Involvement of p38 Mitogen-activated Protein Kinase Signaling Pathway in the Rapid Induction of the 78-kDa Glucose-regulated Protein in 9L Rat Brain Tumor Cells*

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We have previously shown that treatment with okadaic acid (OA) followed by heat shock (HS) (termed OA–HS treatment) leads to rapid transactivation of the 78-kDa glucose-regulated protein gene (grp78) in 9L rat brain tumor cells. A cAMP-responsive element-like (CRE-like, TGACG{TGA}) promoter sequence and a protein kinase A signaling pathway are involved in this induction, and activation of both CRE binding protein (CREB) and activating transcription factor-2 (ATF-2) is required in the above process. Herein, we report that transactivation of grp78, as well as phosphorylation/activation of ATF-2, can be completely annihilated by transactivation of protein kinase A signaling pathway are involved in this induction, and activation of both CRE binding protein (CREB) and activating transcription factor-2 (ATF-2) is required in the above process. Herein, we report that transactivation of grp78, as well as phosphorylation/activation of ATF-2, can be completely annihilated by OA–HS treatment. The involvement of p38MAPK by OA–HS is also substantiated by its own phosphorylation as well as the phosphorylation and activation of MAPK activating protein kinase-2 in cells subjected to this treatment. The involvement of p38MAPK in the activation of ATF-2, which leads to the transactivation of rat grp78, is confirmed by electrophoretic mobility shift assay using a probe containing the CRE-like sequence as well as by transient transfection assays with a plasmid containing a 710-base pair stretch of the grp78 promoter. Together with our previous studies, these results led us to conclude that phosphorylation/activation of CREB upon OA–HS treatment is mediated by cAMP-dependent protein kinase, whereas that of ATF-2 is mediated by p38MAPK. The transcription factors may bind to each other to form heterodimers that in turn transactivate grp78 by binding to the CRE-like element. This suggests that distinct signaling pathways converge on CREB-ATF-2, where each subunit is individually activated by a specific class of protein kinases. This may allow modulation of grp78 transactivation by diverse external stimuli.

The 78-kDa glucose-regulated protein (GRP78) is a calcium-binding molecular chaperone expressed in the endoplasmic reticulum of eucaryotic cells (1, 2). GRP78 is constitutively expressed, and its expression is enhanced up to 20-fold in cells under a variety of stressful conditions that deplete glucose or intracisternal calcium or otherwise disrupt glycoprotein trafficking (3–8). The above changes lead ultimately to accumulation of underprocessed or misfolded proteins in the endoplasmic reticulum and result in the induction of GRP78 (9). This protein is also induced by ethanol via a mechanistic pathway different from that of the “classical inducers” (10). By contrast, GRP78 is usually not induced by heat shock (HS) alone (11, 12).

The mammalian grp78 promoter, lacking any heat shock element, is regulated by a complex interplay of several cis-elements and protein factors binding to these sites (6, 11, 13–17). It has been shown that the highly conserved 36-base pair region within the mammalian grp78 promoter consists of CCAAT-like sequences flanked by GC-rich motifs that are important for basal and enhanced expression of this gene. Using this region as a probe for electrophoretic mobility shift assay (EMSA), at least two specific protein-DNA complexes are detected in the nuclear extracts of HeLa cells (14, 16). Moreover, transactivation of grp78 by several chemical stressors is found to correlate with changes in the activities of protein kinases and phosphatases (18–20). It should also be noted that the induction of grp78 expression by the classical inducers is slow and requires several hours of sustained treatment with lag phases of 3 h to more than 24 h.

We have demonstrated that okadaic acid (OA), a potent inhibitor of protein phosphatases 1 and 2A, enhances the expression of GRP78 (8) as well as potentiates the expression of GRP78 in heat-shocked 9L rat brain tumor (RBT) cells (12). When followed by heat shock at 45 °C for 15 min, GRP78 is induced within 60 min treatment with a low dose (200 nM) of OA compared with prolonged treatment of several hours with classical inducers (12). To understand the signal transduction mechanism involved in the rapid induction of GRP78, we determined the functionality of the cis-acting CRE-like element of rat grp78 promoter in cells treated sequentially with OA and heat shock (OA → HS, treatment of 200 nM OA followed by heat shock at 45 °C for 15 min). We found that OA → HS-induced GRP78 expression is regulated by binding of protein factors in nuclear extract to the CRE-like element. The transcription factors that interact with the grp78 CRE-like element have been identified as activating factor-2-cAMP responsive element binding (AF2-CREB) proteins (21–23). The present article clearly shows that OA–HS treatment is required for the induction of this gene.

