Hypertonicity-induced phosphorylation and nuclear localization of the transcription factor TonEBP

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Dahl, Stephen C., Joseph S. Handler, and H. Moo Kwon. Hypertonicity-induced phosphorylation and nuclear localization of the transcription factor TonEBP. Am J Physiol Cell Physiol 280: C248–C253, 2001.—The accumulation of compatible osmolytes during osmotic stress is observed in virtually all organisms. In mammals, the hypertonicity-induced expression of osmolyte transporters and synthetic enzymes is conferred by the presence of upstream tonicity-responsive enhancer (TonE) sequences. Recently, we described the cloning and initial characterization of TonE-binding protein (TonEBP), a transcription factor that translocates to the nucleus and associates with TonE sequences in a tonicity-dependent manner. We now report that hypertonicity induces an increase in TonEBP phosphorylation that temporally correlates with increased nuclear localization of the molecule. TonEBP phosphorylation is not affected by a number of kinase inhibitors, including the p38 inhibitor SB-203580. In addition, in vitro binding assays show that the association of TonEBP with TonE sequences is not affected by phosphorylation. Thus TonEBP phosphorylation is an early step in the response of cells to hypertonicity and may be required for nuclear import or retention.

aldose reductase; organic osmolytes; sodium-chloride-betaine/γ-aminobutyric acid cotransporter; sodium/myo-inositol cotransporter; NFAT5

MAMMALIAN CELLS RESPOND TO hypertonic conditions by accumulating small organic molecules known as compatible osmolytes (9, 34). It has been suggested that the presence of these compounds balances increased extracellular tonicity and thus protects the cell from the deleterious effects of elevated intracellular electrolyte concentrations (34). The major compatible osmolytes in mammalian systems are glycerophosphocholine, sorbitol, betaine, myo-inositol, and taurine. With the exception of glycerophosphocholine, increased levels of these molecules result from the upregulated expression of synthetic enzymes, e.g., aldose reductase (AR) (27), which converts glucose to sorbitol, or specific transporters, e.g., the betaine/γ-aminobutyric acid transporter (BGT1) (31), the sodium/myo-inositol transporter (SMIT) (33), and the taurine transporter (30). Analysis of the promoter regions of the AR (8, 14), BGT1 (28, 19), and SMIT (24) genes has revealed an 11-bp tonicity-responsive enhancer, TonE (also known as the osmotic regulatory element or ORE). This enhancer sequence drives the expression of reporter genes in a hypertonicity-inducible manner and is protected in DNA footprinting assays when cells are exposed to hypertonic conditions (19, 20, 24).

Recently, we reported the identification and cloning of TonE-binding protein (TonEBP), a tonicity-responsive transcription factor that associates with the TonE sequence (21). TonEBP is a ubiquitously expressed protein that appears to bind TonE through a rel-like NH₂-terminal domain. Studies in Madin-Darby canine kidney (MDCK) cells have shown that TonEBP localizes to the cytoplasm and the nucleus in isotonic conditions and gradually shifts to a predominantly nuclear localization when cells are cultured in hypertonic medium. This redistribution occurs over the course of 8–10 h and is accompanied by a fourfold increase in TonEBP expression.

We have continued our analysis of TonEBP and report that the switch of MDCK cells from isotonic to hypertonic conditions results in an increase in TonEBP phosphorylation. This change occurs within 30 min of exposure to hypertonicity and precedes observable accumulation of TonEBP in the nucleus. The data suggest that phosphorylation may promote the nuclear localization or retention of TonEBP.

EXPERIMENTAL PROCEDURES

Cell culture. MDCK cells (type II) were obtained from American Type Culture Collection and cultured in a defined medium as previously described (32). The culture medium was made hypertonic by the addition of sterile 5 M NaCl to a final concentration equal to 100 mM over isotonic medium. Metabolic labeling with [³²P]orthophosphate (NEN/DuPont) was performed by incubating the cells in phosphate-free medium (Biofluids) containing 500 μCi [³²P]/ml for 2 h before hypertonic shift.

