JNK is a volume-sensitive kinase that phosphorylates the Na-K-2Cl cotransporter in vitro

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Klein, Janet D., S. Todd Lamitina, and W. Charles O'Neill. JNK is a volume-sensitive kinase that phosphorylates the Na-K-2Cl cotransporter in vitro. *Am. J. Physiol.* 277 (*Cell Physiol.* 46): C425–C431, 1999.—Cell shrinkage phosphorylates and activates the Na-K-2Cl cotransporter (NKCC1), indicating the presence of a volume-sensitive protein kinase. To identify this kinase, extracts of normal and shrunken aortic endothelial cells were screened for phosphorylation of NKCC1 fusion proteins in an in-the-gel kinase assay. Hypertonic shrinkage activated a 46-kDa kinase that phosphorylated an NH_2-terminal fusion protein, with weaker phosphorylation of a COOH-terminal fusion protein. This cytosolic kinase was activated by both hypertonic and isosmotic shrinkage, indicating regulation by cell volume rather than osmolarity. Subsequent studies identified this kinase as c-Jun NH_2-terminal kinase (JNK). Immunoblotting revealed increased JNK activity in shrunken cells; there was volume-sensitive phosphorylation of NH_2-terminal c-Jun fusion protein; immunoprecipitation of JNK from shrunken cells but not normal cells phosphorylated NKCC1 in gel kinase assays; and treatment of cells with tumor necrosis factor, a known activator of JNK, mimicked the effect of hypertonicity. We conclude that JNK is a volume-sensitive kinase in endothelial cells that phosphorylates NKCC1 in vitro. This is the first demonstration of a volume-sensitive protein kinase capable of phosphorylating a volume-regulatory transporter.

cell volume; c-Jun NH_2-terminal kinase; stress-activated protein kinase; phosphorylation; endothelia

ACUTE REGULATION OF CELL VOLUME is accomplished through the activation of specific ion transporters, but the mechanism by which cell volume regulates these transporters remains a mystery. NKCC1, the ubiquitous "secretory" or "basolateral" isoform of the Na-K-2Cl cotransporter, is phosphorylated and activated by cell shrinkage. Protein phosphorylation has also been implicated in the activation by shrinkage of the Na/H antiporter (NHE1), another important volume-regulatory transporter, although phosphorylation of the transporter itself is not increased (5, 6). The K-Cl cotransporter, which is activated by cell swelling and regulates cell volume in the opposite direction, appears to be inhibited by phosphorylation. These data suggest that protein phosphorylation may play a central role in the sensing and regulation of cell volume.

It is of interest that several kinases exhibit activation by hyperosmolarity, most notably members of the mitogen-activated protein kinase (MAPK) family, including p42,44 MAPK (2, 10), p38 MAPK (19), and c-Jun NH_2-terminal kinase (JNK), also known as stress-activated protein kinase (4). However, it is not known whether activation of these serine/threonine kinases is due to increased osmolarity or to decreased cell volume. Recently, two members of the Src family of tyrosine kinases were shown to be activated specifically by shrinkage in neutrophils (14). None of these osmosensitive or volume-sensitive kinases have been implicated in the activation of volume-regulatory transporters. We have previously presented evidence that cell shrinkage activates myosin light chain kinase (MLCK), another serine/threonine kinase (12, 21). Although MLCK activation is required for activation of NKCC1 and NHE1 in shrunken cells, this does not occur through transporter phosphorylation. A specific volume-sensitive kinase that phosphorylates a volume-regulatory transporter has yet to be identified.

