Calcineurin-NFATc signaling pathway regulates AQP2 expression in response to calcium signals and osmotic stress

Song-Zhe Li,1 Bradley W. McDill,1 Paul A. Kovach,1 Li Ding,2 William Y. Go,3 Steffan N. Ho,3 and Feng Chen1

1Renal Division, Department of Internal Medicine and Department of Cell Biology and Physiology, and 2Genome Sequencing Center, Washington University School of Medicine, St. Louis, Missouri; and 3Department of Pathology, University of California-San Diego, La Jolla, California

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Calcineurin-NFATc signaling pathway regulates AQP2 expression in response to calcium signals and osmotic stress. Am J Physiol Cell Physiol 292: C1606–C1616, 2007. First published December 13, 2006; doi:10.1152/ajpcell.00588.2005.—The aquaporin (AQP)2 channel mediates the reabsorption of water in renal collecting ducts in response to arginine vasopressin (AVP) and hypertonicity. Here we show that AQP2 expression is induced not only by the tonicity-responsive enhancer binding protein (TonEBP)/nuclear factor of activated T cells (NFAT)5-mediated hypertonic stress response but also by the calcium-dependent calcineurin-NFATc pathway. The induction of AQP2 expression by the calcineurin-NFATc pathway can occur in the absence of TonEBP/NFAT5. Mutational and chromatin immunoprecipitation analyses revealed the existence of functional NFAT binding sites within the proximal AQP2 promoter responsible for regulation of AQP2 by NFATc proteins and TonEBP/NFAT5. Contrary to the notion that TonEBP/NFAT5 is the only Rel/NFAT family member regulated by tonicity, we found that hypertonicity promotes the nuclear translocation of NFATc proteins for the subsequent induction of AQP2 expression. Calcineurin activity was also found to be involved in the induction of TonEBP/NFAT5 expression by hypertonicity, thus further defining the signaling mechanisms that underlie the TonEBP/NFAT5 osmotic stress response pathway. The coordinate regulation of AQP2 expression by both osmotic stress and calcium signaling appears to provide a means to integrate diverse extracellular signals into optimal cellular responses.

AQP2 expression can regulate the long-term expression of AQP2 (35), regulation of AQP2 by AVP-independent mechanisms has also been reported (14, 20, 42).

Although the hypertonic environment within the kidney allows for reabsorption of water, it also subjects cells to a potentially lethal osmotic stress. The cellular response that permits cells of the renal medulla to adapt and survive within a hypertonic environment is mediated by the tonicity-responsive enhancer binding protein (TonEBP)/nuclear factor of activated T cells (NFAT)5 transcription factor (13). TonEBP/NFAT5 is activated in response to hypertonic stress and regulates the transcription of genes that allow for the accumulation of compatible osmolytes within the cell, thus establishing intracellular water homeostasis within a hypertonic environment. Although TonEBP/NFAT5 shares amino acid sequence similarity with the NFATc transcription factors NFATc1–NFATc4, this similarity is limited to the DNA binding domain (17). NFATc proteins are a substrate for calcineurin, a calcium-regulated serine/threonine phosphatase comprised of a catalytic subunit, calcineurin A (CnA), and a regulatory subunit, calcineurin B (Cnb) (22). Calcineurin regulates the nuclear localization of NFATc proteins through the calcium-dependent dephosphorylation of residues within a serine-proline-rich NFATc homology region that is not present in TonEBP/NFAT5. Thus, unlike NFATc proteins, TonEBP/NFAT5 is not subject to direct dephosphorylation by calcineurin. Previous studies suggest that TonEBP/NFAT5 is constitutively localized to the nucleus (29). Other studies, however, indicate that the nuclear localization of TonEBP/NFAT5 is highly regulated (7, 27, 33) and may involve calcineurin via a mechanism distinct from that regulating NFATc proteins (29, 33, 41, 43). The similarity between TonEBP/NFAT5 and NFATc proteins within the DNA binding domain and the resulting similarity in their consensus target DNA recognition sequences (32, 37) suggest that there could be overlap in the target genes regulated by these proteins. However, while TonEBP/NFAT5 binds to DNA as a homodimer, NFATc proteins usually require other cofactors, such as AP-1, for effective DNA binding (17).

The highly specific calcineurin inhibitors cyclosporin A (CsA) and FK506 (tacrolimus) have been utilized clinically as potent immunosuppressive agents (5). CsA and FK506 form complexes with immunophilins cyclophilin A and FK506-binding protein (FKBP)12, respectively, before binding to and
inhibiting calcineurin (6, 17). These drugs exhibit significant nephrotoxicity, suggesting a critical role for calcineurin in normal renal function (1). A recent study found that long-term treatment with CsA reduced the expression of multiple AQPs by decreasing the Gs protein level, but the involvement of calcineurin was not directly addressed (25). CsA was also found to inhibit the hypertonicity-induced upregulation of osmoprotective genes, including aldose reductase (AR) and betaine/γ-amino-n-butyric acid transporter 1 (BGT1) (41). It is worth noting that AQPs have been reported to be a substrate of calcineurin within an AK inosin anchoring proteins (AKAP) complex (19). Furthermore, the expression of AQPs is significantly decreased in mice deficient for TonEBP/NFAT5 (26).

