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Purification, Identification, and Characterization of an Osmotic Response Element Binding Protein[1]

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Kidney cells, especially the epithelial cells lining the collecting tubules in the inner medulla, are constantly exposed to concentrated urine. They are protected from the osmotic effect of high levels of sodium ion and urea by accumulating compatible osmolytes such as sorbitol, betaine, and myo-inositol. These osmolytes are involved in maintaining cell volume and electrolyte contents because they do not perturb the protein structure and function over a wide range of concentrations. Sorbitol is produced via the reduction of glucose by aldose reductase (AR), while betaine and myo-inositol are transported into the cells through specific transporters. Under hyperosmotic stress, transcriptions of genes encoding these proteins are highly induced. The induction of transcription was found to be mediated through the osmotic response elements (OREs) located in the 5′ flanking sequences of these genes. We had earlier identified the OREs in human AR gene. In this study we purified and identified the osmotic response element binding protein (OREBP). OREBP is a transcription factor of approximately 200 kDa in size, characterized by a Rel-like DNA binding domain and a glutamine-rich transactivation domain. Dominant negative OREBP significantly diminished hyperosmotic AR gene induction. Immunohistochemical analysis showed that this transcription factor is rapidly translocated into the nucleus upon hyperosmotic stress.

The epithelial cells lining the collecting tubules of the mammalian kidney medulla are constantly exposed to hyperosmotic stress because of the urinary concentrating mechanism. These cells are protected from the osmotic effect of concentrated sodium ion and urea in the interstitium by accumulating compatible osmolytes (1). These osmolytes, including sorbitol, betaine, myo-inositol, glycerophosphocholine and taurine, preserve the cell volume and osmolality without perturbing macromolecular structure and function (2). In mammals, a specific class of protein is responsible for the accumulation of these compatible osmolytes: sorbitol is produced within the cell via the reduction of glucose by aldose reductase (AR); betaine, myo-inositol, and taurine are transported into the cells by betaine/γ-aminobutyric acid transporter (BGT1), Na^+-dependent myo-inositol transporter (SMIT), and taurine transporter respectively (3). The expression of the AR, BGT1, and SMIT genes is tightly regulated by extracellular osmolality. Hyperosmolality increased the transcription of these genes, leading to increased mRNA abundance, enzyme levels and accumulation of compatible osmolytes (4).

The protective role of the compatible osmolytes in the kidney is well characterized (1, 5–7). In addition, there are suggestions that the brain also adapts to changes in the osmolality of body fluid by modulating the amount of compatible osmolytes, presumably because maintenance of brain cell volume is of crucial importance for normal central nervous system function (8–10). However, abnormally high level of osmolytes can be deleterious to cells. In diabetic animals, large accumulation of sorbitol causes cataract (11).

Among the eukaryotic organisms, the mechanism of osmoregulation is better understood in yeast. *Saccharomyces cerevisiae* utilizes a two-component signaling system as an osmolality sensor (12–14). When exposed to hyperosmotic medium, the stress signal is transduced through the HOG1 kinase cascade to induce the

Abbreviations used: AR, aldose reductase; BGT1, betaine/γ-aminobutyric acid transporter; SMIT, Na^+-dependent myo-inositol transporter; OREs, osmotic response elements; NFAT, nuclear factor of activated T cells; TonE, tonicity-responsive enhancer; OREBP, osmotic response element binding protein.

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synthesis of glycerol-3-phosphate dehydrogenase. Consequently the level of glycerol is increased to serve as the major organic osmolyte (15–17). In contrast, in higher eukaryotes, how the signal of external hyperosmolality is relayed to the nucleus to induce the expression of genes responsible to increase cellular osmolytes is still unclear. We and others have previously identified the osmotic response elements (ORE) of AR and the tonicity-responsive enhancer (TonE) in the 5′ flanking sequences of SMIT and BGT-1 gene (18–22). The ORE and TonE, which share a putative consensus sequence NGGAAAWDHMC(N), are responsible for mediating hyperosmotic induction of these genes (23). In this study, we report the purification and cloning of the osmotic response element binding protein (OREBP). OREBP shares some similarities with other transcription factors in that it contains a Rel-like DNA-binding domain and a glutamine-rich transactivation domain. We demonstrated that OREBP interacts with the ORE and mediates the hyperosmotic expression of aldose reductase gene *in vivo*. The identification of OREBP would allow us to further understand the mechanism of osmotic regulation of gene expression.

MATERIALS AND METHODS

Cell culture. Chang liver cells (American Type Culture Collections, Rockville, MD) were maintained in isosmotic growth medium (Eagle's basal medium supplemented with 10% calf serum and 2 mM L-glutamine; ≈300 mosmol/kg of H_2O) at 37°C in the presence of 5% CO_2. Hyperosmotic induction was achieved by replacing the isosmotic medium with hyperosmotic medium (isosmotic medium supplemented with 5 M NaCl to final concentration of 100 mM, ≈500 mosmol/kg of H_2O). COS 7 cells (American Type Culture Collections, Rockville, MD) were maintained in DMEM medium supplemented with 10% fetal bovine serum and 4 mM L-glutamine. HeLa cells (American Type Culture Collections, Rockville, MD) and Hela Tet-Off cells (Clontech) were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml G418, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 100 ng/ml anhydrotetracycline (Clontech).

UV-crosslinking and SDS–PAGE. Nuclear extracts (10 μg) were incubated with ^{32}P-labeled OreC (5′GGACCAAATGGAAAATCACCGGCA3′) in 50 μl of binding reaction mixture containing gel mobility shift assay buffer (see below) and poly(dI · dC). After incubated at room temperature for 10 min, the samples were put on ice and irradiated with ultraviolet light (UV) for 10–40 min using UV-crosslinker (Spectronic Corporation). Samples were digested with DNase I, heat denatured for 5 min, electrophoresed on an 8% SDS–polyacrylamide gel and autoradiographed.

