Activator Protein-1 Contributes to High NaCl-induced Increase in Tonicity-responsive Enhancer/Osmotic Response Element-binding Protein Transactivating Activity*

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Tonicity-responsive enhancer/osmotic response element-binding protein (TonEBP/OREBP) is a Rel protein that activates transcription of osmoprotective genes at high extracellular NaCl. Other Rel proteins NFAT1–4 and NF-κB complex with activator protein-1 (AP-1) to transactivate target genes through interaction at composite NFAT/NF-κB/AP-1 sites. TonEBP/OREBP target genes commonly have one or more conserved AP-1 binding sites near TonEBP/OREBP cognate elements (OREs). Also, TonEBP/OREBP and the AP-1 proteins c-Fos and c-Jun are all activated by high NaCl. We now find, using an ORE/AP-1 reporter from the target aldose reductase gene or the same reporter with a mutated AP-1 site, that upon stimulation by high extracellular NaCl, 1) the presence of a wild type, but not a mutated, AP-1 site contributes to TonEBP/OREBP-dependent transcription and 2) AP-1 dominant negative constructs inhibit TonEBP/OREBP-dependent transcription provided the AP-1 site is not mutated. Using supershifts and an ORE/AP-1 probe, we find c-Fos and c-Jun present in combination with TonEBP/OREBP. Also, c-Fos and c-Jun coimmunoprecipitate with TonEBP/OREBP, indicating physical association. Small interfering RNA knockdown of either c-Fos or c-Jun inhibits high NaCl-induced increase of mRNA abundance of the TonEBP/OREBP target genes AR and BGT1. Furthermore, a dominant negative AP-1 also reduces high NaCl-induced increase of TonEBP/OREBP transactivating activity. Inhibition of p38, which is known to stimulate TonEBP/OREBP transcriptional activity, reduces high NaCl-dependent transcription of an ORE/AP-1 reporter only if the AP-1 site is intact. Thus, AP-1 is part of the TonEBP/OREBP enhancerome, and its role in high NaCl-induced activation of TonEBP/OREBP may require p38 activity.

Among Rel-proteins, TonEBP/OREBP activates target genes that are osmoprotective at elevated extracellular NaCl. Transcriptional targets of TonEBP/OREBP include aldose reductase (AR). 2

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2 The abbreviations used are: AP-1, activator protein-1; AR, aldose reductase; ARRE-2, antigen receptor-response element-2; BGT1, betaine/γ-aminobutyric acid transporter (BGT1), the sodium-γ-aminobutyric acid co-transporter (SMIT), the taurine transporter (TauT), heat shock protein 70 (HSP70), and aquaporin 2 (AQP2). AR converts glucose to the osmoregulatory solute sorbitol, whereas BGT1, SMIT, and TauT transport the organic osmolytes betaine, inositol, and taurine, respectively, into cells (1). HSP70, a protein chaperone, also serves a protective function at elevated NaCl (2). AQP2 increases water permeability of the renal collecting duct during adaptation to dehydration (3). TonEBP/OREBP target genes often have multiple TonEBP/OREBP cognate DNA elements known as osmotic response elements (OREs) or tonicity enhancer-responsive elements (TonEs) (Tables 1 and 2). Additionally, all of the above noted genes except TauT have one or more activator protein-1 (AP-1) sites within 35 bp of an ORE (Tables 1 and 2). In the SMIT gene, an AP-1 site overlaps an ORE in the antisense direction (Table 1). In the AR gene, which has been mapped in multiple species, the AP-1 site is highly conserved across all species (Table 2).

TonEBP/OREBP, also known as NFAT5, has characteristics intermediate between NFAT1–4 and NF-κB (4, 5). Like other Rel proteins, the DNA binding domain (DBD) of TonEBP/OREBP is in an N-terminal rel-homology region. The TonEBP/OREBP DBD is highly similar to the DBD in NFAT1–4 (up to 43% sequence identity), which was the basis for the cloning of this protein by one group of investigators (6). Although the TonEBP/OREBP rel-homology region is similar to those of NFAT1–4, it binds DNA as a dimer, similar to NF-κB, whereas NFAT1–4 bind as monomers (5). Both NFAT1–4 and NF-κB interact with AP-1 transcription factors in the transactivation of downstream genes. The AP-1 factors c-Jun and c-Fos interact with NFAT1–4 proteins at multiple rel-homology region residues resulting in strong stabilization of the ternary complex on DNA (7). In the transactivation of many genes, the activity of the NFAT1–4 transcription factors depends on physical cooperativity with active AP-1 factors at composite NFAT/AP-1 sites found in promoter and regulatory regions. NF-κB also interacts with AP-1 in activation of target genes. This interac-
TABLE 1
ORE and associated AP-1 sites and their relative positions in BGT1, SMIT, and HSP70 genes
Numbering is relative to transcription start site = +1. Functional ORE positions in BGT1 (42), SMIT (55–57), HSP70 (58), and AQP2 (59).