The purpose of this study was to investigate the regulation of AQP2 expression by the Ca-calcineurin-NFATc pathway and the TonEBP/NFAT5 osmotic stress response pathway. These studies not only reveal the regulation of AQP2 expression by both the Ca-calcineurin-NFATc pathway and the TonEBP/NFAT5-mediated hypertonic response pathway but also demonstrate a novel function of calcineurin-NFATc signaling in the osmotic stress response.

MATERIALS AND METHODS

Cell lines and DNA constructs. mpkCCDc14 cells are immortalized murine collecting duct principal cells originally generated by Dr. A. Vandewalle (45). These mpkCCDc14 cells were seeded on permeable filters (0.4-μm pore size; Corning) in DMEM-F-12 containing 10% FBS, 20 nM dexamethasone, 1 nM triiodothyronine, insulin-transferrin-sodium (ITS) medium supplement (Sigma), 2% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2,95% air. A 600-bp DNA fragment from the mouse AQP2 proximal promoter (nucleotides 8923–9522, GenBank AY055468) was amplified by PCR with KpnI and BglII sites attached. The PCR products were cloned into the KpnI and BglII sites in pGL3-Basic (Promega) to generate pAQP2-WT (wild type) (TonEBP/NFAT5+/+) and pAQP2-NFAT5 knockout (KO) (TonEBP/NFAT5-/-) mouse embryonic fibroblasts (MEFs) (11) were cultured in DMEM (Sigma) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2,95% air. A 600-bp DNA fragment from the mouse AQP2 proximal promoter (nucleotides 8923–9522, GenBank AY055468) was amplified by PCR with KpnI and BglII sites attached. The PCR products were cloned into the KpnI and BglII sites in pGL3-Basic (Promega) to generate pAQP2-WT (wild type). We further generated pAQP2-MT1, pAQP2-MT2, and pAQP2-MT3 mutants in which the putative NFAT sites were mutated by PCR-directed mutagenesis. The pTonELuc reporter (43) has 2 TonE sites: ACCACGGTAAATTTTCCCAAGCTAGCTAGTTGGGTGGAAAATATTACCGCTGTT (TonE sites underlined).

Luciferase, β-galactosidase, and protein assay. Cells were transfected (0.5–1 μg DNA/well) with FuGENE6 (Roche). One day after transfection, the cells were subcultured into 24-well plates. Treatments were applied 24 h later in fresh medium containing 0.2% FBS. After 6 h of treatment, the cells were harvested in Reporter Lysis Buffer (Promega) and the supernatant of cell lysate was subjected to luciferase assays (BD Bioscience). Luciferase activity was standardized by the corresponding β-galactosidase activity (β-galactosidase assay; Tropix) in most cases and by total cellular protein levels (determined by the Bradford method) when treatments were performed in samples originated from the same transfection. Data were analyzed by one- or two-way analysis of variance (ANOVA) with SAS (SAS Institute) software.

Reverse transcription-polymerase chain reaction. Total RNA was isolated from cultured cells with an RNeasy Mini Kit (Qiagen).

The primers used for reverse transcription-polymerase chain reaction (RT-PCR) were AQP2 (5′-CAGCATCACCCTGGCTGT-GAC-3′ and 5′-CAGCTGGCTGTGGTACTCCAG-3′), C1607 (5′-CAGGAGGTGTCTAGTTCC-3′ and 5′-CAAGGCCACAAAATACAGAC-3′), C1665 (5′-CTGCTGATGTTACAGCAGAAC-3′ and 5′-GATGGTGCCACCATACATC-3′), NFATc1 (5′-CATTCTGGTCTCATCG-3′ and 5′-tgaggagttcctcaag-3′), NFAT-2 (5′-GAGTCCATCTCTGGTGAAC-3′ and 5′-CTGCTGG-CATCCATGAG-3′), NFATc3 (5′-CGCAAGTTGGTGACTGG-3′ and 5′-AAGCCTGGTGTATGAGG-3′), NFATc4 (5′-CGCAGTGGATCTACCTAGCA-3′) and GAPDH (5′-CGTCCCCGATAGAATAAGGTTGG-3′ and 5′-AGGTTTTCGCTAGTGGATGTC-3′). Cycling conditions were 45°C for 30 min, 95°C for 5 min, and 30 cycles of 95°C for 20 s, 58°C for 35 s, and 72°C for 45 s.

