Fyn and p38 Signaling Are Both Required for Maximal Hypertonic Activation of the Osmotic Response Element-binding Protein/ Tonicity-responsive Enhancer-binding Protein (OREBP/TonEBP)*


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When cells are challenged by hyperosmotic stress, one of the crucial adaptive responses is the expression of osmoprotective genes that are responsible for raising the intracellular level of compatible osmolytes such as sorbitol, betaine, and myo-inositol. This is achieved by the activation of the transcription factor called OREBP (also known as TonEBP or NFAT5) that specifically binds to the osmotic response element (ORE) or tonicity-responsive enhancer that enhances the transcription of these genes. Here we show that p38, a subgroup of the mitogen-activated kinases activated by hypertonic stress, and Fyn, a shrinkage-activated tyrosine kinase, are both involved in the hypertonic activation of OREBP/TonEBP. Inhibition of p38 by SB203580 or by the dominant negative p38 mutant partially blocked the hypertonic induction of ORE reporter (reporter gene regulated by ORE). Similarly, hypertonic activation of ORE reporter was partially blocked by pharmacological inhibition of Fyn or by a dominant negative Fyn and was attenuated in Fyn-deficient cells. Importantly, inhibiting p38 in Fyn-deficient cells almost completely abolished the hypertonic induction of ORE reporter activity, indicating that p38 and Fyn are the major signaling pathways for the hypertonic activation of OREBP/TonEBP. Further, we show that the transactivation domain of OREBP/TonEBP is the target of p38- and Fyn-mediated hypertonic activation. These results indicate a dual control in regulating the expression of the osmoprotective genes in mammalian cells.

When cells are exposed to hypertonic environment, the immediate effect is the loss of water and increase in the level of electrolytes. The cells adapt to this deleterious situation by gradually replacing the excess electrolytes with compatible osmolytes such as sorbitol, betaine, and myo-inositol (1) to preserve the normal cellular functions (2). The accumulation of these osmolytes is brought about by increased expression of the gene for aldose reductase (AR) (3), a key enzyme for sorbitol synthesis, and genes for the specific transporters for betaine and myo-inositol, the betaine/-aminobutyric acid transporter (BGT-1) (4), and the Na+-dependent myo-inositol transporter (SMIT) (5), respectively. The hypertonic induction of these genes is controlled at the transcriptional level, and it is mediated by a common regulatory element called the osmotic response element (ORE) or by the TonE (tonicity-responsive enhancer) (6–8). The transcription factor that binds to ORE/ TonE, known as OREBP/TonEBP, was identified by yeast one-hybrid assay and affinity chromatography purification (9, 10). This protein is also called NFAT5 because of its sequence homology with the NFAT family of transcription factors (11).

OREBP/TonEBP is subjected to different levels of regulation. Hypertonicity rapidly induces its phosphorylation and induces its translocation into the nucleus (9, 10, 12). This is followed by an increase in the levels of OREBP/TonEBP mRNA and protein (9, 10). Recently, it was shown that hypertonic activation of OREBP/TonEBP involves phosphorylation of its transactivation domain (TAD) (13) and that this activation can be blocked by a tyrosine kinase inhibitor and a protein kinase CK2 inhibitor. However, the upstream signaling pathway(s) leading to its activation remains obscure.

The osmotic signaling pathway in yeast is better understood. When yeast cells are exposed to hypertonic environment, a mitogen-activated protein kinase, Hog1, is activated (14, 15). Activated Hog1 induces adaptive responses by directly participating in chromatin binding (16), and up-regulates the expression of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase isoform 2 (17, 18), which are essential for the production of glycerol, the principal compatible osmolyte in yeast (19, 20). Yeast hog1 null mutant cells are not viable in hypertonic environment, but the expression of p38, the mammalian homologue of Hog1, allowed them to survive such conditions (14). This finding has suggested that p38 may be involved in osmoregulation in mammalian cells. Indeed, p38 was found to be activated by various environmental stresses including osmotic stress (14). However, whether p38 is involved in mediating the hypertonic activation of OREBP/TonEBP remains controversial. Hypertonic induction of AR, BGT-1, and SMIT gene overexpression has been shown to be abrogated by SB2030580, a potent inhibitor of p38 (21, 22). Whether p38 exerts its effect through OREBP/TonEBP is less clear. One report showed that hypertonic induction of an ORE reporter
activity was blocked by SB203580 (23), whereas another report showed that overexpression of a dominant negative form of MKK3, which blocked p38 activation, has no effect on hypertonic ORE reporter activity (24).

