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MEKK3-mediated signaling to p38 kinase and TonE in hypertonically stressed kidney cells

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Padda, Ranjit, Ann Wamsley-Davis, Michael C. Gustin, Rebekah Ross, Christina Yu, and David Sheikh-Hamad. MEKK3-mediated signaling to p38 kinase and TonE in hypertonically stressed kidney cells. *Am J Physiol Renal Physiol* 291: F874–F881, 2006. First published May 9, 2006; doi:10.1152/ajprenal.00377.2005.—Mitogen-activated protein kinase (MAPK) cascades contain a trio of kinases, MAPK kinase kinase (MKKK) → MAPK kinase (MKK) → MAPK, that mediate a variety of cellular responses to different signals including hypertonicity. The signaling response to hypertonicity is conserved across evolution from yeast to mammals in that it involves activation of p38/SAPK. However, very little is known about which upstream protein kinases mediate activation of p38 by hypertonicity in mammals. The MKKKs, MEKK3 and MEKK4, are upstream regulators of p38 in many cells. To investigate these signaling proteins as potential activators of p38 in the hypertonicity response, we generated stably transfected MDCK cells that express activated versions of MEKK3 or MEKK4, utilized RNA interference to deplete MEKK3, and employed pharmacological inhibition of p38 kinase. MEKK3-transfected cells demonstrated increased betaine transporter (BGT1) mRNA levels and upregulated tonicity enhancer (TonE)-driven luciferase activity under isotonic (basal) and hypertonic conditions compared with empty vector-transfected controls; small-interference RNA-mediated depletion of MEKK3 downregulated the activity of p38 kinase and decreased the expression of BGT1 mRNA. p38 Kinase inhibition abolished the effects of MEKK3 activation on BGT1 induction. In contrast, the response to hypertonicity in MEKK4-kA-transfected cells was similar to that observed in empty vector-transfected controls. Our data are consistent with the existence of an input from MEKK3 →→ p38 kinase →→ TonE.

osmotic stress; tonicity enhancer; betaine transporter; TonEBP

MANY ORGANISMS, INCLUDING bacteria, yeast, plants, and animals, adapt to sustained hypertonic stress by the preferential accumulation of compatible organic osmolytes (56). In water-deprived mammals, for example, the extracellular osmolality of the kidney medulla may exceed 4,000 mosmol/kgH_2O (38). Roughly one-half of the prevailing medullary interstitial solutes consist of urea, whereas the other half is composed of NaCl (15). Urea easily equilibrates across biological membranes and does not cause water shift between the intracellular and extracellular compartments. However, NaCl remains confined to the extracellular space, owing to the action of the Na-K-ATPase. An increase in the extracellular concentration of NaCl contributes to dehydration of the intracellular milieu (hypertonic stress), and restoration of intracellular volume in hypertonically stressed kidney cells requires the induction of a group of genes that lead to the accumulation of organic osmolytes intracellulary [*BGT1* for betaine transporter (54), *SMIT* for inositol transporter (55), taurine transporter (49), and the aldose reductase enzyme (*AR*), which catalyze the reduction of D-glucose to the organic solute sorbitol (3)]. The transcriptional "machinery" that drives the expression of these genes [*SMIT*, *BGT1*, taurine transporter, and *AR*] under hypertonic conditions is similar (13, 19, 36, 44) and involves interaction between the *cis*-element [tonicity enhancer (TonE) (36), also known as osmotic response element (ORE) (13); referred to herein as TonE] and transcription factor TonE binding protein [TonEBP; (37), also known also as ORE binding protein (OREBP) (24) as well as NFAT5 (34); referred to herein as TonEBP]. Activation of TonE-mediated gene expression by hypertonicity is not unique to kidney cells [Madin-Darby canine kidney (MDCK) (36); rabbit kidney papillary epithelial cells (PAP-HT25) (25); mouse inner medullar collecting duct cells (mIMCD) (48)], as it has been shown to occur in neurons (35), human liver-derived HepG2 (41), Chang liver, Cos-7, and HeLa cells (24). Deletion of the TonEBP gene in mice blocks the expression of TonE-mediated gene expression in the kidney medulla almost completely, as evidenced by the diminished expression of the *BGT1*, *SMIT*, and *AR* genes. Remarkably, mice lacking TonEBP show atrophy of the renal medulla, which contains smaller cells and displays increased apoptosis (33).

While transcriptional control of hypertonicity-induced genes in mammalian cells is reasonably well characterized, the signaling pathways leading to TonE-mediated gene expression need further delineation. In yeast, the adaptation to osmotic stress is dependent on the p38 MAPK homolog high-osmolarity glycerol 1 (HOG1) (7). Similarly, the induction of TonE-mediated gene expression in mammalian cells is p38 kinase dependent (41, 46) but requires cooperative action of Fyn, the catalytic subunit of PKA and the DNA damage-inducible kinase ATM (reviewed in Ref. 47). While ERK and JNK are induced by hypertonicity, the significance of their activation is not clear, as JNK and ERK do not appear to have an effect on TonE-mediated gene expression (reviewed in Ref. 47).