Purification of OREBP. Column chromatography was carried out in AKTAexplorer system (Amersham Pharmacia Biotech). Chang liver cells were maintained in hyperosmotic medium for 24 h and the nuclear extracts were prepared according to the procedure of Dignam *et al.* (24). 400 mg of the nuclear extract was concentrated by ultrafiltration using a YM10 membrane (Amicon, Inc). The concentrated extract was then applied to a Sephacryl S-300 column (2.6 by 60 cm) (Amersham Pharmacia Biotech) equilibrated with TM buffer (12 mM Hepes, pH 7.9, 150 mM KCl, 5 mM $MgCl_2$, 0.12 mM EDTA). Protein elution was monitored by absorbance at 280 nm, the OREBP activity was determined by gel mobility shift assay. The probe used in this gel mobility shift assay was a 132-bp fragment containing the previously described osmotic response region of human aldose reductase gene (18). Fractions containing OREBP were pooled and applied to a Resource S column at pH 6.0 (Amersham Pharmacia Biotech). OREBP was eluted with 120 ml linear gradient of NaCl (0 to 450 mM). Fractions containing OREBP were pooled.

Affinity chromatography was carried out as described (25). The osmotic response element (ORE)-containing double-stranded oligonucleotides (5′CACCAAATGGAAAATCACCGGCATGG3′) were utilized for the preparation of the DNA-affinity column (18). Tandem copies of this double-stranded oligonucleotides were covalently attached to CNBr-activated Sepharose 4 (Amersham Pharmacia Biotech). This ORE DNA affinity resin (1 ml) was equilibrated in a Econo-Column (Bio-Rad) with buffer Z (12 mM Hepes, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.3 mM dithiothreitol and 0.05% Nonidet P-40). The pooled extract from above was equilibrated with buffer Z containing sonicated pGEM-7zft (Promega) and poly(dI · dC). The DNA-protein complex was allowed to stand for 10 min at room temperature and then pass through the affinity resin by gravity flow. The resin was washed with buffer Z containing 0.1 M KCl. OREBP was recovered by using 0.1 to 1 M KCl gradient. The OREBP activity containing fractions were pooled and analyzed by SDS–PAGE followed by Coomassie blue staining.

Protein microsequencing. The Coomassie blue-stained protein band of about 200 kDa was excised from the gel, cut into small pieces and subjected to an in-gel digestion with Endoproteinase Lys-C (Boehringer-Mannheim, Indianapolis, IN). After overnight digestion the peptides were extracted from the gel and fractionated on a Vydac (The Separations Group, Hesperia, CA) microbore column. Individual peptides were subjected to Edman degradation on a Proicise 492 protein sequencer (PE Biosystems, Foster City, CA).

Plasmids. Human cDNA clone KIAA0827 was a gift from Dr. Takahiro Nagase of the Kazusa DNA Research Institute, Japan. pFLAG-OREBP plasmid was derived by in frame insertion of coding region of KIAA0827 cDNA into pFLAG-CMV-2 mammalian expression vector (Sigma, St. Louis, MO). pFLAG-OREBP-DN plasmid was derived by in frame insertion of KIAA0827 cDNA corresponding to amino acids 1–562 into pFLAG-CMV-2. pBL-OREBP-DN was constructed by subcloning the pFLAG-OREBP-DN cDNA into pBI-L expression vector (Clontech). pFLAG-OREBP-2RL was derived by substitution of codons for Arg 298 and Arg 302 of pFLAG-OREBP to Leu 298 and Leu 302 respectively using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of all constructs were verified by DNA sequence determination.

Protein expression in COS 7 cells. Plasmid DNAs were purified with CsCl ultracentrifugation steps. COS 7 cells were seeded into 100-mm culture dishes (2×10^6/dish) and incubated for 24 h before transfection. Cells were then transfected with pFLAG-OREBP, pFLAG-OREBP-DN and pFLAG-OREBP-2RL respectively using FuGENE 6 transfection reagent (Boehringer-Mannheim, Indianapolis, IN) according to manufacturer's protocol. The cells were harvested after 24 h. The protein extracts were either subjected to immunoblotting or affinity purification using anti-FLAG M2 affinity gel (Sigma, St. Louis, MO) according to manufacturer's protocol.

Stable transfection. To generate stable cell lines that express FLAG-OREBP-DN, Hela Tet-Off cells were transfected with pBL-OREBP-DN and pTK-Hyg (Clontech) using FuGENE 6 transfection reagent (Boehringer-Mannheim). After selection in medium containing 200 μg/ml hygromycin (Clontech), stable transfectants were assayed for the expression of the transgene by Western blot analysis of cell lysates using anti-FLAG M2 antibody.

SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting. SDS–PAGE was performed on a 7.5% acrylamide gel according to Laemmli (26). Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell) using Mini Tank Transphor Unit (Amersham Pharmacia Biotech). To detect the FLAG epitope-tagged