Immunoprecipitations. Chemicals were obtained from Sigma Chemical unless otherwise stated. MDCK cells were cultured in 60-mm dishes and metabolically labeled as described above. Cell layers were washed once with ice-cold PBS (isotonic or hypertonic as appropriate) containing 2 mM

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phenylmethylsulfonyl fluoride (PMSF) and lysed on ice for 10 min in 1 ml of NET-400 (5 mM Tris, pH 8.0, 400 mM NaCl, 5 mM EDTA, 1% NP-40, 2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM Na3VO4, 50 mM β-glycerophosphate, and 50 mM NaF). The lysate was passed through a 1-ml pipetman tip multiple times to dislodge the cell layer from the dish, transferred to a 1.5-ml microfuge tube, and incubated on ice for an additional 30 min. The lysate was then centrifuged at 5 min, 16,000 g to pellet insoluble material. The resulting supernatant was incubated for 60 min with sufficient TonEBP antiserum (21) to quantitatively immunoprecipitate the protein (10 μl of antiserum per 1 ml of lysate) and agitated with continuous end-over-end mixing at 4°C. The immune complexes were collected by a 30-min incubation as described above in the presence of excess Osmisorb (Calbiochem). The collected complexes were then washed twice in NET-400, resuspended in 50 μl of sample buffer (25 mM Tris, pH 6.8, 1% SDS, 1% mercaptoethanol, 10% glycerol, and 0.25 mg/ml bromphenol blue), and heated at 100°C for 3 min. The samples were separated on 9% low-bis (6) or 2–15% gradient (Owl Separation Systems) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (MSI/Osmonics) by the method of Towbin et al. (29) with substitution of ethanol for methanol.

Phosphatase digestion. TonEBP was immunoprecipitated as described above. The collected immune complexes were then washed in NET-400 and resuspended in 100 μl of a buffer containing 50 mM Tris, pH 8.0, and 1 mM MgCl2. After a 10-min preincubation at 30°C, 30 μl of calf intestinal phosphatase (New England Biolabs) were added, and the immunoprecipitates were incubated at 30°C for an additional 15 min. The samples were then washed and processed as described above and resolved on 8% low-bis SDS-polyacrylamide gels.

Phosphoamino acid analysis. Cell lysates were immunoprecipitated from equal cell numbers of isotonic and hypertonic MDCK cells as described above and transferred to Immobilon polyvinylidene difluoride membranes as directed by manufacturer’s instructions (Millipore). The membrane was exposed to a PhosphorImager screen (Molecular Dynamics), and the image was printed onto transparency acetate and used as a mask to slice out pertinent bands. After the sections of membrane containing the bands of interest were removed, the strips were sliced into 2-mm2 pieces and incubated in 6 M HCl at 80°C for 2 h. The liquid was transferred to a new tube and lyophilized in a Speed-Vac (Savant). The resultant pellet was resuspended in 10 μl of water and dotted onto nitrocellulose-coated, glass-backed TLC plates (Merck) along with phosphoserine, phosphothreonine, and phospho-tyrosine standards (Sigma Chemical). TLC was performed as described previously (11). Briefly, dotted samples were dried and incubated in the first dimension in a 1:1:1 solution of ethanol-acetic acid-water. The plate was then run in the second dimension in an 8:3:4 solution of isobutanol-formic acid-water. Phosphoamino acid standards were resolved by spraying the plates with 0.2% ninhydrin in 95% ethanol and incubating them at 70°C for 10 min. The plates were then exposed to PhosphorImager cassettes, and the resultant images were printed on transparency acetate and superimposed on the ninhydrin-developed TLC plates. Since material from equal cell numbers was used in these experiments, the difference in intensity observed on the autoradiogram between isotonic- and hypertonic-derived material is due to the reduced number of phosphorylated residues in TonEBP from isotonic cells.

Kinase inhibitor studies. Kinase inhibitors were prepared in DMSO according to the manufacturer’s instructions. Four inhibitors were used, including genistein (100 μM final concentration; Calbiochem), PD-98059 (50 μM final concentration; New England Biolabs), PP-2 (30 μM final concentration; Calbiochem), and SB-203580 (100 μM final concentration; Calbiochem). MDCK cells were cultured in the presence of kinase inhibitors or carrier alone for 1 h and then incubated for an additional 4 h in isotonic or hypertonic media containing [32P]orthophosphate and kinase inhibitor. The cells were lysed, immunoprecipitated for TonEBP, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The blots were exposed to a PhosphorImager cassette and then probed for TonEBP.