| ORE     | Sequence        | Position | AP-1    | Position |
|---------|-----------------|----------|---------|----------|
| BGT1-canine |                 |          |         |          |
| TonE2   | AGGAAAAATCCC    | −144, −134 |         |          |
| TonE1   | TGAAAAAGTCC     | −62, −52  | TGAATTC | −45, −39 |
| SMIT-human |               |          |         |          |
| TonEA   | TGGAAAAACTAC    | −15 kb   | TGAATGC | 10 bp 5’ |
| TonEB2  | TGGAAAAATCC     | −15 kb   | TGAATCA | 34 bp 3’ |
| TonEC1  | TGGAAAAATAG     | −15 kb   | TGAATTAA| overlap 4-bp antisense |
| TonEC2  | TGGAAAAATTG     | −15 kb   |         |          |
| TonEP   | TGGAAAAATGC     | −331, −321|         |          |
| HSP70-mouse |              |          |         |          |
| TonEA   | TGGAATGTTTT     | −1070, −1060|         |          |
| TonEB   | TGGAAAAATTTT    | −2326, −2316|         |          |
| TonEC   | TGGAAAAATCC     | −3698, −3688|         |          |
| TonED   | TGGAAAAACAC     | −3715, −3705|         |          |
| AQP2-mouse |             |          |         |          |
| TonE    | TGGAATTGTGT     | −489, −479| TGATTAA | −528, −522|

TABLE 2
Relative positions of OREs and associated AP-1 sites in AR genes across multiple species
Numbering is relative to transcription start site = +1. Shown are functional ORE positions in human (10), rabbit (30–31), mouse (60) and rat (61).

| ORE     | Sequence        | Position | AP-1    | Position |
|---------|-----------------|----------|---------|----------|
| AR-human |                 |          |         |          |
| ORE-A   | TGAAAATATTTA    | −1230, −1220|         |          |
| ORE-B   | TGAAAATTTTA     | −1198, −1188|         |          |
| ORE-C   | TGAAAATTTAC     | −1157, −1147| TGAATCA | −1117, −1111|
| AR-rabbit |              |          |         |          |
| ORE-A   | CGAAAATATTTA    | −1181, −1171|         |          |
| ORE-B   | CGAAAATTTTA     | −1148, −1138|         |          |
| ORE-C   | CGAAAATTTAC     | −1105, −1095| TGAATCA | −1072, −1066|
| AR-mouse |            |          |         |          |
| ORE-A   | TGGAATTTATTA    | −1128, −1118|         |          |
| ORE-B   | CGGAATTTTTA     | −1108, −1098|         |          |
| ORE-C   | CGGAATTTAC      | −1053, −1043| TGAATCA | −1012, −1006|
| AR-rat   |                |          |         |          |
| ORE-A   | TGGAATTTATTA    | −1146, −1136|         |          |
| ORE-B   | CGGAATTTTTA     | −1106, −1116|         |          |
| ORE-C   | TGGAATTTAC      | −1071, −1061| TGAATCA | −1031, −1025|

by 37% when the level of extracellular salt is high and by 23% when it is not (10). Noting that neither of these negative studies (6, 10) utilized a composite ORE-AP-1 DNA sequence in its native context, which may have affected the results, we determined to reinvestigate the question using native constructs that contain the cognate DNA element of TonEBP/OREBP.

Using the ORE-AP-1 DNA sequence from the AR gene, we now find that mutating the AR-1 DNA site reduces TonEBP/OREBP-dependent reporter activity at elevated extracellular NaCl. Also, when NaCl is high, dominant negative AP-1 constructs reduce transcriptional and transactivating activity of TonEBP/OREBP, and siRNA knockdown of c-Fos or c-Jun reduces the mRNA abundance of its target genes, AR and BGT1. Furthermore, inhibition of p38, which is known to stimulate TonEBP/OREBP transcriptional activity (14), reduces high NaCl-dependent transcription of an ORE-AP-1 reporter only if the AR-1 site is intact. We conclude that TonEBP/OREBP requires AP-1 for its full high NaCl-dependent increase in transactivation, and its role in high NaCl-induced activation of TonEBP/OREBP may require p38 activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—PAP-HT25 cells (passages 60–78) were cultured in 300 mosmol/kg medium as previously described (15). HEK293 cells (passages 38–48) were cultured in 300 mosmol/kg medium according to ATCC instructions. At experiment-specific time points, medium was replaced with medium that was 300, 200 (NaCl added to NaCl-free medium, Biofluids, Rockville, MD), or 500 mosmol/kg (NaCl added). For inhibitor experiments cells were pretreated with 10 μM of the p38 inhibitor, SB203580 (10 μM) in 300 mosmol/kg medium. After 1 h, fresh 300 or 500 mosmol/kg (NaCl added) medium containing either Me2SO or SB203580 was substituted. Me2SO was 0.01% in all inhibitor experiments.

Plasmids and siRNAs—The ORE reporter construct contains a luciferase reporter gene driven by the Photinus pyralis luciferase gene (described previously as ARLuc9) (16). The sequence −3497 to +27 of the rabbit aldose reductase gene upstream of the Photinus pyralis luciferase gene (described previously as ARLuc9) (16). The sequence −3497 to +27 includes the aldose reductase promoter, three OREs (−1105, −1095; −1181, −1171; −1148, −1138), and an AP-1 site (−1072, −1066) in native gene context (GenBankTM U12317). The promoter
reporter construct is the same but includes only bp −209 to +27. The mutant AP-1 construct reporter is the same as the ORE reporter with bp −1070 to −1068 changed from AGT to CTG. The mutation was made using site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA), and the construct was sequence-verified. The ORE-X luciferase reporter construct contains two copies of human ORE-X (17) within a minimal interleukin-2 promoter (18) (bTonE-GL3, a gift from S. N. Ho, University of California, San Diego, CA), as previously described (19).