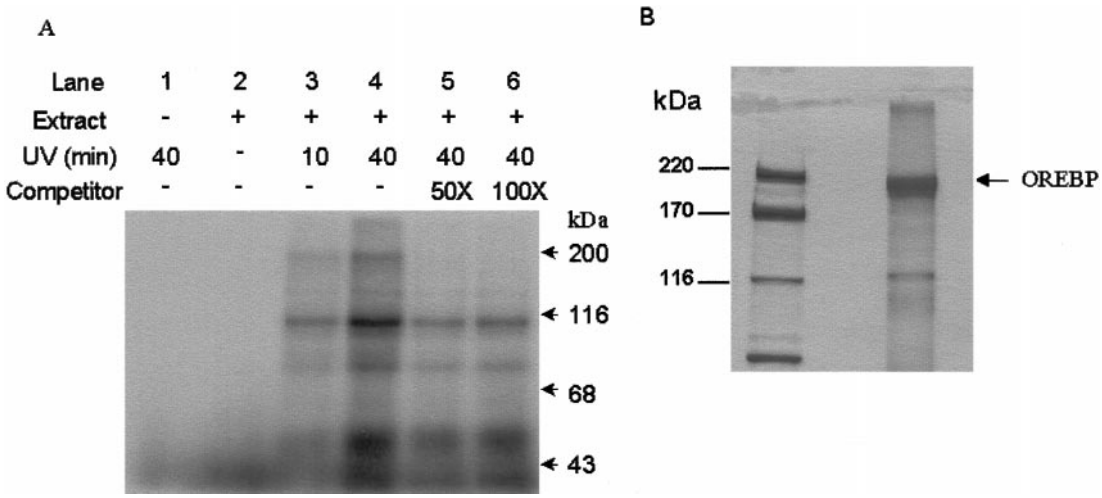

FIG. 1. Identity of OREBP. (A) UV cross-linking of ORE to nuclear extract. Nuclear extracts, isolated from cells exposed to hyperosmotic medium (≈500 mosmol/kg of H_2O) for 24 h, were incubated with ^{32}P-labeled OreC and irradiated with UV light. After the reaction, the samples were digested with DNase I, heat-denatured, electrophoresed on an 8% SDS–polyacrylamide gel, and autoradiographed. Size markers are shown in the right. (B) Coomassie blue gel of the protein products purified by size exclusion, ion exchange, and OreC-affinity column chromatography.

OREBP, anti-FLAG M5 monoclonal antibody (Sigma, St. Louis, MO) was used as the primary antibody and anti-mouse IgG peroxidase conjugate as the secondary antibody. The hybridization signals were visualized by ECL Western blot detection reagent (Amersham Pharmacia Biotech).

mRNA isolation and Northern blot hybridization. Total RNA was extracted from tissue culture using Tri Reagent (Molecular Research Center, Inc). Poly A^+ mRNA was then prepared from total RNA preparations using Oligotex mRNA kits (Qiagen). Northern blot hybridization was carried out as described (18). The human AR cDNA exon 10 was isolated from a Bluescript subclone (27).

Gel mobility shift assay. The anti-FLAG M2 affinity gel purified proteins were incubated with 20,000 cpm of the [α-^{32}P]dCTP labeled oligonucleotide probe in 20 μl of binding reaction mixture containing 12 mM Hepes-KOH, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride and 0.3 mM dithiothreitol. After incubation at room temperature for 10 min, the mixture was electrophoresed on a 4% polyacrylamide gel (79:1 acrylamide/bisacrylamide) in 45 mM Tris borate, 45 mM boric acid, and 2 mM EDTA at 4°C.

Double-stranded oligonucleotides OreA, OreB, OreC and mOreC were used as probes in the gel mobility shift assay. These double-stranded oligonucleotides were labeled with [α-^{32}P]dCTP using Klenow fragment. Oligonucleotide sequences were: OreA: (5′GGTTACATGGAAAAATATCTGGG3′); OreB: (5′GGCTGTATAAATTTTTCCAGGAGG3′); OreC: (5′GGACCAAATGGAAAATCACCGGCA3′); and mOreC: (5′GGACCAAATAGAAAATCACCGGCA3′).

Fluorescence immunocytochemistry and laser scanning confocal microscopy. Transfected HeLa cells were grown to 70% confluence on 18-mm glass coverslip in 60-mm diameter tissue culture dishes. Cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS (pH = 7.4) for 15 min at room temperature. The cells were washed three times with PBS, permeabilized by absolute methanol for 2 min at room temperature, and rehydrated by three washes with PBS. Sixty microliters of diluted mouse anti-FLAG antibodies (Sigma) were beaded on tissue culture dishes and coverslips with fixed cells were inverted onto the antibodies for 1 h. Excess antibodies were removed by multiple washes with PBS. A fluorescein-conjugated rabbit anti-mouse immunoglobulin G (Zymed) was used as the secondary antibody. Coverslips were mounted on slides with Fluormount (Virotech). Immunofluorescence was observed using a confocal microscope (Zeiss Axiovert).

RESULTS

Ultraviolet (UV)-Crosslinking of OREBP to ORE

We have previously shown that hyperosmotic induction of human AR gene is mediated by three osmotic response elements (OREs), namely OreA, OreB, and OreC, located in the 5′ flanking sequence of the gene. These OREs interact with a putative transcription factor under hyperosmotic induction (18). To determine the size of the transcription factor that interacts with the OREs, OreC was covalently crosslinked to the transcription factor by ultraviolet. The molecular mass of the cross-linked protein was determined by SDS–polyacrylamide gel. As shown in Fig. 1A, two major binding proteins of approximately 200 kDa and 116 kDa in size were observed when the mixtures of OreC and nuclear extracts taken from hyperosmotically induced Chang liver cells were irradiated by UV (lane 3). The intensity of the bands increased with time of irradiation (lane 4). However, only the 200 kDa protein band can be competed out by the addition of excess unlabeled OreC (lanes 5 and 6). This protein band cannot be observed in the absence of nuclear extract (lane 2) or without UV irradiation (lane 1). Collectively, these results indicate that a 200 kDa protein specifically interacts with OreC.