Immunofluorescence. Cells were grown on 18 × 18-mm glass coverslips and cultured in isotonic or hypertonic media as appropriate. Cells were washed once with PBS and fixed in ice-cold 10% Formalin in PBS for 10 min. The cells were then extracted for 5 min in room temperature Tris-buffered saline (50 mM Tris, pH 8.0, and 150 mM NaCl) containing 0.1% Triton X-100. Nonspecific binding sites were blocked with 5% BSA in Tris-buffered saline for 15 min; then the coverslips were incubated in a 1:100 dilution of TonEBP antiserum in blocking solution for 30 min at 37°C. After incubation with primary antibody, the coverslips were washed three times in PBS and incubated in a 1:100 dilution of rhodamine-conjugated donkey anti-rabbit antisemur (Zymed) in blocking solution for 30 min at 37°C. The coverslips were then washed three times with PBS, mounted in Fluoromount-G (Southern Biotechnology), and observed on a Nikon microscope equipped for epifluorescence illumination.

TonE binding assay. TonEBP was immunoprecipitated from MDCK cells cultured for 18 h in isotonic or hypertonic medium as described above, except the lysis and wash buffers contained 150 mM NaCl (NET-150). One-half of the immu...
noprecipitated material was treated with phosphatase as described above. The phosphatase-treated and untreated immunoprecipitates were then washed in NET-150 and incubated at room temperature for 10 min in 20 μl of HKMEG buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, and 5% glycerol) supplemented with 1.5 μg of poly(dA-dT) (Amersham), 1 mM dithiothreitol, and 10 μg/ml nonfat dry milk (Carnation). After this incubation, 10 fmol of 32P-labeled hTonE (TGGAAAATTAC) or a mutated TonE, M2, which has a lower affinity for TonEBP (TTGAAAATTAC) (18), were added to the immunoprecipitates, and the incubation was continued for an additional 20 min. The immunoprecipitates were then washed once in HKMEG buffer containing 100 mM KCl, resuspended in 50 μl of HKMEG buffer, and cross-linked with ultraviolet light (Stratalinker, Stratagene) in 96-well dishes (Corning) on ice for 1 h. The immunoprecipitates were then washed once in NET-150 and processed as described above for SDS-PAGE.

RESULTS

Exposure of cells to hypertonicity activates a variety of protein kinases (4, 15). To determine whether TonEBP is a target for hypertonicity-induced phosphorylation, MDCK cells were labeled with [32P]orthophosphate and exposed to hypertonic conditions. Figure 1 depicts immunoblots and corresponding autoradiograms of TonEBP immunoprecipitated from isotonic cells and cells exposed to hypertonicity for 15, 30, 60, and 120 min. No change in the total amount of TonEBP was observed during the time points studied, as is evident in the immunoblot. Radiolabeled phosphate was incorporated into TonEBP under both isotonic and hypertonic conditions. Exposure to hypertonicity resulted in an increase in phosphorylated TonEBP within 30 min of treatment, as noted by the

The data presented are representative of three or more independent experiments.

Fig. 2. Phosphorylation alters TonEBP mobility in SDS-PAGE. Immunoblot and autoradiogram of TonEBP immunoprecipitated from MDCK cells were labeled with [32P]orthophosphate for 4 h in isotonic or hypertonic medium. Immunoprecipitates were incubated in the presence (Ic and Hc) or absence (I and H) of calf intestinal phosphatase and resolved by SDS-PAGE on 8% low-bis gels that were run until the 66-kDa marker was off the gel. Phosphatase treatment yielded TonEBP protein bands of similar size.

Fig. 3. Phosphoamino acid analysis of TonEBP. TonEBP from 32P-labeled isotonic and hypertonic MDCK cells was hydrolyzed, and the amino acids were separated by 2-dimensional TLC. The position of phosphoamino acid standards phosphoserine (Ser), phosphothreonine (Thr), and phosphotyrosine (Tyr) are indicated.