Western blot and immunofluorescent staining assays. Whole cell lysates in lysis buffer (0.3 M sucrose, 25 mM imidazole, and 1 mM EDTA, pH 7.2) containing a cocktail of protease inhibitor (Roche) and 1 mM phenylmethylsulfonyl fluoride were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed by an anti-AQP antibody (Chemicon), an anti-CnA antibody (Chemicon, detecting CnAα, -β, and -γ), an anti-CnB antibody (Upstate, detecting CnB1 and CnB2), an anti-NFATc1 antibody (PharMingen) or an anti-NFAT5 antibody (Santa Cruz), and an anti-β-tubulin antibody (Developmental Study Hybridoma Bank, University of Iowa), respectively. After incubation with Alexa Fluor 680-conjugated rabbit anti-IgG (Molecular Probes), Alexa Fluor 680-conjugated rabbit anti-goat IgG (Molecular Probes), or IRDye 800CW-conjugated goat anti-mouse IgG (Rockland) secondary antibodies, the antibody complexes were revealed by the Odyssey Infrared Imaging System (LI-COR). Immunostaining was carried out as previously described (4). The antibodies used were an anti-NFATc1 antibody (PharMingen) and an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Molecular Probes). Nuclei were counterstained with DAPI (Sigma).

Chromatin immunoprecipitation assays. For the chromatin immunoprecipitation (ChIP) assay, mpkCCDc14 cells were treated for 30 min, harvested, and incubated with 1% formaldehyde (final concentration) for 15 min to cross-link proteins and DNA. Cross-linking was stopped by the addition of glycine at a final concentration of 0.125 M. Cells were washed with ice-cold PBS and resuspended in 500 μl of lysis buffer (Santa Cruz) containing a cocktail of protease inhibitors (Roche) and 1 mM phenylmethylsulfonyl fluoride were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed by an anti-AQP antibody (Chemicon), an anti-CnA antibody (Chemicon, detecting CnAα, -β, and -γ), an anti-CnB antibody (Upstate, detecting CnB1 and CnB2), an anti-NFATc1 antibody (PharMingen) or an anti-NFAT5 antibody (Santa Cruz), and an anti-β-tubulin antibody (Developmental Study Hybridoma Bank, University of Iowa), respectively. After incubation with Alexa Fluor 680-conjugated rabbit anti-IgG (Molecular Probes), Alexa Fluor 680-conjugated rabbit anti-goat IgG (Molecular Probes), or IRDye 800CW-conjugated goat anti-mouse IgG (Rockland) secondary antibodies, the antibody complexes were revealed by the Odyssey Infrared Imaging System (LI-COR). Immunostaining was carried out as previously described (4). The antibodies used were an anti-NFATc1 antibody (PharMingen) and an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Molecular Probes). Nuclei were counterstained with DAPI (Sigma).

1 In this report, we use “NFATc site” for cis elements known to interact with NFATc1–c4, “TonE” for cis elements known to interact with TonEBP/NFAT5, and “putative NFAT sites” for cis elements containing the GGAAA core sequence but not yet examined experimentally for binding specificity.
RESULTS

NFAT expression in collecting duct epithelial cells and NFAT sites within AQP2 promoter. All four NFATc genes, TonEBP/NFAT5, Calcineurin Aα, and Calcineurin B1, were found to be expressed in the collecting duct epithelial cell line mpkCCDc14 as well as in the kidney (Fig. 1A and data not shown). Interestingly, inspection of the AQP2 proximal promoter sequence revealed multiple potential NFAT binding sites including a putative TonE consensus sequence (A site in Fig. 1B; Refs. 13, 42). The potential NFAT sites have the core GGAAA sequence (Fig. 1C). To investigate the potential regulation of AQP2 expression by the NFAT family of proteins, especially the NFATc proteins, we cloned a 600-bp fragment of the mouse AQP2 proximal promoter into the pGL3-basic luciferase reporter vector to generate pAQP2-WT (Fig. 1B). Transfection of pAQP2-WT into mpkCCDc14 cells demonstrated significant promoter activity (Fig. 1C).

AQP2 promoter is stimulated by hypertonicity and by ionomycin plus PMA. The activity of the AQP2 proximal promoter is significantly induced by higher osmolality (~500 mosmol/kgH2O) mediated by 220 mM NaCl (final NaCl concentration is significantly induced by higher osmolality (197, 192, 212, and 235 bp, respectively. (Fig. 1)). Transfection of B1B; Refs. 13, 42). The potential NFAT sites have the core GGAAA sequence (Fig. 1C). To investigate the potential regulation of AQP2 expression by the NFAT family of proteins, especially the NFATc proteins, we cloned a 600-bp fragment of the mouse AQP2 proximal promoter into the pGL3-basic luciferase reporter vector to generate pAQP2-WT (Fig. 1B). Transfection of pAQP2-WT into mpkCCDc14 cells demonstrated significant promoter activity (Fig. 1C).

AQP2 proximal promoter sequence contains cis elements responsive to hypertonic stress. Given the presence of several potential NFAT binding sites within the AQP2 promoter, regulation of AQP2-WT in response to pharmacological agents that induce NFATc-dependent transcription was investigated. Ionomycin, a calcium ionophore, activates calcineurin by increasing intracellular Ca²⁺ through the release of stored Ca²⁺ from the endoplasmic reticulum. Phorbol 12-myristate 13-acetate (PMA) activates AP-1, a known cofactor for NFATc proteins through its effects on PKC (17). Ionomycin plus PMA (I+P) treatment of mpkCCDc14 cells transfected with pAQP2-WT significantly induced reporter activity (Fig. 2B). Western blotting demonstrated a significant increase in the endogenous AQP2 protein level in response to hypertonic stress (Fig. 2, C and D), consistent with the observed induction of the AQP2 reporter construct (Fig. 2, A and B). In addition, the transcription of the endogenous AQP2 gene was increased under hypertonic conditions and with I+P treatment (Fig. 2, E and F). Interestingly, such an effect was significantly inhibited by the calcineurin inhibitors CsA plus FK506 (C+FK), indicating a potential involvement of calcineurin in these processes (Fig. 2, C–F).