Purification and Microsequencing of OREBP

To purify the OREBP, we applied a three-step chromatographic procedure using the gel mobility shift as-

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   1 MPSDFISLLS ADLDLESPKS LYSRESVYDL LPKELQLPPS RETSVASMSQ TSGGEAGSPP
  61 PAVVAADASS APSSSSMGGA CSSFTTSSSP TIYSTSVTDS KAMQVESCSS AVGVSNRGVS
 121 EKQLTSNTVQ QHPSTPKRHT VLYISPPPED LLDNSRMSCQ DEGCGLESEQ SCSMWMEDSP
 181 SNFSNMSTSS YNDNTEVPRK SRKRNPKQRP GVKRRDCEES NMDIFDADSA KAPHYVLSQL
 241 TTDNKGNSKA GNGTLENQKG TGVKKSPMLC GQYPVKSEGK ELKIVVQPET QHRARYLTEG
 301 SRGSVKDRTQ QGFPTVKLEG HNEPVVLQVF VGNDSGRVKP HGFYQACRVT GRNTTPCKEV
 361 DIEGTTVIEV GLDPSNNMTL AVDCVGILKL RNADVEARIG IAGSKKKSTR ARLVFRVNIM
 421 RKDGSTLTLQ TPSSPILCTQ PAGVPEILKK SLHSCSVKGE EEVFLIGKNF LKGTKVIFQE
 481 NVSDENSWKS EAEIDMELFH QNHLIVKVPP YHDQHITLPV SVGIYVVTNA GRSHDVQPFT
 541 YTEDPAAAGA LNVNVKKEIS SPARPCSFEE AMKAMKTTGC NLDKVNIIPN ALMTPLIPSS
 601 MIKSEDVTPM EVTAEKRSST IFKTTKSVGS TQQTLENISN IAGNGSFSSP SSSHLPSENE
 661 KQQQIQPKAY NPETLTTIQT QDISQPGTFP AVSASSQLPN SDALLQQATQ FQTRETQSRE
 721 ILQSDGTVVN LSQLTEASQQ QQQSPLQEQA QTLQQQISSN IFPSPNSVSQ LQNTIQQLQA
 781 GSFTGSTASG SSGSVDLVQQ VLEAQQQLSS VLFSAPDGNE NVQEQLSADI FQQVSQIQSG
 841 VSPGMFSSTE PTVHTRPDNL LPGRAESVHP QSENTLSNQQ QQQQQQQQVM ESSAAMVMEM
 901 QQSICQAAAQ IQSELFPSTA SANGNLQQSP VYQQTSHMMS ALSTNEDMQM QCELFSSPPA
 961 VSGNETSTTT TQQVATPGTT MFQTSSSGDG EETGTQAKQI QNSVFQTMVQ MQHSGDNQPQ
1021 VNLFSSTKSM MSVQNSGTQQ QGNGLFQQGN EMMSLQSGNF LQQSSHSQAQ LFHPQNPIAD
1081 AQNLSQETQG SLFHSPNPIV HSQTSTTSSE QMQPPMFHSQ STIAVLQGSS VPQDQQSTNI
1141 FLSQSPMNNL QTNTVAQEAF FAAPNSISPL QSTSNSEQQA AFQQQAPISH IQTPMLSQEQ
1201 AQPPQQGLFQ PQVALGSLPP NPMPQSQQGT MFQSQHSIVA MQSNSPSQEQ QQQQQQQQQQ
1261 QQQQQQSILF SNQNTMATMA SPKQPPPNMI FNPNQNPMAN QEQQNQSIFH QQSNMAPMNQ
1321 EQQPMQFQSQ STVSSLQNPG PTQSESSQTP LFHSSPQIQL VQGSPSSQEQ QVTLFLSPAS
1381 MSALQTSINQ QDMQQSPLYS PQNNMPGIQG ATSSPQPQAT LFHNTAGGTM NQLQNSPGSS
1441 QQTSGMFLFG IQNNCSQLLT SGPATLPDQL MAISQPGQPQ NEGQPPVTTL LSQQMPENSP
1501 LASSINTNQN IEKIDLLVSL QNQGNNLTGS F
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FIG. 2. Deduced peptide sequence of KIAA0827. The endoproteinase Lys-C-digested protein sequences determined by Edman degradation are underlined. The putative bipartite nuclear localization sequences are in bold, the Rel homology DNA binding domain is in the open box, and the two long stretches of glutamine residues in the C-terminus are highlighted by broken lines.

say to verify the presence of the protein. Nuclear extracts were prepared from Chang liver cells maintained in hyperosmotic medium for 24 h. The extracts were concentrated by ultrafiltration and subjected to gel filtration chromatography on a Sephacryl S-300 column. Samples containing OREBP activity were pooled and subsequently applied to a Resource S cation exchange column. The proteins bound to the column were eluted by increasing NaCl concentration. The final step in the purification was achieved by applying the pooled Resource S fractions to a DNA affinity column prepared by covalently linking tandemly ligated synthetic oligonucleotides containing OreC to Sepharose. The proteins bound to the OreC-affinity column were eluted with 0.1 M to 1 M KCl gradient buffer. The fractions containing the OREBP activity were pooled and analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Fig. 1B, the Coomassie blue gel of the fractions eluted from OreC-affinity fraction revealed a prominent polypeptide of approximately 200 kDa in size, which is consistent with the result obtained from the UV-crosslinking experiment.

Protein microsequencing of the purified OREBP was performed in order to reveal the identity of this protein. Amino acids sequences of several peptides derived from an endoproteinase Lys-C digestion of OREBP were determined. The sequences of these peptides were as follows: Pep 1: RTQQGFPTVK, Pep 2: NADVEARIGIA, Pep 3: VVQPETQLAV, Pep 4: APHYVLSQLTTDNK, and Pep 5: VEDVTPMEVTAELD. A search of these amino acid sequences against GenBank database (NCBI, Bethesda, MD) revealed that they are identical with the *Homo sapiens* brain cDNA clone KIAA0827 (Accession No. AB020634), of unknown function (28). The length of this cDNA clone is 6019 bp with the putative open reading frame encoding a protein of 1531 amino acids as shown in Fig. 2.