In the current experiments, we sought to determine upstream signaling molecules in the p38 kinase cascade that "drive" the expression of hypertonicity-induced genes (represented by the betaine transporter BGT1) and affect TonE-mediated gene expression in kidney cells. The activity of p38 kinase is dependent on MAPK kinases (MKKs) and their activators, the

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MAPK kinase kinases (MKKKs; see review in Ref. 52). MEK kinase 1 (MEKK1; 1 of the MKKKs) is linked to JNK activation, whereas MEKK2 is linked to JNK and ERK activation (reviewed in Ref. 27). On the other hand, MEKK3 may activate ERK, JNK, and p38, whereas MEKK4 may activate JNK and p38 kinase (27). Hence, we hypothesized that MEKK3 and/or MEKK4 are likely mediators of p38 kinase activation in kidney cells under hypertonic conditions. Our data are consistent with the existence of MEKK3 $\rightarrow \rightarrow \rightarrow$ p38 kinase input to drive TonE-mediated gene expression.

EXPERIMENTAL PROCEDURES

Constructs. For stable transfections, hemaglutinin antigen (HA)-tagged kinase active MEKK3 [HA-MEKK3-kA; corresponding to nt 1257–2200 of mouse MEKK3 (accession no. U43187); aa 309–622] and HA-tagged kinase active MEKK4 [HA-MEKK4-kA; corresponding to nt 3936–5153 of mouse MEKK4 (accession no. BC058719; aa 1302–1597)] were cloned in pcDNA3 (a generous gift from Dr. Gary Johnson, Univ. of Colorado, Denver, CO) (6, 16). For TonE-driven luciferase reporter assays, we used a 132-bp human aldose reductase promoter fragment (nt 2032–2163, accession no. AF032455) containing TonE (23), and its surrounding sequence (51) or an insert spanning 1.5 kb of the human *AR* gene (nt 1801–3300, accession no. AF032455), which includes the TonE and other identified elements of the AR promoter (23, 41, 45, 51) to drive the pGL3 basic vector (constructs were a generous gift from Dr. Kurt Bohren, Baylor College of Medicine).

Tissue culture and transfections. MDCK cells (ATCC, Manassas, VA), within *passages 63–68*, were grown to confluence in isotonic medium [315 mosmol/kgH_2O; made of equal volumes of DMEM, low glucose and Coon's-F12 media, supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 2 mM glutamine (GIBCO BRL)]. Hypertonic conditions were generated by the addition of NaCl to basic isotonic media, lacking serum, betaine, inositol, or choline (a precursor for the organic solute glycerophosphorylcholine), bringing the final osmolality to 565 mosmol/kgH_2O. Cells were exposed to hypertonic media for 16 h. In some experiments, we utilized 50 μM SB203580 or vehicle (equal volume of DMSO) for inhibition of the p38 kinase pathway. While lower concentrations of the inhibitor (5–10 μM) are usually sufficient for p38 kinase inhibition in most cells, MDCK cells require higher concentrations of the inhibitor for optimal p38 kinase inhibition, as we have shown previously (46). All cultures were maintained in 5% CO_2-95% air at 37°C.

For stable transfection of MEKK3 or MEKK4, cells (0.25×10^6) were seeded into 10-cm-diameter polystyrene plates and grown for 24 h. The cells were transfected with 8 μg DNA/dish using FuGENE 6 as per the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Cells were transferred to regular media 24 h later, and selection was initiated by the addition of G418 at a concentration of 1,000 μg/ml. Following colony selection, cells were maintained in media containing 500 μg/ml G418. Surviving colonies were propagated and tested for incorporation of the desired constructs by PCR amplification of the construct sequence from genomic DNA, using vector-specific primers (data not shown). Transfected cells appeared morphologically similar to empty vector-transfected controls and formed domes after reaching confluence, a typical feature of MDCK cells. To eliminate inherent bias related to the integration site of the plasmid, we used pooled stable transfectants for all experiments. These pooled colonies exhibited stable phenotype and demonstrated highly reproducible results, even after multiple passages.

For small-interference (si)RNA experiments, human embryonic kidney-derived cells (HEK-293) were transfected (using Dharma-*FECT*1; Dharmacon, Chicago, IL) with scrambled siRNA or SMARTpool MEKK3 siRNAs (total of 3; Dharmacon) for 80 h before experimental manipulation with hypertonicity and analysis of MEKK3 mRNA and protein abundance, BGT1 mRNA, p38 kinase, and phospho-p38 kinase. These cells were chosen for this experiment due to the ready availability of effective siRNAs for human MEKK3 sequences and the difficulty we encountered in establishing effective siRNAs for canine MEKK3. According to data sheets provided by the manufacturer, the scrambled siRNA (proprietary sequence, Dharmacon catalog no. D-001210–01-20) does not affect gene expression, as determined by gene array experiments. SMARTpool MEKK3 siRNAs were as follows: *1*) 5′-GAU AGA AGC UCA AGC AUG AUU-3′; *2*) 5′-AAA CUC AGC UUU AUG ACA AUU-3′; and *3*) 5′-CCA AGC AGG UCC AAU UUG AUU-3′.