We have previously shown that endothelial cells cultured from bovine aortas exhibit abundant NKCC1 activity and rapid volume recovery after shrinkage (18). Cell shrinkage increases the phosphorylation of NKCC1 (16), and kinetic data suggest activation of a protein kinase rather than inhibition of a protein phosphatase (13) as the regulatory event. NKCC1 contains 12 putative membrane-spanning domains flanked by a 280-amino acid NH_2 terminus and a 400-amino acid COOH terminus, both of which appear to be cytoplasmic (23) and phosphorylated (15). Recent data point to regulatory phosphorylation of a specific threonine residue in the NH_2 terminus of the shark NKCC1 (1). To identify the volume-sensitive kinase that phosphorylates NKCC1, we developed an in vitro assay of NKCC1 phosphorylation within polyacrylamide gels of electrophoretically separated endothelial proteins, using both cytoplasmic domains of NKCC1 as substrates.

MATERIALS AND METHODS

Cell cultures and treatments. Endothelial cells were cultured from bovine aortas using DMEM containing 10% fetal bovine serum as previously described (11). All studies were performed on confluent cells in plastic culture flasks or multiwell plates, with fresh medium applied 12–24 h before experiments. For experiments, cells were incubated for 30 min in a HEPES-buffered, balanced salt solution with or without 150 mM sucrose or 2.8 nM human tumor necrosis factor-α (TNF-α).

Fusion proteins. Two overlapping cDNAs encompassing the full-length cDNA for human NKCC1 (TEF1–1 and TEF11a) were obtained from Dr. John Payne (Univ. of California, Davis, CA). TEF11a was excised with *Pst* I and cloned into the pQE-30 expression plasmid (Qiagen, Valencia, CA), yielding a hexahistidine protein that encodes the first 277 amino acids

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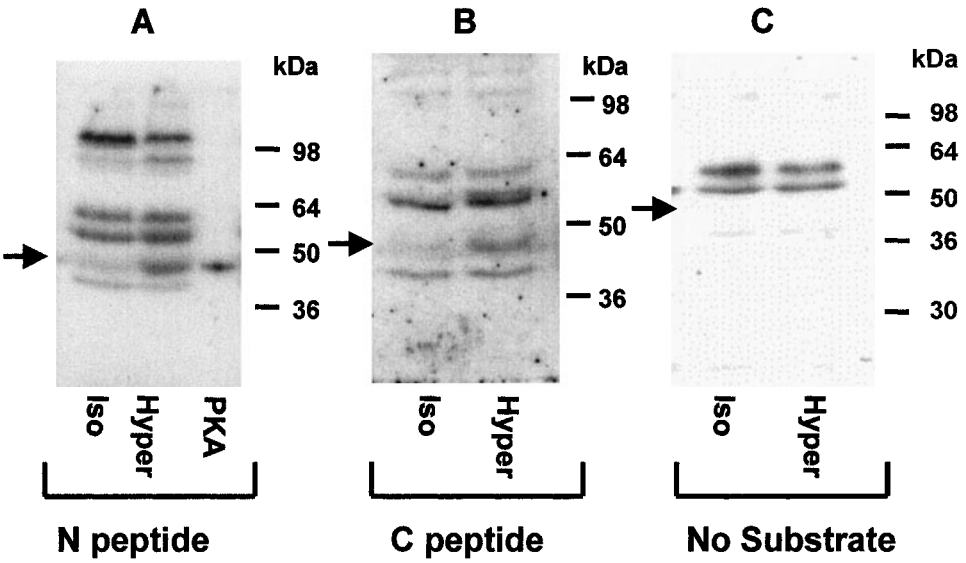

Fig. 1. Gel kinase assays of SDS extracts from control (Iso) and hypertonically shrunken (Hyper) cells and of protein kinase A (PKA) catalytic subunit, in gels containing NH_2-terminal NKCC1 fusion protein (*A*), COOH-terminal NKCC1 fusion protein (*B*), or no added protein (*C*). Exposure time for *C* was increased 3-fold to visualize the bands. Each autoradiogram is representative of at least 4 additional experiments.

