Proteomic identification of proteins associated with the osmoregulatory transcription factor TonEBP/OREBP: functional effects of Hsp90 and PARP-1

Ye Chen, Michael P. Schnetz, Carlos E. Irazarrabal, Rong-Fong Shen, Chester K. Williams, Maurice B. Burg, and Joan D. Ferraris

1Laboratory of Kidney and Electrolyte Metabolism; 2Proteomics Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

Submitted 8 December 2005; accepted in final form 22 November 2006

Chen Y, Schnetz MP, Irazarrabal CE, Shen RF, Williams CK, Burg MB, Ferraris JD. Proteomic identification of proteins associated with the osmoregulatory transcription factor TonEBP/OREBP: functional effects of Hsp90 and PARP-1. Am J Physiol Renal Physiol 292: F981–F992, 2007. First published December 5, 2006; doi:10.1152/ajprenal.00493.2005.—Hypertonicity (e.g., high NaCl) increases transcriptional activity of TonEBP/OREBP by several mechanisms. It causes TonEBP/OREBP to translocate to the nucleus (23, 36). It increases the mRNA and protein abundance of TonEBP/OREBP (23, 36), and it increases activity of the TonEBP/OREBP transactivation domain (TAD) (15). It increases phosphorylation of TonEBP/OREBP (11), including the region containing its COOH-terminal TAD (15), which presumably is involved in signaling its activation. Further, several different protein kinases are known to contribute to activation of TonEBP/OREBP, namely p38 MAP kinase (22, 46), tyrosine kinase Fyn (22), protein kinase A (PKAc) (14) and Ataxia Telangiectasia Mutated kinase (ATM) (21). All contribute to high-NaCl-induced activation of TonEBP/OREBP, but no individual one is sufficient for full activation.

TonEBP/OREBP is part of a large protein complex (23, 36). Some of the other proteins in this complex are already known, based on coimmunoprecipitation with TonEBP/OREBP, namely PKAc (14), ATM (21), and RNA Helicase A (RHA) (9). PKAc (14) and ATM (21) are activated by high NaCl, contributing to the activation of TonEBP/OREBP. Overexpression of RHA inhibits the TonEBP activity, independent of its catalytic activity. High NaCl inhibits the interaction between TonEBP/OREBP and RHA, which also contributes to the stimulation of TonEBP/OREBP (9).

Any additional proteins that physically associate with TonEBP/OREBP are candidates for participation in the transcriptional complex or signaling cascade. To identify such proteins in the present studies, we immunoprecipitated recombinant TonEBP/OREBP from HEK 293 cells stably transfected with a construct coding for TonEBP/OREBP amino acids 1-547, identified a number of coimmunoprecipitated proteins by mass spectrometry, and, using immunoblots and electromobility shift assays, we confirmed the associations between TonEBP/OREBP and most of the identified proteins. Finally, we examined the effects of two of the coimmunoprecipitated proteins, PARP-1 and HSP90 on the function of TonEBP/OREBP.

METHODS

Plasmids. Human TonEBP/OREBP cDNA clone KIAA0827 was a gift from T. Nagase, Kazusa DNA Research Institute (Chiba, Japan). Sequence coding for TonEBP/OREBP amino acids 1-547 from KIAA0827 was amplified by PCR and cloned into

Address for reprint requests and other correspondence: M. B. Burg, National Heart, Lung, and Blood Institute, Bldg 10, Rm 6N260, Bethesda, MD 20892-1603 (e-mail: maurice_burg@nih.gov).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
pcDNA6 V5-His (Invitrogen, Carlsbad, CA) expression vector (which also codes for blasticidin resistance) to generate 1-1531 V5-His and 1-547 V5-His. The -1233/-1105 IL2 min GL3 (ORE-Luc) reporter of TonEBP/OREBP transcriptional activity, and the mutated luciferase reporter counterpart eliminating TonEBP/OREBP binding were as described previously (15, 60). The binary GAL4 reporter of the activity of the TonEBP/OREBP TAD was also described previously (15). PARP-1 constructs (wild type, catalytically inactive E988K, DNA binding inactive C21,125G, along with the control empty vector) (18) were gifts of M. O. Hottiger, University of Zurich, Switzerland. To distinguish between effects on transcription in general and transcription directed by TonEBP/OREBP-specific DNA elements (OREs), ORE-Luc was mutated at -1178, -1176, -1143, -1141, -1102, -1100, -1070, -1068, -1044, and -1042, i.e., at all 3 ORE sites and an API site, as previously described (60) which eliminates binding by TonEBP/OREBP and results in greatly reduced response to high NaCl (see RESULTS).

Cell culture. HEK 293 cells (American Type Culture Collection, Manassas, VA) were cultured at 300 mosmol/kgH2O, as instructed by the suppliers. Osmolality was increased to 500 mosmol/kgH2O by adding NaCl, as indicated. To prepare stably transfected cells, TonEBP/OREBP 1-547 V5-His or 1-1531 V5-His was transfected into HEK 293 cells using Effectene (Qiagen, Valencia, CA) and antibiotic resistant cells were selected and cloned in the presence of 10 μg/ml blasticidin (Calbiochem, San Diego, CA), according to Invitrogen instructions. Two clones expressing TonEBP/OREBP 1-547 V5-His and one expressing TonEBP/OREBP 1-1531 V5-His were identified by anti-V5 immunoblot and expanded in the presence of the antibiotic. The cultures were maintained in the presence of 5 μg/ml blasticidin until just before each experiment. Stable reporter cell lines for measuring TonEBP/OREBP transcriptional activity and the activity of its TAD were previously described (60). Mouse fibroblast PARP-1 +/− cells (gifts from M. E. Smulson, Georgetown University, Washington, D.C.) were cultured at 300 mosmol/kgH2O as instructed by the suppliers.

Geldanamycin, an Hsp90 inhibitor (gift of the Drug Synthesis & Chemistry Branch, National Cancer Institute, Bethesda, MD), was dissolved in DMSO as 2 mM stock solution. Cells to be used for immunoprecipitation were treated with DMSO (control, 0.05%) or 1 μM geldanamycin in an equal concentration of DMSO for 2 h before altering medium osmolality. Cells to be used for reporter assays were treated with DMSO (control, 0.005%) or 100 nM geldanamycin in an equal concentration of DMSO for 1 h before altering medium osmolality.

To study the effect of inhibition of PARP-1 enzymatic activity, HEK 293 cells were transfected with ORE-Luc reporter, using Effectene (Qiagen, Valencia, CA) according to supplier’s instructions. Then, 24 h later, 3 mM 3-amino-benzamide (Sigma, St. Louis, MO), an inhibitor of PARP-1 enzymatic activity (41), was added at 300 mosmol/kgH2O for 1 h, then osmolality was either maintained at 300 or increased to 500 mosmol/kgH2O by adding NaCl for 16 h in the continued presence of the inhibitor. Luciferase activity was measured with the Dual Luciferase assay System (Promega, Madison, WI).