Human TonEBP/OREBP cDNA clone KIAA0827 was a gift from Dr. Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan). Sequence coding for amino acids 1–547 or 1–1531 of KIAA0827 was cloned into expression vector pcDNA6V5-His (Invitrogen) to generate 1–547 or 1–1531V5-His as previously described (19, 20).

The binary GAL4 reporter system has been described (21). In brief, plasmid pFR-Luc (Stratagene) contains the yeast GAL4 binding site (upstream activating sequence) upstream of a minimal promoter and the P. pyralis luciferase gene. Expression plasmid pFA-CMV (Stratagene) contains sequence coding for the yeast GAL4 DNA binding domain. A fusion protein was generated by in-frame insertion of the sequence coding for amino acids 548–1531 of clone KIAA0827 into pFA-CMV to generate GAL4dbd-548–1531. TonEBP/OREBP amino acids 548–1531 contain a NaCl-dependent transactivation domain (21). GAL4dbd contains no transactivation domain but expresses the GAL4dbd (pFC2-dbd, Stratagene).

A-Fos dominant negative construct (22) generously was provided by Dr. Charles Vinson (NCI, National Institutes of Health, Bethesda, MD). Tam-67, lacking the major transactivation domain of c-Jun, was previously described (23).

We designed the siRNA against c-Jun (24) as a synthetic double-stranded RNA Dicer substrate to enhance the RNA interference potency and efficacy (25). Duplex sequences were: sense, 5′-Phos-AGUCAUGAACCACGUUAAUCUCdAdG-3′ and antisense 5′-CUAGAACGUUAAAGCUGGUUCAUGACUGCGG-3′ (Integrated DNA Technologies, Coralville, IA). The siRNA against c-Fos was a pool of 4 target-specific 20–25-nucleotide siRNAs (sc-29221 Santa Cruz Biotechnology, Santa Cruz, CA). The control (nontargeting) siRNA duplex sequences were: sense, 5′-Phos-UGAACCUGACCAGGGGAGGGAAdTdT-3′ and antisense sequence 5′-AACUCCCUCCCGUAGGGGGCUUU-3′ (26) (Integrated DNA Technologies).

Transfection and Luciferase Assays—PAP HT25 cells were grown in 300 mosmol/kg medium in 6-well plates and transfected with 0.5 μg of wild type ORE or mutant AP-1 reporter using CellPhect (GE Healthcare) according to supplier instructions. HEK293 cells were grown in 300 mosmol/kg medium. Five million cells were transfected by electroporation (Gene Pulser, Bio-Rad) according to manufacturer’s instructions. For ORE-X reporter assays, cells were transfected with 5 μg of ORE-X reporter. For ORE reporter assays, cells were co-transfected with 5 μg of ORE or mutant AP-1 reporter and 4 μg of TAM-67, 10 μg of A-Fos, or empty vector. For the GAL4 binary assay, cells were co-transfected with 5 μg of the GAL4 upstream activating sequence reporter, 0.5 μg of GAL4dbd or GAL4dbd-548–1531, and 10 μg of A-Fos or empty vector. Twenty-four hours after transfection at 300 mosmol/kg, fresh 200, 300, or 500 mosmol/kg medium was substituted. Luciferase activity was measured 16 h later with the Bright-Glo Luciferase assay system (Promega, Madison, WI). Total protein was measured (BCA protein assay kit; Pierce). Luciferase activity was expressed in relative light units per μg of total cell protein.

Electrophoretic Mobility Shift Assay—HEK293 cells stably expressing recombinant TonEBP/OREBP 1–1531V5-His were grown at 300 mosmol/kg. Fresh medium at 300 or 500 mosmol/kg (NaCl added) was substituted. Two hours later, nuclear pellets were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to supplier instructions. Nuclear pellets were resuspended in lysis buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 with protease (Complete Mini, Roche Applied Science) and phosphatase (Phosphatase Inhibitor Cocktails 1 and 2, Sigma) inhibitors (27) and centrifuged 10 min at 15,000 × g, and the supernatant was retained. Double-stranded ORE probe (bp −1238 to −1,104 of the human aldose reductase gene), containing three OREs and an AP-1 site in native gene context, was generated by annealing complementary 5′-biotinylated oligonucleotides (Integrated DNA Technologies). The AP-1 mutant probe is the same as the wild type ORE probe with the 5′ to 3′ bp substitutions in the AP-1 site −1115 to −1113 from AGT to CTG. We combined 0.25–1 μg of nuclear extract with 0.5–1 μg of poly(dA·dT) in binding buffer (LightShift chemiluminescent electrophoretic mobility-shift assay kit, Pierce). Anti-TonEBP/OREBP (1 μg, NFAT5, Affinity Bioreagents, Neshanic Station, NJ), anti-c-Jun (4 μg), or anti-c-Fos (2 μg) (Santa Cruz Biotechnology) was added to some reactions and incubated at 4 °C for 1 h. This was followed by the addition of 100 fmol of ORE or AP-1 mutant probe with or without 1000 fmol of non-biotinylated ORE probe and incubation for 20 min. Total binding reaction volume was 20 μl. Reaction products were separated by gel electrophoresis in 0.4% SeaKem Gold agarose in 0.5× Tris borate/EDTA buffer at 4 °C, transferred to nylon membranes, and UV cross-linked (Stratalinker, Stratagene). Biotinylated probes were detected using the LightShift chemiluminescent electrophoretic mobility-shift assay kit (Pierce) according to manufacturer instructions.

