Osmotic Response Element Enhancer Activity

REGULATION THROUGH p38 KINASE AND MITOGEN-ACTIVATED EXTRACELLULAR SIGNAL-REGULATED KINASE KINASE

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Hypertonicity induces a group of genes that are responsible for the intracellular accumulation of protective organic osmolytes such as sorbitol and betaine. Two representative genes are the aldose reductase enzyme (AR, EC 1.1.1.21), which is responsible for the conversion of glucose to sorbitol, and the betaine transporter (BGT1), which mediates Na⁺-coupled betaine uptake in response to osmotic stress. We recently reported that the induction of BGT1 mRNA in the renal epithelial Madin-Darby canine kidney cell line is inhibited by SB203580, a specific p38 kinase inhibitor. In these studies we report that the hypertonic induction of aldose reductase mRNA in HepG2 cells as well as the osmotic response element (ORE)-driven reporter gene expression in transfected HepG2 cells are both inhibited by SB203580, suggesting that p38 kinase mediates the activation and/or binding of the transcription factor(s) to the ORE. Electrophoretic gel mobility shift assays with cell extracts prepared from SB203580-treated, hypertonic stress HepG2 cells further show that the binding of trans-acting factors to the ORE is prevented and is thus also dependent on the activity of p38 kinase. Similarly, treatment of hypertonicity stressed cells with PD098059, a mitogen-activated extracellular regulated kinase kinase (MEK1) inhibitor, results in inhibition of the hypertonic induction of aldose reductase mRNA, ORE-driven reporter gene expression, and the binding of trans-acting factors to the ORE. ORE-driven reporter gene expression was not affected by p38 kinase inhibition or MEK1 inhibition in cells incubated in iso-osmotic media. These data indicate that p38 kinase and MEK1 are involved in the regulation of the hyperosmotic stress response.

Many organisms, including bacteria, yeast, plants, and animals, adapt to sustained hyperosmotic stress by the preferential accumulation of compatible organic osmolytes (1). The induction of osmoprotective genes has been shown to be part of the protective response of the kidney medulla tubular cells to exposure to hypertonicity during urinary concentration (2). The accumulation of these osmolytes is facilitated by the induction of specific proteins as follows: betaine/γ-amino-n-butyric acid transporter (BGT1) for betaine (3); Na⁺-dependent myo-inositol transporter (SMIT) for inositol (4); taurine transporter for the amino acid taurine (5), as well as the aldose reductase enzyme (AR) that catalyzes the NADPH-mediated reduction of aldehydes such as d-glucose to sorbitol (6, 7).

AR is a high Km (30–80 mM) enzyme for glucose and other sugars (6), and it was shown to play a major role in the pathogenesis of the hypertonicity response to osmotic stress (10–12). Studies in the MDCK renal epithelial cell line showed that a specific p38 kinase inhibitor, SB203580, blocked the induction of BGT1 mRNA in response to hypertonic stress (13) suggesting that the p38 kinase pathway is essential for the hypertonicity response. Additional studies in monocytes showed that SB203580 also blocked the hypertonic induction of mRNA of both SMIT and BGT1 (14). However, the role of p38 kinase in the hypertonic induction of these genes remains unknown.

The current studies were undertaken to define the role of p38 kinase and other signal cascades in the possible activation of transcription factors binding to the ORE, by transfection of ORE plasmid constructs containing reporter genes into HepG2 cells. Gel mobility shift studies were also performed to deter-

