Multiple Domains of TonEBP Cooperate to Stimulate Transcription in Response to Hypertonicity*

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TonEBP, also known as NFAT5, belongs to the Rel family of transcriptional activators. In the kidney medulla and thymus, TonEBP plays a major role in protecting renal cells and T cells from the deleterious effects of ambient hypertonicity. TonEBP is stimulated by hypertonicity via several pathways: increased expression of protein, nuclear translocation, and increased transactivation. In this study, we identified five domains of TonEBP involved in transactivation. The two conserved glutamine repeats were not involved in transactivation. There were three activation domains that could stimulate transcription independently. In addition, there were two modulation domains that potentiated the activity of the activation domains. One of the activation domains is unique to a splice isoform that is more active than others, indicating that alternative splicing can affect the activity of TonEBP. Another activation domain and one of the modulation domains were stimulated by hypertonicity. All the five domains acted in synergy in every combination. Although overall phosphorylation of TonEBP increased in response to hypertonicity, phosphorylation of the activation and modulation domains did not increase in isolation. In sum, TonEBP possesses far more elaborate domains involved in transactivation compared with other Rel proteins.

The function of TonEBP was initially cloned from the kidney medulla as a transcriptional activator that stimulates genes in response to ambient hypertonicity, i.e. hyperosmotic salt (1). Osmolality of the mammalian kidney medulla is very high, often 10 times that of the blood or higher, because of high concentrations of salt and urea. The hyperosmolality provides the driving force for water reabsorption and urinary concentration and, thereby, contributes to maintenance of the body fluid volume and blood pressure (6). Because of the hypertonicity, TonEBP activity in the kidney medulla is high (7). By stimulating its target genes, TonEBP contributes to the function of the kidney medulla. TonEBP enhances expression of the vasopressin-regulated urea transporter, a key molecule in the generation of the high urea concentration in the kidney medulla (8). Unfortunately, the hyperosmolality imposes a great deal of stress to cells, causing double-stranded DNA breaks (9) and cell death (10). TonEBP also plays a major role in the protection of cells in the kidney medulla in two ways (see Ref. 11 for a recent review). First, TonEBP stimulates several genes to encode plasma membrane transporters and aldose reductase that catalyze the cellular accumulation of organic osmolytes. Organic osmolytes protect cells from the deleterious effects of hypertonicity by lowering the intracellular ionic strength. Second, TonEBP stimulates expression of heat shock protein 70 that protects cells from the deleterious effects of high urea (12).

TonEBP is abundantly expressed throughout development (13) and in many adult tissues outside the kidney such as brain, heart, and thymus (14). The function of TonEBP is understood in the thymus. T cell receptor activation leads to an increased TonEBP expression in T cells (14). When TonEBP activity is inhibited in the thymus, the number of T cells decreases (15). It appears that the osmoprotective function of TonEBP is required for proliferation/survival of T cells in the thymus. In mature T cells, TonEBP stimulates the expression of tumor necrosis factor-α and lymphotixin β (16). The function of TonEBP in other organs is unknown, except that TonEBP seems to participate in the integrin-mediated carcinoma invasion (17). In Drosophila, recessive lethal mutations of MSER1 have been reported (18). Overexpression of MSER1 resulted in the suppression of RAS signaling (5) and axon guidance/synaptogenesis phenotypes (19).

How TonEBP is stimulated by hypertonicity is of great interest. This involves increased TonEBP abundance caused by

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The abbreviations used are: TonE, tonicity-responsive enhancer; TonEBP, TonE binding protein; NFAT, nuclear factor of activated T cells; RHD, Rel-homology domain; AD, activation domain; MD, modulation domain; GAL4DBD, GAL4 DNA binding domain.
increased transcription (20), nuclear translocation (21, 22), and increased transactivation (23). Although phosphorylation of TonEBP is increased by hypertonicity (21), the role of phosphorylation in the stimulation of TonEBP is not known. Recent studies implicated the p38 mitogen-activated protein kinase, Fyn (24), and protein kinase A (25) in transactivation. In this study, we defined five domains of TonEBP involved in transactivation. Our data do not provide evidence that stimulation of TonEBP transactivation involves phosphorylation of TonEBP.