Immunoprecipitation—HEK293 cells stably expressing recombinant TonEBP/OREBP 1–1531-V5-His, 1–547-V5-His, GAL4dbd-548–1531, or GAL4dbd were grown in 300 mosmol/kg medium. Fresh medium at 200, 300, or 500 mosmol/kg was substituted. Two hours later cells were trypsinized and pelleted by centrifugation. Subsequent steps were at 4 °C. For whole cell extracts, the pellet from one 10-cm dish was extracted for 5 min with 1 ml of lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, protease (Complete Mini, Roche Applied Science), and phosphatase inhibitor cocktails (Phosphatase Inhibitor Cocktails 1 and 2, Sigma) and centrifuged (15,000 × g, 10 min). Nuclear and cytoplasmic extracts were prepared using NE-PER reagents (Pierce) according to supplier instructions. Nuclear and cytoplasmic extracts were diluted with lysis buffer as above. For immunoprecipitation of 1–1531-V5-His or 1–547-V5-His and any associated proteins, samples were precleared with 1 mg of
Dynabeads (Invitrogen) and 2 μg of rabbit IgG biotin-conjugated (Santa Cruz Biotechnology) for 1 h and centrifuged. Precleared supernatants were incubated overnight with 4 μg of rabbit anti-V5 biotin-conjugated (Immunology Consultants Laboratory, Inc.) and 1 mg of Dynabeads (Invitrogen). For immunoprecipitation of GAL4dbd or GAL4dbd-548–1531 and any associated proteins, the samples were precleared with 1 mg of Dynabeads (Invitrogen) and 2 μg of mouse IgG biotin-conjugated (Santa Cruz Biotechnology) for 1 h and centrifuged. Precleared supernatants were incubated overnight with 5 μg of mouse anti-Gal4dbd biotin-conjugated (Immunology Consultants Laboratory) and 1 mg of Dynabeads (Invitrogen). As negative controls, IgG-biotin conjugate (Santa Cruz Biotechnology) was substituted for anti-V5 or anti-Gal4dbd. Ethidium bromide (100 μg/ml), which disrupts protein-DNA association (28), was included in some immunoprecipitations to test whether DNA is involved in the coimmunoprecipitation of associated proteins. For immunoprecipitation of c-Fos or c-Jun and any associated proteins, samples were precleared with 20 μl of protein A/G-Plus-agarose beads (Santa Cruz Biotechnology) and 0.5 μg of rabbit IgG (Santa Cruz Biotechnology) for 1 h and centrifuged. Precleared supernatants were mixed overnight with 2 μg of rabbit anti-c-Jun (Santa Cruz Biotechnology) and 2 μg of rabbit anti-c-Fos (Santa Cruz Biotechnology) and 20 μl of protein A/G-Plus-agarose beads (Santa Cruz Biotechnology). As negative controls, IgG (Santa Cruz Biotechnology) was substituted for anti-c-Fos or c-Jun. Beads were resuspended in Laemmli sample buffer and incubated for 5 min at 95 °C, and after centrifugation, supernatant proteins were separated on a Tris-HCl, 4–15% polyacrylamide gel (Bio-Rad). Proteins were transferred to a nitrocellulose membrane, which was then cut. One part of the membrane was incubated at 4 °C overnight with mouse anti-v-5 (Invitrogen), anti-Gal4dbd (Santa Cruz Biotechnology), or anti-TonEBP/OREBP (NFAT5, Affinity Bioreagents); the others were incubated with rabbit anti-c-Fos or c-Jun (Santa Cruz Biotechnology). Blots were visualized using a LI-COR Odyssey Infrared Imager.

siRNA Knockdown of c-Jun and c-Fos and Quantitative Real Time PCR—HEK293 cells were grown in 300 mosmol/kg medium and transfected with 20 nm control, c-Jun, or c-Fos siRNA using Lipofectamine 2000 according to supplier instructions. After 48 h, fresh medium at 300 or 500 mosmol/kg (NaCl added) was substituted. Sixteen hours later, total RNA was isolated (RNeasy, Qiagen, Valencia, CA), and cDNA was prepared using a TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) according to supplier instructions. PCR was performed on 8 and 80 ng of cDNA samples/20-μl reaction in triplicate for each experiment (Taqman PCR master mix, Applied Biosystems). Amplicons were detected with an ABI Prism 7900HT sequence detection system (Applied Biosystems). Primers directed against the human sequence of the cyclophilin gene were 5′-TGTCGCAAGGTTGACTT-3′ and 5′-TCACATCTCTCCGTAGATGGACTT-3′. The 6-carboxyfluorescein-labeled probe was 5′-CCACCACTGCATTA-TGGCGTGT-3′.