Reporter gene assays. For reporter gene assays, cells were grown to 50% confluence in 10-cm-diameter polystyrene dishes and were transiently cotransfected with pRL-TK (*Renilla* luciferase) and either 132-bp TonE-pGL3 (firefly luciferase) or 1.5-kb AR promoter-driven pGL3 (41), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and subjected to isotonic or hypertonic medium for 16 h. Transfection efficiency was determined using green fluorescent protein-expressing plasmids and averaged 40–50%. After hypertonic stress, cells were harvested by scraping into 1 ml PBS, pH 7.3, collected by centrifugation, and lysed at 4°C in 1 ml Triton lysis buffer (TLB; see kinase assay). The lysate was cleared from debris by centrifugation for 10 min (microcentrifuge, 14,000 rpm, 4°C), and 20 μl of the supernatant were analyzed for dual firefly and *Renilla* luciferase activity (10), using a dual luciferase kit (Promega) and a TD-20/20 luminometer (Turner Design Instruments, Sunnyvale, CA). Firefly luciferase activity was normalized to *Renilla* luciferase activity in each sample, and each experiment was carried out in triplicate.

Northern blot analysis. MDCK cells, stably transfected with MEKK3-kA, MEKK4-kA, or empty vector were grown to confluence and exposed to isotonic or hypertonic medium for 16 h as described above. The cells were scraped and harvested by centrifugation (5,000 *g* for 5 min at 4°C). Total RNA was isolated from the cell pellet using RNAzol (Tel-test). Equal amounts of RNA per lane were loaded onto a 1% agarose-2.2 M formaldehyde gel. The gel was electrophoresed and transferred to GeneScreen membrane (New England Nuclear, Boston, MA). Canine BGT1 cDNA (a gift from Dr. Moo Kwon, Johns Hopkins School of Medicine) (54) and full-length human β-actin cDNA (Clontech) were labeled with [α-^{32}P]dCTP (Random Primed DNA Labeling Kit, Boehringer Mannheim) for use as probes. Probes were hybridized to the blots overnight at 42°C in a solution containing 40% formamide, 5× SSC (0.75M NaCl, 75 mM trisodium citrate, pH 7), 5× Denhardt's solution [0.5% (wt/vol) polyvinylpyrolidone, 0.5% (wt/vol) Ficoll 400], 0.5% SDS, 250 μg/ml salmon sperm DNA, 10 mM Tris, pH 7.5, and 10% dextran sulfate. The blots were washed under high stringency at 65°C as follows: twice in 3× SSC, 0.5% SDS for 30 min, and twice in 0.3× SSC, 0.5% SDS for 30 min. The blots were autoradiographed, and band intensities were quantitated using Image Tool software (University of Texas Health Science Center, San Antonio, TX). Relative band intensities were normalized to β-actin.

Real-time RT-PCR. Total RNA was isolated from the HEK-293 cell pellet using RNAzol (Tel-test), and real-time RT-PCR was performed to determine the abundance of MEKK3 and BGT1 mRNAs relative to GAPDH mRNA, essentially as was previously described (31). One-step RT was carried out using Maloney murine leukemia virus reverse transcriptase and oligo-dT to generate cDNAs (Invitrogen), followed by PCR using specific primers, SYBR Green PCR Reagents (Bio-Rad, Hercules, CA), and the Opticon DNA Engine (MJ Research, Watertown, MA) according to the manufacturers' instructions. Five micrograms of total RNA were reverse transcribed followed by PCR (denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min). Primers used in this study included MEKK3, forward 5′-GAT GGC AGA AGA ACA TTT-3′, reverse 5′-ACC CAT GTT CTC GCC ATT-3′; BGT1, forward 5′-AGC CAG TTT GTC TGT GTG GAG T-3′, reverse 5′-ACA GCA ATG GCA AGG ATG AGG A-3′; and GAPDH, forward 5′-CAA TGA CCC CTT CAT TGA

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CC-3′, reverse 5′-GTT CAC ACC CAT GAC GAA CAT G-3′. Reaction specificity was confirmed by electrophoretic analysis of products before real-time PCR, and bands of expected size were detected. Ratios for MEKK3/GAPDH and BGT1/GAPDH mRNAs were calculated for each sample and expressed as means ± SD.

Kinase assays. MAPK activity was determined as previously described (18, 39, 43) with slight modifications. The cells were exposed to isotonic or hypertonic medium for 16 h, scraped into the experimental medium, and harvested by centrifugation. The cell pellet was lysed in TLB, consisting of 20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 2 mM Na pyrophosphate, 10% glycerol, 1 mM PMSF, and 1 μg/ml leupeptin. The supernatant was collected by centrifugation at 15,000 *g* for 10 min. Anti-ERK1/2 (Upstate Biotechnology; the antibody cross reacts with ERK1 and ERK2), anti-MEKK3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MEKK4 (Santa Cruz Biotechnology), or anti-HA antibodies (Santa Cruz Biotechnology) were bound to protein A and G-agarose. Cell lysates (100 μg protein) were then added to the agarose beads, and the immobilized kinases were precipitated by the antibody-agarose complex. The beads were washed three times in TLB, followed by three additional washes in kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM DTT, 0.1 mM Na vanadate). Myelin basic protein (Sigma, St. Louis, MO) in kinase buffer containing 25 μM [γ-^{32}P]dATP was added to the beads and incubated for 20 min at 30°C. The kinase reaction was stopped by centrifugation at 12,000 *g* for 2 min. The supernatant was resolved on a 15% SDS-PAGE, and the gel was dried and autoradiographed. Kinase activity was determined by the extent of incorporation of ^{32}P into the myelin basic protein substrate. For JNK1, JNK2, p38α, or p38β assays, the above procedure was used except for the following: anti-p38α, anti-p38β, anti-JNK1 (Santa Cruz Biotechnology), or anti-JNK2 (StressGen, Victoria, AB) antibodies were used for immunoprecipitation, and Pansorbin (Calbiochem) was used to immobilize the antibodies; ATF-2 (Santa Cruz Biotechnology) was used as a substrate. The reaction supernatant was resolved on 10% SDS-PAGE. Each experiment was carried out in triplicate. Band intensities were quantitated using Image Tool software (University of Texas Health Science Center, San Antonio, TX), and statistical analysis was carried out using ANOVA.