The abbreviations used are: GRP78, 78-kDa glucose-regulated protein; OA, okadaic acid; HS, heat shock; HSP, heat shock protein; RBT cells, rat brain tumor cells; CRE, cyclic-AMP responsive element; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; ATF-2, activating factor-2; CREB, CRE binding protein; PKA, protein kinase A or cyclic AMP-dependent protein kinase; p38MAPK, p38 mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase-2; IL, interleukin; ECLI, enhanced chemiluminescence; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid.

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Western blotting detection kit were from Amersham Corp. (Buckinghamshire, UK). Polyclonal antibody against phosphorylated CREB (specific for Ser133-CREB) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Antibodies for GRP78, p38MAPK, phosphorylated p38MAPK, CREB, and ATF-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Chemicon. Chemotherapy were from Bio-Rad. Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma. SB203580 was a kind gift from Smith-Kline Beecham Pharmaceuticals (King of Prussia, PA).

Cell Culture—9L RBT cells, derived from rat gliosarcoma (48), were a gift of Dr. M. L. Rosenbaum (University of California at San Francisco) and were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Prior to each experiment, stock cells were plated in 25-cm² flasks or 6-well plates at a density of 4–6 × 10⁶ cells per cm². Exponentially growing cells at 80–90% confluency were used.

Drug and Heat Treatment—For drug treatments, OA and SB203580 stock solutions were diluted with culture medium to the desired concentration before adding to the cells, and the treatment was performed at 37 °C. For heat shock, cells in flasks or plates were sealed with Parafilm and submerged in a water bath pre-set at 45 ± 0.1 °C for 15 min. To study the combined effects of OA and heat shock (OA → HS treatment), cells were preincubated with 200 μM OA for 1 h and then heated at 45 °C for 15 min in the presence of the drug. Alternatively, the cells were treated according to various protocols as described below and in the figure legends.

Metabolic Labeling and SDS-PAGE—Alterations of protein synthesis and phosphorylation were revealed by [³²P]methionine and [³⁵S]orthophosphate labeling, respectively. In vivo ³²P labeling was performed with 1 mCi of [³²P]orthophosphate in 1 ml of labeling medium (phosphate-free DMEM containing 10% FCS) for 1 h prior to various treatments in the presence of the isotopic. Synthesis of GRP78 protein in the treated cells was revealed by [³²P]methionine labeling. After treatments, cells were washed twice and incubated with fresh medium at 37 °C for 8 h before labeling with 20 μCi of [³²P]methionine in 1 ml of medium for 1 h. After labeling, cells were washed with PBS, lysed in sample buffer (49), and subjected to electrophoresis as described previously (8, 50). The gels were fixed, dried, and processed for autoradiography.

Immunoblot Analysis—For immunoblot analysis, cell lysates were resolved using a mini-gel apparatus (Hoeffer, San Francisco, CA). After electrophoresis, the proteins were electro-transferred onto a nitrocellulose membrane (Hybond-C Super, Amersham Corp.) and probed with antibodies against p38MAPK, phosphorylated p38 MAPK, GRP78, CREB, phosphorylated CREB, and ATF-2, separately. The immune complexes were visualized using enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham Corp.).