The detection system records the number of PCR cycles (Ct) required to produce an amount of product equal to a threshold value, which is a constant. From the Ct values we calculated the mRNA abundance in each experimental condition relative to that of control cells at 300 mosmol/kg, as described (21).

Statistical Analysis—Data were compared by repeated measures of analysis of variance followed by Bonferroni multiple comparison test for separation of significant means. Normalized data were log-transformed before analysis of variance. Differences were considered significant for p ≤ 0.05.

RESULTS

The 5′-flanking region of the rabbit AR gene contains three OREs (16, 30) and an AP-1 site (31) (Table 2). We used an ORE luciferase reporter and the same reporter mutated at the AP-1 site to determine the relative contribution of this binding site to TonEBP/OREBP-mediated transcriptional activity in PAP-HT25 and HEK293 cells at 300 and at 500 mosmol/kg (NaCl added) (Fig. 1). When osmolality is raised from 300 to 500 mosmol/kg by adding NaCl, transcriptional activity of the ORE reporter increases significantly. Mutation of AP-1 reduces transcriptional activity by 46% in PAP-HT25 cells and 44% in HEK293 cells. Transcriptional activity at 300 mosmol/kg is unaffected by mutation at the AP-1 site. We conclude that at 500 mosmol/kg, AP-1 is a potentiating element.

To determine whether the AP-1 transcription factors c-Fos and c-Jun associate with TonEBP/OREBP in the protein complex that binds to the wild type DNA probe containing three OREs and an AP-1 site, we performed electrophoretic mobility shift assay binding reactions using HEK293 nuclear protein extracts with and without added antibody to c-Fos or c-Jun.
AP-1 Enhances TonEBP/OREBP-dependent Transcription at High NaCl

Electrophoretic mobility shift assays using nuclear extracts from cells at 300 mosmol/kg required 4× more extract to generate a mobility shift than nuclear extracts from cells at 500 mosmol/kg because high NaCl causes TonEBP/OREBP to translocate to the nucleus, which results in more TonEBP/OREBP in the nucleus at 500 mosmol/kg (Fig. 2, A and B). The addition of either anti-c-Fos or c-Jun results in a supershift with extracts from cells in 300 or 500 mosmol/kg medium (Fig. 2, A and B). The addition of anti-TonEBP/OREBP results in a similar supershift. The addition of a 10-fold molar excess of non-biotinylated competitor eliminates the TonEBP/OREBP supershift with DNA containing the AP-1 site (Fig. 2, A and B). Mutation of the AP-1 site does not affect the anti-c-Fos or c-Jun supershift (Fig. 2C). The mobility shift with the AP-1 mutant probe is specific since the TonEBP/OREBP supershift is abolished by the addition of a 10-fold molar excess of non-biotinylated competitor. We conclude that c-Fos and c-Jun are part of the protein complex associated with TonEBP/OREBP at its binding sites, that the association is not tonicity-dependent, and that it does not require the AP-1 binding site that lies 3′ of the ORE.

TonEBP/OREBP transcriptional activity increases with increased tonicity (Fig. 3A). To determine whether c-Fos and c-Jun physically associate with TonEBP/OREBP, we immunoprecipitated proteins from HEK293 cells stably transfected with recombinant TonEBP/OREBP expressing either full-length (1–1531–V5-His), N-terminal (1–547–V5-His), or C-terminal (Gal4dbd-548–1531) amino acids. Cells were treated with 200, 300, or 500 mosmol/kg medium. Anti-V5 antibody coimmunoprecipititates TonEBP/OREBP amino acids 1–1531 or 1–547 along with c-Fos and c-Jun regardless of medium osmolality, but the IgG control does not (Figs. 3, B and C, upper panels). Anti-c-Fos and c-Jun reciprocally coimmunoprecipitate TonEBP/OREBP amino acids 1–1531 or 1–547 regardless of tonicity, but the IgG control does not (Figs. 3, B and C, middle and lower panels). Attempts to coimmunoprecipitate the C terminus of TonEBP/OREBP (Gal4dbd-548–1531), c-Jun, and c-Fos yielded mixed results. Anti-c-Fos and anti-c-Jun do coimmunoprecipitate TonEBP/OREBP amino acids 548–1531 from whole cell extracts regardless of tonicity (Fig. 3D, middle and lower panels). However, from whole cell extracts, anti-Gal4dbd did not consistently coimmunoprecipitate c-Fos or c-Jun regardless of medium osmolality (Fig. 3D, upper panels). Because the Gal4dbd-548–1531 fusion protein locates to the nucleus (21), we reasoned that an immunoprecipitation from nuclear extracts might be more revealing. In fact anti-Gal4dbd antibody coimmunoprecipititates TonEBP/OREBP amino acids 548–1531 along with c-Fos and c-Jun from nuclear (but not from cytoplasmic) extracts regardless of medium osmolality, and the IgG control does not (Fig. 3E). However, when ethidium bromide, which interferes with DNA protein associations, is added to the nuclear extracts, the Gal4dbd antibody does not coimmunoprecipitate c-Fos and c-Jun (Fig. 3F). As an additional control, anti-Gal4dbd antibody does not coimmunoprecipitate c-Fos or c-Jun from nuclear or cytoplasmic extracts of Gal4dbd stably transfected cells, which do not express the C terminus of TonEBP/OREBP (amino acids 548–1531) (data not shown). We conclude that c-Fos and c-Jun are part of the protein complex associated with both the N terminus and the C terminus of TonEBP/OREBP but that the interaction with the C terminus appears to require the presence of DNA.