of NKCC1. A 1.4-kb portion of cotransporter cDNA encoding most of the putative COOH-terminal cytoplasmic region including a unique *Bam*H I site was amplified from TEF1–1 by PCR, with a *Sal* I restriction site added to the 3′ end. This was then cloned into the pQE-30, yielding a protein that contains amino acids 758–1,157 (full-length NKCC1 is 1,212 amino acids). The cDNA for smooth muscle myosin light chain 2 from chicken gizzard (kindly provided by Dr. Paul Zavodny, Schering-Plough Pharmaceutical) was cloned into the expression plasmid as a 759-bp fragment containing the entire coding region. The pQE plasmid containing the sequence for dihydrofolate reductase was purchased from the vendor. The structures of all constructs were confirmed by sequencing. The resulting NH_2-terminal hexahistidine proteins were produced in bacterial strain M15[pRep4], extracted with guanidinium hydrochloride, and purified in a denatured form on an Ni-NTA-agarose column according to the vendor's instructions. A cDNA encoding a glutathione *S*-transferase fusion protein containing c-Jun_{1-135} was kindly provided by Dr. S. R. Price and transformed into *Escherichia coli* (DH5 strain). The expressed protein was purified on a hexylglutathione column (Sigma Chemical, St. Louis, MO) and eluted with glutathione.

Kinase assay. Medium was removed and cells were extracted with 2% SDS and 1% β-mercaptoethanol. After solubilization by scraping and shearing through a 27-gauge needle, the samples were boiled for 1 min and then electrophoresed into a 10% polyacrylamide gel containing 1 mg/ml fusion protein. Kinase activity was then determined within the gel as described by Hutchcroft et al. (9) with minor modifications. Briefly, gels were washed free of SDS with 40 mM HEPES, pH 7.5, with exchanges every 30 min for ~4 h. Gels were then incubated with phosphorylation buffer (10 mM $MgCl_2$, 0.1 μM cold ATP, and [γ-^{32}P]ATP at 5 μCi/ml and 25 mM HEPES, pH 7.5) for 4 h with gentle agitation. Unincorporated ATP was removed by repetitive 30-min washes with 40 mM HEPES, pH 7.5, 1% $Na_4P_2O_7$ over 4–6 h. After fixation in 50% methanol and 10% acetic acid, gels were reequilibrated in water, dried, and exposed to X-ray film.

Immunoprecipitations. Endothelial cells were solubilized in RIPA buffer (10 mM Tris, pH 7.4, 2.5 mM EDTA, 50 mM NaF, 1 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 1 μg/ml aprotinin, 0.18 mg/ml phenylmethylsulfonyl fluoride, 0.18 mg/ml orthovanadate) and centrifuged at 4,000 *g*, and the supernatant fractions were