Immunoprecipitation of TonEBP/OREBP and mass spectrometry. HEK 293 cells stably transfected with TonEBP/OREBP 1-547 V5-His were grown in 15-cm dishes. Osmolality was increased to 500 mosmol/kgH2O by adding NaCl for 6 h. Protein extraction and immunoprecipitation were performed, as above, except using either control IgG or anti-V5 and protein A/G plus-agarose beads (sc-2003, Santa Cruz). Proteins were separated on 4–12% Novex Tris-Glycine gels, transferred to nitrocellulose membranes (Invitrogen), then probed with anti-V5 and other antibodies. Blots were analyzed using the Odyssey infrared imaging system (LI-COR, Lincoln, NE) (59) or using ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ).

To test further the association of TonEBP/OREBP with Hsp90, antibodies for immunoprecipitation, namely IgG (control, Santa Cruz Biotechnology, Santa Cruz, CA), anti-V5 (Invitrogen), or anti-Hsp90 (SPA-845, Stressgen, Victoria, BC Canada), were added together with A/G plus-agarose beads (sc-2003, Santa Cruz Biotechnology). Protein blots were prepared as above, then probed with anti-V5, anti-Hsp90 (SPA-830, Stressgen), or anti-Hsp90α (SPA-771, Stressgen). Blots were analyzed using the Odyssey infrared imaging system (LI-COR, Lincoln, NE) (59).

To test further the association of TonEBP/OREBP with PARP-1, HEK 293 cells, grown in 10-cm dishes, were transfected with TonEBP/OREBP 1-1531 V5-His using Effectene. After 24 h, osmolality was either maintained at 300 or increased to 500 mosmol/kgH2O by adding NaCl for 6 h. Protein extraction and immunoprecipitation were performed, as above, except using either control IgG, anti-V5, or anti-PARP-1 (06-557, Upstate, Waltham, MA) antibody, and protein A/G plus-agarose beads (sc-2003, Santa Cruz Biotechnology). Proteins were separated on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen), then probed with anti-V5 and other antibodies. Blots were analyzed using the Odyssey infrared imaging system (LI-COR, Lincoln, NE) (59).
EMSA. HEK 293 cells stably transfected with TonEBP/OREBP 1-1531 V5-His were treated 2 h at 500 mosmol/kgH$_2$O (NaCl added). Nuclear pellets were prepared using nuclear and cytoplasmic extraction reagents (NE-PER, Pierce), according to the manufacturer’s instructions. Nuclear pellets were resuspended in lysis buffer (31), centrifuged 10 min at 15,000 g, and the supernatant retained. Double-stranded ORE probe (bp -1238 to -1104 of the human aldose reductase gene) containing three OREs in native gene context was generated by annealing complementary $5'$ biotinylated oligonucleotides (Integrated DNA Technologies, Coralville, IA). We preincubated 0.5 µg of nuclear extract with 0.5 µg of poly(dA-dT) in binding buffer (31) with or without added antibodies against associated proteins for 1 h at 4 °C. Antibodies were against TonEBP/OREBP (1 µg, NTA5, Affinity Bioreagents, Golden, CO), Hsc70 (1 µg, sc-7298); DNA-PKcs (1 µg, sc-9051); Ku80 (1 µg, sc-9034); hnRNP F (1 µg, sc-10045); hnRNP K (1 µg, sc-2537); hnRNP M (1 µg, sc-20002); Hsp90β (2 µg, sc-1057); PARP-1 (0.5 µg, 611038 BD Pharmingen), RNA helicase A (2.5 µl serum), and RNA helicase II/Gu (2.5 µl serum). One hundred femtomoles of ORE probe were added and reactions included an additional 20 min at room temperature. Total binding reaction volume was 20 µl. Reaction products were separated by electrophoresis on 0.4% SeaKem Gold agarose gels in 0.5 X Tris-borate/EDTA buffer at 4 °C, transferred to Hybond-N+ membranes (Amersham Biosciences), and crosslinked (UV Stratalinker, Stratagene, La Jolla, CA). Probe was detected using the LightShift chemiluminescent electrophoretic mobility-shift assay kit (Pierce) according to the manufacturer’s instructions.

Effects of Hsp90 and PARP-1 on TonEBP/OREBP function. To test for effects of Hsp90 on TonEBP/OREBP transcriptional activity and the activity of its TAD, HEK 293 cells stably expressing the respective reporters were treated with either DMSO or 100 nM geldanamycin at 300 mosmol/kgH$_2$O for 1 h, then osmolality was either maintained at 300 or increased to 500 mosmol/kgH$_2$O by adding NaCl in the presence of either DMSO or geldanamycin for 16 h. Luciferase activity was measured with the Dual Luciferase Assay System (Promega) (15). To test for effects of Hsp90 on TonEBP/OREBP protein abundance, native HEK 293 cells were treated with DMSO or 1 µM geldanamycin at 300 or 500 mosmol/kgH$_2$O for the indicated times followed by immunoblot of whole cell lysates, performed, as above, except using an antibody against TonEBP/OREBP itself (Affinity Bioreagents, Golden, CO). To test for effects of Hsp90 on TonEBP/OREBP intracellular localization, HEK 293 cells were treated with DMSO or 100 nM geldanamycin at 300 mosmol/kgH$_2$O for 1 h, then for another hour at 200, 300, or 500 mosmol/kgH$_2$O, followed by separate immunoblots of cytosolic and nuclear lysates prepared with NE-PER following vendor’s instructions (Pierce). Immunoblots were analyzed using the Odyssey infrared imaging system (LI-COR) and nuclear to cytoplasmic ratios of TonEBP/OREBP were calculated as previously described (59).

Transfection and luciferase assays. To measure the effect of expression of PARP-1 on TonEBP/OREBP transcriptional activity, PARP-1 −/− cells were cotransfected with 1) ORE-Luc or mutated ORE-Luc (as control) and 2) either of various PARP-1 constructs or the empty vector control.

To measure the combined effects of the ATM inhibitor, wortmannin (Calbiochem, San Diego, CA), and expression of PARP-1 on TonEBP/OREBP transcriptional activity, PARP-1 −/− cells were doubly transfected by electroporation (Gene Pulser II Electroporator, Bio-Rad, Hercules, CA) with 1) ORE-Luc reporter, 2) either wild-type PARP-1 construct or the empty vector control, then seeded in a 96-well cell culture plate. After 24 h, osmolality was increased to 500 mosmol/kgH$_2$O for 16 h in the presence or absence of wortmannin and luciferase activity was measured.

To measure the activity of the TonEBP/OREBP TAD, cells were triply transfected by electroporation with 1) pFR-Luc, which contains a Gal4 upstream activating sequence and reports activity of TADs, 2) Gal4dbd-TonEBP/OREBP 548-1531, which drives the reporter depending on the activity of the TonEBP/OREBP TAD sequence that it contains, or, as a control, Gal4dbd, which does not contain a TAD, and 3) various PARP-1 constructs or the empty vector control, then seeded in 96-well cell culture plate. After 24 h the osmolality was either maintained at 300 or increased to 500 mosmol/kgH$_2$O for 16 h cells and luciferase activity was measured.