RNA Isolation and Northern Blotting—Total RNA was isolated from 9L cells according to Chomczynski and Sacchi (51). After washing with PBS, cells (5 × 10⁶) were trypsinized, collected by centrifugation, and lysed in 1.5 ml of RNA extraction buffer (0.5% sarcosyl, 4 μg/ml dimethylthiourea, 0.1 μM β-mercaptoethanol, and 25 mM sodium citrate). Total RNA was extracted by sequential addition of one-tenth volume of 2 μM sodium acetate, pH 4.0, an equal volume of water-saturated phenol, and two-tenths volume of chloroform/isooamyl alcohol (24:1) followed by precipitation of the aqueous phase with an equal volume of isopropl alcohol. The RNA pellets were then re-dissolved in 0.3 ml of extraction buffer (without β-mercaptoethanol) and precipitated again with isopropl alcohol. The pellets were washed with 70% ethanol and dissolved in diethyl pyrocarbonate-treated water. RNA concentrations were determined spectrophotometrically at 260 nm. An equal amount of total RNA isolated from different treatment conditions was blotted onto the nylon membrane and fixed by using an ultraviolet (UV) cross-linker (Stratagen). Template for the GRP78 hybridization probe was a polymerase chain reaction product from isolated 9L genomic DNA. Polymerase chain reaction primers for producing the GRP78 probe were 5’-TCG-GCCCTCTCGT-3’ (forward) and 5’-CAACGACATCTCCA-3’ according to the exon 1 of rat grp78 gene. Rat glyceraldehyde-3-phosphate dehydrogenase oligonucleotide probe was purchased from CLONTECH (Palo Alto, CA). GRP78 and glyceraldehyde-3-phosphate dehydrogenase oligonucleotide probes were labeled with [³²P]dCTP by Rediprime DNA labeling system (Amersham Corp.). Following prehybridization, hybridization, and autoradiography, membranes were stripped off probes by boiling in 1.0 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) containing 0.01% SDS for 20 min and then rehybridized with other probes. Autoradiograms were quantified by densitometric scanning in two-dimensional mode (Molecular Dynamics).

Construction of Plasmids and Transient Transfection Assays—The
construction of pGRP78-BGL plasmid was derived from ligation of a rat grp78 promoter fragment with the pCAG-MAPK reporter vector (CLONTECH). The pCAG-MAPK Promoter vector contains the SV40 early promoter inserted upstream of the lacZ gene. A 0.7-kilobase pair Blul/HpaI fragment derived from rat grp78 promoter containing the CRE-like by LipoTAXI™ maximal transfection kit (Stratagene, Jolla, CA). Briefly, cells were seeded at a density of 1.2-3.0 × 10⁶ cells per 100-mm dish 16 h before transfection and were incubated in 3 ml of serum-free DMEM containing LipoTAXI transfection reagent for 4 h under standard growth conditions (5% CO₂ in humidified incubator at 37 °C). After transfection, DMEM containing 20% serum was added to the tissue culture dish and further incubated for 24 h. The cells were then subjected to OA → HS treatment with or without preincubation with SB203580. Subsequently, the cells were solubilized in extraction buffer (100 mM NaH₂PO₄, pH 7.5, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol), and the cell lysates were assayed for β-galactosidase with o-nitrophenyl β-D-galactopyranoside as substrate (Sigma, 4 mg/ml in 100 mM NaH₂PO₄, pH 7.5). The reaction was terminated by the addition of 1 N Na₂CO₃, and the enzyme activity was determined spectrophotometrically at 420 nm. β-Galactosidase activity is expressed as units (nmol of o-nitrophenol formed per min) per mg of cell lysate. The averaged value obtained from three negative controls was subtracted from each experimental result before presentation.

Nuclear Extract Preparation—Nuclear extracts were prepared as described by Roy et al. (52) with some modifications (17). In brief, approximately 5 × 10⁶ cells were trypsinized, collected by centrifugation at 1,000 × g for 8 min at 4 °C, washed with PBS, and centrifuged. Cells were suspended in 4 ml of nuclear extraction buffer I (250 mM sucrose, 15 mM Tris-HCl, pH 7.9, 140 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 1 mM dithiorthiol, 0.4 mM phenylmethylsulfonyl fluoride, 25 mM KCl, and 2 mM MgCl₂) and homogenized by a Dounce grinder with 3 strokes. Nonidet P-40 was then added to a final concentration of 1% to stabilize the nuclei, and the mixture was incubated on ice for about 5 min. Following another round of homogenization (6 more strokes), nuclei and cell debris were collected by centrifugation at 1,000 × g for 8 min as described previously. Nuclei were washed with 5 ml of buffer I and centrifuged as above. The nuclei were then lysed by incubating the sample one on ice for 5 min in 1 packed cell volume of nuclear extraction buffer II (150 mM NaCl, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 20% glycerol) for 90 min at 4 °C and stored frozen at −70 °C until use.