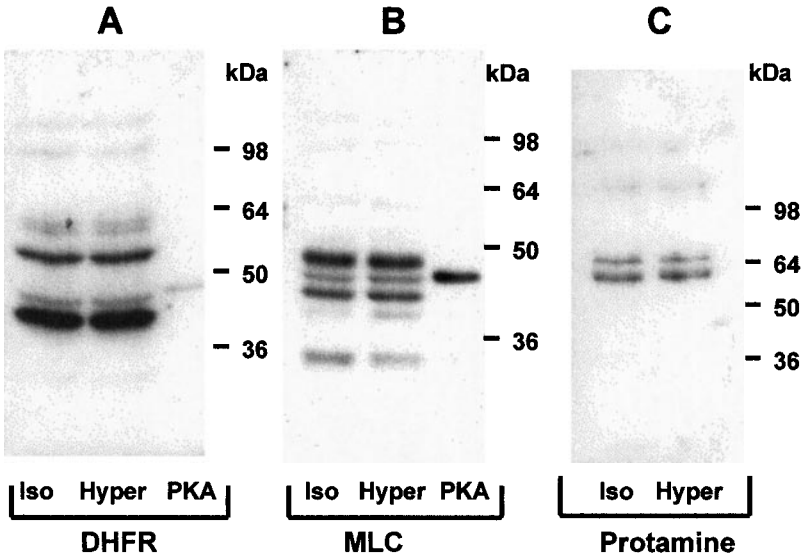

Fig. 2. Gel kinase assays of extracts from control (Iso) and hypertonically shrunken (Hyper) cells using substrates unrelated to NKCC1. *A*: hexahistidine fusion protein containing dihydrofolate reductase (DHFR). *B*: hexahistidine fusion protein containing myosin light chain (MLC). *C*: protamine at 1 mg/ml. PKA, catalytic subunit of protein kinase A. Each autoradiogram is representative of at least 1 additional experiment. Concentration of each substrate was 1 mg/ml.

incubated with 10 μg of anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, KY) or 20 μg of anti-JNK bound to agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Beads were washed three times with RIPA buffer, SDS-PAGE sample buffer was added, and the samples were boiled for 1 min. Proteins were separated on 10% gels containing NKCC1 NH_2-terminal fusion protein and assayed in the gel for kinase activity.

Western blots. Samples were prepared as above, followed by separation on a 10% polyacrylamide gel and electroblotting onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) and incubated overnight with anti-ERK1/2 (Transduction Laboratories) or anti-activated JNK (Promega, Madison, WI). After the membrane was washed with TBS containing 0.5% Tween 20, it was incubated with horseradish peroxidase-linked donkey anti-rabbit antibody for 2 h. Proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

RESULTS

Extracts of untreated endothelial cells exhibited several bands when polyacrylamide gels containing either NKCC1 fusion protein were exposed to [^{32}P]ATP (Fig. 1, *A* and *B*, *left lanes*). The catalytic subunit of protein kinase A (PKA), which phosphorylates many proteins, was also electrophoresed into the gel as a positive control, resulting in a single band at the appropriate size (Fig. 1*A*, *right lane*). This phosphorylation by PKA is probably not physiologically relevant, since a relatively large amount of PKA was used and since no region of NKCC1 matches the consensus motif for PKA phosphorylation (15). One kinase in the cell extracts, migrating slightly higher than the PKA catalytic subunit at ~46 kDa, was consistently activated in hypertonic medium and phosphorylated both the NH_2-terminal fusion protein (Fig. 1*A*, *middle lane*) and COOH-terminal fusion protein (Fig. 1*B*, *right lane*). This band was always more prominent with the NH_2-terminal NKCC1 fusion protein, suggesting that it is a better substrate. Figure 1*A* also shows a faint, hypertonically activated kinase at ~90 kDa. This kinase was only occasionally seen in other assays. When fusion protein was omitted from the gel, bands were observed only when exposure time was substantially increased (Fig. 1*C*). None of these bands were volume sensitive, and they most likely represent autophosphorylating kinases. Kinase assays were performed with other, unrelated proteins as substrates to determine the specificity of this kinase and to confirm that phosphorylation was occurring on the fusion proteins and not on a