Calcineurin-NFATc pathway regulates AQP2 expression. Increases in intracellular calcium mediated by ionomycin may induce transcriptional responses independent of calcineurin and NFATc. To further confirm that calcineurin plays a role in the regulation of AQP2 expression, a constitutively active form of CnA (CACnA) lacking the autoinhibitory peptide and wild-type CnB were overexpressed in mpkCCDc14 cells together with pAQP2-WT. CACnA markedly increased the transcriptional activity of the AQP2 proximal promoter (Fig. 3A), indicating that calcineurin is capable of regulating AQP2 transcription. Furthermore, overexpression of NFATc1, but not NFATc2, NFATc3, and NFATc4, significantly induced pAQP2-WT in the absence of CACnA (Fig. 3B). In the presence of CACnA, the induction by overexpression of NFATc1 protein was significantly higher than the simple sum of the effects of CACnA and NFATc1 protein (Fig. 3B).
NFAT sites within the AQP2 proximal promoter mediate regulation of AQP2 expression in response to hypertonicity and calcium signaling. To investigate the involvement of the putative NFAT sites in the hypertonicity induction and calcium induction of AQP2 expression, three additional reporter constructs, pAQP2-MT1, pAQP2-MT2, and pAQP2-MT3, were generated in which the first, the last five, or all six putative NFAT sites within the proximal AQP2 promoter were mutated (Fig. 4A). We found that the induction of pAQP2-WT by overexpression of NFATc1 is sensitive to calcineurin inhibitors (Fig. 4B). Mutating all six sites (pAQP2-MT3) resulted in a complete loss of this induction (Fig. 4B), suggesting that the NFATc1 effect is mediated by the putative NFAT sites. Interestingly, this induction was significantly reduced, but not lost, when either the first (pAQP2-MT1) or the last five (pAQP2-MT2) NFAT sites were mutated, suggesting that NFATc1 can utilize not only the last five sites but also the first site (A site) for transcription activation. While it appeared surprising at first since the A site has been suggested as a TonE site (13, 42), a close examination of the A site revealed the sequence TGGAAATTTGT, which is identical to the known NFATc binding sites in the IL-4 promoter and the IFN-γ promoter (17). In parallel to the NFATc1 study, we found that promoter activity induced by the overexpression of TonEBP/NFAT5 was lost when all the putative NFAT sites were mutated (pAQP2-MT3). Interestingly, mutation of the first NFAT site (pAQP2-MT1) caused a decrease, but not a complete loss, of the induction. Furthermore, mutation of the last five sites (pAQP2-MT2) led to an even greater decrease in promoter activity induced by TonEBP/NFAT5. These results suggest that the effect of TonEBP/NFAT5 on the AQP2 promoter is not solely mediated by the A site. A possible mechanism underlying this observation is described in DISCUSSION.

To further determine whether regulation of the AQP2 promoter by extracellular stimuli known to induce TonEBP/NFAT5 or calcineurin-NFATc signaling was also dependent...
on the putative NFAT sites, mpkCCDc14 cells transfected with the pAQP2-WT, pAQP2-MT1, pAQP2-MT2, and pAQP2-MT3 constructs were treated with 220 mM NaCl (~500 mosmol/kgH₂O), I-P, or both. The induction of the AQP2 promoter (pAQP2-WT) by the combined NaCl and I-P treatment is greater than those from either of the two individual treatments. This result indicates that TonEBP/NFAT5 and NFATc1 do not have completely redundant functions in AQP2 induction, because the combined treatment, in theory, would not lead to any increase above the highest level of the individual inductions if NFATc1 and TonEBP/NFAT5 have complete overlap of site usage and activity. The induction by NaCl, I-P, or both was completely lost in pAQP2-MT3 with all six sites mutated, confirming the importance of these sites in the induction of AQP2 promoter activity by hypertonicity and I-P. In addition, the mutation of site A in pAQP2-MT1 and the mutation of the last five sites in pAQP2-MT2 both reduced, but neither eliminated, the response to NaCl, I-P, and the combined stimulation (Fig. 4D). This is consistent with the observations in Figs. 4, B and C, and with the observation that the combined treatment produced a higher induction that is smaller than the sum of those from the individual treatments, most obviously in treatment produced a higher induction that is smaller than the sum of the individual inductions, near the sum of the individual inductions. Therefore, these results suggest that NFATc1 and TonEBP/NFAT5 have partial overlap in their binding site usage. However, these are simplistic models, and more studies are required to reveal the detailed mechanism of these events.