Electrophoretic Mobility Shift Assays (EMSA)—CRE-like element binding activities of the nuclear extracts were determined by EMSA using double-stranded oligonucleotide as probes. The CRE-like oligonucleotide probe was prepared by annealing 5'-GCGTACGAGTACGAGT- GAGTTCCGGAGG-3' with its complementary strand, followed by end labeling with T4 polynucleotide kinase. Each gel shift reaction was carried out in a 20-μl volume in the presence of binding buffer (15 mM Hepes, pH 7.9, 100 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 2 μg of poly(dI-dC)). Nuclear extract was added to the binding buffer, and the samples were incubated on ice for 15 min. After incubation, the DNA probe (2 × 10⁶ cpm for each reaction) was added and incubated at room temperature for 20 min. The reaction mixtures were loaded onto a 5% (40:1) polyacrylamide gel which had been pre-run for 30 min in 0.5 × TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 7.0) at 150 V. 4% nondenaturing PAGE was run at 150 V for 1.5 h at 4 °C. After electrophoresis, the gels were dried and exposed to x-ray film.

In Vivo Labeling and Immunoprecipitation—For in vivo metabolic labeling, 9L cells were incubated with 1 μCi of [³²P]orthophosphate in 1 ml of phosphate-free DMEM, 10% fetal bovine serum for 1 h. After treatment, cells were rapidly chilled on ice, washed twice with ice-cold PBS, and lysed in lysis buffer (20 mM Hepes, pH 7.9, 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg/ml microcystin-LR, 10% glycerol, 1 mM dithiothreitol, 0.4 mM KCl, 0.4% Nonidet-P-40 and protease inhibitors as follows: 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM benzamidine, 50 μg/ml phenylmethylsulfonyl fluoride) for 10 min on ice. Insoluble material was removed by centrifugation (10,000 × g, 20 min, 4 °C). The protein concentration of the cell lysate was determined by the Bradford assay (Pierce). An equal amount of cell lysate was incubated with 6 μg of anti-MAPKAPK-2 or anti-ATF-2 antibody for 2 h at 4 °C. Immune complexes were precipitated with protein G-Sepharose (Pharmacia Biotech Inc.) washed twice with lysis buffer and then mixed with equal volume of 2 × sample buffer (49). The phosphorylation of the MAPKAPK-2 or ATF-2 was examined after 10% SDS-PAGE followed by autoradiography and densitometric analysis (Molecular Dynamics).

Assays for MAPKAPK-2 Activity—The MAPKAPK-2 activity in cell-free lysate prepared from 9L cells was assayed. After OA → HS treatment and recovery, cell lysate was prepared by sonication in lysis buffer and then clarified by centrifugation. Protein concentration of the cell lysate was determined by Bradford assay (Pierce). An equal amount of cellular proteins was incubated with 6 μg of anti-MAPKAPK-2 or anti-CREB antibody for 2 h at 4 °C. Immunocomplexes were precipitated with protein G-Sepharose and washed twice with lysis buffer and once with assay buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na₃VO₃, 1 mM dithiothreitol). For the kinase assay, 5 μg of substrate peptide (KKNRNR7SV) or 1 μg of immunoprecipitated CREB, 0.1 mM ATP, 15 mM MgCl₂, and 10 μCi/ml [γ-³²P]ATP were incubated in a total volume of 100 μl, and the reaction was allowed to proceed at 30 °C for 10 min. The labeled proteins were analyzed by autoradiography and densitometric analysis.