RNA isolation, cDNA preparation, and real-time PCR. PARP-1 −/− cells were transfected with wild-type PARP-1 or empty vector by electroporation. After 24 h osmolality was maintained at 300 or increased to 500 mosmol/kgH$_2$O by adding NaCl for 16 h. Total RNA was isolated (RNasy, Qiagen) and cDNA was prepared (TaQMan reverse transcription kit, Applied Biosystems, Foster city, CA) according to suppliers’ instructions. Real-time PCR was performed using ABI Prism 7900HT sequence detection system (Applied Biosystems) (14), primers and probes were designed and relative differences were calculated, as previously described (15).

Statistical analysis. Data were compared by a one-way ANOVA followed by Dunnett’s multiple comparison test for separation of significant means. Logarithmic transformation was applied first to data calculated as ratios. Results are expressed as mean ± SE (n = 3). Differences were considered significant for P < 0.05.

RESULTS

Identification by LC-MS/MS of proteins that coinmunoprecipitate with TonEBP/OREBP. To identify proteins that associate with and, thus, possibly regulate or support TonEBP/OREBP activity we immunoprecipitated recombinant TonEBP/OREBP from nuclear extracts of HEK 293 cells, stably transfected with TonEBP/OREBP 1-547 V5-His, 6 h after medium osmolality had been increased from 300 to 500 mosmol/kgH$_2$O by adding NaCl. We used cells stably transfected with TonEBP/OREBP 1-547 V5-His because, like other transcription factors, the abundance of native TonEBP/OREBP protein is low. We started with plasmids containing either TonEBP/OREBP 1-547, 548-1531, or 1-1531. Since we able to immunoprecipitate enough recombinant protein for successful analysis only from the cells stably transfected with TonEBP/OREBP 1-547 V5-His (data not shown), we continued with those cells. Anti-V5 antibody immunoprecipitates a number of proteins from the TonEBP/OREBP 1-547 transfected cells that are not immunoprecipitated by control non-specific antibody (IgG), as shown by markedly different SDS-PAGE profiles (Fig. 1A). Considering only proteins represented by two or more unique peptides, we identified by LC-MS/MS of tryptic peptides, digested from gel slices, 14 different coinmunoprecipitated proteins, ranging from 30 to 350 kDa (Table 1 and Supplementary Table). Representative spectra for two peptides from each protein are shown as Supplementary Figures. The spectra are arranged according the gel piece, S1, S2, etc., which is the order they appear in table I and the Supplementary Table. The spectrum of each peptide is displayed in two charge states, Charge +1 and Charge +2. The sizes of the identified proteins match closely with the molecular masses expected in the gel slices (Table 1). As might be expected, TonEBP/OREBP 1-547 is represented by a strong protein band and the most peptides, namely 14.

These 14 proteins fall into several classes: 1) DNA dependent protein kinase, both its catalytic subunit (DNA-PKcs) and regulatory subunit, Ku86; 2) Helicases, namely RNA helicase A, nucleolar RNA helicase II/Gu, and RNA helicase p72; 3) Small or heterogeneous nuclear ribonucleoproteins (snRNPs or hnRNPs), namely U5 snRNPs-specific 116 kDa protein, U5
snRNP-specific 200 kDa protein, hnRNP U, hnRNP M, hnRNP K, and hnRNP F; 4) Heat shock proteins, namely Hsp90/H9251 and Hsc70; and 5) PARP-1. Among those 14 proteins, PARP-1 (113 kDa) is predominant, being represented by a clear protein band in the 120 kDa gel slice, from which 14 specific peptides were identified.

Confirmation of protein identifications by immunoblot. Proteins from whole cell lysates of HEK 293 cells that were transiently transfected with TonEBP/OREBP 1-1531 V5-His were maintained at 300 or increased to 500 mosmol/kgH2O (NaCl added) for 6 h. Immunoprecipitation from whole cell lysates was with IgG or anti-V5. Immunobots used primary antibodies against the proteins that are indicated and were analyzed by the Odyssey imaging system or, for RNA helicase II/Gu, by ECL chemiluminescence. Images are representative of 3 experiments. C: reciprocal coimmunoprecipitation of TonEBP/OREBP and PARP-1. HEK 293 cells transiently transfected with TonEBP/OREBP 1-1531 V5-His were maintained at 300 or increased to 500 mosmol/kgH2O (NaCl added) for 6 h. Immunoprecipitation from whole cell lysates was done with IgG (control), anti-V5, or anti-PARP-1. Immunobots used the primary antibodies that are indicated and were analyzed by ECL chemiluminescence. Images are representative of 3 experiments. D: association of PARP-1 with TonEBP/OREBP is independent of DNA binding. HEK 293 cells were transfected with TonEBP/OREBP 1-1531 V5-His using Effectene. Then, they were kept at 300 or increased to 500 mosmol/kgH2O for 6 h. Immunoprecipitation from cell lysates was with either IgG or anti-V5 and protein A/G Plus agarose beads, in the presence of 100 µg/ml ethidium bromide. The blot is representative of those from 3 independent experiments. E and F: association of Hsp90 with TonEBP/OREBP. HEK 293 cells, stably transfected with TonEBP/OREBP 1-1531 V5-His, were maintained at 300 or increased to 500 mosmol/kgH2O (NaCl added) for 1 h. Images are representative of 3 independent experiments. E: coimmunoprecipitation of Hsp90α with TonEBP/OREBP 1-1531-V5-His. Immunoprecipitation from whole cell lysates was done with IgG or anti-V5. Immunobots used primary antibodies against V5 or Hsp90α. F: coimmunoprecipitation of TonEBP/OREBP 1-1531-V5-His with Hsp90. The anti-Hsp antibody does not distinguish between Hsp90α and Hsp90β. Anti-Hsp or IgG was used for immunoprecipitation.
V5-His, were incubated with 100 μg/ml ethidium bromide during immunoprecipitation. Since the presence of ethidium bromide does not interrupt the coimmunoprecipitation of TonEBP/OREBP and PARP-1 (Fig. 1D), we conclude that TonEBP/OREBP and PARP-1 are physically associated by a protein-protein interaction.

Hsp90 has two isoforms, Hsp90α and Hsp90β. Hsp90β associates with TonEBP/OREBP (Table 1 and Fig. 1B). To determine whether Hsp90α also associates with TonEBP/OREBP, we used an antibody specific for Hsp90α in immunoblots of proteins immunoprecipitated along with TonEBP/OREBP (Fig. 1E). Hsp90α, as well as Hsp90β is associated with TonEBP/OREBP. Further evidence for the association is that TonEBP/OREBP, as well as Hsp90, is immunoprecipitated by anti-Hsp90 (Fig. 1F). Although in the particular experiment the amounts of both TonEBP/OREBP and Hsp90 that coimmunoprecipitates with Hsp90 is less at 500 than at 300 mosmol/kgH2O, this was not a consistent finding, and the level of HSP90 was not affected by NaCl concentration (see Fig. 3A).