Hyperosmotic induction of TonEBP/NFAT5 gene expression may involve calcineurin. To investigate the mechanism by which calcineurin-NFATc and TonEBP/NFAT5 mediate the induction of AQP2, we examined the expression of the TonEBP/NFAT5, calcineurin, and NFATc genes by RT-PCR and Western blotting in mpkCCDc14 cells exposed to NaCl-induced hypertonic stress or treated with I-P. TonEBP/NFAT5 mRNA was significantly induced by both NaCl and I-P, while CnAα, CnB1, and NFATc1 mRNA levels were not affected (Fig. 5A). Interestingly, the upregulation of TonEBP/NFAT5, either by NaCl or by I-P, was significantly reduced by CsA/FK506, suggesting a role of calcineurin in the induction of TonEBP/NFAT5 mRNA in response to hypertonicity or I-P. These effects were further confirmed in the Western blot assay (Fig. 5B). The presence of putative NFAT sites in the TonEBP/NFAT5 promoter (data not shown) and the observation that CsA downregulates TonEBP/NFAT5 in the renal medulla (25) and splenocytes (11, 43) are consistent with the possible regulation of TonEBP/NFAT5 expression by calcineurin-NFATc.

Hyperosmotic stress induces nuclear translocation of NFATc proteins. Since hyperosmotic stress did not increase the expression of NFATc1, we investigated whether the nuclear translocation of the NFATc proteins could be modulated by hypertonicity. NFATc1 was transfected into mpkCCDc14 cells. Interestingly, hypertonicity induced the nuclear accumulation of NFATc1 (Fig. 6A). This effect was prevented by treatment with CsA/FK506, indicating that the nuclear accumulation of NFATc1 induced by hypertonicity is dependent on activation of calcineurin. As with the overexpressed NFATc1, hypertonicity also induced the nuclear localization of the endogenous NFATc1 that was again inhibited in the presence of CsA/FK506 (Fig. 6B). These results demonstrate that hyperosmotic stress activates NFATc proteins as transcription factors, further supporting a role for the calcineurin-NFATc pathway in the osmotic stress response.

Calcineurin-NFATc can regulate AQP2 expression independent of TonEBP/NFAT5. Observations made by other laboratories (13, 26) and in the present study strongly indicate that TonEBP/NFAT5 has a role in AQP2 expression. Findings that calcineurin is involved in the regulation of TonEBP/NFAT5 expression (Ref. 19 and Fig. 5) lead to the possibility that the observed effects of calcineurin-NFATc on the AQP2 promoter are indirect and mediated through TonEBP/NFAT5. To answer this question, we investigated the effect of calcineurin activation on the pAQP2-WT construct in MEFs deficient for TonEBP/NFAT5 (11, 43). A pTonE-Luc construct carrying a dimeric consensus TonE site directing the luciferase reporter

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transfected mpkCCDc14 cells were treated with 220 mM NaCl (only), which was set to 1. Activities were standardized by corresponding putative NFAT sites. The 2-day transiently transfected mpkCCDc14 cells were treated with CsA (1 μM) and FK506 (10 ng/ml). pAQP2-MT1, pAQP2-MT2, and pAQP2-MT3 constructs that have the first, the last 5, or all 6 putative NFAT binding sites mutated within the proximal AQP2 promoter. Mutated sites are shown as crossed-out boxes. Actual sequences of the putative NFAT binding sites and engineered mutations are underlined.

METHODS). While the binding of NFATc1 to the endogenous AQP2 promoter depended on the putative NFAT sites. *P < 0.01 vs. reporter only; **P < 0.01 vs. NFATc1 transfection. C: effects of TonEBP/NFAT5 on the AQP2 promoter depended on the putative NFAT sites. *P < 0.01 vs. reporter only; #P < 0.01 vs. TonEBP/NFAT5 transfection. In B and C, luciferase activities were standardized by corresponding β-galactosidase assay results and presented finally with fold changes relative to the respective control (reporter only), which was set to 1. D: mutations in the putative NFAT sites diminished the response of AQP2 proximal promoter to high salt and I+P. Transiently transfected mpkCCDc14 cells were treated with 220 mM NaCl (~500 mosmol/kg H2O), ionomycin (0.1 μM) + PMA (50 ng/ml), or both for 6 h before harvest. *P < 0.01 vs. untreated control; **P < 0.01 vs. NaCl or I+P only. Luciferase activities were standardized by the corresponding protein levels and presented as fold changes relative to the respective controls (nontreatment), which were set to 1. All experiments were performed in triplicate. Results shown are representative of ≥3 independent experiments.

gene (23) was transfected into TonEBP/NFAT5-WT and -KO MEFs (11, 43). pTonE-Luc was markedly induced (360-fold) by hypertonicity in WT cells (Fig. 7A). This response was nearly absent in the KO MEFs but was restored by the transfection of TonEBP/NFAT5 (Fig. 7A), confirming the absence of TonEBP/NFAT5 activity in the TonEBP/NFAT5-KO MEFs. We then transfected the pAQP2-WT reporter into the WT and KO MEFs with or without cotransfection of constitutively active calcineurin. The calcineurin-mediated increase in AQP2 promoter activity observed in WT cells was largely retained in KO cells (Fig. 7B), indicating that regulation of AQP2 promoter by calcineurin-NFATc can be independent of TonEBP/NFAT5.