1 The abbreviations used are: BGT1, betaine/γ-amino-n-butyric acid transporter 1; SB203580, 4-(4-hydrophosphyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole; PD098059 [2-(2-amino-4'-methoxyphenyl)-ox-anapththien-4-one] is a selective inhibitor of MEK1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; p38 kinase, a HOG1 homologue; MEK1, mitogen-activated extracellular regulated kinase kinase; MDCK, Madin-Darby canine kidney; ORE, osmotic response element; TonE, toxicity enhancer; AR, aldose reductase; Me2SO, dimethyl sulfoxide; SMIT, sodium-dependent myo-inositol transporter; bp, base pair; CAT, chloramphenicol acetyltransferase; kb, kilobase pair; NFAT, nuclear factor of activated T lymphocytes; DMEM, Dulbecco’s modified Eagle’s medium; JNK, c-Jun NH₂-terminal kinase.
mine the specificity of responses. Hypertonic induction of AR mRNA in HepG2 cells, the expression of ORE-driven reporter gene products, as well as the binding of trans-acting factors to the ORE are p38 kinase- and MEK1-dependent, yet ERK (extracellular signal-regulated kinase)-independent. These findings indicate that the hypertonicity-induced activation and binding of transcription factors to the ORE are regulated by the p38 kinase- and MEK1-signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Preparation of Constructs**—A 132-bp fragment (GenBank™ accession number L14440, nucleotides 2032–2163) (10) containing the osmotic response element (ORE) and its surrounding sequence was excised from the AR promoter construct pARPs.4.2 and inserted into the pCAT SV40 promoter vector (Promega) using BamHI/PstI cloning sites as described previously (11). Introduction of SacI and NheI sites by polymerase chain reaction using primers 5'-TTTAGGCTCGAGTTCAAATTCTATTACTTGG and 5'-GCTTCGCTCCCCAGACCCC. The resulting fragment was inserted into the pGL3 basic vector using XhoI and HindIII restriction sites. All constructs were sequenced to verify the sequence and orientation (sequencing kit from U. S. Biochemical Corp.).

**Tissue Culture and Transfections**—HepG2 cells were grown at 37 °C and 5% CO₂ in DMEM low glucose medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 2 mM glutamine (Life Technologies, Inc.). Cells (0.25 × 10⁶ per dish) were plated on 10-cm dishes and grown in regular DMEM until they reached 90% confluency. The cells were transfected with 2 µg of DNA per well of pCAT (chloramphenicol acetyltransferase) or pGL3 (luciferase) vectors, driven by either the 12- or 132-bp ORE constructs. The 1.5-kb promoter-driven pGL3 basic vector construct was transfected at 4 µg of DNA/well. A control firefly luciferase vector pRLSVL (Promega, 0.2 µg of DNA/well) was cotransfected with the pRLSVL vector to correct for transfection efficiency. For controls, a plasmid, pRLTK (Promega, 0.2 µg of DNA/well) which expresses the Renilla luciferase, was used as a control for transfection efficiency of the ORE-pGL3 plasmids, which expresses the firefly luciferase. The two luciferase vectors exhibit luminescence under different conditions in the dual luciferase assay, thus allowing independent quantitation. Transfections were done using the calcium phosphate method. The cells were transfected after 6 to 8 h of harvest. After 24 h, the cells were harvested and used directly. This single nucleotide mutation was previously described as being associated with the hypertonicity response of the ORE (11).

**Reporter Gene Assays**—In the CAT reporter gene assays, the cells were harvested by scraping in 200 µl of phosphate buffer (0.1 M potassium phosphate, pH 7.9) and lysed by 3 freeze-thaw cycles at −80 °C and 37 °C. The lysate was centrifuged, and 10 µl of the supernatant was analyzed for luciferase activity (15) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Sparks, MD). Extracts (2–5 µl) containing equal luciferase activity were applied to a 15°C chromatoplates (Amersham Pharmacia Biotech) and 1 mM acetylcarnitine (Sigma) in phosphate buffer. The acetylated carnitamine products were separated by thin layer chromatography and visualized by autoradiography. The bands visualized by autoradiography were then excised from the chromatophate and quantitated by liquid scintillation counting.

**In the dual luciferase transfections, passive lysis buffer (200 µl)** supplied by the manufacturer (Promega) was added to each well, and the plate was shaken on an orbital shaker for 30 min at room temperature. The resulting cell lysate was collected in amber tubes and centrifuged, and 2–5 µl of the supernatant was analyzed for Renilla and firefly luciferase activities in a Monolight 2010 luminometer using the Dual-Luciferase® Assay kit (Promega).