Apart from the activation of p38, one of the earliest cellular responses induced by hypertonicity is a dramatic increase in protein tyrosine phosphorylation (25), which is mediated at least in part by Fyn kinase (26), a ubiquitously expressed member of the Src family of protein tyrosine kinases (27). Fyn is activated by cell shrinkage rather than hypertonicity per se. It is thought to be involved in the reorganization of the cytoskeleton, which may have important consequences in altering the gene expression pattern of the cell. In addition, it has been shown that Fyn expression induces phosphorylation of Hic-5, a paxillin-like protein, and its Fyn-mediated phosphorylation is strongly potentiated by hypertonicity (28). These findings suggested that Fyn might represent an alternative pathway in addition to p38 to regulate gene expression in response to extracellular hypertonicity. Consistent with this hypothesis, the participation of tyrosine kinase pathways in ORE induction has been suggested, but the responsible enzyme(s) has not been identified. Therefore, we were interested to determine whether Fyn is involved in the OREBP/TonEBP-mediated hypertonic response. In this report, we show that p38 and Fyn both participate in the hypertonic activation of OREBP/TonEBP.

EXPERIMENTAL PROCEDURES

Materials—PP2 and SB203580 were purchased from Calbiochem. FuGENE transfection reagent was from Roche Molecular Biochemicals. Monoclonal anti-phosphospecific p38 antibody was from Cell Signaling Technology, Inc. Polyclonal anti-Fyn antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal Anti-Fyn was obtained from Upstate Biotechnology, Inc. Peroxidase-conjugated anti-mouse and anti-rabbit IgG, protein G-Sepharose beads, the Enhanced Chemiluminescence kit, and [α-32P]dCTP and [γ-32P]ATP were from Amersham Biosciences. The Tcr assay kit was from Upstate Biotechnology, Inc. The dual luciferase reporter assay system and the luciferase reporter vector control by SV40 promoter (SVLuc) were purchased from Promega (Madison, WI). pCMV-Script mammalian expression vector was from Stratagene. pCMGAL4 DNA-BD vector (pM) was from Clontech (BD Biosciences). GAL4 reporter plasmids contain 4 tandem repeats of yeast GAL4-binding site upstream of a minimal promoter, and a luciferase gene (GAL4-Luc) is kindly provided by Dr. D. Y. Jin. Dominant negative Fyn vector (pCMV-FynDN) and dominant negative p38α vector (FLAG-p38αAF) were kind gifts from Dr. Marilyn Resh and Dr. Jiahui Han (The Scripps Research Institute), respectively. Human OREBP cDNA clone KIAA0827 was a gift from Dr. Takahiro Nagase (Razusa DNA Research Institute). The identities of all of the clones were confirmed by DNA sequencing.

Cell Culture—Fyn−/− fibroblasts were originally isolated from mouse embryos that were homozygous for disruption in the Fyn gene. The wild type (WT) and Fyn−/− fibroblast cells were immortalized with large T antigen (29) and were kindly provided by Sheila M. Thomas (Fred Hutchinson Cancer Center, Seattle, WA). NIH 3T3 cells, WT, and Fyn−/− fibroblast cells grown in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37 °C in the presence of CO2 (300 mosmol/kg of H2O). Hypertonic medium was prepared by supplementing the growth medium with 100 mosmol/kg of NaCl resulting in a final osmolality of 500 mosmol/kg of H2O.

Plasmid Construction—ORE-SVLuc luciferase reporter constructs containing −1235 to −1104 of the human AR gene, which includes three OREs, was made as described previously (7). pM-TAD fusion proteins were generated by in-frame insertion of the sequence coding for the transactivation domain (amino acids 548–1531) of clone KIAA0827 into GAL4-DNA-BD vector (pM).