Fig. 4. Effect of kinase inhibitors on TonEBP phosphorylation. Immunoblot and autoradiogram of TonEBP immunoprecipitated MDCK from isotonic and hypertonic [32P]orthophosphate-labeled MDCK cells were exposed to kinase inhibitors for 30 min before and during a 4-h incubation in hypertonic medium. Samples treated with calf intestinal phosphatase are shown as markers for dephosphorylated TonEBP. Samples were resolved by SDS-PAGE on 8% low-bis gels. G, 100 μM genistein, a broad-range tyrosine kinase inhibitor; P, 30 μM PP-2, an inhibitor of the src family of kinases; S, 100 μM SB-203580, an inhibitor of p38 kinase; D, 50 μM PD-98059, an inhibitor of mitogen-activated protein kinase.
upward shift of TonEBP on the immunoblot and the increased incorporation of \(^{32}\text{P}\) in the autoradiogram. The reduced mobility of TonEBP in SDS-PAGE was due entirely to phosphorylation, as demonstrated by treatment with calf intestinal phosphatase (Fig. 2). TonEBP from isotonic and hypertonic cells resolved as similar-sized protein bands after phosphatase treatment, as indicated on the immunoblot. The corresponding autoradiogram confirmed that the phosphatase treatment was effective and essentially complete. In the absence of phosphatase, the data suggest that TonEBP from isotonic cells exists in phosphorylated and nonphosphorylated forms, whereas TonEBP from cells exposed to hypertonicity is mostly phosphorylated. Phosphoamino acid analysis of TonEBP immunoprecipitated from isotonic cells or cells subjected to hypertonicity for 4 h showed phosphorylation on serine and tyrosine residues in both conditions (Fig. 3). An apparent increase in serine phosphorylation relative to that for tyrosine is observed for TonEBP from hypertonic cells.

The use of protein kinase inhibitors has implicated a number of signal transduction pathways in the response of cells to hypertonicity. In particular, the p38 kinase inhibitor SB-203580 has been used to suggest a role for p38 in signaling osmotic stress. To determine the effect of kinase inhibitors on TonEBP phosphorylation, cells were pretreated with genistein, PP-2, SB-203580, and PD-98059, shifted to hypertonic medium, and immunoprecipitated for TonEBP. The immunoblot in Fig. 4 shows the amount of TonEBP immunoprecipitated from each culture condition. The corresponding autoradiogram shows that these kinase inhibitors had no effect on the phosphorylation of TonEBP, although changes were observed for other nonspecific proteins brought down with the immunoprecipitates.

We previously reported that the hypertonicity-induced nuclear accumulation of TonEBP is a gradual process that proceeds over several hours before reaching a plateau (21). Figure 5 documents the localization of TonEBP during the first few hours of exposure to hypertonicity. An increase in nuclear staining was apparent within 60 min (Fig. 5C) of induction of hypertonicity. In addition, the overall appearance of the nuclear/cytoplasmic boundary sharpened, perhaps as early as 30 min (Fig. 5B) after induction. Maximum nuclear staining was observed 8–10 h after the shift to hypertonic medium (data not shown).

Since the TonEBP of hypertonic cells is phosphorylated and localized to the nucleus, phosphorylation may play a role in the association of TonEBP with the TonE sequence. To investigate this possibility, immunoprecipitates of TonEBP from isotonic cells or cells exposed to hypertonicity for 4 h were treated with or without alkaline phosphatase, incubated with radiolabeled TonE or mutant TonE DNA, cross-linked with ultraviolet light, and resolved by SDS-PAGE. Figure 6 shows that the phosphorylation state of TonEBP had no effect on its association with TonE DNA in vitro. The immunoblot confirms that similar amounts of TonEBP were used within each assay condition, i.e., isotonic- or hypertonic-derived material. As expected on the basis of earlier studies (18), the autoradiogram
shows that an active TonE sequence, hTonE, bound to TonEBP efficiently while an inactive mutant, mTonE2, did not.