EXPERIMENTAL PROCEDURES

DNA Constructs—All constructs were generated by using standard cloning procedures and verified by restriction enzyme digestion and DNA sequencing. Each TonEBP cDNA fragment was generated by PCR or by using restriction enzymes and cloned into pCMV-Tag or pFA-CMV (Stratagene, La Jolla, CA), which allows the expression of TonEBP fragments fused with the GAL4 DNA binding domain (GAL4DBD). Where indicated (see Fig. 3), the polyglutamine repeats were deleted by using two independent PCRs, which led to the insertion of glycine and serine in place of the glutamines. Serial deletion mutants from the C terminus of 618–1531 fragments were made by using the Erase-a-Base System (Promega, Madison, WI).

Cell Culture and Transfection—COS7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 100 unit/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). COS7 cells were transfected using LipofectAMINE 2000 as instructed by the manufacturer (Invitrogen). To examine the effects of protein kinase inhibitors (see “Discussion”), NIH3T3 cells were maintained in the same conditions as COS7 cells except that bovine calf serum (Invitrogen) was used and FuGENE6 (Roche Applied Science) was used for transfection, as reported earlier (24).

Luciferase Assay—Cells grown in each well of a 24-well cluster were transfected with 0.2 μg of GAL4 upstream activation sequence-driven photinus luciferase expression construct (pFR-Luc); 0.2 μg of pFA-CMV plasmids directing expression of the TonEBP fragments in fusion with GAL4DBD, and 2 ng of pRL-CMV (Promega) directing expression driven by the cytomegalovirus promoter. In some experiments (see Figs. 6C and D and 10), TonE-driven photinus luciferase expression construct (1) and pCMV-Tag plasmids directing expression of myc- or FLAG-tagged TonEBP fragments were used. The cells were cultured for 20 h, and then some of the wells were switched to hypertonic medium made by the addition of 100 mM NaCl. Activity of luciferase was measured 4 h or 20 h later from cell lysates using a commercial kit, Dual-Luciferase Assay System (Promega). For each extract, activity of the photinus luciferase was divided by the activity of the renilla luciferase to correct for transfection efficiency.

Immunoblot Analysis—To detect the expression of the fusion proteins, immunoblot analysis was performed on cells transfected as described above. To prepare whole cell extracts, cells were washed once with ice-cold phosphate-buffered saline and lysed in lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, protease inhibitor mixture (Roche Applied Science), and phosphatase inhibitor mixture (Roche Applied Science) for 30 min at 4 °C. The extracts were then cleared by centrifugation for 5 min at 15,000 × g. Whole cell extracts were separated on a 4–15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with 5% nonfat milk in Tween-Tris-buffered saline (25 mM Tris, pH 7.4, 137 mM NaCl, 3 mM KCl, 0.05% Tween-20) for 30 min at room temperature. The membrane was then incubated with 1/200 dilution of antibody (Santa Cruz Biotechnologies Inc) or anti-FLAG M2 antibody (Stratagene, La Jolla, CA), which allows the expression of TonEBP fragments fused with the GAL4 DNA binding domain (GAL4DBD). Where indicated (see Fig. 3), the polyglutamine repeats were deleted by using two independent PCRs, which led to the insertion of glycine and serine in place of the glutamines. Serial deletion mutants from the C terminus of 618–1531 fragments were made by using the Erase-a-Base System (Promega, Madison, WI).