SDS-PAGE. This method is based on Laemmli (28) with slight modifications. Briefly, equal amounts of protein were run on 12% reducing SDS-PAGE. Proteins were transferred overnight (4°C, 40 V) onto Hybond-ECL membrane (Amersham, Arlington Heights, IL), in Laemmli buffer (25 mM Tris, 52 mM glycine, pH 8.3). Blots were then blocked for 1 h at room temperature (RT) in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween 20) containing 5% dry milk. This was followed by overnight incubation at 4°C with anti-p38 kinase (at a dilution of 1:500; Santa Cruz Biotechnology), anti-phospho-p38 kinase (at a dilution of 1:200; Santa Cruz Biotechnology), or rabbit anti-p-ERK5 polyclonal antibody (anti-Thr218/Tyr220 at a dilution of 1:1,000; Cell Signaling Technology, Beverly, MA). Incubations were carried out in TBST containing 5% fraction V bovine serum albumin. After a 15-min wash in TBST, blots were incubated for 1 h (RT) with peroxidase-conjugated secondary antibody and diluted (1:2,000) in TBST containing 5% milk. Blots were then washed for 15 min in TBST, and protein bands were visualized using the ECL-Plus detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) as per the manufacturer's instructions.

For HA, MEKK3, and GAPDH detection, proteins were transferred overnight (4°C, 40 V) onto Hybond-ECL membrane in Laemmli buffer containing 10% methanol. Blots were blocked for 1 h at RT in PBST (50 mM $NaPO_4$, pH 7.5, 100 mM NaCl, 0.05% Tween 20) containing 5% dried milk. This was followed by 1-h incubation at RT with polyclonal anti-HA (at a dilution of 1:50; Santa Cruz Biotechnology), goat anti-MEKK3 (C-20; at a dilution of 1:500; Santa Cruz Biotechnology), or anti-GAPDH (at a dilution of 1:1,000; Santa Cruz Biotechnology) suspended in PBST containing 5% milk. After a 15-min wash in PBST, blots were incubated for 1 h (RT) with peroxidase-conjugated secondary antibody and diluted (1:1,000) in PBST containing 5% milk. Blots were then washed for 15 min in PBST and protein bands were visualized as discussed above.

Statistical analysis. Statistical analysis was carried out using Student's *t*-test and ANOVA. A *P* value of <0.05 was considered statistically significant.

RESULTS

MEKK3 (but not MEKK4) activation upregulates the expression of BGT1 mRNA under isotonic and hypertonic conditions, whereas siRNA-mediated MEKK3 depletion diminishes BGT1 mRNA expression. Expression of MEKK3 and MEKK4 constructs was verified by Western blotting using anti-HA antibodies (Fig. 1). To document upregulation of MEKK3- and MEKK4-specific kinase activities, we examined the in vitro phosphorylation of myelin basic protein by HA-MEKK3-kA and HA-MEKK4-kA, using anti-HA antibodies to pull down the respective kinases (Fig. 2). The activity of HA-MEKK3-kA, but not HA-MEKK4-kA, was slightly higher under hypertonic conditions compared with isotonic conditions, consistent with hypertonicity-mediated activation of MEKK3, but not MEKK4. Of interest, stimulation of native MEKK3 activity by hypertonicity was not detected. This observation may reflect negative autoregulation. The NH_2-terminal portions of MEKK3 contain PB1 domains, which function in an inhibitory capacity (42). Thus native MEKK3 activity 16 h into hypertonic stress may be attenuated, reflecting negative autoregulation; this autoregulation is absent in HA-MEKK3-kA as the NH_2-terminal portion containing the PB1 is not included in the construct, and hence, sustained activation of MEKK3 signaling is expected in MEKK3-kA-transfected cells. Similarly, native MEKK4 activity was not altered in HA-MEKK4-kA-transfected cells (data not shown; because the results in MEKK4-kA-transfected cells were negative, we focused our illustrations in Fig. 2 on the MEKK3-relevant data). To determine the effect of HA-MEKK3-kA and HA-MEKK4-kA on the expression of hypertonic stress-relevant genes, we examined mRNA abundance of betaine transporter BGT1 under isotonic and hypertonic conditions. MDCK cells, stably transfected with kinase-active MEKK3, but not kinase active MEKK4 or empty vector (pcDNA3.1), showed upregulation of BGT1 under isotonic (Fig. 3*A*) and hypertonic (Fig.

Fig. 1. Expression of hemaglutinin antigen (HA)-tagged MEK kinase 3 (MEKK3) and MEKK4. Pooled clones of Madin-Darby canine kidney (MDCK) cells, stably transfected with kinase-active HA-MEKK3-kA, HA-MEKK4-kA, or empty vector (pcDNA3.1), were lysed in Triton lysis buffer (TLB). Fifty micrograms of protein were run on SDS-PAGE, and blots were reacted with anti-HA antibodies. HA-tagged MEKK3-kA and MEKK4-kA protein fragments were detected at 43 and 41 kDa, respectively.