Both NFATc1 and TonEBP/NFAT5 bind to AQP2 proximal promoter in response to hypertonicity and calcium signaling at physiological concentrations. To assess whether or not NFATc1 and TonEBP/NFAT5 can bind to the AQP2 promoter at physiological concentrations, we performed a ChIP assay using antibodies against NFATc1 and TonEBP/NFAT5. The precipitated DNA-protein complexes were used to amplify, by PCR, a 366-bp DNA segment of the AQP2 proximal promoter containing the six NFAT binding sites (see MATERIALS AND METHODS). While the binding of NFATc1 to the endogenous AQP2 proximal promoter was minimal in isotonic medium, it was significantly increased in hypertonic medium with 220 mM NaCl (Fig. 8, lane 8). This increase was greatly attenuated by the addition of calcineurin inhibitors CsA and FK506 (Fig. 8, lane 9). Similarly, the binding of NFATc1 to the endogenous AQP2 proximal promoter was increased in the presence of I+P (Fig. 8, lane 10). This increase was again reversed by the addition of CsA and FK506 (Fig. 8, lane 11). These results demonstrate that both hypertonicity and calcium signaling increase the binding of endogenous NFATc1 to the promoter of the AQP2 gene in a calcineurin-dependent manner. The binding of TonEBP/NFAT5 to the proximal promoter region of the AQP2 gene was also induced not only by hypertonicity but also by an increase in intracellular calcium (Fig. 8, lanes 12, 13, and 15). This increase was again sensitive to calcineurin inhibitors CsA and FK506 (Fig. 8, lanes 14 and 16). Since the average chromatin fragments are ~500 bp in the ChIP assay we performed, it is not feasible to assess the binding of NFATc1 and TonEBP/NFAT5 to individual putative NFAT sites with this method. Future experiments will aim at investigating the properties of the individual candidate NFAT sites in terms of binding with NFATc and TonEBP/NFAT5, as well as the relative functional importance of these sites.
and TonEBP/NFAT5 can facilitate transcellular transfer of water from the apical membrane of the collecting duct epithelial cells to the medullary interstitium when interstitial osmolarity rises. This regulation is likely to provide the collecting duct epithelial cells a means to react to local osmotic change independent of the systemic water and electrolyte balance. Nephrotoxicity is a major side effect of calcineurin inhibitors such as CsA and FK506 (1, 12, 34). Calcineurin inhibitor-induced nephrotoxicity is characterized by, among other pathological changes, tubular atrophy, especially in the outer and inner regions of the medulla that are typically exposed to a hypertonic environment (34). Since hypertonicity regulates the expression of multiple AQPs (18, 30, 44) and CsA treatment decreases the expression of AQP1-s4 in rat kidney (25), it is possible that calcineurin-NFATc signaling is involved in the osmotic regulation of other AQPs. Besides the AQPs, CsA treatment has been found to reduce the expression of multiple osmoprotective genes (41). Therefore, calcineurin may play a key role in water homeostasis and osmoprotection in collecting duct epithelial cells. The chronic toxicity of calcineurin inhibitors on renal tubules may partially result from the loss of a calcineurin-mediated regulatory mechanism necessary for water homeostasis and the accumulation of protective osmolytes. In fact, the calcineurin-NFATc pathway has been found to be involved in osmotic protection of lymphoid cells through the regulation of TonEBP/NFAT5 (43).

The high degree of conservation among the mammalian AQP2 promoters with regard to the putative NFAT sites especially in the first, second, and last positions (data not shown) strongly suggests the functional importance of these elements. While a previous study by Storm et al. (42) sug-

**Fig. 5.** Hypertonicity induces TonEBP/NFAT5 gene expression in a calcineurin-dependent manner. mpkCCDc14 cells were treated with 220 mM NaCl ($\sim$500 mosmol/kgH2O) or ionomycin (0.1 mM) + PMA (50 ng/ml) alone, as well as with CsA (1 $\mu$g/ml) + FK506 (10 ng/ml), for 20 h. A: total RNA was isolated from the cells at the end of the treatment period and was subjected to RT-PCR. Columns represent fold changes of the level of amplified RT-PCR products relative to the nontreatment control (Ctrl). Quantification of the products was performed by densitometric analysis with NIH ImageJ. The level of transcripts for each gene tested was normalized with the respective GAPDH level and is presented as fold change relative to the respective control (Ctrl), which was set to 1. Primers for CnA are specific for CnAa. Primers for CnB are specific for CnB1. B: whole cell lysates were harvested after 20 h of treatment for Western blotting. Levels of these proteins were normalized to the amount of $\beta$-tubulin in each sample. *P < 0.01 vs. Ctrl; #P < 0.01 vs. NaCl only; **P < 0.01 vs. I+P only. The antibody for CnA reacts to all 3 CnA isoforms, but the testis-specific CnAa is not expected to be present. The antibody for CnB reacts to both CnB1 and CnB2, but again the testis-specific CnB2 is not expected to be present in these cells. Each experiment was repeated 3 times independently.