Association of proteins with TonEBP/OREBP in a complex that binds specifically to its cognate DNA element, ORE/TonE. To determine whether the identified proteins are members of the TonEBP/OREBP-containing protein complex that binds to its cognate DNA element (ORE/TonE), we performed electrophoretic mobility-shift assays using a probe containing the ORE/TonE of the aldose reductase gene (Fig. 2). Antibodies against the 10 putative associated proteins for which antibodies were available were added to the binding buffer. The mobility shift in the absence of added antibody is specific since it is eliminated by addition of 10-fold excess of non-biotinylated probe (Fig. 2). Binding of an antibody against a protein contained in the complex retards mobility of the complex, resulting in a “supershift.” Clear supershifts occur with addition of anti-TonEBP/OREBP or antibody against any of the other tested proteins, except for PARP-1 for which the result is equivocal (Fig. 2). We conclude that at least 9 of the identified proteins associate in vitro with TonEBP/OREBP in a complex that binds to its cognate DNA element, ORE/TonE.

It seemed likely that association of these proteins with TonEBP/OREBP could affect its function. We have tested this possibility, starting with Hsp90 and PARP-1, as follows.

**Effect of Hsp90 on function of TonEBP.** Geldanamycin is an inhibitor of Hsp90 (17). Geldanamycin decreases the association of proteins with TonEBP/OREBP in a complex that binds specifically to its cognate DNA element, ORE/TonE.

**Table 1. Proteins that coimmunoprecipitate with TonEBP/OREBP 1-547 V5-His**

<table>
<thead>
<tr>
<th>Slice</th>
<th>Gel Slice, kDa</th>
<th>Protein Identification</th>
<th>Expected Mass, kDa</th>
<th>Accession Number</th>
<th>Number of Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>over 300</td>
<td>DNA dependent protein kinase catalytic subunit</td>
<td>460</td>
<td>NP_008835</td>
<td>2</td>
</tr>
<tr>
<td>S2</td>
<td>250</td>
<td>U5 small nuclear ribonucleoprotein-specific protein 200 kDa</td>
<td>200</td>
<td>NP_054733</td>
<td>5</td>
</tr>
<tr>
<td>S4</td>
<td>180</td>
<td>RNA helicase A</td>
<td>140</td>
<td>NP_001348</td>
<td>3</td>
</tr>
<tr>
<td>S6</td>
<td>120</td>
<td>Heterogeneous nuclear ribonucleoprotein U</td>
<td>120</td>
<td>NP_114032</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U5 small nuclear ribonucleoprotein-specific protein 116 kDa</td>
<td>116</td>
<td>NP_004238</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(ADP-ribose) polymerase-1</td>
<td>116</td>
<td>NP_001609</td>
<td>13</td>
</tr>
<tr>
<td>S8</td>
<td>100</td>
<td>Nucleolar RNA helicase II/Gu</td>
<td>100</td>
<td>NP_004719</td>
<td>6</td>
</tr>
<tr>
<td>S9</td>
<td>90</td>
<td>Heat shock protein 90β</td>
<td>90</td>
<td>NP_031381</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ku86</td>
<td>86</td>
<td>NP_060694</td>
<td>2</td>
</tr>
<tr>
<td>S10</td>
<td>80</td>
<td>Heterogeneous nuclear ribonucleoprotein M</td>
<td>77</td>
<td>NP_005959</td>
<td>2</td>
</tr>
<tr>
<td>S11</td>
<td>70</td>
<td>RNA helicase p72</td>
<td>72</td>
<td>NP_006377</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heat shock cognate protein 70</td>
<td>70</td>
<td>NP_006588</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TonEBP/OREBP 1-547</td>
<td>63</td>
<td>NP_006590</td>
<td>14</td>
</tr>
<tr>
<td>S13</td>
<td>60</td>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
<td>65</td>
<td>NP_112553</td>
<td>4</td>
</tr>
<tr>
<td>S15</td>
<td>45</td>
<td>Heterogeneous nuclear ribonucleoprotein F</td>
<td>53</td>
<td>NP_004957</td>
<td>2</td>
</tr>
</tbody>
</table>
tion between Hsp90 and TonEBP/OREBP at both 300 and 500 mosmol/kgH2O (Fig. 3A). This result is consistent with previous studies that show geldanamycin disrupts the interaction between Hsp90 and its client proteins (17), and with the association between HSP90 and TonEBP/OREBP being a specific protein-protein interaction, which requires that Hsp90 is in its active conformation.

To test for an effect of geldanamycin on TonEBP/OREBP transcriptional activity, we treated HEK 293 cells that stably express a TonEBP/OREBP transcriptional reporter with either 100 nM geldanamycin or vehicle (DMSO, “Control”). Geldanamycin reduces the transcriptional activity of TonEBP/OREBP by 33% at 300 mosmol/kgH2O and by 66% at 500 mosmol/kgH2O when the reporter contains wild-type ORE/TonE sequence (Fig. 3B). In contrast, geldanamycin does not have a significant effect when the ORE/TonE element in the reporter is mutated so that TonEBP/OREBP cannot bind to it (Fig. 3B), indicating that the effect is not due to general inhibition of transcription.

The inhibition of TonEBP/OREBP transcriptional activity is explained, at least in part, by the fact that geldanamycin inhibits activity of the TonEBP/OREBP TAD. When HEK 293 cells stably reporting activity of the TonEBP/OREBP TAD are treated with 100 nM geldanamycin, activity of the of TonEBP TAD is reduced by 43% at 300 and 48% at 500 mosmol/kgH2O (Fig. 3C). As a control for specificity, Geldanamycin has no effect on a reporter (Gal4-DBD) that lacks the TonEBP/OREBP TAD (TAD) (Fig. 3C).

High NaCl increases the amount of TonEBP/OREBP mRNA and protein (7, 23, 36). TonEBP/OREBP mRNA increases because high NaCl stabilizes it, reducing the rate at which it is degraded (7). The greater mRNA abundance results in increased translation of TonEBP/OREBP, accounting for the greater protein abundance (56). In HEK 293 cells the increase in TonEBP/OREBP protein peaks between 8 and 24 h after osmolality is increased to 500 mosmol/kgH2O by adding NaCl (Fig. 4A). Geldanamycin significantly inhibits this increase at 500 mosmol/kgH2O but does not affect the level of TonEBP/OREBP protein at 300 mosmol/kgH2O (Fig. 4A), implying that HSP90 either supports the increased translation or inhibits the degradation of the protein.