KIAA0827 cDNA Clone Encoded the OREBP

Analysis of the protein encoded by KIAA0827 reveals the presence of a DNA binding domain (amino acid 263 to 543), which shows significant similarity to the Rel-

like DNA-binding domain of nuclear factor of activated T cells (NFAT) (Fig. 7) (29). A stretch of 18 amino acids near the N-terminus displays significant homology to the bipartite nuclear localization signal (amino acid 199 to 216). The C-terminus, which consists of about 1000 amino acids, is glutamine-rich (19%) and characterized by two long stretches of glutamine residues (Fig. 2). In many transcription factors, the glutamine-rich region functions as a transactivation domain (30, 31). The first 200 amino acid portion of this protein does not have any homology to the conserved N-terminal region among NFAT isoforms in which it is thought to function as a regulatory domain (32).

To see if this protein is the OREBP, we expressed the KIAA0827 cDNA in mammalian cells to see if the expressed protein exhibits ORE binding activity. We constructed pFLAG-OREBP, containing region of KIAA0827 coding for the putative OREBP under the control of a CMV promoter. The OREBP was expressed as a FLAG-tagged fusion protein such that the expression can be monitored by an anti-FLAG monoclonal antibody. Western blot analysis of extracts of COS 7 cells transfected with pFLAG-OREBP showed that the KIAA0827 cDNA encoded a polypeptide of approximately 200 kDa (Fig. 3A, lane 1), which is in good agreement with the size of the OREBP determined by UV-crosslinking experiment. The size of the expressed protein deviates from the calculated molecular mass of 166 kDa, suggesting the possibility of post-translational modification. To test if the expressed protein interacts with the ORE, we purified the expressed protein using anti-FLAG affinity column and subsequently performed gel mobility shift assay using OreC as a probe. As shown in Fig 3B, a prominent band was observed when OreC was incubated with the purified protein (lane 1). The band could be competed out by the addition of excess unlabeled OreC (lanes 2 and lane 3), indicating the specificity of the DNA/protein interaction. These results strongly indicated that the KIAA0827 cDNA clone encodes the OREBP.

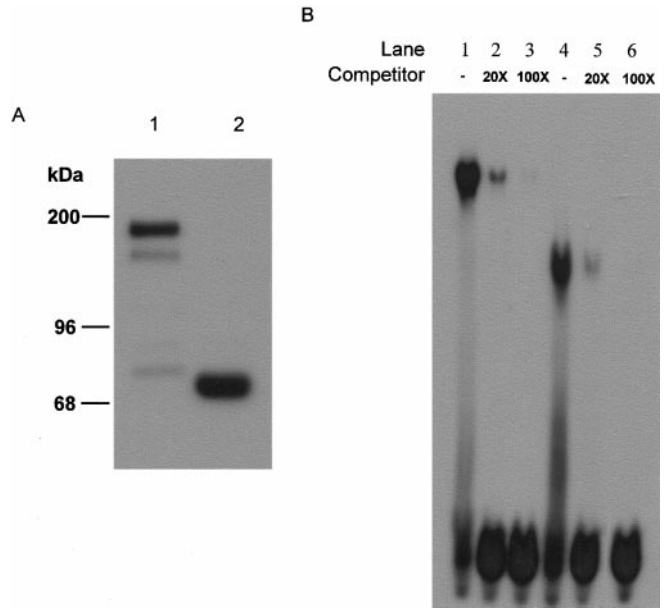

FIG. 3. Expression of cloned OREBP and dominant negative OREBP. (A) Expression of the putative OREBP and dominant negative OREBP in COS 7 cells is described under Materials and Methods. The FLAG-tagged polypeptides were electrophoresed on SDS gel. Western blot analysis was performed using anti-FLAG monoclonal antibody. Lane 1, OREBP. Lane 2, dominant negative OREBP. (B) Gel mobility shift assay of the anti-FLAG affinity column purified OREBP (lanes 1–3) and dominant negative OREBP (lanes 4–6). Affinity purified FLAG-tagged proteins were incubated with ^{32}P-labeled OreC in the absence (−) or presence of 20- and 100-fold molar excesses of the unlabeled OreC probe.

Characterization of the OREBP

Our earlier finding had indicated that OreA, OreB and OreC work in concert to confer a full transcriptional response to hyperosmotic induction (18). To test if the OREBP interacts with these three osmotic response elements *in vitro*, FLAG-tagged OREBP was purified and gel mobility shift assay was performed using OreA, OreB or OreC as probe. As shown in Fig. 4A, OREBP is able to interact with all three osmotic response elements (lanes 1–3), suggesting that OREBP may interact with these three elements *in vivo*. In previous study we also showed that a single base substitution made in OreC, changing the core sequence from TGGAAAA to TAGAAAA (mOreC), completely abolished the osmotic response (18). We asked if OREBP interacts with the mOreC *in vitro*. Results from the gel mobility shift assay showed that when the FLAG-tagged OREBP was incubated with OreC probe, a prominent band was observed as expected (Fig. 4B, lane 1). However, we do not observe a similar band shift when mOreC was used as a probe (Fig. 4B, lane2), further verifying the specificity of interaction between OREBP and ORE.