Transfection and Luciferase Assay—NIH 3T3, WT, and Fyn−/− were grown in medium (300 mosmol/kg) in 24-well plates. For ORE activity assay, the cells were transfected with 100 ng of ORE-SVLuc. For OREBP-TAD activity assay, the cells were co-transfected with 300 ng of GAL4-Luc and 600 ng of pM-TAD. In co-transfection experiments, the total amounts of plasmid being transfected were made constant by adjusting with plasmid DNA PBS-2KII (Stratagene). Transfection was carried out using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. 24 h after transfection, the cells were replaced into isotonic or hypertonic medium. In the experiments, where the effects of inhibitors were studied, the cells were placed into isotonic medium with the inhibitor for 30 min, and then this medium was changed to a hypertonic one containing the inhibitor. 4–6 h after switching the medium, the cells were harvested by addition of 100 μl of passive lysis buffer, and luciferase activity was determined using the dual luciferase reporter assay system. Luciferase activity was expressed in relative light units/μg of total cell protein.

Immunoprecipitation and in Vitro Kinase Assay—Lysates containing equivalent amounts of protein (400 μg) were clarified by centrifugation at 12,000 × g for 10 min and further processed for immunoprecipitation. Immunoprecipitates were prepared for 1 h using 40 μl of 50% suspension of protein G-Sepharose beads and then incubated with the polyclonal anti-Fyn antibody for overnight at 4 °C. Immunocomplexes were captured using 20 μl of protein G-Sepharose, and the beads were either washed twice with lysis buffer and once with kinase buffer (20 mM Hepes, 10 mM MnCl2, 0.25 mM Na3VO4, pH 7.1) for determination of kinase activity. Kinase activity was measured as the activation of the phosphorylation of the Src family-specific substrate peptide Cdc2 (6–20) according to the manufacturer’s instructions. Briefly, the immunocomplexes were incubated with 20 μl of reaction buffer (100 mM Tris-HCl, pH 7.2, 125 mM MgCl2, 25 mM Na3VO4, 2 mM EGTA, 0.25 mM Na2VO4, 2 mM dithiothreitol and 10 μl of substrate peptide (0.6 mM stock) at 30 °C, and the reaction was initiated by the addition of manganese/ATP mixture (0.5 mM ATP, 75 mM MgCl2 in reaction buffer) containing 10 μl of γ-32P[ATP]/sample. After 20 min at 30 °C, 20 μl of trichloroacetic acid was added, and 25 μl of the mixture was layered on P81 phosphocellulose squares. After extensive washing with 0.85% phosphoric acid, the radioactivity bound to the filters was determined by scintillation counting. Non-specific binding of radioactivity to the filters was determined in each experiment by measuring the activity of samples to which 10 μl of H2O was added instead of the peptide. The low activity measured in these samples was subtracted as background.

Western Blotting—Protein extracts or immunoprecipitated proteins were electrophoresed with Laemmli sample buffer, boiled for 5 min, and subjected to electrophoresis on 10% SDS-polyacrylamide gels. The separated proteins were transferred to nitrocellulose using a Bio-Rad Mini Protein II apparatus. The blots were blocked in Tris-buffered saline containing 5% bovine serum albumin and then incubated with the antibody. The binding of the antibody was visualized by peroxidase-coupled secondary anti-mouse antibody using enhanced chemiluminescence.

Northern Blotting—20 μg of each total RNA sample was loaded on 1.2% agarose gel containing 3% formaldehyde. After electrophoresis, the RNA was transferred onto Hybond-N membrane (Amersham Biosciences) by capillary blotting in 20% SSC. The membrane was first incubated with prehybridization buffer (7% SDS and 0.25 M sodium phosphate) at 65 °C for 1 h and then with hybridization buffer containing [α-32P]dCTP-labeled mouse AR cDNA probe and incubated at 65 °C for 12 h. After hybridization, the filter was washed with 0.1× SSC and 0.5% SDS at 65 °C for 50 min and exposed to X-Omat AR film (Eastman Kodak Co.). The filter was then stripped in 0.1× SSC and 0.5% SDS at 100 °C and rehybridized to an [α-32P]dCTP-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech, BD Biosciences).