High NaCl also causes TonEBP/OREBP to move from cytoplasm to nucleus, making more available for transcriptional regulation. This was previously found in MDCK (36, 56), Hela (23), Hep G2, COS-7 cells, and fibroblasts (59). In HEK 293 cells at 300 mosmol/kgH2O the amount of TonEBP/OREBP in the nucleus approximately equals that in the cytoplasm, but after one h at 500 mosmol/kgH2O (NaCl added), there is four times as much TonEBP/OREBP in the nucleus as in the cytoplasm (Fig. 4B). Despite its effects on other aspects of TonEBP/OREBP activity, geldanamycin does not affect the nuclear to cytoplasmic ratio of TonEBP/OREBP at 200, 300, or 500 mosmol/kgH2O (Fig. 4B).

Effect of PARP-1 on function of TonEBP/OREBP. To test for specific effects of PARP-1 on the function of TonEBP/OREBP, we reconstituted PARP-1−/− cells with expression vectors that either are empty (Control), contain wild-type PARP-1 or contain PARP-1 mutated to eliminate either its enzymatic or DNA-binding activity (18). We measured TonEBP/OREBP transcriptional activity by cotransfecting a luciferase reporter (Fig. 5A). Increasing osmolality from 300 to 500 mosmol/kgH2O by adding NaCl for 16 h increases transcriptional activity more than 20-fold in PARP−/− cells. Expression of PARP-1 reduces TonEBP/OREBP transcriptional activity by about 30–40% both at 300 and 500 mosmol/
kgH₂O. Mutants of PARP-1 that lack DNA binding or enzymatic activity have an effect similar to wild-type PARP-1. To exclude the possibility that this effect of PARP-1 is on transcription in general, rather than transcription directed by TonEBP/OREBP, we repeated the experiment, using a reporter in which the ORE elements to which TonEBP/OREBP binds are mutated (Fig. 5A). This inhibition presumably contributes to the inhibition of transcriptional activity at 500 mosmol/kgH₂O, but leaves unexplained the inhibition of transcriptional activity at 300 mosmol/kgH₂O (Fig. 5A). As a control, PARP-1 has no effect on an otherwise identical reporter system that lacks the TonEBP/OREBP TAD (Fig. 5D), making it unlikely that PARP-1 inhibits transactivation in general.

High NaCl-induced increase in TonEBP/OREBP activity raises transcription and mRNA abundance of its transcriptional targets, including aldose reductase and BGT1. In the PARP-1−/− cells, raising osmolality from 300 to 500 mosmol/kgH₂O by adding NaCl for 16 h increases aldose reductase mRNA 42-fold and BGT1 mRNA 216-fold (Fig. 5E). Expression of wild-type PARP-1 reduces the abundance of both mRNAs at 500 mosmol/kgH₂O, by 45% for aldose reductase and 52% for BGT1, but has no effect at 300 mosmol/kgH₂O. These findings support a contribution of PARP-1 to TonEBP/OREBP transcriptional activity.

ATM contributes to high NaCl-induced increase of TonEBP/OREBP transcriptional activity and activity of its TAD (21). PARP-1 inhibits activation of ATM by the DNA damaging agent, necrostatin (52). That raised the possibility that PARP-1 might inhibit TonEBP/OREBP transcriptional activity indirectly, by inhibiting ATM. To determine whether the effect of PARP-1 on TonEBP/OREBP is mediated by ATM, we added wortmannin, which inhibits ATM (44). Expression of PARP-1 reduces TonEBP/OREBP transcriptional activity at 500 mosmol/kgH₂O even when ATM is already inhibited by wortmannin (Fig. 5F). We conclude that inhibition of high NaCl-induced increase in TonEBP/OREBP transcriptional activity by PARP-1 is not mediated by ATM.

**DISCUSSION**

**Effects of Hsp-90 on function of TonEBP/OREBP.** We found that both HSC70 (a member of the HSP70 family) and HSP90 are associated with TonEBP/OREBP (Figs. 1, 2, 5, and 6). Hsp90 is an abundant and highly conserved molecular chaperone (39). It exists as homodimers (35, 48) of two isoforms, α (inducible form/major form) and β (constitutive form/minor form). Hsp90α and Hsp90β are expressed abundantly in both the renal cortex and the medulla, with expression being higher in the renal medulla (42). Further, HSP90α protein abundance increases in TALH cells when they are adapted to medium in which osmolality is increased to 600 mosmol/kgH₂O by adding NaCl (12). HSP90 (20, 40) in cooperation with several cochaperones, one of which is HSP70, is important for maintaining folding, trafficking and stability of client proteins, many of which are involved in cellular signaling networks. These include steroid hormone receptors, protein kinases and transcription factors (40). HSP90 is known to regulate nearly 100 transcription factors (40).

### References

1. HSP90 and its Role in Cellular Signaling.
2. HSP70 and Its Role in Cell Survival.
3. HSP90 and Its Role in Cell Death.
4. HSP90 and Its Role in Cancer.
5. HSP90 and Its Role in Inflammation.
6. HSP90 and Its Role in Aging.
7. HSP90 and Its Role in Neurodegeneration.
8. HSP90 and Its Role in Metabolism.
9. HSP90 and Its Role in Immunity.
10. HSP90 and Its Role in Reproduction.

**AJP-Renal Physiol • VOL 292 • MARCH 2007 • www.ajprenal.org**
it to 500 mosmol/kgH2O (NaCl added) either in the absence or presence of wortmannin for 16 h. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with “None.” B: same as A, except that the luciferase reporter was either wild-type ORE or mutated to prevent binding of TonEBP/OREBP. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with “None.” C: inhibition of PARP-1 enzymatic activity does not decrease ORE reporter activity. HEK 293 cells were transfected with ORE-Luc. Twenty-four hours later medium was changed for 1 h, keeping it the same or adding 3-aminobenzamide. Then, otherwise identical media were substituted at 300 or 500 mosmol/kgH2O (NaCl added) for 16 h. Values are normalized to control cells at 300 mosmol/kgH2O. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with control. D: PARP-1 inhibits high NaCl-induced increase in the activity of the TAD of TonEBP/OREBP. PARP-1 \(-/-\) cells were transfected with pFR-Luc, Gal4dbd-TonEBP/OREBP 548-1531 or Gal4dbd (lacking the TonEBP/OREBP TAD), and various PARP-1 constructs or empty vector (“None”), as indicated. After 24 h, medium was changed for 16 h, keeping it at 300 or increasing to 500 mosmol/kgH2O (NaCl added). Values are normalized to empty vector control at 300 mosmol/kgH2O for Gal4dbd-TonEBP/OREBP 548-1531. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with “None.” E: PARP-1 reduces aldose reductase and BGT-1 mRNA abundance at 500 mosmol/kgH2O. PARP-1 \(-/-\) cells were transfected with wild-type PARP-1 or empty vector (“None”). After 24 h, osmolality was kept at 300 or increased to 500 mosmol/kgH2O by adding NaCl for 16 h. mRNA abundance was measured by RT-PCR. Values are normalized to empty vector at 300 and 500 mosmol/kgH2O, respectively. With empty vector elevation to 500 mosmol/kgH2O increased aldose reductase mRNA abundance 142-fold and BGT1 216-fold. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with empty vector. F: effects of PARP-1 and wortmannin are additive. PARP-1 \(-/-\) cells were transfected with the ORE-Luc reporter and either wild-type PARP-1 or empty vector (“None”), as indicated, then seeded in 96-well cell culture plates. Twenty-four hours later medium was changed, keeping it at 300 or increasing it to 500 mosmol/kgH2O (NaCl added) either in the absence or presence of wortmannin for 16 h. Means ± SE \( (n = 3) \). * \( P < 0.05 \), compared with no wortmannin.