**Kinase Assays—MAPK activity was determined as described previously (16, 17) with slight modifications.** The cells were exposed to hyperosmotic conditions in the presence or absence of inhibitors as described above, scraped into the experimental medium, and harvested by centrifugation. The cell pellet was lysed in Triton Lysis Buffer (TBL) containing 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml leupeptin. The supernatant was collected by centrifugation at 15,000 × g for 10 min. Anti-ERK antibody (Upstate Biotechnology Inc. NY; the antibody cross-reacts with ERK1 and ERK2) was bound to protein A- and G-agarose. Cell lysate (100 µg of protein) was then added to the agarose beads, and ERK was precipitated by the antibody-agarose complex. The beads were washed 3 times in TBL, followed by 3 additional washes in kinase buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM sodium vanadate). Myelin basic protein (Sigma) in kinase buffer containing 25 µM [α-32P]dATP was added to the beads and incubated for 20 min at 30 °C. The kinase reaction was stopped by centrifugation at 12,000 × g for 2 min. The beads were washed in SDS-PAGE, and the gel was dried and autoradiographed. ERK1 and -2 activity was determined by the extent of incorporation of 32P in the myelin basic protein substrate. For JNK1, p38, and p38β assays, the above procedure is used except for the following: anti-p38, anti-p38β, or anti-JNK1 antibodies (Santa Cruz, CA) are used for immunoprecipitation, and Fos (Calbiochem, CA) is used to immobilize the anti-ERK antibodies; activated transcription factor 2 is used as substrate for the p38 kinase, whereas the NH₂-terminal peptide (1–79) derived from c-Jun (Santa Cruz Biotechnology) was used as substrate for JNK1. The reaction supernatant is resolved on 10% SDS-PAGE.

**Gel Shift Assays—**The oligonucleotides 5'-GTGAAACGACAATTGGAATAATCAGCATGGAGT and 5'-GTGACTCCATGGCGTGATTTTCATGCATGGTTCCCT containing the minimal ORE (in bold) were annealed, and the 5'-overhangs were labeled using Klenow polymerase (Roche Molecular Biochemicals), [α-32P]dCTP, and [α-32P]dATP (ICN Radiochemicals). The labeled probe was purified using a Select G-25 spin column (5 Prime—3 Prime, Inc., Boulder, CO). Specific competitor fragments were generated in a similar fashion using non-radioactive oligonucleotides and dATP. For the non-specific competitor, a single base (underlined and bold) was changed in the ORE element to yield oligonucleotides 5'-AACGGACCAATTGGAATAATCAGCATGGAGT and 5'-ACTCCATGGCGTGATTTTCATGCATGGTTCCCT which were annealed and used directly. This single nucleotide mutation was previously shown in transfection studies to abolish the hypertonicity response of the ORE (11).

**Western Blot Analysis**—Western blot analyses were performed on HepG2 cells seeded into 10-cm dishes and grown in regular DMEM until they reached 90% confluency. The cells were then exposed to isotonic or hypertonic media containing the appropriate concentrations of kinase inhibitors (10 µM SB203580 or 10 µM PD98059) for 16 h. The plates were washed with phosphate-buffered saline, and the cells were scraped and collected in extraction buffer (1% Nonidet P-40, 5 µg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 100 µM leupeptin, and 100 µM benzamidine in phosphate-buffered saline) and incubated on ice for 30 min. The lysate was centrifuged, and the supernatant was stored in aliquots at −70 °C. Aliquots containing 5 µg of total protein were incubated for 30 min in gel shift buffer (10 mM Tris, 50 mM NaCl, 10% glycerol) containing radiolabeled probe (50,000 cpm), 100 ng of poly(dI/ dC) and cold competitor (0, 10, or 50%). The reaction products were then separated on a 4% acrylamide gel at 4 °C and visualized by autoradiography.

**Northern Blot Analysis—**HepG2 cells at 90% confluency were exposed to iso-osmotic or hyperosmotic medium (with and without 10 µM SB203580 or PD98059) for 16 h as described above. The cells were harvested, pelleted by centrifugation at 5000 × g for 10 min, and Total RNA was isolated from the cell pellet using RNAzol (Tel-Test, Friendswood, TX). Equal amounts of RNA per lane were loaded onto 1% agarose, 2.2 x formaldehyde gel. The gel was electrophoresed and transferred to GeneScreen membrane (NEN Life Science Products). Human AR cDNA and introns 6–8 from the human AR gene (6) and human full-length β-actin cDNA (CLONTECH) were labeled with [α-32P]dCTP (Random Primed DNA Labeling Kit, Roche Molecular Bio-
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FIG. 1. Effect of SB203580 and PD098059 on the hypertonic induction of AR mRNA in HepG2 cells. Northern blot analysis of AR mRNA from HepG2 cells is shown. Bar graph shows the relative intensity of AR mRNA bands in the autoradiograph normalized to β-actin. Three determinations were made for each treatment, and the means normalized to β-actin are plotted. Inset shows representative blot: lanes 1 and 2, isotonic media; lanes 3 and 4, hypertonic media; lanes 5 and 6, hypertonic media plus PD098059; lanes 7 and 8, hypertonic media plus SB203580.