RESULTS

Effect of PP2, Dominant Negative Fyn, and Fyn Deficiency on the Hypertonic Induction of ORE Reporter Activity—To assess whether Fyn is involved in relayed hypertonic signals to activate OREBP/TonEBP, we determined the effect of the tyrosine kinase inhibitor PP2 on the hypertonic activation of OREBP/TonEBP using a luciferase reporter gene under the control of ORE (ORE-SVLuc). PP2, a pyrazolopyrimidine derivative, is a potent and specific inhibitor of the Src family of protein kinases (30). Fig. 1 shows that hypertonicity induced a 14-fold increase in the ORE reporter activity in 4 h. The addition of PP2 caused a concentration-dependent reduction in the hypertonic response of the ORE reporter. When 10 μM of PP2 was used, the reporter activity was reduced to one-third of the maximal level. This tyrosine kinase inhibitor had no effect on the reporter gene activity when cells were cultured in isotonic medium. It
was similar in both cell lines. However, there was a 2.3-fold (Fig. 2C) hypertonicity-induced ORE reporter activity was determined in NIH 3T3 cells were transfected with ORE-luciferase activity and protein concentration were determined as described under "Experimental Procedures." The values are the means ± S.D. of three independent transfections. The data shown are representative of three independent experiments. The values are expressed as percentages of change in luciferase activity relative to the ORE-SVLuc transfected cell under isotonic conditions without PP2 (lane 1, 100%). Me2SO (0.25%) was added to cells that are not treated with PP2 (lanes 1, 2, 7, and 8). H, hypertonic. I, isotonic. *, p < 0.001.

also did not affect the reporter gene activity under isotonic or hypertonic conditions when the reporter gene was not under the control of ORE (SVLuc). These results suggested that PP2 specifically inhibits hypertony-induced, ORE-mediated transcription.

Because PP2 inhibits the activity of other members of the Src family protein kinase besides Fyn (30), a dominant negative Fyn (FynDN) (31) was used to verify the specific role of this tyrosine kinase in the hypertonic activation of OREBP/TonEBP. The FynDN contains a point mutation in the ATP-binding site where lysine 299 was replaced by methionine. As a result, the FynDN cannot bind ATP and becomes catalytically inactive (32). A plasmid containing FynDN (pCMV-FynDN) was co-transfected with ORE-SVLuc. As shown in Fig. 2A, whereas hypertonic induction for 4 h greatly stimulated the ORE-SVLuc activity in mock-transfected cells, co-transfection with increasing amounts of pCMV-FynDN resulted in an incremental reduction in the hypertonicity-induced ORE-Luc activity. Consistent with the results obtained after pharmacological inhibition of Src-type kinases by PP2, FynDN did not affect the ORE-SVLuc activity under isotonic conditions or the SVLuc activity under isotonic and hypertonic conditions.

To further explore the requirement of Fyn in hypertonic ORE reporter activity, we utilized a fibroblast cell lines derived from WT or Fyn-deficient (Fyn−/−) mice (29). Immunoprecipitation with Fyn-specific antibodies confirmed that Fyn is present in the WT cells and not found in the Fyn−/− cells (Fig. 2B). WT and Fyn−/− cells were transfected with ORE-SVLuc, and the hypertonicity-induced ORE reporter activity was determined (Fig. 2C). Under isotonic conditions the ORE reporter activity was similar in both cell lines. However, there was a 2.3-fold decrease in the hypertonic induction of ORE reporter activity in the Fyn-deficient cells. On the other hand, hypertonic induction of ORE reporter activity was not suppressed in Src-deficient cells (data not shown). Taken together, these findings showed that Fyn plays a significant role in mediating the hypertonic induction of ORE reporter activity.