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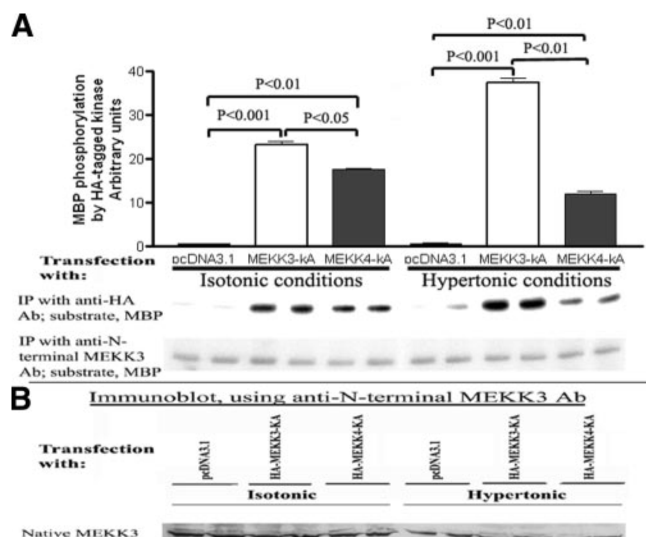

Fig. 2. *A*: assay of native MEKK3, MEKK3-kA, and MEKK4-kA activities. MDCK cells, stably transfected with kinase-active HA-MEKK3-kA, HA-MEKK4-kA, or empty vector (pcDNA3.1), were subjected to isotonic or hypertonic medium for 16 h, harvested, and lysed in TLB. Anti-HA antibodies were used to "pull down" the HA-tagged MEKK3-kA and MEKK4-kA. Anti-NH_2-terminal MEKK3 antibody was used to pull down native MEKK3. Kinase activity was determined using myelin basic protein (MBP) as the substrate. Bar graph shows kinase activity of HA-pulled down protein, and data represent 4 independent determinations (statistical analysis using Student's *t*-test and ANOVA). Representative blot is shown. *B*: native MEKK3 protein abundance is shown. Total cell lysates were resolved on a SDS-PAGE, and immunoblot was reacted with anti-NH_2-terminal MEKK3 antibody (Santa Cruz Biotechnology).

3*B*) conditions. While the activity of HA-MEKK4-kA was not as robust as that of HA-MEKK3-kA, we believe that MEKK4 activation was sufficient for detection of gene-target effects. We observed a small decrease in the BGT1 mRNA level in MEKK4-kA-transfected cells under hypertonic conditions compared with pcDNA3.1-transfected cells, suggesting negative input from MEKK4 to BGT1, or potential interference by MEKK4-kA in native MEKK3-mediated signaling; however, this observation requires further analysis before definite conclusions are drawn. Incubation of HA-MEKK3-kA-transfected cells with SB203580 (a highly-specific p38 kinase inhibitor) blocked the induction of BGT1 mRNA in MEKK3-kA-transfected cells (Fig. 3*C*). The data suggest that BGT1 is a downstream target for MEKK3 and that the effect of MEKK3 on BGT1 is p38 kinase mediated.

To confirm the involvement of MEKK3 in the expression of BGT1 under hypertonic conditions, we examined BGT1 expression in HEK-293 cells treated with MEKK3 siRNA. These cells were chosen for this experiment because of the ready availability of effective siRNAs for human MEKK3 (but not canine MEKK3) and the fact that HEK-293 cells (thought to be of distal nephron origin) are osmotically active and have been previously utilized to study hypertonicity responses (14, 29, 30). As shown in Fig. 4*A*, treatment of HEK-293 cells with pooled MEKK3 siRNAs provides a >95% reduction in MEKK3 mRNA and an ~70% reduction in MEKK3 protein level (Fig. 4*B*) compared with scrambled siRNA-treated cells. Scrambled siRNA had no effect on MEKK3 protein abundance compared with nontreated control (data not shown). MEKK3 siRNAs-treated cells demonstrated a 75% reduction in p38 kinase activity (phospho-p38α kinase; Fig. 4*B*) and diminished BGT1 mRNA expression under isotonic and hypertonic conditions compared with scrambled siRNA-treated cells (BGT1 mRNA was 15% of control under hypertonic conditions and 60% of control under isotonic conditions). These data confirm the involvement of MEKK3 in p38 kinase activation and BGT1 expression.

MEKK3-kA enhances the expression of TonE under isotonic and hypertonic conditions. The regulation of many genes by hypertonicity, including those that contribute to the accumulation of organic osmolytes in hypertonically stressed cells, is similar. Promoter analyses of *BGT1* (betaine transporter), *SMIT* (inositol transporter), taurine transporter, and *AR* suggest that the transcription of these genes is dependent on the