Crystal structure of the NFAT DNA-binding domain showed that a number of amino acid residues are in close contact with the antigen–receptor response element (ARRE2) (33). Two amino acid residues, Arg 430 and Arg 421 of the NFAT, were shown to participate in bidentate hydrogen-bond interaction with the two guanine nucleotides in the NFAT core sequence (GGAAAA). These two residues are conserved in the corresponding DNA binding domain of OREBP. They are, respectively, Arg 293 and Arg 302 (Fig. 7). Since the ORE core sequence (TGGAAA) closely resembles that of NFAT (32), we questioned if these two residues are also important for OREBP-DNA interaction. To this end, we generated pFLAG-OREBP-2RL, in which the codons for Arg 293 and Arg 302 were selectively changed to encode Leu 293 and Leu 302 respectively. As shown in Fig. 4C, lane 2, the Arg to Leu substitution completely abolished the DNA binding activity of

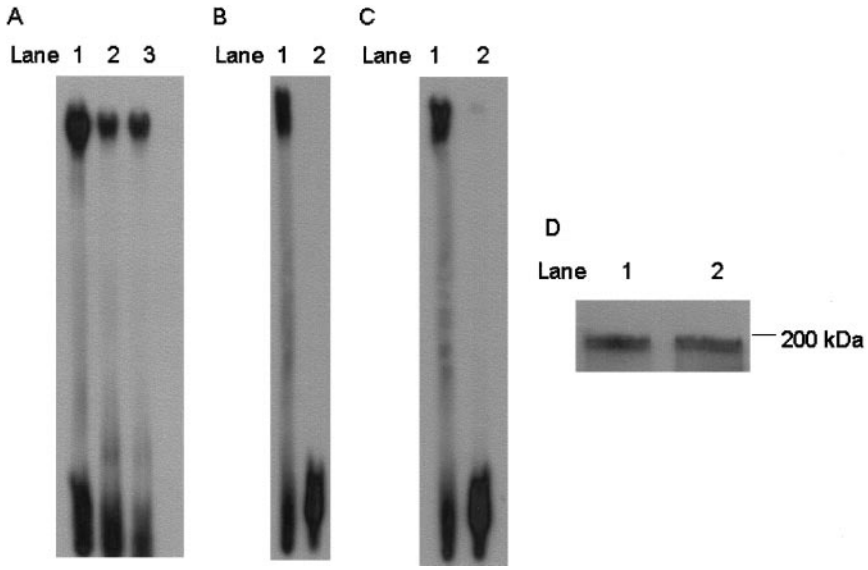

FIG 4. Characterization of OREBP. (A) Gel mobility shift assays of purified FLAG-tagged OREBP using OreC (lane 1), OreA (lane 2) and OreB (lane 3) as probes. (B) Gel mobility shift assays using OreC (lane 1) and mOreC (lane 2) as probes. (C) Gel mobility shift assays of FLAG-tagged OREBP (lane 1) and FLAG-tagged mutant OREBP (lane 2) using OreC as probe. (D) Equal amount of purified FLAG-tagged OREBP (lane 1) and FLAG-tagged mutant OREBP (lane 2) were electrophoresed on SDS gel and subjected to Western blot analysis using anti-FLAG monoclonal antibody.

OREBP. Western blot analysis showed that equal amounts of the normal and mutant OREBP were used in the assay (Fig. 4D). These results indicated that the two arginine residues are crucial to the OREBP-ORE interaction.

Dominant Negative Form of OREBP Blocks Hyperosmotic Induction of AR in Vivo

As mentioned previously, the glutamine-rich region of transcription factors may function as a transactivation domain (31). We asked if a truncated form of OREBP devoid of the glutamine-rich region would diminish hyperosmotic induction of AR gene in a dominant negative manner. We tested whether the truncated OREBP retains the ability to interact with DNA. As shown in Fig. 3A expression of pFLAG-OREBP-DN (encoding amino acids 1–562 of the OREBP), produced a polypeptide of ≈70 kDa in size (Fig. 3A, lane 2). This truncated form of OREBP is able to interact with OreC as revealed by gel mobility shift assay (Fig. 3B, lanes 4–6).

To see if the truncated OREBP functions in a dominant negative manner, HeLa Tet-off cells were transfected with pBI-OREBP-DN to generate a stable cell line in which the truncated OREBP is expressed under the control of a tetracycline-responsive promoter. Although we failed to obtain a tetracycline-responsive cell line, one of the stable transfectants (clone 49) showed constitutively higher level of expression of the truncated OREBP (Fig. 5A, lane 1) when compared to the control cell line (lane 2). The expression of AR mRNA under hyperosmotic stress was determined. Fig 5B shows the change in AR mRNA expression when these cell lines were maintained in isosmotic medium or medium supplemented with 100 mM (≈500 mosmol/kg of H_2O) NaCl for 14 h. Under isosmotic condition, the expression of AR mRNA in both cell lines is undetectable (lanes 1 and 3). When control cells were maintained in hyperosmotic medium, a high level of induction of AR mRNA is observed within 14 h (lane 2). However, the hyperosmotic induction of AR mRNA was

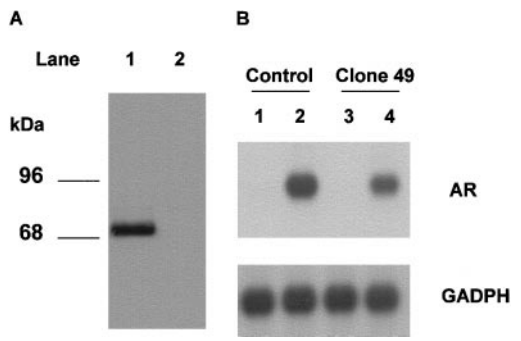

FIG. 5. Dominant negative OREBP diminished hyperosmotic induction of AR gene *in vivo*. (A) Western blot analysis using anti-FLAG monoclonal antibody showing the constitutive expression of dominant negative FLAG-tagged OREBP of clone 49 (lane 1) compared to the control cell line (lane 2). The control cell line is a stable transfectant where the transgene was not expressed. (B) Northern blot analysis of the control cell line and clone 49 showing the expression of AR gene under isosmotic conditions (lanes 1 and 3), and maintained in hyperosmotic medium for 12 h (lane 2 and lane 4). The blot was rehybridized with GADPH cDNA.