Effect of SB203580 and Dominant Negative p38 on Hypertonicity-induced ORE Reporter Activity—Although these results showed that Fyn significantly contributes to the hypertonic activation of OREBP/TonEBP, inhibition of Fyn by co-transfection of FynDN or in Fyn-deficient cells resulted in only ~50% reduction in the hypertonic induction of ORE reporter activity, suggesting the involvement of other signaling pathway(s) in this process. As mentioned earlier, p38 has been implicated in the cellular adaptation to hypertonic stress, but there was also a report that contradicted that interpretation. To determine the contribution of p38 to the hypertonic activation of OREBP/TonEBP, first we measured the ORE reporter activity in the presence of SB203580. As shown in Fig. 3A, hypertonicity resulted in a 15-fold increase in ORE reporter activity. The addition of 5 and 10 μM SB203580 elicited a dose-dependent decrease in hypertonic ORE reporter activity suppressing the level of induction to 11- and 5-fold, respectively. The presence of SB203580 did not affect the ORE reporter activity under isotonic conditions, nor did it affect the reporter gene activity, which was not controlled by ORE (SVLuc) under both isotonic and hypertonic conditions. These results confirm the previous findings using SB203580.

To substantiate that SB203580 exerted its effect on ORE reporter activity by inhibiting p38 and not by interfering with other pathways, we tested the effect of a dominant negative mutant p38 (p38DN) as well. As shown in Fig. 3A, in the control experiment where NIH 3T3 cells were co-transfected with ORE-SVLuc and pCMV-Script, hypertonicity resulted in a 19-fold increase in ORE reporter activity. Co-transfection with ORE-SVLuc and the p38DN (FLAG-p38αAF) resulted in a reduction in hypertonic induction of ORE reporter activity to 3.5-fold, confirming the results obtained by SB203580 to inhibit p38. Increasing the dosage of p38DN led to a slightly but not significantly greater decrease in the hypertonic induction of ORE reporter activity, suggesting that the expression of p38DN sufficiently suppressed the effect of the endogenous p38 even when the lower DNA plasmid concentration was used for transfection. Consistent with the results obtained with SB203580, p38DN affected neither the ORE-SVLuc expression under isotonic conditions nor the SVLuc expression under isotonic and hypertonic conditions.

The Additive Effect of p38 and Fyn Inhibition on the Hypertonic Induction of ORE Reporter Activity—Inhibition of p38 by SB203580 or by p38DN did not completely block the hypertonic induction of ORE reporter activity. Notably, a 5-fold induction was still observed in the presence of p38 inhibition, and significant residual induction of ORE reporter expression remained after Fyn inhibition. These findings suggest that neither p38 nor Fyn alone is sufficient to fully activate OREBP/TonEBP upon hypertonic induction and support the possibility that both p38 and Fyn are required for the complete induction of ORE reporter activity. To test this hypothesis, the ORE-SVLuc was transfected into WT and Fyn-deficient fibroblasts, and the hypertonic induction of this reporter gene was determined in the presence or absence of SB203580. As shown in Fig. 4A, the addition of SB203580 resulted in an incomplete but marked decrease of hypertonicity-induced ORE reporter activity in the WT fibroblasts in a dose-dependent manner. Hypertonic induction of ORE reporter in the Fyn−/− fibroblasts was much lower than that of the WT fibroblasts, and the induction of the ORE reporter was further attenuated by the addition of SB203580.
Importantly, in Fyn−/− cells, 10 μM of SB203580 almost completely blocked the hypertonic induction of ORE reporter expression. Collectively these results indicate that p38 and Fyn are the major contributors to the hypertonic activation of OREBP/TonEBP.

To determine whether p38- and Fyn-mediated hypertonic
activation of OREBP/TonEBP leads to the induction of osmoprotective genes like AR, SMIT, and BGT, WT and Fyn-deficient fibroblasts were treated with SB203580, and the expression of AR gene was examined under isotonic and hypertonic conditions. As shown in Fig. 4B, in the WT cells hypertonicity dramatically increased AR mRNA under hypertonic conditions, and this effect was markedly decreased by 10 μM SB203580. In the Fyn-deficient cells, hypertonic induction of the AR mRNA was less prominent, and the induction was further attenuated in the presence of 10 μM SB203580. None of these treatments affected the expression level of glyceraldehyde-3-phosphate dehydrogenase. These results demonstrated that p38- and Fyn-mediated hypertonic activation of OREBP/TonEBP leads to the induction of osmoprotective genes exemplified by AR.