RESULTS

SB203580 Abolishes OA → HS-induced GRP78 Expression and grp78 Promoter Activity—To evaluate the possible role of p38MAPK in the rapid induction of GRP78 by OA → HS, we tested the effect of SB203580, a highly specific inhibitor of p38MAPK, on OA → HS-induced GRP78 production and grp78 gene expression in 9L cells by metabolic labeling with [³⁵S]methionine and Western blotting. We found that, in the presence of SB203580, GRP78 synthesis and its accumulation were almost completely diminished in OA → HS-treated cells (Fig. 1, A and B). The cell viability was unaffected by the dose of SB203580 used (data not shown), but protein translation was significantly inhibited at higher concentrations (up to 50 μM) (Fig. 1A). We have previously demonstrated that OA → HS augments GRP78 production primarily by an increase in the transcription rate. To investigate the level at which SB203580 affects production of GRP78 in OA → HS-treated 9L cells, we performed a Northern blot analysis of total cytoplasmic RNA using a [³²P]-labeled rat grp78 probe. Cells were pretreated for 1 h with an increasing concentration of SB203580 (up to 100 μM), followed by OA → HS treatment. The induction of grp78 mRNA increased markedly at 1 h recovery after OA → HS treatment and could be completely prevented by SB203580 (Fig. 2, A and B). A 50% inhibition was observed at about 5 μM concentration of the drug and almost complete inhibition at 20 μM (Fig. 2B). We also determined whether the transcriptional activity of rat grp78 promoter was activated by OA → HS treatment and whether the activation was affected by prior treatment of SB203580. The T10-base pair grp78 promoter-β-galactosidase reporter construct (designated as pGRP78-BGL) was, therefore, tested in the transient transfection assay. pGRP78-BGL showed much higher activity in cells after 1 h recovery of OA → HS stimulation (69.19 ± 7.46 units/mg protein) compared with either OA or heat shock alone (8.36 ± 2.17 and 23.47 ± 3.27 units/mg protein, respectively). The degree of promoter activation induced by OA → HS was markedly reduced to that of heat shock stimulation in cells subjected to prior treatment with SB203580 (27.63 ± 6.51 units/mg protein) (Fig. 3). However, we normally did not observe enhanced expression of grp78 mRNA in heat-shocked cells, and the increase of β-galactosidase activity upon heat shock may be due to the temperature effect on β-galactosidase. These data clearly demonstrate that the induction of GRP78 by OA → HS treatment can be completely prevented by SB203580, indicating the involvement of p38MAPK in this process.
OA3HS Activates the p38MAPK Signal Transduction Pathway in 9L RBT Cells—To demonstrate that OA3HS activates the p38MAPK pathway in 9L rat brain tumor cells, we studied the effect of OA3HS on phosphorylation of p38 MAPK in cells recovering from the treatment. The tyrosine phosphorylation of p38MAPK was determined by double immunoblotting using a phospho-p38MAPK antibody and anti-p38 MAPK antibody to assess the changes in phosphorylation and total expression of p38MAPK. The OA3HS treatment resulted in a 7.5-fold increase in p38 MAPK phosphorylation on Tyr-182 and was reached maximum at 30 min of recovery (Fig. 4, A and B), whereas the amount of p38MAPK remained constant during the treatment (Fig. 4A). Furthermore, preincubation of the cells with 20 μM SB203580 had negligible effect on the phosphorylation of p38 MAPK, demonstrating that the inhibitor does not interfere with the upstream activators of p38MAPK (Fig. 4). This experiment provides unique evidence that in 9L cells, OA3HS treatment results in Tyr-182 phosphorylation of p38MAPK.

To determine whether OA → HS activates the p38MAPK pathway, we examined the phosphorylation of MAPKAPK-2, a downstream effector of p38MAPK, in [32P]orthophosphate-labeled OA → HS-treated cells. MAPKAPK-2 was immunoprecipitated from 9L cell lysates and subjected to SDS-PAGE. As shown in Fig. 5, treatment with OA → HS caused a 5-fold increase in MAPKAPK-2 phosphorylation, which was completely abolished by preincubation of the cells with SB203580 (Fig. 5A). To confirm

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**Fig. 1.** Inhibitory effect of SB203580 on GRP78 expression in OA → HS-treated 9L RBT cells. 9L cells were incubated for 1 h in the absence or presence of SB203580, followed by OA → HS treatment. The cells were then allowed to recover for 8 h in the presence of SB203580 and labeled with [35S]methionine for 1 h before lysing. Equal amounts of cell lysates were electrophoresed on SDS-PAGE followed by autoradiography (A). As additional controls, cells were subjected to heat shock at 45 °C for 15 min and then allowed to recover for 8 h (lanes 5). Time-dependent accumulation of GRP78 protein was determined by Western blotting, using anti-GRP78 antibody (B). Similar results are observed in three independent experiments.