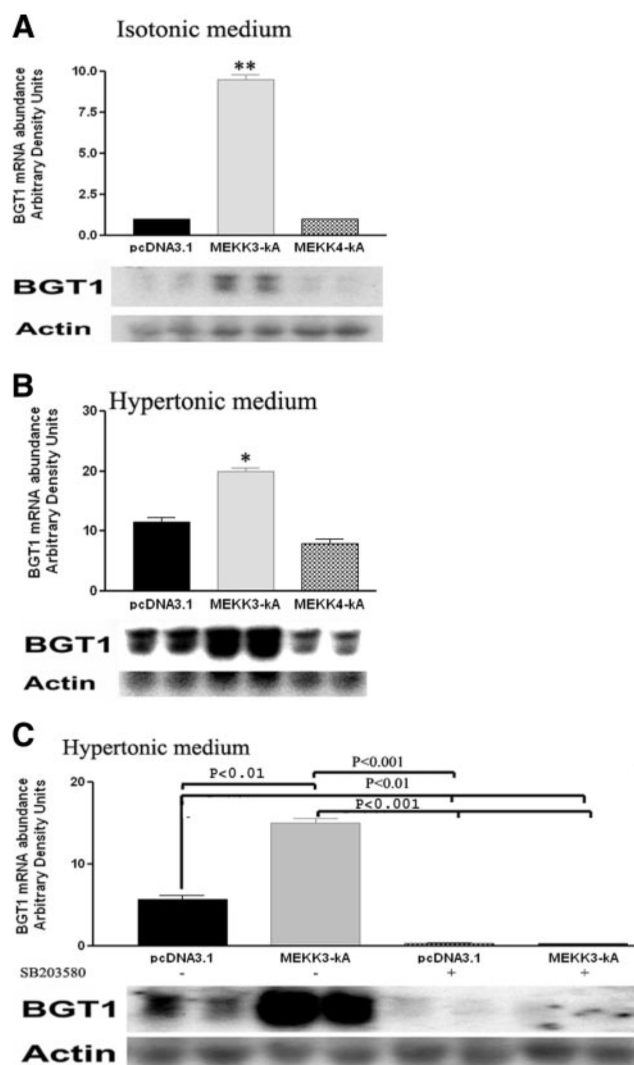

Fig. 3. MEKK3 (but not MEKK4) activation in MDCK cells upregulates the expression of betaine transporter (BGT1) mRNA under isotonic and hypertonic conditions. MDCK cells, stably transfected with MEKK3-kA, MEKK4-kA, or empty vector (pcDNA3.1), were treated for 16 h with isotonic (*A*), hypertonic (*B*), or hypertonic medium containing 50 μM p38 kinase inhibitor SB-203580 or vehicle (*C*), and were examined for BGT1 and β-actin mRNA abundance using Northern hybridization. Bar graph represents data from 4 independent determinations. Representative blots are shown. $*P < 0.01$, $**P < 0.001$: MEKK3-kA vs. pcDNA3.1 and/or MEKK4-kA using Student's *t*-test and ANOVA.

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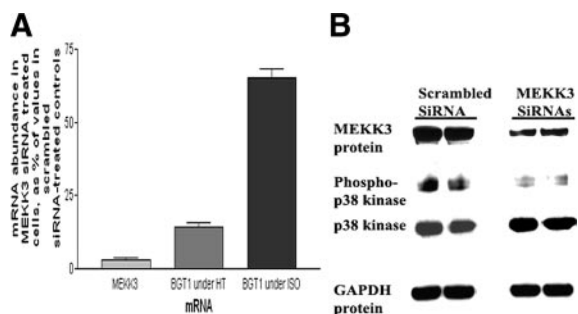

Fig. 4. Small-interference (si)RNA-mediated depletion of MEKK3 decreases p38 kinase activity and the expression of BGT1 mRNA. HEK 293 cells were treated with scrambled siRNA (SiControl) or smartPool MEKK3 siRNA for 80 h followed by incubation in isotonic or hypertonic medium for 16 h. The cells were then scraped, harvested by centrifugation, and subjected to RNAzol (for RNA extraction) or TLB (for protein analysis). *A*: bar graph shows mRNA abundance of MEKK3 and BGT1 (under isotonic and hypertonic conditions), measured using real-time RT-PCR in MEKK3 siRNA-treated cells, and expressed as percentage of values observed in scrambled siRNA-treated controls. Results were normalized to GAPDH and represent the means of 3 independent determinations. *B*: total native MEKK3 protein, p38 kinase, phospho-p38 kinase, and GAPDH protein abundance in cells treated with scrambled siRNA vs. cells treated with MEKK3 siRNAs.

interaction between the transcription factor TonEBP and TonE in the respective promoters. To elucidate the mechanism of BGT1 induction by MEKK3, we examined MDCK cells stably transfected with empty vector, MEKK3-kA, or MEKK4-kA for the expression of luciferase reporter constructs (transient transfection) driven by the 1.5-kb AR promoter or the AR promoter-derived 132-bp fragment containing TonE. TonE-driven luciferase reporter gene expression in MEKK3-kA-transfected cells was 1.5-fold higher under isotonic conditions and 7-fold higher under hypertonic conditions compared with empty vector-transfected control (Fig. 5*A*). Similarly, the expression of a luciferase reporter construct driven by the 1.5-kb AR promoter was 1.5-fold higher under isotonic conditions and 3-fold higher under hypertonic conditions in MEKK3-kA-transfected cells, compared with empty vector-transfected controls (Fig. 5*B*). However, the expression of both reporter constructs in MEKK4-kA-transfected cells was similar to that seen in empty vector-transfected controls. Our data are consistent with the existence of MEKK3-mediated input to TonE and thus suggest that TonEBP is subject to MEKK3 regulation.