**DISCUSSION**

The results presented here indicate that expression of AQP2 is regulated by the Ca-calcineurin-NFATc pathway in addition to the TonEBP/NFAT5 osmotic stress response pathway. Remarkably, besides activating TonEBP/NFAT5-dependent transcriptional responses, hypertonicity also induces nuclear localization of NFATc1 (Fig. 6), thus adding to the pleiotropic effects of hypertonicity on intracellular signaling (15). It has been reported that hypertonicity can increase intracellular calcium concentration (8, 24, 36, 38). Therefore, it is possible that hypertonicity regulates the calcineurin-NFATc pathway through its effect on intracellular calcium level, independent of TonEBP/NFAT5 (Fig. 7). Furthermore, inhibition of calcineurin specifically reduced the induction of TonEBP/NFAT5 mRNA in response to hypertonicity (Fig. 5), consistent with the observation that expression of TonEBP/NFAT5 could be regulated by calcineurin during T cell activation (27, 43). Thus the coordinate regulation of AQP2 expression could conceivably occur not only through direct effects of NFATc and TonEBP/NFAT5 on the AQP2 promoter but also by cross talk between the intracellular signaling events that regulate the TonEBP/NFAT5 and calcineurin-NFATc pathways. While calcineurin-NFATc can be activated by hypertonicity, it can also be induced by intracellular calcium increase mediated by other extracellular stimuli. The coordinate regulation of AQP2 expression by both osmotic stress and calcium signaling is another example of integration of diverse extracellular signals for optimal cellular responses.

Transcriptional regulation of AQP2 by calcineurin-NFATc and TonEBP/NFAT5 can facilitate transcellular transfer of
Fig. 6. NaCl-mediated hypertonicity promotes nuclear translocations of NFATc proteins. A: high osmolality induces nuclear translocation of NFATc1. mpkCCDc14 cells were transfected with NFATc1 and treated with 220 mM NaCl alone or together with CsA (1 μg/ml) + FK506 (10 ng/ml) for 20 h. Green staining is NFATc1. Nuclei were counterstained with DAPI (blue). B: high osmolality promotes nuclear translocation of the endogenous NFATc1 in mpkCCDc14 cells. Transiently transfected mpkCCDc14 cells were treated with 220 mM NaCl alone or together with CsA (1 μg/ml) + FK506 (10 ng/ml) for 20 h. Green staining reveals the NFATc1. Nuclei were counterstained with DAPI (blue). Each experiment was repeated ≥2 times independently.
ggested that the putative TonE site in the proximal AQP2 promoter (designated the 1st binding site in this study) subjects the expression of AQP2 to osmotic control, the study by Kasono et al. (20) discounted the importance of this proximal putative TonE site and suggested that an unknown cis element for tonicity response resides in a 2-kb region located 4.3 kb upstream to the site of transcription initiation (20). A more recent study by Hasler et al. (13) demonstrated that the proximal AQP2 promoter (including the proximal putative TonE site) responds to hypertonicity. Discrepancy between these studies may result from the distinct culture systems being used and possible differences in experimental procedures. Our results support a role for the proximal AQP2 promoter in osmotic stress response and further indicate that the regulation of AQP2 expression is a complex process involving not just TonEBP/NFAT5 but also calcineurin/NFATc. Similar results were obtained in both mpkCCD14 cells and mIMCD3 cells, suggesting that such regulation is likely common in kidney collecting duct epithelial cells of different origins (data not shown). While the importance of TonEBP/NFAT5 and calcineurin/NFATc in the hypertonic induction of AQP2 expression is indicated by previous reports and the present study, there is possible involvement of other factors in this process, especially considering the likely contributions of cofactors for NFATc and the discovery of putative osmotic responsive elements with no resemblance to the NFAT sites (20).

The observation that not only the A site but also the other five sites (B–F) can mediate TonEBP/NFAT5 effects on the AQP2 proximal promoter (Fig. 4) may seem surprising at first, since only the A site has been suggested as a TonE site (13, 42). However, the examination of the DNA sequence provides clues on the possible biological basis for the other sites to mediate TonEBP/NFAT5 effects. Although the A site has the sequence TGGAAATTTGT matching the NFAT consensus sequence TGGAANNYNY proposed by Miyakawa et al. (32), it has mismatches in two nucleotides when compared against the NGGAADNHMC consensus sequence proposed by Ferraris et al. (9, 10) (keys to degenerate nucleotides are R: A+G, M: A+C, W: A+T, K: G+T, S: G+C, Y: C+T, H: A+T+C, B: G+T+C, D: G+A+T, N: A+C+G+T, V: G+A+C). It also has two nucleotide mismatches in the sequence TGGAAYADWMD derived from PCR clones selected by TonEBP/NFAT5 binding by Lopez-Rodriguez et al. (28). On the other hand, the corresponding sequence in the B site (TGGAAAACACAT) matches the consensus by Ferraris et al. (9, 10), and has only one nucleotide mismatch when compared against the other two proposed consensus sequences (29, 32). In addition, site C (CGGAAAGGCAG) is very similar to the first two proposed consensus sequences, with one mismatch only in each comparison, and site E (AGGAAAACAG) is very similar to the last two proposed consensus sequences, with one mismatch only in

