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Alternative Isoforms of TonEBP with Variable N-termini are Expressed in Mammalian Cells

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Hypertonicity imposes a great deal of stress to cells since it causes rise in cellular ionic strength, which can be reduced by the accumulation of compatible osmolytes. TonEBP plays a central role in the cellular accumulation of compatible osmolytes via transcriptional stimulation of membrane transporters and aldose reductase. Alternatively spliced forms of TonEBP mRNA have previously been reported and two of them showed different transcriptional activity. In the present study, isoform-specific antibodies were produced to confirm the translation of the spliced mRNA to protein. TonEBP was immunoprecipitated by using anti-TonEBP antibody and then immunoblotted using anti-TonEBP or isoform specific antibodies to find out the expression profile of TonEBP isoforms in basal or stimulated condition. From these results, we conclude that all TonEBP isoforms are expressed in mammalian cells and their expression patterns are not same in every cells.

Key Words: TonEBP, Hypertonicity, Alternative splicing

INTRODUCTION

TonEBP plays a central role in the cellular accumulation of compatible osmolytes via transcriptional stimulation of membrane transporters and aldose reductase (Ko et al, 1997; Rim et al, 1998; Miyakawa et al, 1999a; Cha et al, 2001). The critical importance of TonEBP in mediating adaptation to hyperosmotic stress within the kidney has been demonstrated by targeted disruption of the TonEBP gene in mouse resulting in a null allele (Lopez-Rodriguez et al, 2004). Although the majority of homozygous TonEBP$^{-/-}$ animals die late in gestation or perinatally, approximately 3% of the homozygous progeny survive past weaning. These animals exhibit marked atrophy of the renal medulla that is associated with impaired activation of several TonEBP-regulated osmoprotective genes. Consistent with this result, transgenic over-expression of a dominant inhibitory form of TonEBP in the epithelial cells of the renal collecting tubules results in an impaired capacity to concentrate urine, leading to progressive hydronephrosis, that is also associated with defects in the expression of osmo-regulatory genes (Lam et al, 2004). Therefore, as expected from the unique physiology of mammalian kidney, which is characterized by the generation of a hyperosmotic environment within the renal medulla during antidiuresis, the osmotic stress response pathway which is defined by the TonEBP transcription factor is clearly essential for normal renal function.

TonEBP plays also a role in T-cell growth, cell migration and invasion. TonEBP is activated by T-cell receptor activation (Trama et al, 2000), and murine transgenic line expressing a dominant negative form of TonEBP results in lymphoid hypocellularity and impaired T lymphocyte growth (Trama et al, 2002). TonEBP is also activated by the cross-linking of $\alpha 6\beta 4$ integrin, and the expression of an inhibitory form of TonEBP in cancer cells impairs cell invasion (Jauliac et al, 2002). In addition, overexpression of TonEBP results in enhanced cell migration.

The human TonEBP gene is made up of 16 exons over 130 kbp (Maouyo et al, 2002). Due to alternative splicing involving exons 2~4, three different polypeptides are predicted. All the deduced splice isoforms share the same 1,455 amino acids of the "a" form (Miyakawa et al, 1999b) that includes the DNA binding domain. Other isoforms have additional 76~96 amino acids at the N-terminus. In the present study, we produced isoform-specific antibodies and showed that the isoforms are expressed as proteins.

METHODS

DNA constructs

All constructs were generated using standard cloning procedures and verified by restriction enzyme analysis and

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ABBREVIATIONS: TonEBP, Tonicity-responsive enhancer (TonE) binding protein; RHD, Rel Homology dornain.

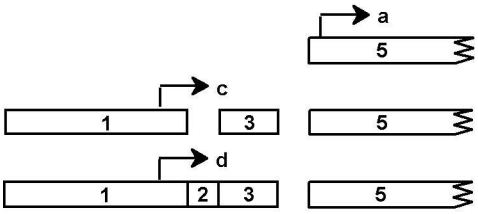
DNA sequencing. Each full-length human TonEBP cDNA was cloned into a mammalian expression vector pCMV-Tag2 (Stratagene, La Jolla, CA) which allows the expression of TonEBP fused with FLAG epitope. Each Y1 fragments of TonEBP were generated by PCR or using restriction enzyme and cloned into pCMV-Tag2.

Cell culture and transfection

COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Woodland, CA), 100 μg/ml penicillin, and 100 μg/ml streptomycin (Life Technologies Inc. Grand Island, NY). HeLa cells were maintained in DMEM supplemented with 10% horse serum (Quality Biological, Inc. Gaithersburg, MD), 100 μg/ml penicillin, and 100 μg/ml streptomycin. Jurkat cells were maintained in RPMI 1640 supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. mIMCD cells were maintained in DMEM/F-12 supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. CHO cells were maintained in MEM-alpha supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using Lipofectamine 2000 as instructed by the manufacturer (Life Technologies Inc. Grand Island, NY).