Activation of ERK5 and p38α kinase in HA-MEKK3-kA-transfected cells. Unlike the Raf MKKKs, which do not normally activate JNK or p38 kinase, MEKKs are relatively promiscuous. Downstream input from MEKK3, for instance, has been shown to activate ERK, JNK, p38, and ERK5. Therefore, we examined the activities of these kinases in MEKK3-kA-transfected cells compared with empty vector-transfected controls. In MEKK3-kA-transfected cells, we found increased activity of p38α and ERK5, whereas that of ERK1/2 was decreased (under both isotonic and hypertonic conditions) (Fig. 6). The activities of p38β and JNK2 were not altered. Of interest, the activity of JNK1 was suppressed under hypertonic conditions independent of MEKK3 activation. The significance of this finding remains to be determined. We observed no change in protein level of p38α, ERK5, ERK1/2, or JNK1 (data not shown). The activities of p38γ and p38δ were not examined, as p38γ is expressed exclusively in muscle (32), whereas p38δ is expressed predominantly in the lungs and glomeruli (21). It should be emphasized that kinase activity in this experiment represents the steady-state condition after 16-h incubation in isotonic or hypertonic media, which is different from the response of these kinases to acute (minutes) hypertonic stress, where activation of all MAPKs normally occurs (20, 53). In summary, our data are consistent with an MEKK3-mediated input to drive ERK5 and p38 kinase.

DISCUSSION

These data on gene expression in MEKK3-transfected cells provide novel insight into the mechanism of p38 kinase activation in the context of hypertonicity. MDCK cells expressing MEKK3-kA show increased BGT1 mRNA and TonE-mediated reporter gene expression, a response correlated with upregulation of p38α and ERK5 and downregulation of ERK1/2. However, our data do not suggest a role for MEKK4 in the adaptive response of kidney cells to hypertonic stress. Thus induction of TonE-mediated gene expression in MDCK cells is consistent with an input from MEKK3 to p38 kinase or

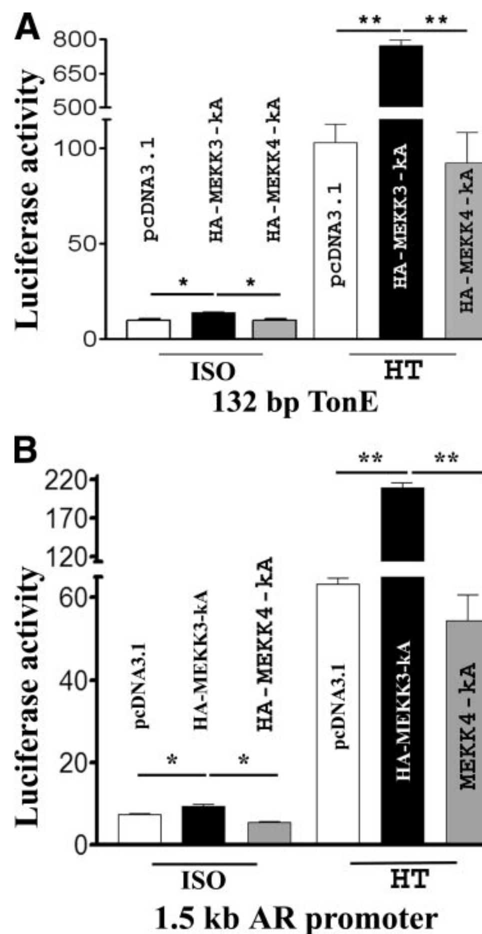

Fig. 5. Kinase active MEKK3 enhances tonicity enhancer (TonE)-mediated reporter gene expression under isotonic (ISO) and hypertonic (HT) conditions. MDCK cells, stably transfected with MEKK3-kA, MEKK4-kA, or empty vector (pcDNA3.1), were transiently cotransfected with pRL-TK plus 132-bp TonE-pGL3 (*A*) or pRL-TK plus 1.5-kb AR promoter-driven pGL3 (*B*) and subjected to isotonic or hypertonic conditions for 16 h. Cells were harvested, lysed in TLB, and the ratio of firefly to *Renilla* luciferase activity was determined. Results represent the means of 3 independent determinations. $*P < 0.05$, $**P < 0.001$, using Student's *t*-test and ANOVA.

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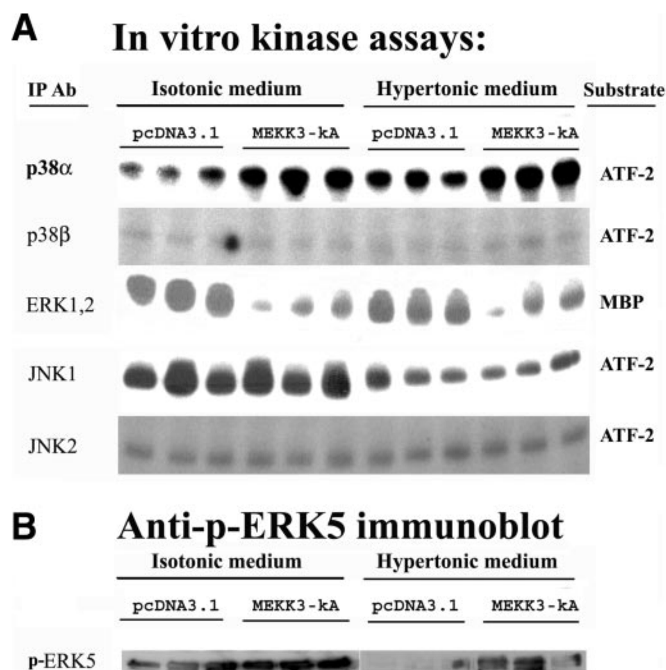