**Fyn and p38 Activate OREBP/TonEBP by Acting on Its Transactivation Domain**—Recently, it was shown that hypertonic activation of OREBP/TonEBP is mediated by phosphorylation of its TAD (13). To test whether p38 and Fyn regulate OREBP/TonEBP by modifying its TAD, a chimeric expression vector called pM-TAD was constructed by fusing the TAD of OREBP (amino acid 548–1531) to the GAL4 DNA-binding domain expression vector (pM). When co-transfected with a luciferase reporter vector containing the GAL4-binding site (GAL4-Luc), the activity of the TAD can be quantified by measuring luciferase activity. To test whether Fyn-mediated regulation targets the TAD and activates OREBP/TonEBP in response to extracellular hypertonicity, NIH 3T3 cells were co-transfected with pM-TAD and increasing amounts of pCMV-FynDN, or the control vector pCMV-Script. As shown in Fig. 5A, there was an 8.6-fold increase in GAL4-Luc reporter activity when cells were exposed to hypertonic conditions. The increase in the reporter activity is attributed to OREBP-TAD because co-transfection of GAL4-Luc with pM, which lacks the TAD, resulted in very low levels of luciferase activity both under isotonic and hypertonic conditions. Co-transfection with increasing amounts of pCMV-Fyn DN resulted in a progressive reduction of hypertonic reporter activity, indicating that FynDN has an inhibitory effect on TAD activity. The expression of FynDN did not affect the TAD under isotonic conditions, suggesting that activation of TAD by Fyn occurs only under hypertonic conditions. The effect of p38 on TAD was also examined. As shown in Fig. 5B, co-transfection of increasing amounts of FLAG-p38αAF also resulted in a progressively greater reduction of the hypertonic induction of luciferase reporter activity from 13-fold to a minimum of about 5-fold. Expression of the p38DN had no effect on the luciferase reporter activity when the cells were under isotonic conditions. It is noteworthy that, similar to the results of the experiments on the endogenous OREBP/TonEBP described above, neither the dominant negative Fyn nor the dominant negative p38 mutant alone was able to completely block the transactivation activity of OREBP-TAD.

**Fyn and p38 Activate OREBP/TonEBP through Independent Osmotic Signaling Cascades**—We have demonstrated that both Fyn and p38 are required for the maximal hypertonic activation of OREBP/TonEBP. Given that both Fyn and p38 are rapidly activated by hypertonic stress (14, 26), we wished to determine whether Fyn and p38 belong to different signaling pathways that converge at OREBP/TonEBP or whether they are elements of the same osmotic signaling cascade. If the latter was the case, inhibition of the upstream kinase should prevent the activation of the downstream member. To distinguish between these two possibilities, hypertonic activation of p38 in Fyn-deficient cells and activation of Fyn in the presence of SB203580 were examined. As shown in Fig. 6A, using antibodies specific for the activated form of p38 as a probe, a large increase in the phosphorylated p38 was observed in both WT and Fyn-/- fibroblasts as p38 was rapidly activated by hypertonic stress (14, 26). These results indicate that the hypertonic activation of p38 was not affected by the lack of Fyn, and p38 inhibition did not reduce hypertonic activation of Fyn activity, supporting the notion that p38 and Fyn are members of independent signaling pathways, and they act in concert to fully activate OREBP/TonEBP in response to hypertonic stress.
AR, BGT-1, and SMIT are a group of genes that are responsible for increasing the levels of cellular osmolytes. Hypertonic stress strongly stimulates the expression of these osmoprotective genes, the products of which serve to raise the intracellular osmolyte concentration, thereby countering the deleterious effects of the hypertonic environment. Hypertonic induction of these genes is mediated by the binding of the activated OREBP/TonEBP to ORE/TonE associated with these genes. However, the signaling mechanisms that link the hypertonic challenge to OREBP/TonEBP activation remained to be elucidated. In mammalian cells, hypertonicity activates three mitogen-activated protein kinase pathways: c-Jun N-terminal kinases, extracellular signal-regulated kinases, and p38 (21, 34). Of these, p38 has been implicated in relaying the hypertonic signal to OREBP/TonEBP because its inhibitor SB203580 reduced the activation of these genes.