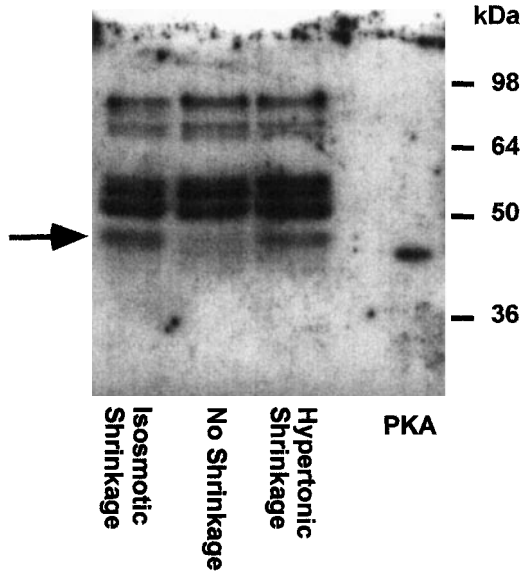

Fig. 3. Comparison of isosmotic and hypertonic shrinkage. In-the-gel kinase assay with NH_2-terminal NKCC1 fusion protein as substrate, using extracts from isosmotically shrunken cells (15-min incubation in isosmotic Na-free, K-free medium), control cells (no shrinkage), and hypertonically shrunken cells (150 mM sucrose for 15 min). PKA, catalytic subunit of protein kinase A. Arrow indicates location of volume-sensitive kinase. Identical results were obtained in 2 additional experiments.

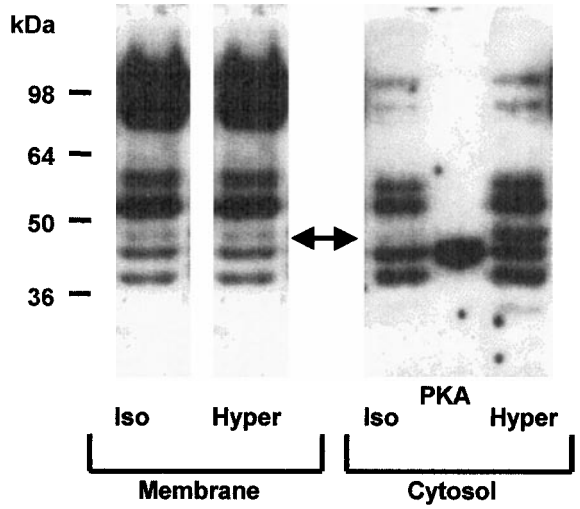

Fig. 4. Cellular localization of NKCC1 kinase. In-the-gel kinase assay of cytosol or membranes from normal (Iso) or hypertonically shrunken (Hyper) cells using NH_2-terminal NKCC1 fusion protein as substrate. Approximately 15% of membrane fraction and 2% of cytosol were loaded on the gel. PKA, catalytic subunit of protein kinase A. Identical results were obtained in 1 additional experiment. Arrow indicates location of volume-sensitive kinase.

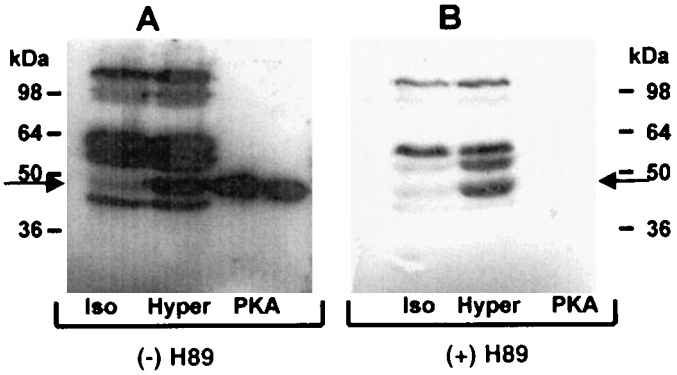

Fig. 5. Effect of PKA inhibition in vitro on phosphorylation of NH_2-terminal NKCC1 fusion protein. Duplicate samples of extracts from normal (Iso) and hypertonically shrunken (Hyper) cells and of PKA catalytic subunit were electrophoresed into a gel containing NH_2-terminal NKCC1 fusion protein. The gel was then divided and each half was incubated with [^{32}P]ATP in absence (*A*) or presence (*B*) of 10 μM H-89. PKA, catalytic subunit of protein kinase A. Arrows indicate location of volume-sensitive kinase. Results are representative of 2 additional experiments.