OA → HS Activates the p38MAPK Signal Transduction Pathway in 9L RBT Cells—To demonstrate that OA → HS activates the p38MAPK pathway in 9L rat brain tumor cells, we studied the effect of OA → HS on phosphorylation of p38MAPK in cells recovering from the treatment. The tyrosine phosphorylation of p38MAPK was determined by double immunoblotting using a phospho-p38MAPK antibody and anti-p38MAPK antibody to assess the changes in phosphorylation and total expression of p38MAPK. The OA → HS treatment resulted in a 7.5-fold increase in p38MAPK phosphorylation on Tyr-182 and was reached maximum at 30 min of recovery (Fig. 4, A and B), whereas the amount of p38MAPK remained constant during the treatment (Fig. 4A). Furthermore, preincubation of the cells with 20 μM SB203580 had negligible effect on the phosphorylation of p38MAPK, demonstrating that the inhibitor does not interfere with the upstream activators of p38MAPK (Fig. 4). This experiment provides unique evidence that in 9L cells, OA → HS treatment results in Tyr-182 phosphorylation of p38MAPK.

To determine whether OA → HS activates the p38MAPK pathway, we examined the phosphorylation of MAPKAPK-2, a downstream effector of p38MAPK in [32P]orthophosphate-labeled OA → HS-treated cells with or without pretreatment with SB203580. MAPKAPK-2 was immunoprecipitated from 9L cell lysates and subjected to SDS-PAGE. As shown in Fig. 5, treatment with OA → HS caused a 5-fold increase in MAPKAPK-2 phosphorylation, which was completely abolished by preincubation of the cells with SB203580 (Fig. 5A). To confirm further that SB203580 specifically blocks OA → HS-induced activation of p38MAPK, we measured the phosphorylation of MAPKAPK-2 peptide substrate by incubating with lysates of cells recovering from OA → HS treatment (Fig. 5B). Prior incubation of 9L cells with 20 μM SB203580 completely suppressed the phosphorylation of peptide substrate caused by OA → HS treatment. The effect of SB203580 on the phosphorylation of MAPKAPK-2 peptide substrate was highly specific because this drug had little effect on the phosphorylation state of any of the other major [32P]-labeled proteins in 9L cells that were resolved by one-dimensional SDS-PAGE (Fig. 5B).

**Fig. 2.** Inhibitory effect of SB203580 on the induction of grp78 mRNA in OA → HS-treated 9L RBT cells. 9L cells were preincubated in the presence of 1–100 μM SB203580 for 1 h and then treated with OA → HS in the presence of SB203580. Total cytoplasmic RNA was extracted and analyzed for the expression of grp78 mRNA by Northern blotting with a [32P]-labeled grp78 probe. Repeated hybridization of the filters with a glyceraldehyde-3-phosphate dehydrogenase (G3PDH)-specific probe confirmed a similar amount of RNA loading in all lanes (A). Concentration dependence of inhibitory effect of SB203580 on grp78 mRNA accumulation was obtained by densitometric scanning of the Northern blots (B).

**Fig. 3.** SB203580 prevents the up-regulation of the promoter activity of rat grp78 gene activated by OA → HS. 9L cells transfected with a pβgal-Basic reporter vector (C, BGL) or a recombinant plasmid containing a grp78 promoter fragment (pGRP78-BGL) were subjected to 200 nM OA, HS (45 °C for 15 min), or OA → HS treatment with or without 1 h preincubation with SB203580. β-Galactosidase activity in cell lysates was quantified by spectrophotometry at 420 nm. Data are the means ± S.D. of triplicate experiments.

**Fig. 4.** Dependence of OA → HS-induced ATF-2 Phospho-
rylation—We have previously found that, in OA3 HS-treated cells, ATF-2 protein is markedly increased, and it binds to the CRE-like element of rat grp78 promoter. To test whether ATF-2 is phosphorylated by p38MAPK in vivo, we examined the effect of OA3 HS in ATF-2 phosphorylation. We measured the phosphorylation level of ATF-2 in [32P]orthophosphate-labeled 9L cells with or without pretreatment of 20 μM SB203580. ATF-2 was immunoprecipitated from 9L cell lysates and subjected to SDS-PAGE. We found that treatment with OA3 HS caused a significant increase in ATF-2 phosphorylation (Fig. 6), which was completely abolished by preincubation of the cells with SB203580. However, prior treatment of SB203580 did not affect the increase in ATF-2 induced by OA → HS (Fig. 6, lane 3).