Antibodies, Immunoprecipitation, and Immunoblot

Each peptides corresponding to exon 2 (for anti-E2 antibody) or N-terminus of exon 3 (for anti-E3 antibody) were synthesized, purified, and then used to raise antibodies in rabbits through a commercial service (Covance, Denver, PA). For immunoprecipitation, cells were washed once with ice-cold phosphate-buffered saline (PBS) 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, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) for 30 min at 4°C. Lysates were centrifuged for 5 min at 15,000 g to pellet insoluble materials. Resulting supernatants (whole cell extracts) were incubated for 60 min with 10 μl of anti-TonEBP serum and 40 μl of protein A agarose (Life Technologies Inc. Grand Island, NY) to immunoprecipitate endogenous TonEBP at 4°C. The immune complexes were then washed four times in lysis buffer, resuspended in the sample buffer [62.5 mM Tris (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol], and heated at 95°C for 5 min. For immunoblot, whole cell extracts or immune complexes were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Nonspecific binding sites were blocked with 5% nonfat milk in TTBS [25 mM Tris (pH 7.4), 137 mM NaCl, 3 mM KCl, 0.05% Tween20] for 30 min at room temperature. The membrane was incubated with 1 : 200 dilution of anti-E3 antibody, with 1 : 600 dilution of anti-E2 antibody, with 1 : 2,000 dilution of anti-FLAG M5 antibody (Sigma, Saint Louis, MO) or anti-TonEBP antibody in blocking solution for 60 min at room temperature. The membrane was then incubated with 1 : 5,000 dilution of anti-mouse IgG or anti-rabbit IgG conjugated with alkaline phosphatase in the same way as described above (Jackson Immunoresearch, West Grove, PA). Alkaline phosphatase activity was visualized using a commercial kit (Sigma, Saint Louis, MO).



▲ amino acid sequences of exon 2

SLKLHPSQTFHRAGLLE
Exon2-specific => α-E2

▲ amino acid sequences of exon 3

SVYDLLPKELQLPPPRETSVASMSQTSGGEAGSPPPAVVAA
Exon3-specific => α-E3

Fig. 1. Schematic representation of TonEBP isoform, based on previous reports. Amino acid sequences corresponding to exon 2 and exon 3 of TonEBP are shown. Each peptides used in antibody production is underlined.

RESULTS

We previously reported that widespread alternative splicing in exons 2~4 was detected during development as well as in different adult tissues. Therefore, it is highly possible that different polypeptides with different lengths at the N-terminus might be produced. Two of the isoforms have been shown to be different in their ability to stimulate transcription (Maouyo et al, 2002). In the present study, therefore, isoform-specific antibodies were prepared to confirm the translation of the spliced mRNA to protein. Each peptides corresponding to exon 2 for TonEBP-d isoform or N-terminus of exon 3 for TonEBP-c and -d isoform were synthesized, and they were then used to raise antibodies as described in materials and method (Fig. 1). Affinity of each antibodies produced was tested using cell lysates which express FLAG-fused N-terminal fragment of TonEBP-d isoform (FLAG-Yd1) as antigen (data not shown). FLAG-fused full-length or Y1 fragments of each TonEBP isoforms were expressed in COS7 cells and then used for characterization of each antibodies (Fig. 2). Anti-FLAG antibody or anti-TonEBP antibody which recognizes all isoforms of TonEBP were used as a positive control to confirm the expression of each protein. TonEBP-c isoform (FLAG-Yc1 or FLAG-TonEBP-c) was detected by anti-E3 antibody, but not by anti-E2 antibody, whereas TonEBP-d isoform (FLAG-Yd1 or FLAG-TonEBP-d) was detected by both antibodies as expected. From these results, isoform specificity of each antibodies was confirmed; that is to say, anti-E2 antibody detects only TonEBP-d isoform, whereas anti-E3 antibody detects both TonEBP-c and -d isoforms.

To elucidate the expression profile of TonEBP isoforms in basal or stimulated condition, endogenous TonEBP was immunoprecipitated by using anti-TonEBP antibody and then immunoblotted using anti-TonEBP antibody or isoform specific antibodies (Fig. 3). In Jurkat T-cells and HeLa cells, TonEBP was not detected by both anti-E2 antibody and anti-E3 antibody, indicating that only TonEBP-a iso-

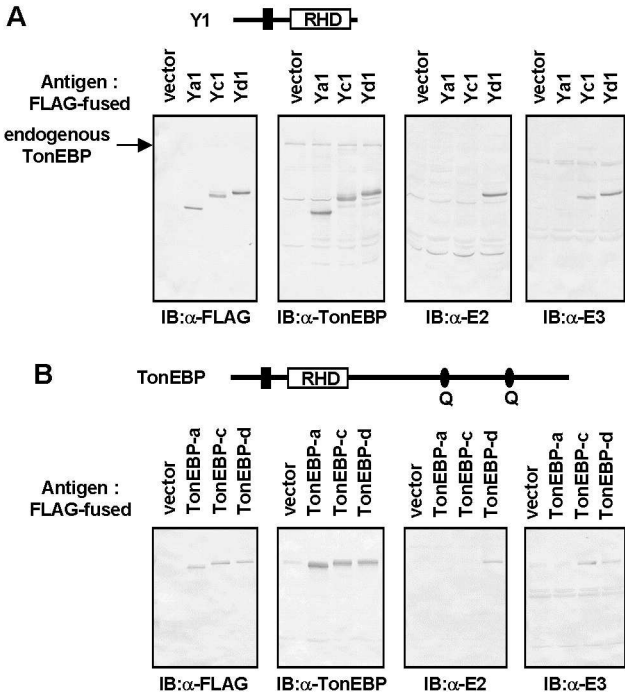


Fig. 2. Antibodies are specific to each TonEBP isoform. FLAG-fused full-length (B) or Y1 fragments (A) of each TonEBP isoform were used for characterization of each antibody. Anti-FLAG antibody and anti-TonEBP antibody were used as a positive control to confirm the expression of each protein.