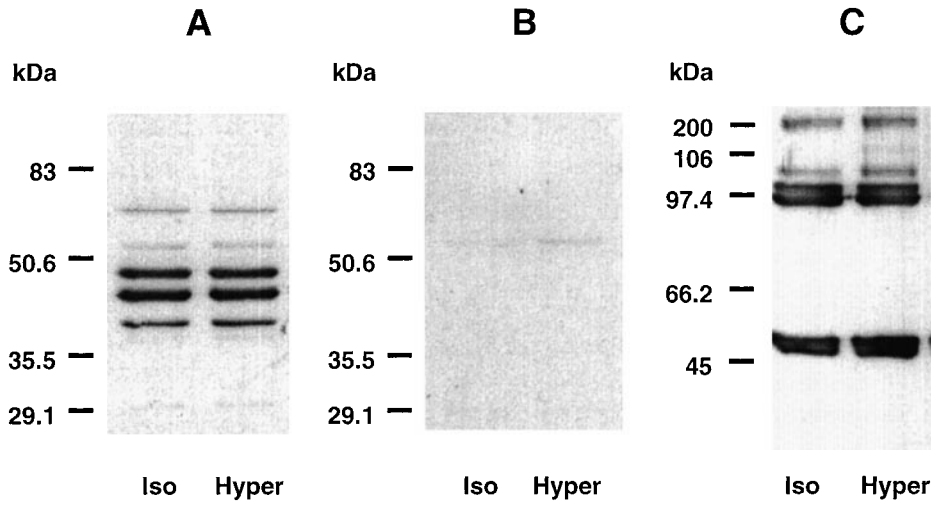

Fig. 6. Effect of cell volume on tyrosine phosphorylation of p42,44 mitogen-activated protein kinase. Cells were exposed to isotonic (290 mosM) or hypertonic (440 mosM) media for 20 min before extraction for immunoprecipitation and/or immunoblotting with anti-phosphotyrosine antibody or anti-ERK1/2 antibody as described in MATERIALS AND METHODS. *A*: cell extracts probed with anti-ERK1/2 antibody. *B*: anti-phosphotyrosine immunoprecipitates of same extracts probed with anti-ERK1/2 antibody. *C*: same anti-phosphotyrosine immunoprecipitates probed with anti-phosphotyrosine antibody (7.5% acrylamide vs. 10% acrylamide in the previous blots).

contaminating bacterial protein. There was no hypertonically induced phosphorylation of fusion proteins containing dihydrofolate reductase or myosin light chain (prepared using the same expression and purification procedure) or of protamine (Fig. 2). With myosin light chain as substrate, a faint band is observed at ~40 kDa but was not reproducible. Although we have previously shown that cell shrinkage activates MLCK, we would not expect to see this in the gel assays, since MLCK requires calmodulin as a cofactor. There was no volume-sensitive phosphorylation of histone II AS or casein (not shown).

NKCC1 is activated by isosmotic shrinkage as well as hypertonic shrinkage, indicating regulation by cell volume rather than osmolarity (18). To determine whether the NKCC1 kinase is similarly regulated, cells were shrunk isosmotically by incubation in isosmotic Na-free, K-free solution containing *N*-methyl-D-glucamine for 15 min as previously described (18). This reduces endothelial cell volume by ~30% and produces an activation of NKCC1 similar to that seen with the addition of 150 mM sucrose (18). As shown in Fig. 3, activation of the NKCC1 kinase is similar with the two types of shrinkage, indicating that it is cell volume and not osmolarity that activates the kinase. To determine the intracellular location of the volume-sensitive kinase, endothelial monolayers were scraped into suspension and sonicated, and fractions were separated by low-speed centrifugation followed by ultracentrifugation of the supernatant (100,000 *g* for 60 min). The resulting supernatant (cytosol) was concentrated by centrifugation through a 10,000-mol-wt cutoff membrane and subjected to SDS-PAGE along with the ultracentrifugation pellet (membranes). The resulting kinase assay (Fig. 4) demonstrates that the volume-sensitive NKCC1 kinase is present exclusively in cytosol.