Fig. 7. Calcineurin-NFATc can regulate AQP2 expression independent of TonEBP/NFAT5. A: immortalized TonEBP/NFAT5-null (WT) and -knockout (KO) mouse embryonic fibroblasts (MEFs) were transfected with TonE-Luc, pSV-Bgal, and with or without TonEBP/NFAT5. Transfected cells were treated with 220 mM NaCl for 20 h before harvest for luciferase and β-galactosidase assays. Luciferase activities were standardized by corresponding β-galactosidase activities and are presented as fold changes relative to the respective control (nontreatment). B: TonEBP/NFAT5-WT and TonEBP/NFAT5-KO MEFs were transfected with the luciferase reporters (pAQP2-WT or pAQP2-MT), pSV-Bgal, and with or without TonEBP/NFAT5. Luciferase activities were standardized by corresponding β-galactosidase activities and are presented as fold changes relative to the controls (the luciferase reporter-alone samples in each set of experiments). All experiments were performed in triplicate. Results shown are representative of ≥3 independent experiments.

Fig. 8. Both NFATc1 and TonEBP/NFAT5 bind to the AQP2 proximal promoter in response to hypertonicity and calcium signaling at physiological concentrations. mpkCCD14 cells were treated for 30 min with NaCl (Na+, 220 mM), or ionomycin + PMA (I/P; 0.1 μM and 50 ng/ml, respectively), and with or without CsA + FK506 (C/F; 1 μM and 10 ng/ml, respectively) in fresh culture medium containing 0.2% FBS. Cells were collected for chromatin immunoprecipitation (ChIP) analysis using NFATc1- and TonEBP/NFAT5-specific antibodies. Normal mouse IgG1 and goat IgG were used as background control. PCR was performed as described in MATERIALS AND METHODS. After gel electrophoresis, ethidium bromide-stained PAGE gels were photographed. M, DNA molecular weight ladder; −, untreated; Negative, PCR reaction using DNA elution buffer as template control.
each comparison. While confirming that the TonEBP/NFAT5 can act through the A site, these results also suggest that the other putative NFAT sites in the AQP2 proximal promoter are potential TonEs capable of mediating TonEBP/NFAT5 functions.

Comparison of NFATc and TonEBP/NFAT5 function in the mouse collecting duct cells used in this study with similar studies performed in the Jurkat lymphoid cell line (43) reveals several similarities. In both systems, TonEBP/NFAT5 activity, measured by the same pTonE-Luc reporter construct, could be induced not only by hypertonicity but also by the pharmacological agents I+P (Fig. 7A and data not shown). In addition, TonEBP/NFAT5 expression (either mRNA or protein) in both systems exhibited calcineurin dependence in the form of sensitivity to inhibition by CsA. Several known targets of calcineurin-NFATc, including IL-8 and TNF-α, have been shown to be regulated by hypertonicity, especially by the TonEBP/NFAT5-mediated osmotic stress response (27, 39, 40). Together, these observations further support the hypothesis that, similar to AQP2, other targets of the calcineurin-NFATc pathway may also be subject to regulation by the TonEBP/NFAT5 osmotic stress response and vice versa. Besides the apparent regulation of TonEBP/NFAT5 by calcineurin-calcineurin, this overlap in target genes between NFATc and TonEBP/NFAT5 may result from the potential overlap in binding specificity between NFATc and TonEBP/NFAT5 (9, 10, 29, 31). Furthermore, these observations also imply that changes in cellular function induced by receptor-mediated signaling events that activate the calcineurin-NFATc pathway under isotonic conditions may affect intracellular water homeostasis in a manner similar to extracellular osmotic stress (15, 16).

Contrary to the notion that TonEBP/NFAT5 is the only Rel/NFAT family member regulated by tonicity, the results presented here demonstrate that hypertonicity promotes the nuclear translocation of both calcineurin and NFATc proteins and the subsequent induction of AQP2 transcription. Calcineurin activity was also found to be involved in the induction of TonEBP/NFAT5 expression by hypertonicity, suggesting that calcineurin may play an essential role in osmotic stress responses mediated by both TonEBP/NFAT5 and NFATc proteins. The coordinate regulation of AQP2 expression by both osmotic stress and calcium signaling appears to provide a means to integrate diverse extracellular signals into optimal cellular responses. Additional studies are necessary to further define the functional specificity of NFATc proteins and TonEBP/NFAT5 under various physiological conditions, both in the presence and absence of overt extracellular osmotic stress.

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Present address for S. N. Ho: Molecular Discovery, Biogen Idec, 5200 Research Place, San Diego, CA 92122.

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