Fig. 5. Effect of PARP-1 on function of TonEBP. A: PARP-1 \(-/-\) cells were cotransfected with ORE-Luc reporter and various PARP-1 constructs or empty vector (“None”), as indicated. 24 h later medium was changed, keeping it at 300 or increasing it to 500 mosmol/kgH2O (NaCl added) for 16 h, before measuring luciferase activity. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with “None.” B: same as A, except that the luciferase reporter was either wild-type ORE or mutated to prevent binding of TonEBP/OREBP. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with “None.” C: inhibition of PARP-1 enzymatic activity does not decrease ORE reporter activity. HEK 293 cells were transfected with ORE-Luc. Twenty-four hours later medium was changed for 1 h, keeping it the same or adding 3-aminobenzamide. Then, otherwise identical media were substituted at 300 or 500 mosmol/kgH2O (NaCl added) for 16 h. Values are normalized to control cells at 300 mosmol/kgH2O. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with control. D: PARP-1 inhibits high NaCl-induced increase in the activity of the TAD of TonEBP/OREBP. PARP-1 \(-/-\) cells were transfected with pFR-Luc, Gal4dbd-TonEBP/OREBP 548-1531 or Gal4dbd (lacking the TonEBP/OREBP TAD), and various PARP-1 constructs or empty vector (“None”), as indicated. After 24 h, medium was changed for 16 h, keeping it at 300 or increasing to 500 mosmol/kgH2O (NaCl added). Values are normalized to empty vector control at 300 mosmol/kgH2O for Gal4dbd-TonEBP/OREBP 548-1531. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with “None.” E: PARP-1 reduces aldose reductase and BGT-1 mRNA abundance at 500 mosmol/kgH2O. PARP-1 \(-/-\) cells were transfected with wild-type PARP-1 or empty vector (“None”). After 24 h, osmolality was kept at 300 or increased to 500 mosmol/kgH2O by adding NaCl for 16 h. mRNA abundance was measured by RT-PCR. Values are normalized to empty vector at 300 and 500 mosmol/kgH2O, respectively. With empty vector elevation to 500 mosmol/kgH2O increased aldose reductase mRNA abundance 142-fold and BGT1 216-fold. Means ± SE \( (n = 3) \). * \( P < 0.05 \) compared with empty vector. F: effects of PARP-1 and wortmannin are additive. PARP-1 \(-/-\) cells were transfected with the ORE-Luc reporter and either wild-type PARP-1 or empty vector (“None”), as indicated, then seeded in 96-well cell culture plates. Twenty-four hours later medium was changed, keeping it at 300 or increasing it to 500 mosmol/kgH2O (NaCl added) either in the absence or presence of wortmannin for 16 h. Means ± SE \( (n = 3) \). * \( P < 0.05 \), compared with no wortmannin.

substrates or “client proteins” (40). Most of these client proteins have been shown to interact directly with the chaperone site(s) in hsp90 as part of substrate-hsp90 heterocomplexes, formed in an ATP-dependent process by the multiprotein hsp90/hsp70-based chaperone machinery.

Although Hsp90 resides mostly in the cytoplasm where it is the main functional component of an important cytoplasmic chaperone complex, it is also found inside the nucleus. Most studies of the role of Hsp90 in transcriptional regulation have focused on how it shifts the glucocorticoid receptor into the nucleus and regulates its nuclear retention. However, Hsp90 also recruits the basic transcriptional factor Sp1 to the promoter of 12(S)-lipoxigenase, where it regulates gene transcription by modulating binding of Sp1 to the promoter (20). HSP90 also physically associates with the transcription factor, STAT3, and disruption of this association inhibits transcriptional activity of STAT3 (45). Hsp90 client proteins also include many protein kinases (40), which is of significance since protein kinases are involved in high-NaCl induced activation of TonEBP/OREBP. Thus, there are several different ways in which Hsp90 might regulate transcriptional activity of TonEBP/OREBP.

High NaCl increases transcriptional activity of TonEBP/OREBP by raising its protein abundance, causing it to move into the nucleus, and increasing activity of its TAD. We tested for possible effects of Hsp90 on each of these regulating mechanisms by using the Hsp90 inhibitor, geldanamycin, which disrupts the association between Hsp90 and its client proteins (17) by competitive inhibition at the NH2-terminal ATP binding domain of Hsp90 (49). Geldanamycin inhibits TonEBP/OREBP transcriptional activity both at 300 and 500 mosmol/kgH2O (Fig. 3B). It reduces high-NaCl-induced increase of TonEBP/OREBP protein abundance (Fig. 4A), and of activity of its TAD (Fig. 3C), but not its nuclear localization (Fig. 4B).
High NaCl-induced increase of TonEBP/OREBP protein abundance peaks between 8 and 24 h in HEK 293 cells (Fig. 4A). High NaCl increases TonEBP/OREBP protein abundance by stabilizing its mRNA (7), which raises abundance of the mRNA (7, 56), resulting in increasing translation of the protein (56). Hsp90 could support this process by stabilizing TonEBP/OREBP protein. Geldanamycin disrupts HSP90-mediated stabilization of proteins, increasing the rate of their degradation (17), which could explain its effect on TonEBP/OREBP. It is striking that geldanamycin reduced TonEBP protein at 500 mosmol/kgH2O, but not at 300. Perhaps, TonEBP/OREBP is in an activated conformation at 500 mosmol/kgH2O, which increases its dependence on stabilization by HSP90. That is the case with Lck kinase, which activation makes more susceptible to geldanamycin-induced degradation (17). Destabilization of Hsp90 client proteins by geldanamycin normally leads to ubiquitination and subsequent degradation by proteosomes (17). However, since we did not find that geldanamycin significantly increases ubiquitination of TonEBP/OREBP (data not shown), we cannot support that possibility. The reduction of TonEBP/OREBP protein at 500 mosmol/kgH2O (Fig. 4A) could contribute to lowering its transcriptional activity at that osmolality by reducing the amount of TonEBP/OREBP available for binding to its ORE/TonE DNA element (Fig. 3B). However, geldanamycin also lowers TonEBP/OREBP transcriptional activity at 300 mosmol/kgH2O (Fig. 3B), without lowering its protein abundance (Fig. 4A), which we suggest is because of its other action, which is to inhibit activity of its TAD at both osmolalities.