The apparent molecular weight of the volume-sensitive kinase suggested several candidate kinases that were investigated in subsequent studies. To determine whether volume-sensitive phosphorylation was mediated by PKA, duplicate samples were electrophoresed into a gel containing NKCC1 NH_2-terminal fusion protein, which was then divided so that the gel kinase assay could be performed in the absence (Fig. 5*A*) and presence (Fig. 5*B*) of H-89, an inhibitor of PKA. The intensity of several bands was reduced and the PKA band was completely eliminated by H-89. However, the 46-kDa, volume-sensitive band persisted despite complete inhibition of PKA, indicating that PKA was not the volume-sensitive kinase. In the presence of H-89, another volume-sensitive kinase is apparent at ~55 kDa. Despite the fact that the p42,44 MAPKs (ERK1 and ERK2) are reported to be activated by hypertonicity (2, 10), there was no evidence of this in endothelial cells. Neither ERK1 nor ERK2 was precipitated by anti-phosphotyrosine antibodies in shrunken cells, despite the fact that both kinases were present in cell extracts (Fig. 6). Furthermore, pretreatment of cells with PD-98059, which blocks activation of p42,44 MAPK or with tyrphostin A23, an inhibitor of tyrosine kinases, did not prevent activation of the volume-sensitive kinase and did not block the stimulation of

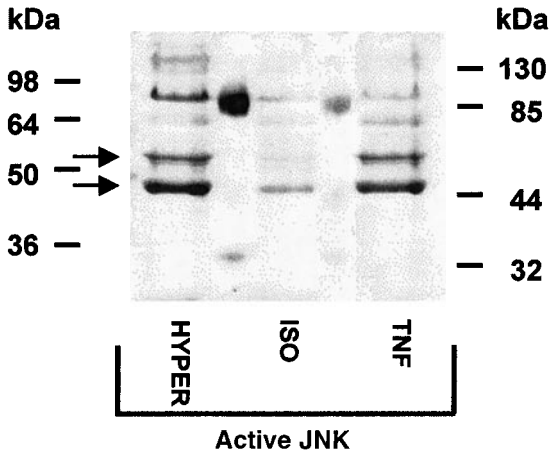

Fig. 7. Immunoblot of endothelial cell extracts separated by SDS-PAGE, using antibody against activated c-Jun NH_2-terminal kinase (JNK). Hyper, cells treated with hypertonic medium for 30 min; Iso, isotonic control; TNF, cells treated with 2.8 nM tumor necrosis factor-α for 30 min. Arrows indicate 46- and 55-kDa forms of JNK. Similar results were obtained in 1 additional experiment.

