments revealed that cadmium treatment induces HSE binding activity and phosphorylation of HSF1. However, the constitutive binding of HSF1 to HSE could be abolished only by staurosporine and not by SB203580, and it is also shown that neither SB203580 nor PD98059 could affect the constitutively phosphorylated form of HSF1. It appeared that the constitutive and inducible phosphorylation forms of HSF1 were regulated by multiple mechanisms and that distinct kinases were involved in different stages. We have demonstrated the involvement of ERK1/2 and p38 MAPK pathways in the inducible phosphorylation and thus transcription activity of HSF1; however, the candidates responsible for constitutive phosphorylation of HSF1 and the detailed regulatory mechanism for HSF1 phosphorylation need to be elucidated.

In conclusion, we have demonstrated that the p38 MAPK and ERK1/2 signaling pathways are differentially activated by cadmium at different concentrations and that the activation of these MAPKs will converge to the phosphorylation and activation of HSF1, which in turn transactivates the heat-shock genes. However, the upstream activation mechanisms still await further elucidation.

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Differential Activation of p38MAPK and ERK1/2 by Cadmium

Y.-K. (1998) J. Cell. Biochem. 71, 21–35
34. Weizsaecker, M., Deen, D. F., Rosenblum, M. L., Hoshino, T., Gutin, P. H., and Baker, M. (1991) J. Neuro. 224, 183–192
35. Laemmli, U. K. (1970) Nature 227, 680–685
36. Hannighausen, L., and Lubon, H. (1987) Methods Enzymol. 152, 721–735
37. Mestril, R., Chi, S. H., Sayen, M. R., and Dillmann, W. H. (1994) Biochem. J. 298, 561–569
38. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14846
39. Mestril, R., Chi, S. H., Sayen, M. R., and Dillmann, W. H. (1994) Biochem. J. 298, 561–569
40. Derijard, B., Raingeaud, J., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Science 267, 682–685
41. Han, J., Lee, J.-D., Jiang, Y., Li, Z., Feng, L., and Ulevitch, R. J. (1996) J. Biol. Chem. 271, 2886–2891
42. Chen, K.-D., Chen, L.-Y., Huang, H.-L., Liu, C.-H., Chang, Y.-N., Chang, M. D.-T., and Lai, Y.-K. (1998) J. Biol. Chem. 273, 749–755
43. Kummer, J. L., Rao, P. K., and Heidenreich, K. A. (1997) J. Biol. Chem. 272, 20490–20494
44. Frasch, S. C., Nick, J. A., Fadok, V. A., Bratton, D. L., Worthen, G. S., and Heuson, P. M. (1998) J. Biol. Chem. 273, 8389–8397
45. Chu, B., Sonein, F., Price, B. D., Stevenson, M. A., and Calderwood, S. K. (1990) J. Biol. Chem. 275, 30847–30857
46. Sheikh-Hamad, D., Di Mari, J., Suki, W. N., Safirstein, R., Watts, B. A., III, and Rouse, D. (1998) J. Biol. Chem. 273, 1832–1837