RESULTS

Isoform-specific Activation Domain (AD)—Because of alternative splicing in exons 2–4, there are 4 forms of mouse TonEBP named the a-, b-, c-, and d-forms (13). The b-, c-, and d-forms have an additional 52, 76, and 94 amino acids, respectively, at the N terminus of the a-form, which is the shortest. In various human cells, we have found the a-, c-, and d-forms but not the b-form of TonEBP (not shown). The c-form of TonEBP is more active in the stimulation of transcription than the a-form (13). We suspected that there might be an additional transactivation domain in the N-terminal 76 amino acids of...
TonEBP-c. With this in mind, we decided to search the entire TonEBP-c for domains involved in transactivation by fusing TonEBP fragments with the GAL4DBD, as shown in Fig. 1A. Three of the four TonEBP fusions stimulated transcription of the reporter gene by virtue of their binding to the GAL4 binding sites upstream (Fig. 1C). The C-terminal fragment, amino acids 1044–1531, was most active, whereas the N-terminal fragment, amino acids 1–548, was least active.

The lack of activity by the 406–876 fragment was not due to low level of expression. When the expression of the fusion protein was increased up to 4 times by increasing the amount of the expression vector, expression of luciferase was not affected (not shown). In several other fusion constructs tested, variations in the level of expression (up to 4-fold) did not influence their transcriptional activity (measured by expression of luciferase, not shown). Activity of transfected TonEBP isoforms was also independent of its level of expression in COS7 cells (13). In the experimental conditions used in this study, it seems that the constructs expressed at levels above the functionally maximum point.

We examined the N-terminal fragment to locate precisely the region responsible for transactivation. As shown in Fig. 2A, the TonEBP fragment containing amino acids 1–76 or 1–171 was active, whereas other fragments devoid of the N-terminal 76 amino acids were inactive. These data indicate that the N-terminal 76 amino acids contain a transactivation domain. This domain is AD1 (see Fig. 5). Because AD1 is unique to TonEBP-c, it should be at least partially responsible for the higher activity of TonEBP-c compared with TonEBP-a (13).

**The Glutamine Repeats of TonEBP Do Not Stimulate Transcription**—In human or mouse TonEBP, there are two glutamine repeats in the C terminus (Fig. 1A). MSER1, the Drosophila homolog of TonEBP, also contains two glutamine repeats (5). Because 10 or more repeats of glutamines were known to stimulate transactivation (26), we asked whether the glutamine repeats in TonEBP were responsible for the transactivation by the C-terminal fragments (Fig. 1). Deletion of either the glutamine repeats did not affect the activity of the C-terminal fragments (Fig. 3). Thus, the evolutionarily conserved glutamine repeats are not involved in transactivation. Although glutamine repeats also play a role in protein-protein interaction (27), they are not involved in the dimerization of TonEBP (28). The function of the glutamine repeats of TonEBP remains unknown.

**Activation Domains 2 and 3; AD2 Is Stimulated by Hypertonicity**—The two overlapping C-terminal fragments of TonEBP shown in Fig. 1 displayed far greater activity than the N-terminal fragments, and their activity was stimulated by hypertonicity. A larger C-terminal fragment with amino acids 618–1531 of TonEBP that covers the two C-terminal fragments displayed tonicity-responsive transactivation as expected (Fig. 4). To define domains of transactivation within the 618–1531 region, serial deletions were made from the C terminus (Fig. 4A). As shown in Fig. 4B, there were two large peaks and a small peak (Fig. 4B, Inset) of activity. From these and other data shown below, we concluded that the large peaks represented activation domains (AD2 in amino acids 1039–1249 rather than 1039–1276 because of the glutamine repeats in 1250–1266, and AD3 in amino acids 1364–1476), and the small peak was due to modulation domains (see below), as summarized in Fig. 5.

Deletion of AD2 or AD3 from the GAL4DBD-TonEBP fusion (Fig. 6B) or TonEBP itself (Fig. 6D) led to a decrease in transcription. When AD2 or AD3 was isolated, on the other hand, it stimulated transcription (Fig. 6F) like AD1 (Fig. 2). The three
activation domains displayed remarkable synergy in all possible combinations (Fig. 7). Of interest, AD2, but not AD1 or AD3, was stimulated by hypertonicity in COS7 cells (see also Figs. 1 and 6F). When AD2 was combined with AD1 or AD3, the response to hypertonicity was maintained. When AD1 and AD3 were combined without AD2, the response to hypertonicity was not observed. We conclude that TonEBP contains three domains, AD1, AD2, and AD3, that stimulate transcription. They act synergistically, and only AD2 is stimulated by hypertonicity in COS7 cells (see below for more).