SB203580 Prevents the Factor Occupancy of the CRE-like Element in grp78 Promoter Induced by OA3 HS Treatment—We further analyzed whether the factor occupancy of CRE-like element induced by OA → HS caused a significant increase in ATF-2 phosphorylation (Fig. 6), which was completely abolished by the treatment with SB203580. We found that treatment with OA → HS caused a significant increase in the phosphorylation level of ATF-2 in [32P]orthophosphate-labeled 9L cells with or without pretreatment of 20 μM SB203580. ATF-2 was immunoprecipitated from 9L cell lysates and subjected to SDS-PAGE. We found that treatment with OA → HS caused a significant increase in ATF-2 phosphorylation, which was completely abolished by the treatment with SB203580. However, prior treatment of SB203580 did not affect the increase in ATF-2 induced by OA → HS (Fig. 6, lane 3).

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OA → HS-induced MAPKAPK-2 Activity Has Little Effect on the Phosphorylation Level of CREB and the Kinase Does Not Phosphorylate CREB in Vitro—To assay the activation of MAPKAPK-2 in OA → HS-treated cells and for studying whether the kinase exhibited activity toward CREB, we immunoprecipitated the enzyme and CREB, the substrate, from 9L cell extracts. As earlier, OA → HS strongly activated MAPKAPK-2

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**FIG. 4.** Activation of the p38MAPK pathway by OA → HS treatment in 9L RBT cells. 9L cells were treated with OA → HS and allowed to recover in the absence or presence of SB203580 for indicated periods. The cell lysates were analyzed by immunoblotting with anti-phospho-p38MAPK (specific for Tyr-182 in p38MAPK) and anti-p38MAPK. The protein-antibody complexes were visualized by ECL (A) and the levels of phosphorylation of p38MAPK were quantified by densitometric scanning (B). Data represent the means ± S.D. of three independent experiments.

**FIG. 5.** Activation of MAPKAPK-2 by OA → HS treatment in 9L cells and its abolishment by SB203580. 9L cells were prelabeled with [32P]orthophosphate in the absence or presence of SB203580 and then subjected to OA → HS treatment. At the end of the indicated recovery period, the cells were lysed, and the cell lysates were allowed to react with anti-MAPKAPK-2 immunoprecipitated. The immunoprecipitated complexes were resolved by 10% SDS-PAGE. The phosphorylation and protein levels of MAPKAPK-2 were determined by autoradiography and Western blotting (A). Alternatively, unlabeled cells were treated as above, and the cell lysates were incubated with the MAPKAPK-2 peptide substrate (KKLNRTLSVA) in the presence of γ-[32P]ATP at 30 °C for 5 min, resolved by 10% SDS-PAGE, and autoradiographed (B). Data represent the means ± S.D. of three independent experiments.

**FIG. 6.** Phosphorylation of ATF-2 in vivo by OA → HS treatment in 9L RBT cells and its abolishment by SB203580. 9L cells were prelabeled with [32P]orthophosphate in the absence or presence of SB203580 for 1 h and then treated with HS or OA → HS. Phosphorylation of ATF-2 was detected by immunoprecipitation and autoradiography. The amount of ATF-2 was found to be increased in cells subjected to OA → HS treatment (lanes 2 and 3). Lane 4 represents cells heat-shocked at 45 °C for 15 min. Similar results were obtained in three independent experiments.
within 15 min, and the activation of the kinase was annihilated by preincubation with SB203580 (Fig. 8). Furthermore, activated MAPKAPK-2, which is strongly activated in OA→HS-treated cells, does not phosphorylate the CREB protein in vitro (Fig. 8, bottom panel).

DISCUSSION

Herein we show that the p38MAPK pathway is activated by OA→HS treatment and that this pathway is involved in the OA→HS-induced rapid transactivation of the grp78 gene and expression of GRP78 in 9L RBT cells. This has been achieved by exploiting SB203580, a highly specific inhibitor of p38MAPK (36). OA→HS increased the phosphorylation of p38MAPK which, in turn, promoted the activation (phosphorylation) of its downstream effector, MAPKAPK-2, and the transcription factor, ATF-2. Both activation of MAPKAPK-2 and phosphorylation of ATF-2 were prevented by SB203580, confirming the OA→HS-induced activation via p38MAPK signaling pathway.