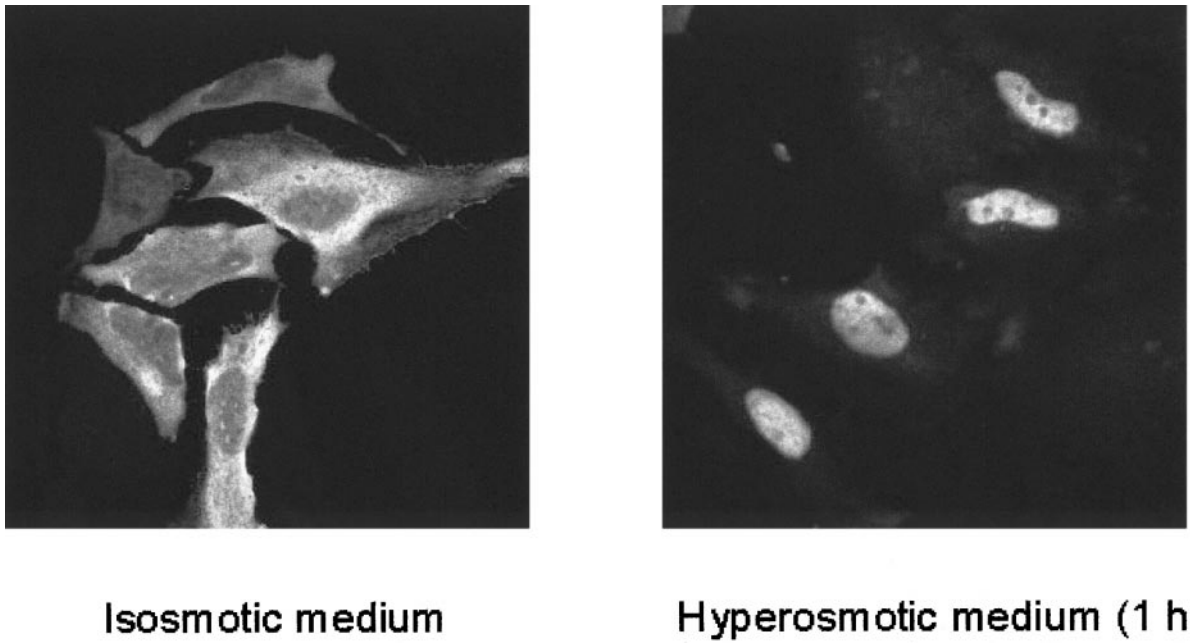

FIG. 6. Rapid translocation of OREBP into nucleus under hyperosmolality. Hela cells transfected with pFLAG-OREBP were either maintained in isosmotic medium or treated with hyperosmotic medium for 1 h. Cells were then fixed and OREBP identified by anti-FLAG antibodies as described under Materials and Methods.

decreased by 1.5-fold when clone 49 was subjected to the same treatment (lane 4), presumably because the truncated OREBP occupies the osmotic response elements without activating transcription machinery. Taken together, these results suggest that OREBP binds to ORE of AR and activates the transcription of the AR gene *in vivo.*

Rapid Translocation of OREBP into Nucleus and Induction of OREBP mRNA under Hyperosmolality

To study the cellular location of OREBP, pFLAG-OREBP was transiently transfected into Hela cells and the expression monitored by immunocytochemistry using anti-FLAG antibodies. As shown in Fig. 6A, when the cells were maintained in isosmotic medium, fluorescence staining was observed in both the nucleus and the cytoplasm. In contrast, when the cells were subjected to hyperosmotic stress for 1 h, the fluorescence signal was primarily found in the nucleus. These results indicate that the OREBP contains nuclear translocation signal that is activated by hyperosmotic stress. To see if OREBP mRNA is induced in response to hyperosmotic stress, Hela cells were maintained in medium containing 100 mM NaCl. As shown in Fig. 6B, there was an induction of OREBP mRNA expression within 2 h, reaching a maximum at 6 h, and decrease to the basal level at 12 h. Taken together, these data suggested that hyperosmotic stress induces a rapid translocation of the OREBP into the nucleus as well as an increase in OREBP mRNA abundance.

DISCUSSION

We have purified OREBP, a transcription factor that binds specifically to the osmotic response elements. Partial peptide sequence of this protein reveals that it is encoded by a cDNA clone called KIAA0827 whose function has not been characterized. This protein contains a Rel-like DNA binding domain and a glutamine-rich transactivation domain, suggesting that it is a transcription factor. We show that when the glutamine-rich carboxyl end is deleted, the protein suppresses the hyperosmotic induction of AR gene indicating that this portion of the protein is indeed involved in the hyperosmotic transcriptional activation of AR gene.

During the preparation of this manuscript, Miyakawa *et al.* reported the cloning of the cDNA of a protein that binds to the TonE of betaine transporter gene by means of yeast one-hybrid screening (34). This protein, named TonEBP, is essentially identical to OREBP described in this report except that OREBP contains 70 more amino acids in the N-terminus. Since the core sequence of ORE and TonE are identical, it is not surprising the same protein is involved in the hyperosmotic induction of AR and betaine transporter. It is reassuring that the two different approaches identified the same protein. The 70 amino acids missing in the N-terminus of TonEBP are most likely the results of cloning artifact. Using antibodies against TonEBP as a probe Miyakawa and his co-workers found that