Our assay of activity of the TonEBP/OREBP TAD should be independent of native TonEBP/OREBP in so far as it utilizes a recombinant TonEBP/OREBP TAD in one of the components of the binary GAL4 luciferase reporter system. Activity of the TonEBP/OREBP TAD is regulated by protein kinases including PKA (14), ATM (21), p38 (22), and Fyn (22). Hsp90 client proteins include numerous kinases (40). Although at this time none of the kinases known to affect TonEBP/OREBP is reported to be an Hsp90 client, we suggest that one or more might be, explaining the effect of geldanamycin on activity of the TonEBP/OREBP TAD.

Hsp90 affects transcriptional activity of the glucocorticoid receptor by facilitating its binding to steroid ligands in the cytosol and facilitating its translocation into the nucleus (20, 40). However, the effect of Hsp90 on TonEBP/OREBP cannot involve an analogous mechanism, since geldanamycin does not affect the subcellular location of TonEBP/OREBP (Fig. 4B).

Effects of PARP-1 on function of TonEBP/OREBP. PARP-1 is an enzyme that catalyzes poly ADP-ribosylation of chromatin-associated proteins involved in the regulation of nucleic acid metabolism and chromatin structure (10, 25). It has roles in DNA repair (33) and cell death (24). It also plays at least two important roles in transcription regulation (26). First, it modifies histones and creates an anionic poly(ADP-ribose) matrix that binds histones, thereby promoting the decondensation of higher-order chromatin structures. Second, it acts as a component of enhancer/promoter regulatory complexes in conjunction with other DNA binding factors and coactivators.

PARP-1 evidently associates with TonEBP/OREBP (Fig. 1, C and D) and inhibits its transcriptional activity (Fig. 5A). That raises the question of what the mechanism might be. One possibility was that the effect might be indirect, mediated by the effect of PARP-1 on ataxia-telangiectasia mutated (ATM). ATM is a DNA damage response protein that is activated by high NaCl, contributing to activation of TonEBP/OREBP (21). PARP-1 inhibits ATM activity in vitro and in cells exposed to the DNA damaging agent, nocazarinostatin (52), which made ATM a plausible mediator of its effect on TonEBP/OREBP. However, PARP-1 inhibits high NaCl-induced activation of TonEBP/OREBP even when ATM is already inhibited by wortmannin (Fig. 5F). In addition, inhibition of ATM by PARP-1 in vitro requires the presence of DNA (52), and PARP-1 mutated so that it cannot bind DNA still reduces TonEBP/OREBP activity (Fig. 5A). We conclude that ATM is not involved in the effects of PARP-1 on TonEBP/OREBP activity.

Another possibility is that PARP-1 might directly repress transcriptional activity of TonEBP/OREBP. Many studies have shown that PARP-1 functions as a transcriptional coactivator at enhancers and promoters, stimulating the activity of DNA binding transcription factors (26). However, there are also a few reports of repression of transcription by PARP-1. For example, PARP-1 binds directly to retinoid X receptors and represses ligand-dependent transcriptional activities mediated by heterodimers of retinoid X receptors and thyroid hormone receptor (37). However, catalytic activity of PARP-1 apparently is involved since enzyme-defective mutant PARP-1 does not produce this inhibition. This differs from our result with TonEBP/OREBP, which does not involve enzymatic activity of PARP-1 (Fig. 5, A and C). Another example is that PARP-1 protein binds to its own chromosomal promoter in vivo, which reduces its own transcription (47). Enzymatic activity of PARP-1 is not involved in this case since vectors expressing a truncated mutant of PARP-1, lacking catalytic activity, also down-regulate the transcription. However, DNA binding is necessary for the effect. This differs from our result with TonEBP/OREBP, which does not involve DNA binding activity of PARP-1 (Fig. 5A). A third example is that PARP-1 negatively regulates HIV-1 transcription by directly competing with Tat-P-TEFb for binding to TAR RNA. This also differs from effects on TonEBP/OREBP since it involves the DNA-binding activity of PARP-1 (38). We conclude that, although PARP-1 is known to repress transcription in several different ways, repression of transcriptional activity of TonEBP by PARP-1 involves a novel mechanism that differs from each of the ones previously described.

Effects of RNA helicases on function of TonEBP/OREBP. We identified 3 RNA helicases in immunoprecipitates of TonEBP/OREBP 1-547 V5-His, namely RNA helicase A (RHA), nucleolar RNA helicase II/Gu, and DEAD-box RNA helicase p72 (Table 1). We confirmed the identity of the first two by immunoblot (Fig. 1B) and showed that they are part of the large protein complex that binds along with TonEBP/OREBP to the ORE/TonE DNA element (Fig. 2). RNA helicases are enzymes that unwind double-stranded RNA (dsRNA) molecules in an energy-dependent fashion through the hydrolysis of ATP (50). They are associated with all processes involving RNA molecules, including transcription, editing, splicing, ribosome biogenesis, RNA export, translation, RNA turnover, and organelle gene expression. Their involvement in transcriptional regulation is through the modulation of chromatin structure or through interactions with the assembly of the transcription-initiation complex.
The association of RHA with TonEBP/OREBP was previously discovered when a GST fusion of the NH2-terminal 548 amino acids of TonEBP pulled RHA out of a lysate of Jurkat cells (9). The association was confirmed by the observation that TonEBP/OREBP and RHA mutually coimmunoprecipitate from Cos7 cells. High NaCl apparently disrupts association of RHA and TonEBP/OREBP because it doubles the abundance of TonEBP/OREBP protein without increasing the amount of RHA immunoprecipitated along with the TonEBP/OREBP. Although siRNA directed reduction in the level of RHA does not affect the activity of TonEBP/OREBP, overexpression of RHA inhibits the activity. Overexpression of a mutant RHA that is defective in nucleotide binding, inhibits TonEBP/OREBP activity as effectively as does wild-type RHA. The conclusion was that high NaCl-induced dissociation of RHA from TonEBP/OREBP stimulates TonEBP/OREBP activity by removing the inhibitory effect of RHA.

We have not determined whether RNA helicase RHI/Gu or DEAD-box RNA helicase p72 affects the function of TonEBP/OREBP. However, roles for both of these RNA helicases in transcription were previously reported. RNA helicase RHI/Gu can affect transcription by acting as a partner of c-Jun (54). The COOH-terminal domain of RHI/Gu interacts with the NH2-terminal transcription activation region of c-Jun, supporting c-Jun-mediated target gene activation through its RNA helicase activity. On the other hand, RNA helicase p72, can act either as a transcriptional coactivator, or in some contexts, as a transcriptional repressor (55). It is an activator of estrogen-receptor alpha, but it also interacts with histone deacetylase 1 to repress transcription in a promoter-specific manner.