A-Fos and Tam-67 are dominant negatives that affect AP-1 transcriptional activity (22, 32). A-Fos forms heterodimers with endogenous binding partners (e.g. c-Jun). The heterodimers are inactive since they do not bind to AP-1 sites, and this interaction inhibits AP-1 activity by removing the binding partners on an equimolar basis (22). Tam-67 similarly forms heterodimers with endogenous binding partners (e.g. c-Fos). Unlike A-Fos heterodimers, Tam-67 heterodimers do bind to AP-1 sites. However, they are inactive since Tam-67 lacks the c-Jun transactivation domain (32). Both A-Fos and Tam-67 act to eliminate AP-1-mediated transactivation. We co-transfected HEK293 cells with the ORE reporter and A-Fos. As controls, empty vector replaced the A-Fos, or the reporter contained only the AR gene promoter without ORE or AP-1 sites. Cotransfection of A-Fos decreases ORE reporter activity relative to vector control ∼40% at 500 mosmol/kg but not at 300 mosmol/kg (Fig. 4A). A-Fos does not affect transcription of the reporter containing only the AR gene promoter at either osmolality (Fig. 4A). A-Fos inhibition of TonEBP/OREBP-mediated transcription requires the AP-1 site that lies 3′ of the ORE. In HEK293 cells, co-transfection of A-Fos decreases transcription
AP-1 Enhances TonEBP/OREBP-dependent Transcription at High NaCl

The transcriptional activity of TonEBP/OREBP varies with tonicity. HEK293 cells were grown in 300 mosmol/kg medium and transfected with an ORE reporter construct. The transcriptional activity of TonEBP/OREBP is dependent on the N terminus of TonEBP/OREBP, which contains two copies of ORE-X. Twenty-four hours after transfection, fresh 300 mosmol/kg medium was substituted for NaCl-free medium, 300, or 500 mosmol/kg medium was substituted. Cells were harvested 2 h later. Results are the mean ± S.E. (*, p < 0.05). B. HEK293 cells were transfected with recombinant TonEBP/OREBP construct 1–1531-V5-His. Whole cell extracts were immunoprecipitated (IP) with anti-V5 (upper panels), anti-c-Fos (middle panels), anti-c-Jun (lower panels), or IgG and immunoblotted with antibodies that recognize V5, c-Fos, or c-Jun. Immunoblots are representative of three or more experiments. C. HEK293 cells stably transfected with the recombinant TonEBP/OREBP construct 1–1531-V5-His were grown at 300 mosmol/kg. Fresh medium at 200 (NaCl added to NaCl-free medium), 300, or 500 mosmol/kg (NaCl added) was substituted for 2 h. Whole cell extracts were immunoprecipitated (IP) with anti-V5 (upper panels), anti-c-Fos (middle panels), anti-c-Jun (lower panels), or IgG and immunoblotted with antibodies that recognize V5, c-Fos, or c-Jun. Immunoblots are representative of three or more experiments. D. HEK293 cells stably transfected with the recombinant TonEBP/OREBP construct 1–1531-V5-His were grown at 300 mosmol/kg. Fresh medium at 200 (NaCl added to NaCl-free medium), 300, or 500 mosmol/kg (NaCl added) was substituted for 2 h. Whole cell extracts were immunoprecipitated (IP) with anti-V5 (upper panels), anti-c-Fos (middle panels), anti-c-Jun (lower panels), or IgG and immunoblotted with antibodies that recognize V5, c-Fos, or c-Jun. Immunoblots are representative of three or more experiments. E. HEK293 cells stably transfected with the recombinant TonEBP/OREBP construct 1–1531-V5-His were grown at 300 mosmol/kg. Fresh medium at 200 (NaCl added to NaCl-free medium), 300, or 500 mosmol/kg (NaCl added) was substituted for 2 h. Whole cell extracts were immunoprecipitated (IP) with anti-V5 (upper panels), anti-c-Fos (middle panels), anti-c-Jun (lower panels), or IgG and immunoblotted with antibodies that recognize V5, c-Fos, or c-Jun. Immunoblots are representative of three or more experiments. F. HEK293 cells stably transfected with the recombinant TonEBP/OREBP construct 1–1531-V5-His were grown at 300 mosmol/kg. Fresh medium at 200 (NaCl added to NaCl-free medium), 300, or 500 mosmol/kg (NaCl added) was substituted for 2 h. Whole cell extracts were immunoprecipitated (IP) with anti-V5 (upper panels), anti-c-Fos (middle panels), anti-c-Jun (lower panels), or IgG and immunoblotted with antibodies that recognize V5, c-Fos, or c-Jun. Immunoblots are representative of three or more experiments.
We used a binary GAL4 assay to measure TonEBP/OREBP transactivating activity. We co-transfected HEK293 cells with a GAL4 upstream activating sequence reporter and GAL4dbd-548–1531 plus empty vector or A-Fos. GAL4dbd, which lacks a functional transactivating domain, was used as the control. The dominant negative A-Fos decreases TonEBP/OREBP transactivation of the luciferase gene by 40% at 500 but not at 300 mosmol/kg (Fig. 4E). We conclude that AP-1 factor increase of TonEBP/OREBP-mediated transcription at elevated extracellular NaCl involves stimulation of TonEBP/OREBP transactivation.