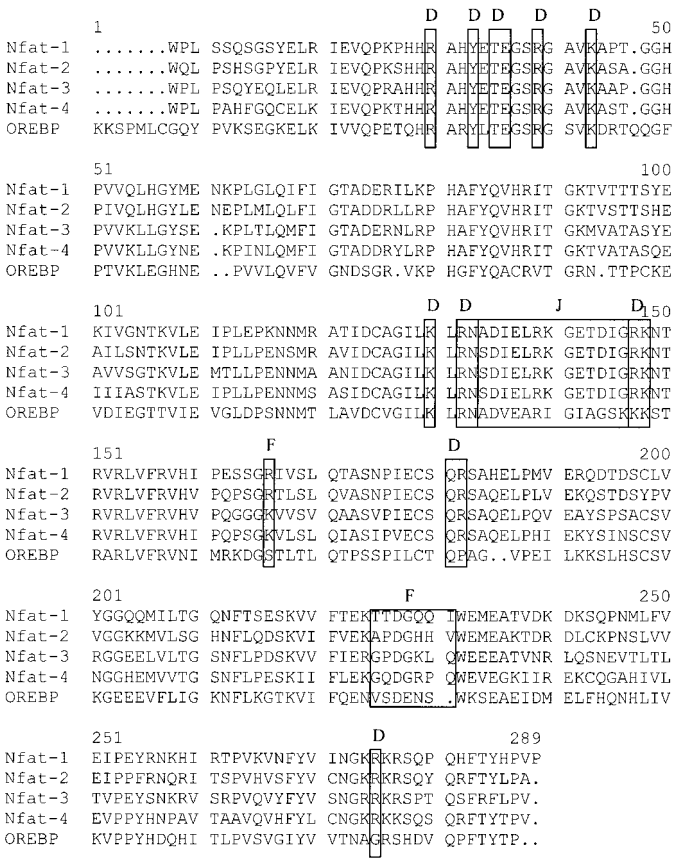

FIG. 7. Comparison of the DNA binding domain between OREBP and NFAT isoforms. Alignment of the Rel homology region of OREPB and NFAT isoforms. Amino acids residues known for DNA (D), Jun (J), and Fos (F) contacts in NFAT isoforms are boxed. The numbers correspond to amino acid position in OREBP.

this protein is distributed throughout the cytoplasm when the cells are cultured in isosmotic medium. Upon hyperosmotic stress, it is translocated to the nucleus. Since the DNA binding domain is homologous to other proteins, it can be argued that the antibodies may have identified other transcription factors. In this report, we showed that the cloned OREBP in transfected cells is translocated to the nucleus upon hyperosmotic induction. This further confirms that OREBP/TonEBP is the hyperosmotic transcription activator.

Comparison of the DNA binding domain between OREBP and NFAT isoforms revealed that the amino acid residues in this region are highly conserved between these two classes of proteins (Fig. 7), presumably because the NFAT and OREBP recognize similar core sequence (GGAAAA for NFAT and TGGAAA for OREBP) (23, 32). We demonstrated that changing the two positively charged arginine residues in the DNA binding domain of OREBP to two uncharged leucine residues abolished the DNA binding activity of this transcription factor. These two arginine residues are known to interact with guanine contact points in the NFAT core sequence, suggesting similar protein-DNA interaction for these proteins. However, the amino acids residues in this region that are responsible for Fos-Jun contact in NFAT isoforms are not found in OREBP (Fig. 7). These residues are highly conserved among different NFAT members, suggesting that unlike NFAT, OREBP is unlikely regulated by Fos and Jun. This supports previous report which showed that inhibition of c-Jun N-terminal kinase does not affect the hyperosmotic induction of BGT and SMIT genes (35, 36).

In our previous report we found that there may be two protein species interacting with OREs (18). Indeed, after the size exclusion chromatography and cation exchange chromatography, we were able to observe two protein species in the gel mobility shift assay. However, the slower migrating protein species was lost after affinity chromatography (data not shown). This observation suggested that OREBP may interact with another protein but this protein does not bind to the ORE directly. This idea is further supported by the fact that in the size exclusion chromatography the native OREBP was eluted close to the void volume (≈1500 kDa), implicating that OREBP is associated with a larger protein complex.

Hyperosmolality provokes translocation of OREPB into the nucleus within 1 h, indicating that the signal of hyperosmolality is quickly relayed to the transcription factor. Despite the rapid translocation of OREBP, AR, BGT-1 and SMIT genes are not induced until 8 to 12 h (4). The striking time lag between the translocation of transcription factor into the nucleus and gene induction cannot be explained at present. It is possible that the full hyperosmotic gene induction requires other accessory factors that are under slow activation kinetics. Since the OREBP mRNA is induced after hyperosmotic challenge, it is also possible that an increase in amount of OREBP through transcription and translation is required before it can mediate hyperosmotic gene induction. Purification of OREBP interacting proteins may help us to clarify this issue.

The OREBP/TonEBP mRNA is ubiquitously expressed (28, 34). It is detectable in the kidney, brain, lung, liver, spleen, gonads and skeletal muscle, suggesting that OREBP may play a role in the protection of the cells against changes in osmolality by modulating the expression of AR, BGT-1 and SMIT gene. These genes are involved in increasing the osmolyte concentrations in the cells. Interestingly, the ORE consensus sequence was also found in the regulatory region of vasopressin gene as well as in the 5′ flanking sequences of cyclooxygenase-2 gene (COX-2) (37, 38), and the transcription of these genes is induced by hyperosmolality. Vasopressin, a peptide hormone secreted by the hypothalamus, is well known to be involved in osmoregulation in the kidney. It induces the translocation of aquaporin 2 from the cytoplasm to the cell

surface of the epithelial cells lining the collecting tubes in the renal medulla to enhance the reabsorption of water from urine (39). On the other hand, the prostaglandins produced by cyclooxygenase act as functional antagonists of vasopressin (35). Vasopressin and prostaglandins are thought to play a key role in the fine tuning of net water transport in collecting ducts. Thus, it appears that OREBP is the key protein responsible for activating genes involved in protecting the animal against dehydration both at cellular level and organismic level.

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