**FIG. 5.** Fyn and p38 activate hypertonic gene transcription through modifying transactivation activity in the OREBP/TonEBP activation domain. A, NIH 3T3 cells were transfected with pM-TAD or pM and GAL4-Luc, with increasing amounts of expression vector pCMV-FynDN (0.2 and 0.4 μg) or control vector pCMV-Script and subjected to isotonic or hypertonic treatment. B, NIH 3T3 cells were transfected with pM-TAD or pM and GAL4-Luc, with increasing amounts of expression vector FLAG-p38α-AF (0.1, 0.2, and 0.4 μg) or control vector pCMV-Script and subjected to isotonic or hypertonic treatment. For A, and B, after 4 h, the cells were harvested, and luciferase activity and protein concentration were determined as described under “Experimental Procedures.” The values are the means ± S.D. of three independent transfections. The data shown are representative of three independent experiments. The values are expressed as the percentages of change in luciferase activity relative to the cells co-transfected with pM-TAD, GAL4-Luc, and pCMV-Script under isotonic conditions (lane 3, 100%).

**FIG. 6.** Fyn and p38 activate OREBP/TonEBP through independent osmotic signaling cascades. A, hypertonic activation of p38 in WT and Fyn−/− fibroblast. WT and Fyn−/− cells were incubated in hypertonic medium for the indicated time. The cells were lysed and aliquots of the containing equal amount of protein were subjected to SDS-PAGE followed by Western blotting. Activated p38 was detected using monoclonal phosphospecific p38 antibody. Total p38 was detected using polyclonal p38 antibody. B, effect of SB203580 on hypertonic Fyn activation in WT fibroblast. NIH 3T3 cells were pretreated with vehicle (DMSO) or 10 μM SB203580 for 30 min and then treated with isotonic or hypertonic solutions in the presence of Me2SO or the inhibitor for 15 min. The cells were then lysed, Fyn was immunoprecipitated from the extracts, and its activity was determined using Cdc2 peptide as substrate (n = 3).
hypertonic induction of AR, BGT-1, and SMIT mRNAs (21, 22) and blocked the hypertonic induction of ORE-regulated reporter gene expression (23). However, a dominant negative mutant of MKK3, an upstream activator of p38, failed to inhibit the hypertonic induction of an ORE-regulated reporter gene (24), casting doubt on the role of p38 in the hypertonic OREBP/TonEBP activation and raising the possibility that SB203580 might have exerted its effect by interfering with other enzymes. In this report, in addition to SB203580, we used p38DN to show that p38 is indeed involved in the hypertonic induction of ORE reporter activity and presumably in the hypertonic activation of OREBP/TonEBP. This conclusion was directly confirmed by experiments showing that SB203580 and p38DN also blocked the hypertonic induction of the transactivation activity of the TAD of OREBP/TonEBP. The failure of the dominant negative mutant MKK3 to block the ORE reporter gene activity reported earlier can be explained by the recent discovery that p38 can be activated by interaction with transforming growth factor-β-activated protein kinase 1-binding protein 1 as well as by other MKK isoforms (35). Thus, it is possible that hypertonic activation of p38 may bypass MKK3 via other MKK isoforms or transforming growth factor-β-activated protein kinase 1-binding protein 1. On the other hand, it is also possible that the relative contribution of the p38 and the tyrosine kinase pathways is different in various cell types. Our experiments using SB203580 and p38DN to inhibit p38 have clearly demonstrated that this signaling enzyme is involved in the hypertonic activation of OREBP/TonEBP. However, the hypertonicity-induced ORE reporter activity was only partially blocked by p38 inhibition, suggesting that other signaling pathways may be involved.