p38 enhances transactivation by TonEBP/OREBP of its target genes (14). p38 also activates c-Jun and c-Fos. p38 increases c-Jun abundance by activating the c-jun promoter (36), and it increases transactivating activity of c-Fos (37). We used the p38 inhibitor, SB203580, to see if the effect of p38 on TonEBP/OREBP transactivating activity involves AP-1. When NaCl is high, SB203580 reduces the wild type ORE reporter activity by 40%, consistent with previously reported results (14) (Fig. 4F). In contrast, SB203580 does not significantly reduce activity of the AP-1 mutated reporter. We conclude that the role of AP-1 in high NaCl-induced activation of TonEBP/OREBP may require p38 activity.

**DISCUSSION**

Gene activation by transcription factors is augmented by cooperative binding to multiple copies of their cognate sequences (38) and participation of multiple transcription factors and other interacting proteins in the enhanceosome that forms (for review, see Refs. 39 and 40). TonEBP/OREBP activates osmoprotective genes, and hypertonicity increases this activity (1). The 5′-flanking regions of TonEBP/OREBP-responsive genes generally contain multiple copies of its cognate DNA element, ORE, as well as an AP-1 site (Tables 1 and 2). AR contains three TonEBP/OREBP cognate elements (Table 2), and BGT1 contains two (Table 1). The ORE is sufficient for induction of transcription by high extracellular NaCl. The hypertonic activation of an isolated ORE is relatively small,
AP-1 Enhances TonEBP/OREBP-dependent Transcription at High NaCl

about 2–3-fold (17, 30, 41), but when it is mutated or deleted, hypertonic induction is completely lost (10, 42). In contrast, in the absence of an intact ORE, the other ORE-like elements are not activated by hypertonicity (10). Nevertheless, their presence potentiates activation of the ORE by high NaCl (10, 42). Thus, although the ORE can independently activate transcription in response to high salt, all of the sites acting in synergy are necessary for full induction of downstream genes. Synergy among the TonEBP/OREBP proteins occupying multiple OREs may involve physical interaction among the transcription factors in an enhancerome, but direct experimental evidence for this is lacking.

We find that the AP-1 site 3′ of the ORE contributes to TonEBP/OREBP-dependent transcription when NaCl is high (Fig. 1). Based on somewhat similar experiments, others previously concluded that the AP-1 site does not play an important role in the osmoregulation of AR gene transcription (10). The actual difference in results is that we now find that mutation of AP-1 reduces TonEBP/OREBP transcriptional activity at 500 but not at 300 mosmol/kg, whereas in the previous study activity was reduced at both osmolalities by 37% at 500 mosmol/kg and by 23% at 300 mosmol/kg (10). The difference may be due to the way reporter constructs were made. In the present study the reporter contained bp −3497 to +27 of the AR gene in native gene context, and the AP-1 site was altered by mutagenesis, whereas in the previous work fragments containing OREs were linked by an introduced restriction site, and the AP-1 site was absent.

AP-1 sites generally occur close to the OREs in the regulatory regions of genes transactivated by TonEBP/OREBP (Tables 1 and 2). In the present study we demonstrate that AP-1 proteins form part of the TonEBP/OREBP transcription complex. This complex or enhancerome is likely to be very large based on the number of factors known to interact with TonEBP/OREBP, as discussed below, as well as empirical estimations. In gel filtration, native TonEBP/OREBP (molecular mass 160 kDa) migrates with an apparent size of greater than 1000 kDa (34), and in size exclusion chromatography it elutes close to the void volume (>1500 kDa) (43). Notably, enhanceromes of the TonEBP/OREBP-related proteins NFAT1–4 and NF-κB also include AP-1. NFAT1–4 and AP-1 bind cooperatively to composite DNA sites (7), whereas NF-κB and AP-1 display various modes of interaction including functional synergy (8) and physical cooperativity (9).

AP-1 transcription factors (Jun, Fos, Fra) comprise a family of proteins that form homodimers or heterodimers with each other or with activating transcription factor proteins (ATF) (44). Jun-Fos dimers bind to AP-1 elements, whereas Jun-ATF dimers bind to cAMP-responsive elements. Both types of elements contain the same AP-1 half-site. AP-1 activity is regulated by dimerization, by external signals, and through interactions with protein kinases and transcriptional coactivators (44). c-Jun and c-Fos are regulated both by their transcription and by modulation of their protein stability. Additionally, post-translational modifications increase the transcriptional activity of c-Jun and c-Fos (37, 44). Accordingly, high extracellular NaCl activates AP-1 transcription factors in multiple ways. High NaCl increases transcription of c-fos and c-jun in cultured cells (11, 13, 45) and increases DNA binding activity of AP-1 proteins, c-Fos/Fra, and c-Jun in vivo (12, 13, 46). Also, hypertonicity increases phosphorylation of c-Jun and increases nuclear phospho-c-Jun (47).