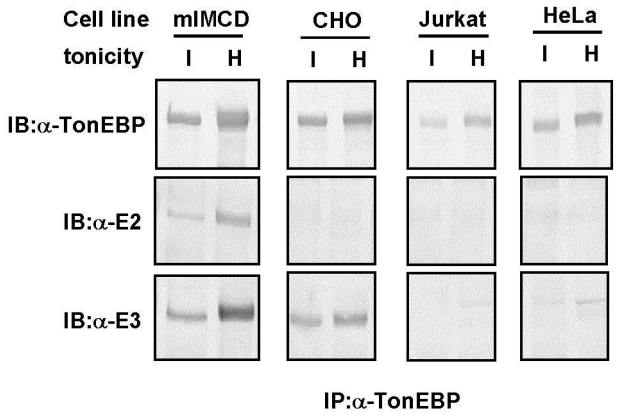


Fig. 3. Each isoforms of TonEBP are expressed in several mammalian cells. Each cell line was treated with 100 mM NaCl for 16 hours. TonEBP was immunoprecipitated from whole cell lysates using anti-TonEBP antibody. Immunecomplexes were then resolved on 6% SDS-polyacrylamide gels and probed using each antibody. I or H represents isotonic or hypertonic conditions, respectively.

form was expressed. On the other hand, TonEBP in mIMCD cells was detected by all antibodies used, suggesting that TonEBP-d isoform was expressed. TonEBP in CHO cells was detected by anti-E3 antibody, but not by anti-E2 antibody, indicating that TonEBP-c isoform was expressed. From these results, we can conclude that all TonEBP isoforms are expressed in mammalian cells and their expression patterns are variable, depending on cell types.

DISCUSSION

The amino acid sequence and gene structure of TonEBP are highly homologous between humans and mice. A genome database search reveals that Rel-like transcription factors are present in Drosophila and mammals, but not in Caenorhabditis elegans, yeast, and plants (Riechmann et al, 2000). Drosophila has one homolog of TonEBP, named as MESR1 (GenBank accession no. AF195496). MESR1 was identified as a modifier of RAS1 signaling involved in eye development (Huang & Rubin, 2000).

We and others previously detected widespread alternative splicing in exon 2~4 during mouse development and in several cell lines (Maouyo et al, 2002; Kim et al, 2005). Consequently, four different mRNA and three possible polypeptides with different lengths at the N-terminus are expected to be produced (Fig. 1). TonEBP-c and TonEBP-d isoform have an additional 76 and 94 amino acids, respectively, at the N-terminus of the TonEBP-a, which is the shortest. Compared with adult human brain, the abundance of the TonEBP-a transcript is much higher in the human fetal brain than other spliced isoforms (Dalski et al, 2000). In other tissue, there is little difference in the relative abundance of TonEBP isoforms between fetuses and adults. By using isoform-specific antibodies, we found that the expression patterns of TonEBP isoforms were different in several cell lines (Fig. 3).

Alternative splicing generates multiple protein isoforms from a single gene and contributes to the diversity of the eukaryotic proteome (Black, 2000; Graveley, 2001; Caceres & Kornblihtt, 2002). By varying the inclusion of functionally significant sequences, such as CoRNR boxes and their flanking sequences, it is possible to generate corepressor isoforms that differ in their affinity for transcription factors (Goodson et al, 2005). This is likely to contribute to the refinement of the regulation of gene expression. Insertion of N-terminal 76 and 94 amino acids in the TonEBP-c and TonEBP-d isoform results in different basal and hypertonicity-induced TonEBP activity (Maouyo et al, 2002; Kim et al, 2005), and TonEBP-c shows the highest activity in both isotonic and hypertonic conditions.

Another insertion of 18 amino acids to the TonEBP-c produces TonEBP-d, which reduces TonEBP activity, although TonEBP-d activity is still higher than TonEBP-a. Expression of TonEBP is known to be induced by T-cell receptor and integrin signaling as well as hypertonicity. Phorbol-ester-induced macrophagic differentiation in U937 cells leads to surface expression of Mac-1 integrin and its activation as well (Hamada & Utiyama, 2005). Expression of TonEBP is also induced during PMA-induced macrophagic differentiation, although it is not known whether Mac-1 integrin is involved in this pathway (data not shown). These observations suggest that different expression profiles of TonEBP isoforms in different tissues and in response to different stimulations, such as hypertonicity, T-cell receptor, and integrin, may be important for the regulation of TonEBP activity.

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