Modulation Domains (MD) 1 and 2; MD1 Is Stimulated by Hypertonicity—When the AD1, AD2, and AD3 were combined (Fig. 7, AD123), their activity was significantly lower than the TonEBP fragment 618–1531 (Figs. 4B and 6B), indicating that other domains were required for full activity. We noticed that the 618–955 fragment stimulated transcription (small peak in Fig. 4B, Inset), and that this activity was stimulated by hypertonicity. Because the 618–820 fragment (Fig. 4B, Inset), the 406–876 fragment (Fig. 1C), and the glutamine repeats at 879–888 (Fig. 3) were not active, we examined the fragment 889–955 named MD2. Unexpectedly, this fragment was not active (Fig. 6F), hence the name modulation domain rather than activation domain. When MD2 was deleted from the GAL4DBD-TonEBP fusion (Fig. 6B) or TonEBP itself (Fig. 6D), transcriptional activity decreased as in the deletion of either AD2 or AD3. When fused to the activation domains, MD2 enhanced their activity dramatically (Fig. 6D). Thus, MD2 does not stimulate transcription by itself, but it potentiates the activity of the activation domains.

To identify the source of the hypertonicity-inducible activity of the 618–955 fragment (Fig. 4B, Inset), we examined the fragment 618–820 (MD1) further. Like MD2, MD1 dramatically potentiates activity of all of the activation domains, whereas MD1 itself is not active in the stimulation of transcription (Fig. 6B). Furthermore, MD1 made AD1 and AD3 responsive to hypertonicity and increased the responsiveness of AD2 to hypertonicity. We suggest that MD1 is responsible for the
hypertonicity responsiveness of the AD2-deleted 618–1531 construct (Fig. 6B/H9004 MD2). This is further supported by the observation that the 618–1531 construct was stimulated by hypertonicity even when amino acids 821–1249 (i.e. MD2 and AD2) were deleted (not shown).

In summary, MD1 or MD2 is inactive in transactivation unless both are fused together. They potentiate the activity of AD1, AD2, and AD3. In addition, MD1 renders AD1 and AD3 responsive to hypertonicity and increases the responsiveness of AD2 (Fig. 8).

**Phosphorylation Does Not Correlate with the Stimulation of Transcription in Response to Hypertonicity**—To explore the potential role of phosphorylation in the hypertonicity-induced transactivation, we examined phosphorylation of the GAL4DBD-TonEBP fragment fusions shown in Fig. 1. Although the active fusion proteins were phosphorylated in isotonic conditions, the level of phosphorylation did not change in response to hypertonicity even in the two C-terminal TonEBP fragments (amino acids 733–1204 and 1044–1531; Fig. 9A) whose activity was tonicity-responsive (Fig. 1). On the other hand, we noticed that phosphorylation of the larger fusion protein containing amino acids 618–1531 of TonEBP increased dramatically in response to hypertonicity (Fig. 9A). To define the regions involved in the tonicity-responsive phosphorylation, we examined phosphorylation of the C-terminal deletion constructs shown in Fig. 4. The hypertonicity-induced phosphorylation disappeared completely when amino acids were deleted to 1233 (Fig. 9B), even though all the fusion constructs displayed hypertonicity-induced transactivation (Fig. 4). Similar results were obtained when the C-terminal deletions were made from TonEBP without the GAL4DBD fusion (Fig. 10). Thus, in the various TonEBP deletion constructs, the level of phosphorylation did not correlate with transactivation.