RESULTS

Hypertonic Induction of AR mRNA in HepG2 Cells Is p38 Kinase- and MEK1-dependent—We examined the effects of SB203580, a specific p38 kinase inhibitor, on the hypertonic induction of AR mRNA in HepG2 cells. Fig. 1 shows that 16 h of exposure to hypertonicity induces a 15-fold increase in AR mRNA levels in comparison with cells incubated in isotonic media. The increase in mRNA is attenuated in the presence of a 5 μM concentration of the p38 kinase inhibitor. These osmotically stressed cells express two specific AR mRNA species in equal abundance (1.5 and 2.5 kb) as compared with cells incubated in isotonic media, where only the smaller mRNA is detected. Northern blots were hybridized with genomic probes derived from introns 6–8 of the human AR gene. Only the larger 2.5-kb mRNA species hybridized to the intron 7 probe (Fig. 1 inset). The blots were autoradiographed, and relative band intensities were quantitated using Image Tool (University of Texas Health Science Center, San Antonio) software. Relative band intensities were normalized to β-actin.

To define better the cis-elements regulated by p38 kinase and MEK1, we examined the expression of luciferase reporter constructs, driven by one of the following enhancer/promoter constructs: the 12-bp basic ORE, TGGAAAATCACC; 132-bp sequence, which contains the ORE complex as well as the full 1.5-kb human AR promoter (10–12). Hypertonicity increases ORE-driven luciferase reporter expression 5.9-, 4.7-, and 1.6-fold with the 1.5-kb, 132-, and 12-bp ORE sequences, respectively. This induction is attenuated in the presence of either MEK1 or p38 kinase inhibitor, in a dose-dependent manner, with complete inhibition at 10 μM SB203580 or 5 μM PD098059 (Table I). However, the expression of the reporter gene was not affected by either inhibitor under isotonic conditions. Although the magnitude of hypertonic induction of the reporter gene product differed among the various constructs (1.5 kb > 132 bp > 12 bp), the effects of the inhibitors were similar (Table I). The data suggest that the hypertonicity-induced regulatory effects of p38 kinase and MEK1 on AR expression are ORE-specific.

p38 Kinase and MEK1 Regulate the Binding of trans-Acting Elements to the ORE—We examined the effects of p38 kinase and MEK1 inhibitors on the interaction between the ORE and transcription factors. The basic 12-bp ORE fragment was radiolabeled and incubated with whole cell extracts derived from isotonic or hypertonically stressed HepG2 cells. Two prominent DNA-protein complexes were observed, designated as complex 1 and 2 (Fig. 4). Complex 2 was seen most prominently in extracts from osmotically stressed cells, whereas complex 1 was observed in all extracts and is intensified 15-fold under hypertonic conditions.

\[ V. \text{ Nadkarni, K. H. Gabbay, and K. M. Bohren, and manuscript in preparation.} \]
Discrete response and cellular specificities may be attained through multiple levels of interactions. In yeast, the induction of GPD1 (glycerol-3-phosphate dehydrogenase 1), which is responsible for the synthesis of glycerol in response to osmotic stress, is dependent on the HOG1 mitogen-activated protein kinase (MAPK), a mammalian p38 homologue, suggesting that the p38 kinase pathway may be important in the regulation of osmoprotective genes by hypertonicity in mammalian cells (21). Although the activation of ERK, JNK, and p38 kinases by osmotic stress has been demonstrated in mammalian cells (22–24), ERK activity was not found to be essential for transcriptional regulation of beta- and inositol transporters (25). In addition, inhibition of MKK3 (one of the upstream activators of p38 kinase) or SEK1 (an upstream activator of JNK) did not affect the induction of osmoprotective genes in mammalian cells (26). However, using a specific p38 kinase inhibitor, SB203580, it was shown that the p38 kinase pathway is essential for the hypertonic induction of BGT1 mRNA in the canine renal epithelial cells, MDCK (13), and the hypertonic induction of both SMT and BGT1 mRNAs in monocytes (14).