DISCUSSION

In this report we have extended our characterization of TonEBP to implicate phosphorylation as a potential modulator of TonEBP activity. Previously, in a study describing the cloning of TonEBP (21), we noted an increased nuclear localization of the molecule in response to hypertonic stress and suggested that TonEBP was activated by hypertonicity. Subsequent to this report, TonEBP was identified as a phosphoprotein by Lopez-Rodriguez et al. (17), who recovered TonEBP (which they named NFAT5) from an expressed sequence tag database screen for NFAT-like molecules. Their data showed that a myc-tagged TonEBP construct was phosphorylated in transfected HEK 293 cells and demonstrated a nuclear localization of endogenous TonEBP in a number of cultured cell lines. Cytoplasmic staining, although not mentioned in the text, was observable, particularly in a comparison of matched immunofluorescence and phase-contrast micrographs, and may reflect an isotonic pattern of TonEBP distribution similar to that described in this report and above (21). In contrast to other NFAT family members, treatment with effectors of NFAT protein activity, such as calcineurin, phorbol esters, cyclosporin A, and ionomycin, had no effect on TonEBP phosphorylation or nuclear localization. Since the nuclear translocation of NFAT proteins is dependent on a loss of phosphorylation (21, 23), the data suggest that, despite a degree of similarity in the rel-homology domain, TonEBP is not a member of the NFAT family.

We have shown that TonEBP phosphorylation and nuclear localization are affected by hypertonicity and their temporal correlation is notable. On the basis of reduced electrophoretic mobility and immunofluorescence localization, the majority of TonEBP in hypertonic cells is phosphorylated and localized to the nucleus. In isotonic cells, TonEBP is less phosphorylated and is diffusely distributed to nuclear and cytoplasmic locations. Since we observed no phosphorylation dependence on the ability of TonEBP to associate with TonE sequences, the data suggest that TonEBP phosphorylation may target the protein for nuclear import. In this case, phosphorylation would act as a binary switch dictating import (phosphorylated form) or export (nonphosphorylated form) (13). Alternatively, TonEBP phosphorylation may be involved in nuclear retention by promoting stable protein-protein interactions with scaffolding proteins or subunits of a transcription factor complex. In this scenario, TonEBP phosphorylation may occur before or after nuclear import. We are in the process of determining which of the 197 serines and 13 tyrosines in TonEBP are phosphorylated in response to hypertonicity.

The signaling pathways leading to the accumulation of compatible osmolytes in response to hypertonic stress have been best elucidated in yeast (18). Yeast accumulates glycerol during hypertonic stress through activation of the high-osmolarity glycerol response (HOG) pathway, a primary component of which is the MAP kinase Hog1 (3). The mammalian homolog of Hog1 is p38, also known as SAPK2 (12). Studies with SB-203580, an inhibitor of p38, have implicated this kinase in the hypertonicity-induced upregulation of AR, BGT1, and SMIT mRNAs (6, 22, 26). These experiments are based on 6- to 18-h incubations of cells in the presence of inhibitor. We observed no effect of SB-203580 on TonEBP phosphorylation in our relatively short-term inhibitor and hypertonicity exposures. Although these data suggest that p38 does not play a role in the phosphorylation and nuclear translocation of TonEBP, SB-203580 exerts an effect on p38α and p38β, but not p38γ (SAPK3) or p38ε (SAPK4) (5, 10). Thus a role for these kinases in the phosphorylation of TonEBP cannot be ruled out. It should be noted, however, that SB-203580 has recently been shown to have inhibitory effects on cyclooxygenase-1 and -2 as well as thromboxane synthase (2). In addition, SB-203580 has also been reported to inhibit phosphoinositide-dependent protein kinase 1 (16). On the basis of these findings, experiments with SB-203580, especially those involving long-term exposure, may be confounded and need to be interpreted with caution.

Experiments with kinase-deficient mutants of p38 have been used effectively to demonstrate a role for p38 in the hypertonicity-induced activation of STAT1 (signal transducer and activator of transcription 1) (1). Similar experiments should delineate the role of p38 in the hypertonic induction of AR, BGT, and SMIT mRNA.

In summary, the phosphorylation of TonEBP and its translocation to the nucleus are early steps in the cellular response to hypertonic stress and may provide a sensitive assay for delineating the signal transduction pathways involved in these processes. In addition, it remains to be determined whether TonEBP is a component of a larger TonE-binding complex or capable of self-association. Experiments designed to address these questions should provide further clues into the regulation of this important biological response.

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