It is possible that phosphorylation of critical residues may be a very small portion of the total phosphorylation because there are 327 serines and threonines plus 15 tyrosines in the c-form of TonEBP. If this is the case, it might be easier to detect the hypertonicity-responsive phosphorylation in smaller TonEBP constructs. The smallest functional constructs, i.e. the GAL4DBD fusions to AD1, AD2, and AD3 (Fig. 7), were examined for phosphorylation. As shown in Fig. 9C, phosphorylation of these proteins (including the GAL4DBD-AD2, which displayed hypertonicity-induced transactivation) did not increase in hypertonicity. In sum, we have not found evidence that hypertonicity-induced phosphorylation of TonEBP plays a role in the stimulation of transactivation.

**DISCUSSION**

Collectively the data demonstrate that multiple domains of TonEBP cooperate to stimulate transcription. Three activation domains, AD1, AD2, and AD3, are distinguished from the two modulation domains, MD1 and MD2, by virtue of their ability to stimulate transcription independently. On the other hand, both activation domains and modulation domains are synergistic in all combinations tested (Figs. 4, 6–8). The amino acid sequence of these domains does not resemble any of the known motifs in current databases. Although 21% of amino acids in TonEBP are serines and threonines, 19–28% of amino acids of the activation and modulation domains are serines and threonines. AD1 is enriched with proline (12%) and acidic residues (13%). The tonicity-responsive AD2 and MD1 have more glutamine residues (22 and 19% of the amino acid residues, respectively) than the whole TonEBP (14%), AD1 (2.6%), AD3 (18%), or MD2 (16%). This is generally in line with the three classical types of transactivation domains: acidic, proline-rich, and glutamine-rich (29).
Transactivation domains of the NFAT family have been characterized mainly in NFAT1. Of the two domains, one is in the N-terminal and 100 residues, which is rich in acidic residues and proline (30). Corresponding regions in other members of the NFAT family are also rich in acidic residues and proline, suggesting that they are also transactivation domains. The other transactivation domain of NFAT1 is located C-terminal to the DNA binding domain (31). It is not clear whether other members also have C-terminal transactivation domains.

More information is available for transactivation of the NFκB family. In the NFκB p65/p50 heterodimer, the transactivation domain is found only in the p65 subunit at the C terminus (32). This domain stimulates transcription by means of interacting with transcriptional coactivators CBP/p300. Of interest, this pathway is regulated by protein kinases. Phosphorylation of the p65 subunit at Ser-276 by protein kinase A enhances transactivation because the phosphorylated Ser-276 binds CBP/p300 independently of the transactivation domain, creating a stronger bivalent interaction (33). The cyclin-dependent kinase 2 that is associated with p300 inhibits the transactivation by p65 (34).

When a cell is exposed to hypertonicity, a number of protein kinases including p38 mitogen-activated protein kinase and Fyn are activated (35). However, our data do not provide support for a model in which protein kinases directly phosphorylate TonEBP and, as a result, influence its transactivation. In many of the truncated constructs of TonEBP, increased transactivation is not associated with increased phosphorylation (36). Stimulation of AD2 by hypertonicity is not accompanied by an increase in phosphorylation of AD2 itself (Fig. 9C). As AD2 contains 44 serines and threonines, however, we cannot exclude the possibility of increased phosphorylation in a small number of residues that would not be detectable with the method used. On the other hand, unlike the previous report (24), we did not observe consistent inhibition of transactivation by p38 mitogen-activated protein kinase and Fyn (not shown). Even though we found that H89, an inhibitor of protein kinase A, consistently inhibited the activation domains in hypertonic conditions (not shown) as reported (25), its effects seemed to be indirect, because AD1 that was not activated by hypertonicity was also inhibited by it (not shown). The role of increased phosphorylation of TonEBP in transactivation is unknown.

We conclude that transactivation of TonEBP is mediated by five discrete domains distributed throughout the TonEBP molecule. Thus, TonEBP displays far more elaborate transactivation domains compared with the other Rel proteins, NFκB and
NFAT. All of the domains cooperate to stimulate transcription. Stimulation of transactivation by TonEBP in response to hypertonicity is mediated by two of the domains, AD2 and MD1. The data do not provide evidence that TonEBP is a direct substrate of the tonicity-responsive protein kinases. The domains provide tools to investigate mechanisms involved in transactivation by TonEBP.

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