In these experiments (Fig. 1), we show that the hypertonicity-induced AR mRNA increase in HepG2 cells is attenuated by the p38 kinase and MEK1 inhibitors, SB203580 and PD098059, respectively. In addition, we show in transfection experiments that the specific locus of action of these inhibitors is the activation of the ORE in response to hypertonicity (Figs. 2 and 3). The data in Table I, describing the effect of the inhibitors on the minimal 12-bp ORE, the more responsive 132-bp ORE, and the full 1.5-kb AR promoter, confirm that the ORE motif is the main effectors, since the inhibitory effects are comparable among the three constructs despite quantitative gradations in the response to hypertonicity.

Our data also demonstrate the involvement of p38 kinase in the binding of trans-acting elements to the ORE. Two specific major complexes were observed in the electrophoretic mobility shift assays (Fig. 4). The faster mobility complex 1, which is also seen with extracts derived from HepG2 cells maintained in isotonic media, increases 15–20-fold with hypertonicity, whereas the slower mobility complex 2 was detected only upon exposure of the cells to hypertonic media. Under hypertonic conditions, treatment with either p38 kinase or MEK1 inhibitor abolishes the formation of complex 2 and diminishes the formation of complex 1. The data suggest that the binding of transcription factors to the ORE upon exposure of the cells to hypertonic stress is dependent on the activities of both MEK1 and p38 kinase.

**DISCUSSION**

Exposure of cells to hypertonicity induces a sequence of events that results in the expression of a variety of genes, some protective, whereas others may have harmful effects. The induction of these genes results from a complex of signal cascades that ultimately affect the promoter of the genes involved. The promoters of AR, BGT1, and other genes that have been shown to be induced by the hypertonic response have a 12-bp minimal enhancer element that has been shown to respond to hypertonicity (11, 12, 18, 19). The minimal osmotic response element (ORE or TonE) appears to be widely distributed in the genome and can be found in the promoters of other genes that respond to stimuli other than hypertonicity. For example, the nuclear factor of activated T lymphocytes (NFAT) binds to a DNA region that contains a consensus 12-bp ORE motif, TG-GAAAATTGTG, that responds to hypertonicity in transfection studies. In activated T lymphocytes, the NFAT transcription factor binds the consensus ORE in conjunction with a Jun-Fos AP-1 heterodimer to form a large NFAT-AP-1-DNA complex (20). It thus appears that discrete response and cellular specificities may be attained through multiple levels of interactions.
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The effect of p38 kinase inhibitor (SB203580, abbreviated as SB) or MEK1 inhibitor (PD098059, abbreviated as PD) on the expression of various ORE-driven luciferase reporter constructs is shown. Values represent the ratio of ORE-driven firefly luciferase expression relative to control Renilla luciferase (see “Experimental Procedures”) and are a mean of six independent transfections (±S.E.) in three separate experiments. I, isotonic medium; H, hypertonic medium.

|          | 1.5-kb PGL3 (n = 6) | H/I     | 132-bp PGL3 (n = 6) | H/I     | 12-bp PGL3 (n = 6) | H/I     | 132-bp CAT (n = 3) | H/I     |
|----------|---------------------|---------|---------------------|---------|-------------------|---------|-------------------|---------|
| I        | 116 ± 10            |         | 48 ± 6              |         | 53 ± 5            |         | 1293 ± 158        |         |
| I + SB (5 μM) | 91 ± 5              | 58 ± 7  | 62 ± 5              |         | 808 ± 94          |         |                   |         |
| I + PD (5 μM) | 93 ± 5              | 63 ± 6  | 53 ± 6              |         |                   |         |                   |         |
| H        | 684 ± 38            | 5.9     | 226 ± 7             | 4.7     | 83 ± 5            | 1.6     | 7517 ± 154        | 5.8     |
| H + SB (1 μM) | 552 ± 27            | 4.7     | 145 ± 9             | 2.5     | 78 ± 3            | 1.3     | 8550 ± 73         | 6.6     |
| H + SB (5 μM) | 260 ± 29            | 2.1     | 91 ± 8              | 1.6     | 62 ± 5            | 1.0     | 4600 ± 527        | 5.7     |
| H + SB (10 μM) | 55 ± 6              | 0.6     | 52 ± 11             | 0.9     | 52 ± 3            | 0.8     | 1551 ± 35         | 1.9     |
| H + PD (0.5 μM) | 579 ± 26           | 5.0     | 149 ± 11            | 3.1     | 81 ± 3            | 1.5     |                   |         |
| H + PD (1 μM) | 392 ± 13            | 4.2     | 129 ± 5             | 2.0     | 74 ± 4            | 1.4     |                   |         |
| H + PD (3 μM) | 267 ± 24            | 2.9     | 86 ± 4              | 1.4     | 59 ± 3            | 1.1     |                   |         |
| H + PD (5 μM) | 55 ± 5              | 0.5     | 60 ± 3              | 1.0     | 46 ± 0.2          | 0.9     |                   |         |