Hypertonicity also activates Fyn, a member of the Src family of tyrosine kinases. Fyn appears to be activated by the hypertonicity-induced cell shrinkage rather than by hypertonicity per se (26). Because cell shrinkage is an immediate consequence of hypertonic exposure, we examined whether Fyn might also be involved in the hypertonic activation of OREBP/TonEBP. Using PP2, an inhibitor of the Src kinases, FynDN, and Fyn-deficient cells, we demonstrated that Fyn is indeed involved in the hypertonic induction of ORE reporter activity via the activation of OREBP/TonEBP. Apparently the nuclear translocation of OREBP/TonEBP under hypertonicity is not affected by the pharmacological inhibition of Fyn nor by Fyn deficiency (Data not shown). Similar to p38, Fyn only contributes to part of the hypertonic OREBP/TonEBP activation, as evidenced by the fact that inhibition of Fyn by PP2 or FynDN and also Fyn deficiency did not completely block the hypertonic induction of ORE reporter activity. However, inhibiting p38 in Fyn-deficient cells almost entirely abolished the hypertonic induction of ORE reporter activity and the induction of AR mRNA. These findings indicate that p38 and Fyn are the major signal-relaying pathways for the hypertonic activation of OREBP/TonEBP. Together these two signaling pathways account for almost the entire hypertonic induction of ORE reporter activity.

Because p38 is activated by a variety of environmental factors including osmotic stress, oxidative stress, cytokines, growth factors, etc., whereas Fyn is also involved in signal transduction of oxidative stress (36, 37) and is activated by osmotic stress, these two kinases may belong to the same signaling pathway. However, we showed that the activation of one was not influenced by the inhibition or the deficiency of the other under hypertonic stress, indicating that these two signaling pathways transmit hypertonic signal to activate OREBP/TonEBP independent of each other. Interestingly, under isotonic conditions, inhibition of p38 by SB203580 activates Fyn activity, suggesting that under isotonic conditions, p38 suppresses Fyn. This suppression is released when cells experience hypertonic stress.

At this point it is not clear whether Fyn and p38 act directly or indirectly on OREBP/TonEBP. Recently it was shown that FER, a kinase presumably downstream of Fyn, directly phosphorylates cortactin, a cortical actin-binding protein that is highly phosphorylated in a Fyn-dependent manner when cells are under hypertonic stress. Cortactin is involved in the reorganization of the cytoskeleton and may mediate signaling to downstream targets (33). FER inhibition mitigates cortactin phosphorylation under hypertonic stress (26). However, we found that the hypertonic induction of ORE reporter activity was not affected in FER-deficient cells, although the osmotic stress-induced cortactin phosphorylation is reduced to marginal levels.

This observation indicates that the Fyn-mediated hypertonic activation of OREBP/TonEBP does not require FER or cortactin. Fyn may mediate the activation of OREBP/TonEBP via other signaling molecules or it may modify OREBP/TonEBP directly. It is still not known whether p38 directly phosphorylates OREBP/TonEBP. Because of the paucity of the transactivation factors including the activating transcription factor, CHOP/GADD153 (growth arrest and DNA damage), several CAMP response element-binding proteins, ELK-1, Ets-1, MAX, MEF-2A, MEF-2C, NF-κB, and heat shock transcription factor 1, were shown to be phosphorylated by p38, it is conceivable that OREBP/TonEBP is directly phosphorylated by p38. In conclusion, we show that p38 and Fyn are involved in the hypertonic activation of OREBP/TonEBP. Furthermore, inhibiting p38 in Fyn-deficient cells almost completely abolished the hypertonic induction of ORE reporter activity, indicating that p38 and Fyn are the major signaling pathways for the hypertonic activation of OREBP/TonEBP. In addition, we show that the transactivation domain of OREBP/TonEBP is targeted by p38- and Fyn-mediated hypertonic activation. These results indicate a dual control in regulating the expression of the osmoprotective genes in mammalian cells.

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