In the present study we show that c-Fos and c-Jun are part of the protein complex associated with TonEBP/OREBP at its DNA binding sites and are necessary for full high NaCl-induced activation of its target sequence in the AR gene (Figs. 1–4). Furthermore, c-Jun activity is also necessary for full high NaCl-induced increase of mRNA abundance of the TonEBP/OREBP transcriptional targets, AR and BGT1 (Fig. 4D). Previous investigations of the possible association of TonEBP/OREBP, c-Fos, and c-Jun in transcriptional complexes yielded conflicting conclusions. On one hand, the DNA binding domain of TonEBP/OREBP (amino acids 252–548) does not bind cooperatively in vitro with recombinant c-Jun or c-Fos to an ARRE-2-AP-1 composite DNA element (ARRE-2 is an NFAT binding site in the interleukin-2 promoter) (6, 7). The conclusion was that, unlike other NFATs, TonEBP/OREBP does not cooperate with c-Jun and c-Fos to activate target genes. However, also unlike other NFATs, TonEBP/OREBP does not activate an ARRE-2-dependent luciferase reporter in vivo (6). On the other hand, TonEBP/OREBP and c-Jun/ATF2 do associate in activating the TNF promoter under hypertonic conditions. In this case two TonEBP/OREBP dimers bind to non-consensus ORE sites, and c-Jun/ATF-2 binds to an adjacent 5′ cAMP-responsive element site (48).

The c-Jun and c-Fos binding sites of NFAT1–4 are found in the N-terminal DNA binding domain. Compared with other NFAT DNA binding domains, that of TonEBP/OREBP has only 3 of 11 conserved c-Jun binding sites and 3 of 10 c-Fos binding sites. Nevertheless, c-Fos and c-Jun are present together with TonEBP/OREBP in the complex that binds to OREs, and the association is independent of the presence of the AP-1 site (Fig. 2). c-Fos and c-Jun coimmunoprecipitate with the N terminus and with the C terminus of TonEBP/OREBP (Fig. 3). However, interaction with the C terminus appears to require the presence of DNA since ethidium bromide abrogates the association. The N terminus of TonEBP/OREBP contains the DNA binding domain (5), whereas the C terminus contains a high NaCl-dependent transactivation domain (21). It is possible that c-Fos/c-Jun bind to a region located near amino acid 547 of TonEBP/OREBP and, thus, are partially present in both 1–547- and 548–1531-TonEBP/OREBP constructs. The cooperative interaction between NFAT1–4 and AP-1 represents direct protein-protein binding, requires the presence of DNA, and is obligatory for transactivation of certain but not all NFAT target genes (7). NFAT1–4 and AP-1 bind cooperatively to their cognate elements in composite NFAT:AP-1 sites, resulting in greatly increased stability of the ternary complex. Physical association of AP-1 with TonEBP/OREBP differs from that with NFAT1–4 in that it occurs in the absence of DNA. The experiments depicted in Fig. 3 were performed in the absence of DNA. Because maximal TonEBP stimulation by hypertonicity requires an intact AP-1 binding site (Figs. 1 and 4), perhaps DNA is required for full association between TonEBP and c-Fos/c-Jun. We do not know if the association is direct or occurs indirectly through another component of the enhanceo-
some. For example, RNA helicase RHH/Gu, which physically associates with TonEBP/OREBP (49), can affect transcription by acting as a partner of c-Jun (50). The C-terminal domain of RHH/Gu interacts with the N-terminal transcription activation region of c-Jun, supporting c-Jun-mediated target gene activation through its RNA helicase activity. Finally, high NaCl-dependent TonEBP/OREBP transactivation of the AR and BGT1 genes is only reduced, not eliminated, when the AP-1 interaction is blocked either by mutation of the AP-1 site or by siRNA knockdown of c-Fos or c-Jun, demonstrating that the association is not obligatory for transcriptional activation of a TonEBP/OREBP target gene.

High extracellular NaCl activates TonEBP/OREBP in several ways. Within 30 min, existing TonEBP/OREBP becomes phosphorylated at serine and tyrosine residues and translocates to the nucleus (51). In the nucleus its greater abundance increases its binding to OREs (34, 43). Later, TonEBP/OREBP protein abundance increases due to enhanced stability of its mRNA (52). Also, the C terminus of TonEBP/OREBP contains its high NaCl-dependent transactivation domain (21). Its phosphorylation is blocked either by mutation of the AP-1 site or by siRNA transactivation domain (21). Its phosphorylation appears to be necessary for full transactivation. For example, in the presence of active ataxia-telangiectasia mutated (ATM), transactivation appears to be necessary for full transactivation. For example, AR

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AP-1 Enhances TonEBP/OREBP-dependent Transcription at High NaCl

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