which is present at the NH₂-terminal end of TonEBP, has a 45% amino acid identity with the corresponding region of the NH₂-terminal DNA binding domains of the NFAT transcription factors (27). The x-ray crystallographic structure of the NFAT-AP-1-DNA complex (20) demonstrates that this region forms part of a large groove, which along with the grooves generated by the DNA-facing surfaces of Fos and Jun bind the DNA and form the large multimer. All of the above data clearly establish that p38 kinase is involved in the activation and/or synthesis of the transcription factor binding to the ORE.

PD098059 is a selective inhibitor of MEK1, without significant inhibitory activity against ERK itself. It has no inhibitory activity against 18 different protein Ser/Thr kinases, including two other MEK1 homologues that participate in stress and interleukin-1-stimulated kinase cascades, suggesting high specificity for MEK1 (29). Inhibition of MEK by PD098059 was shown to prevent the activation of ERK and subsequent phosphorylation of ERK substrates both in vitro and in intact cells (30). Our data with PD098059 indicate that MEK1 is involved in the regulation of hypertonic induction of ORE-mediated gene expression. Inhibition of MEK1 in hypertonically stressed cells results in attenuation of AR mRNA abundance, inhibition of ORE-driven reporter construct expression, and the binding of trans-acting elements to labeled ORE. However, the MEK1 effect does not appear to be ERK-mediated, since the ERK1/ERK2 activity is in fact increased in response to inhibitor treatment. Similarly, the induction of GLUT-1 gene by hyperosmotic stress in L6 muscle cells has been shown to be MEK1-dependent, yet ERK-independent, suggesting that in the context of osmotic stress, the MEK1 effects are ERK-independent (31). In addition, the activities of p38 kinase, p38β kinase, and JNK1 in PD098059-treated and hypertonically stressed cells are not affected, indicating that MEK1 effects are not mediated through these kinases. The basis for MEK1 effect on ORE-mediated gene expression remains to be determined. The pyridyl imidazole group of compounds to which SB203580 belongs demonstrate a highly specific and potent inhibitory activity against p38 kinase. In earlier studies these compounds showed no inhibitory activity against ERK1 and ERK2, JNK, p38β kinase, and MAPK-activated protein kinase-2 (MAPKAPK-2), MAPK, protein kinase C, calmodulin-dependent protein kinase, and cAMP-dependent protein kinase (32, 33). In fact, the binding specificity of these compounds was utilized to identify and affinity purify p38 kinase (33). It should be noted that in Fig. 5 SB203580-treated cells do not show any change in the activities of immunoprecipitated p38 and p38β kinases, since the drug is removed after repeated washes of the precipitates. As was
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previously shown in MDCK cells (13). JNK1 activity is up-regulated in HepG2 cells when p38 kinase is inhibited by SB203580. The significance of this finding remains to be determined.

Our data indicate that the hypertonic induction of AR mRNA in HepG2 cells is regulated by p38 kinase and MEK1 and is mediated by the ORE. Inhibition of either kinase independently results in loss of the hypertonic effects suggesting that both signal pathways are necessary for the hyperosmotic response. It is not known at this time whether these kinases affect only the binding of the transcription factors to the ORE, their nuclear translocation and activation, or both.

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