Thus far three MAPK subgroups have been characterized in mammalian cells as follows: extracellular-regulated-protein kinases, stress-activated protein kinase/Jun-N-terminal protein kinases, and p38MAPKs. These kinases are at the center of three distinct but closely related phosphorylation cascades, playing critical roles in signal transductions (53, 54). p38MAPK was first identified as the signaling system in response to lipopolysaccharide in human monocytes, leading to the production of IL-1 and tumor necrosis factor-α (55, 56). Sequence comparison of p38MAPK with other MAPKs led to identification of a Thr-Gly-Tyr (TGY) dual phosphorylation motif in p38MAPK distinct form other MAPKs having Thr-Glu-Tyr (TEY) or Thr-Pro-Tyr (TPY) dual phosphorylation motifs. This points to the uniqueness of this subgroup of MAPK and their distinct activation mechanism (57–61). In the present study, we have demonstrated that p38MAPK provides a signal necessary for rapid activation of grp78 gene caused by sequential treatment of a relatively low dose of OA (200 nM for 1 h) and heat shock (45 °C for 15 min) in specific order. We also found that OA→HS treatment induces phosphorylation of p38MAPK in 9L cells. Phosphorylation of p38MAPK is detected within 1 h after treatment, and the process is blocked by pretreatment of cells with a protein tyrosine kinase inhibitor herbimycin A (data not shown). Several upstream kinases, including the dual specific kinases MAPK kinase-3 (MKK3), MKK4, and MKK6, have been implicated in the phosphorylation and activation of p38MAPK (62–64). Herein, we have not identified the upstream effector(s) of p38MAPK in the transactivation of rat grp78, and further investigation is warranted.

Activation of the p38MAPK pathway leads to the phosphorylation and activation of MAPKAPK-2 and ATF-2. Currently, three isoforms of p38MAPK have been identified. Although ATF-2 can be phosphorylated by p38MAPKα and p38MAPKβ, in vitro and in vivo experiments show a strong substrate preference for p38MAPKβ for ATF-2. It has been shown that enhancement of ATF-2-dependent gene expression by p38MAPK is approximately 20-fold greater than that of p38MAPKα, and other MAPKs tested (29). On the other hand, transcription factor ATF-2 is phosphorylated in vitro by p38MAPK on Thr-69 and Thr-71 (62, 65). Phosphorylation of ATF-2 at these sites increases the transcriptional activation potential (27, 28). In addition to forming DNA binding homodimers, ATF-2 also efficiently forms heterodimers with numerous other members of the ATF family as well as members of the Jun/Fos family (28, 66–72). Such promiscuity in dimerization makes ATF-2 an important constituent of factor complexes exhibiting subtle differences in DNA binding specificity and regulatory potential. Indeed, a number of studies have demonstrated different binding properties of ATF-2 homodimers, ATF-2/C-Jun heterodimers, and ATF-2/C/EBPα heterodimers (24–26, 72).

Previously, we have shown that both ATF-2/CREB heterodimer and CREB homodimer, referred to as the complex I and II in the gel shift assays, are involved in the transactivation of rat grp78 gene in 9L RBT cells treated by OA→HS. Activation of CREB is apparently mediated by the PKA pathway since binding activity to EMSA probe containing the CRE-like sequence is completely abolished by H-89, a specific inhibitor of PKA (17). Although it has been shown that MAPKAPK-2 phosphorylates CREB at Ser-133 in vitro by p38MAPK on Thr-69 and Thr-71 (62, 65), Phosphorylation of CREB at these sites increases the transcriptional activation potential (27, 28). A simple explanation for these observations is that HS activates CREB via a PKA signaling pathway, and OA activates ATF-2 via a p38MAPK signaling pathway. Phosphorylation of CREB by PKA and ATF-2 by p38MAPK activates the transcription factors which bind to each other to form heterodimers that in turn transactivate the grp78 by binding to the CRE-like element. Taken together, our data demonstrate that distinct signaling pathways converge on CREB-ATF-2, where each subunit is individually activated by a specific class of protein...
kinases. This may allow modulation of grp78 transactivation by a diverse external stimuli. It should be noted that there are other regulatory elements in the promoter of the grp78 gene; therefore, the functions of these elements as well as the cooperativity of the corresponding transcription factors, including the CREB-ATF-2, warrant further investigation. Our system provides a detailed analysis of the mechanism leading to the rapid transactivation of rat grp78 gene.

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