Fig. 6. MEKK3 enhances the activities of p38α and ERK5 and downregulates the activities of ERK1/2. MDCK cells, stably transfected with MEKK3-kA or empty vector (pcDNA3.1), were subjected to isotonic or hypertonic medium for 16 h and lysed using TLB. *A*: kinases were immunoprecipitated (IP) using specific antibodies (Ab), and kinase activity was determined in vitro. *B*: ERK5 activity was determined by immunoblotting, using anti-pERK5. Data represent 3 separate determinations for each treatment (triplicate). The differences between the activities of p38α kinase, ERK1/2, and ERK5 in MEKK3-kA-transfected cells compared with pcDNA3.1-transfected controls were statistically significant ($P < 0.01$, using Student's *t*-test and ANOVA).

MEKK3 to ERK5. Alternatively, it may result from downregulation of an inhibitory input from ERK1/2. However, addition of SB203580 (a specific inhibitor of p38 kinase) to the experimental media abolished the stimulatory effects of MEKK3 on BGT1 induction, suggesting that the effects of MEKK3 on BGT1 are p38 kinase mediated. Early studies suggested that ERK activity was not essential for transcriptional regulation of BGT1 and SMIT (26) or the stimulation of inositol uptake (5), and thus the observed differences in ERK activity in MEKK3-kA-transfected cells may not have a role in TonEBP/TonE regulation.

In yeast, cell survival in a hypertonic environment (7) and the induction of glycerol-3-phosphate dehydrogenase 1, which is responsible for the synthesis of glycerol in response to osmotic stress, are dependent on the MAPK HOG1, a homolog of p38 kinase (2, 7). Consistent with that, p38 kinase is required for induction of BGT1 mRNA as well as TonE-mediated gene expression under hypertonic conditions in mammalian cells (11, 22, 41, 46).

p38 Kinase is activated by the upstream MAPK kinases MKK3 and MKK6 (27). Both enzymes are highly selective for p38 and do not normally activate JNK or ERK (9, 12). They differ, however, in their substrate specificity with regard to p38 isoforms. Whereas MKK3 activates p38α and p38β preferentially, MKK6 can activate all known p38 isoforms (α, β, γ, and δ). Thus both MKK3 and MKK6 may be relevant to TonE-mediated gene expression, and whereas previous data employing MKK3 inhibition did not block TonE-mediated gene expression (25), the negative results may have been related to redundancy in the p38 kinase pathway at the MKK level (discussed in Ref. 46).

At the MKKK level, activation of p38 may be channeled through multiple protein kinase families including the MEKKs (27), Thousand And One kinases (17), apoptosis signal-regulating kinase-1 (18), and TGF-β-activating kinase-1 (40). However, this paper focuses on the involvement of MEKK3 and MEKK4 in the adaptive response of kidney cells to hypertonicity. The MEKKs are the most diverse of eukaryotic MKKKs, and except for the catalytic domain, which is conserved from yeast (*STE11*; which regulates mating pheromone and osmosensing pathways) to mammals, the proteins are dissimilar. Accordingly, these enzymes can catalyze different MAPK pathways and may interact with a wide array of regulatory proteins (27).

Our findings are in agreement with recent discoveries by Uhlik et al. (50) that suggest the existence of an osmotic stress-relevant actin-bound scaffold of proteins, which includes MEKK3, MKK3, and the Rac GTPase. This scaffold contains a newly discovered protein, osmosensing scaffold for MEKK3, which appears to be required for hypertonicity-mediated p38 kinase activation. Depletion of MEKK3 using RNA interference attenuated p38 kinase activation in hypertonically stressed cells. The authors concluded that the Rac-OSM-MEKK3-MKK3 complex is the mammalian counterpart of the osmosensing CDC42-STE50-STE11-PBS2 complex of *Saccharomyces cerevisiae* and is required for p38 kinase activation in response to hypertonicity in mammalian cells. The report by Uhlik et al., however, did not provide functional linkage between the protein scaffold and target gene expression in response to hypertonic stress, and thus our data support and complement the report by Uhlik et al. (50) and suggest signaling from MEKK3 → p38 → TonE in mammalian cells. Taken together, our data and those by Uhlik et al. suggest that signaling by hypertonicity is conserved through evolution from yeast to mammals, and as in yeast, mammalian cells may initiate hypertonic stress-induced signaling from the cell membrane. The "trigger" for activation of membrane-bound MEKK3 under hypertonic conditions and the identity of the "osmotic sensor" remain to be determined.

Last, whereas activation of ERK5 by MEKK3 is evident, the relevance of ERK5 activation to organic osmolyte gene expression remains to be determined. ERK5 is the only known substrate for MEK5 (the MKK in the ERK5 pathway) (8, 57), and activation of ERK5 through MEKK3 has been previously demonstrated (8). In addition, activation of ERK5 by hypertonicity is one of its characteristic features (1). Thus, whereas an input from MEKK3 → → ERK5 leading to TonE induction is possible, our current data indicate that the effects of MEKK3 on BGT1 are p38 kinase mediated. Further clarification of the involvement of ERK5 in the adaptive response of mammalian cells to hypertonicity awaits additional studies.

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