**Known effects of the snRNPs and hnRNPs that associate with TonEBP/OREBP.** We identified two snRNPs and four hnRNPs associated with TonEBP/OREBP, namely U5 snRNP-specific 116 kDa protein, U5 snRNP-specific 200 kDa protein, hnRNP U, hnRNP M, hnRNP K, and hnRNP F. We were able to obtain antibodies against three of the hnRNPs, namely hnRNP M, hnRNP K, and hnRNP F, and, using the antibodies, confirmed their identity in immunoprecipitates of TonEBP/OREBP 1-547 V5-His (Fig. 1B) and in the large protein complex that binds along with TonEBP/OREBP to the ORE/TonE DNA element (Fig. 2). Although we did not investigate their effects on TonEBP/OREBP, they are known to have multiple functions related to other transcription factors.

hnRNPs are nuclear RNA-binding proteins that form complexes with RNA polymerase II transcripts. They function in many cellular activities, ranging from transcription and pre-mRNA processing in the nucleus to cytoplasmic mRNA translation and turnover (27). hnRNP K is involved in transcriptional activation and repression, RNA splicing and stability, translation activation and silencing, signal transduction, and chromatin remodeling (5). Its involvement in so many processes suggests that it acts as a docking platform to integrate multiple signals. hnRNPA participates in regulation of transcription of several genes. It binds to a 65 kDa Yes-associated protein, regulating its co-activation of Bax transcription (19), and it regulates Kruppel-like factor 2 transcription (1). It regulates initiation of transcription in general by associating with both actin and Pol II (28). HnRNP F binds to TATA-binding protein, associates with RNA polymerase II, and interacts with the nuclear cap-binding complex. It binds to the insulin-responsive element of the rat angiotensinogen gene, regulating transcription of that gene (53). U5 snRNPs are important components of spliceosomes (51).

The DNA-PK complex consists of a catalytic subunit, DNA-PKcs and two regulatory subunits, Ku70 and Ku86 (13). We identified both DNA-PKcs and Ku86 in immunoprecipitates of TonEBP/OREBP (Table 1), confirmed their identity by immunoblot (Fig. 1B), and demonstrated that they are part of the large protein complex that binds along with TonEBP/OREBP to the ORE/TonE DNA element (Fig. 2). The DNA-PK complex contributes to repair of DNA double-strand breaks by nonhomologous end joining (NHEJ). Association of the DNA-PK subunits with TonEBP/OREBP is particularly intriguing because of previous findings that high NaCl not only activates TonEBP/OREBP, but also causes DNA double strand breaks (29) and that Ku86 (but not DNA-PKcs) is necessary for cellular adaptation to high NaCl (13). Another DNA damage response protein, ATM, is activated by high NaCl, contributing to the high NaCl-induced activation of TonEBP/OREBP (21). At this point we do not know whether DNA-PK is similarly activated by high NaCl, and, if so, whether that contributes to activation of TonEBP/OREBP. The presence of DNA-PKcs and Ku86 in immunoprecipitates of TonEBP/OREBP was previously reported (9). In those studies the association was interrupted by ethidium bromide, indicating dependance on binding to DNA. We find that the DNA-PK subunits are present along with TonEBP/OREBP in the protein complex formed in vitro that binds specifically to the ORE/TonE DNA (Fig. 2). The question remains, however, whether they are also part of the complex within cells.

**Perspectives**

It is not surprising that our proteomic screen picked up abundant nuclear proteins, many of which were already known to be involved in metabolism of DNA and RNA of numerous genes. What is of interest, however, is that the roles of these proteins differ between different genes and contexts. For example, as discussed above, we find that expression of PARP-1 inhibits TonEBP/OREBP activity. Yet, depending on which transcription factor is studied, PARP-1 cannot only depress, but can also enhance transcriptional activity. The difference presumably depends on the properties of particular transcription factors and on the particular combination of ancillary proteins in each complex. A further complication is that an associated protein may serve more than one role. For example, as also discussed above, it is difficult to explain by a single action of HSP90 its participation in the TonEBP/OREBP transcriptional complex interrelationships. In this study we set out to identify proteins that physically associate with TonEBP/OREBP and, thus, are candidates for participation in the TonEBP/OREBP transcriptional complex or in osmotic signaling to TonEBP/OREBP. We identified 14 such proteins, most of which are novel in this regard. It is striking that the amounts of the associated proteins that coimmunoprecipitate with TonEBP/OREBP generally are not affected by NaCl concentration (Fig. 1, B-F). Thus, any effects that they might have on TonEBP/OREBP function are unlikely to depend on tonicity-induced changes in the amount associated with TonEBP/OREBP. Nevertheless, the two associated proteins whose functions we investigated in detail do affect
TonEBP/OREBP activity. Hsp90 activity supports transcriptional activity of TonEBP/OREBP (Fig. 3B) and activity of its TAD (Fig. 3C), as well as toxicity-induced increase in its protein abundance (Fig. 4A). Also, expression of PARP-1 reduces those activities (Fig. 5, A and B). We anticipate that the other associated proteins will also be found to modulate activity of TonEBP/OREBP.

The use of tandem protein mass spectrometry (LC-MS/MS) to identify by coimmunoprecipitation proteins that are functionally significant suffers from several limitations. First, identification of a protein by MS2 is probabilistic. Certainty depends on the quality of a match of a series of fragmented ions to a database. To select the most likely candidates we chose only those that had highly probable matches (Supplementary figures) to two or more peptides (Supplementary table), and then confirmed the association by immunoblots (Fig. 1) and EMSA supershifts (Fig. 2). Second, since immunoprecipitations are not perfectly specific, an immunoprecipitate will contain at least some irrelevant contaminating proteins. Therefore, we proceeded to confirm directly the functional importance of two of the proteins, namely HSP90 and PARP-1. We cannot know whether the other proteins that we identified are important for the function of TonEBP/OREBP until they are tested. Lastly, we are most interested in how TonEBP/OREBP is activated by hypertonicity. While many of the proteins associated with TonEBP/OREBP may contribute to this regulation, others will be general transcriptional factors that are less interesting in this regard.

Finally, it is also interesting that we did not pick up a protein that we previously found to be associated with TonEBP/OREBP, namely PKA (14). Perhaps, its protein abundance in the nucleus is too low for it to be detected in this type of assay. Or, perhaps the explanation is the different biases that were used. In the present study we used the NH2-terminal third of TonEBP/OREBP, amino acids 1-547 as bait, whereas previously we used the entire protein (1531 amino acids). PKA contributes to high NaCl-induced increase of the activity of the TonEBP/OREBP TAD. The TAD of TonEBP/OREBP is located within amino acids 548 to 1531, not in amino acids 1 to 547, which constituted the bait in the present study. Therefore, the results are consistent with association of PKA being with the region of TonEBP/OREBP that contains its TAD.

ACKNOWLEDGMENTS

We thank Dr. B. C. Valdez of Baylor College of Medicine (Houston, TX) for anti-RNA helicase II/Gu antibody; Drs. M. O. Hottiger and M. E. Smulson for PARP-1 constructs and PARP-1 −/− cells, respectively; and the National Cancer Institute for providing geldanamycin.

GRANTS

This research was supported by the Intramural Research Program of National Heart, Lung, and Blood Institute, National Institutes of Health. Present address of Y. Chen: Dept. of Biochemistry and Molecular Biology, Univ. of Medicine and Dentistry of New Jersey, Newark, NJ 07103.