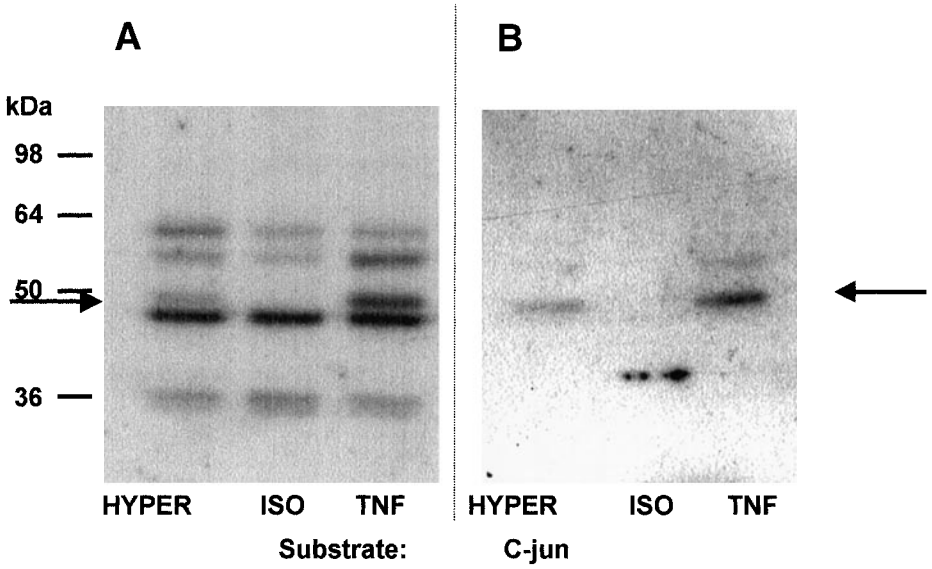

Fig. 8. Volume-sensitive phosphorylation of c-Jun by JNK. In-the-gel kinase assays of extracts (*A*) and JNK1 immunoprecipitates (*B*) from hypertonically shrunken (Hyper), normal (Iso), and TNF-treated (TNF) cells, using c-Jun_{1-135} glutathione *S*-transferase fusion protein as substrate. Treatment with hypertonic medium or TNF (2.8 nM) was 30 min. Arrows indicate location of 46-kDa form of JNK. Identical results were obtained in 1 additional experiment.

bumetanide-sensitive K^+ influx by cell shrinkage (data not shown).

Another member of the MAPK family that is activated by hyperosmolarity in other cells is JNK, also known as stress-activated protein kinase (4). Immunoblotting of cell extracts with an antibody specific for the activated (phosphorylated) form of JNK revealed that this kinase is activated by hyperosmolarity in endothelial cells as well (Fig. 7). This blot demonstrates activation of both the 46-kDa (JNK1) and 55-kDa (JNK2) forms of JNK. The identity of the band at ~90 kDa is unknown. Activation of JNK was confirmed by showing volume-sensitive phosphorylation of c-Jun NH_2-terminal fusion protein at 46 kDa, both by cell extracts and by JNK immunoprecipitates (Fig. 8). Treatment of cells with TNF, which activates JNK in endothelial cells (17), mimicked the effect of shrinkage on c-Jun phosphorylation. The additional, weaker band at 55 kDa in the immunoprecipitates is consistent with the larger form of JNK (7) as observed with Western blotting (Fig. 7). Identical results were obtained with NKCC1 fusion protein as substrate (Fig. 9), demonstrating that JNK is activated in shrunken cells, that activation of JNK by TNF mimics the effect of cell shrinkage, and that JNK can phosphorylate NKCC1 in vitro.

DISCUSSION

Based on phosphorylation of the volume-regulatory Na-K-2Cl cotransporter in vitro, we have detected a 46-kDa, cytosolic protein kinase that is regulated by cell volume. This is the first demonstration of a volume-sensitive kinase that phosphorylates a relevant target protein. Several findings indicate that this kinase is the stress-activated protein kinase JNK. The apparent molecular weight corresponds to that of the smaller form of JNK, and the larger volume-sensitive kinase occasionally observed corresponds to the larger form of JNK (7). The NH_2 terminus of c-Jun is a substrate for the volume-sensitive kinase, and TNF, which activates JNK in aortic endothelial cells (17), also activates a 46-kDa kinase that phosphorylates NKCC1. Last, JNK was activated in shrunken cells, and immunoprecipitates of JNK demonstrated volume-sensitive phosphorylation of NKCC1 at 46 kDa. A volume-sensitive kinase was occasionally observed at ~90 kDa, the identity of which is unknown. Because antibodies against activated JNK also recognized a protein of this size in extracts from shrunken cells, this kinase may be related to JNK and possibly could represent a dimer. Several other kinases phosphorylated NKCC1 in vitro but were not influenced by cell volume.

Both JNK1 and JNK2 are known to be activated by hypertonicity (4, 8) and appear important for osmotic tolerance. Specifically, JNK1 rescues a mutant yeast with defective growth in hyperosmolar medium (4), and inhibition of JNK2 blocks osmotic tolerance of cells cultured from inner medullary collecting ducts (22). However, previous studies have not determined whether activation by JNK is a result of hypertonicity or cell

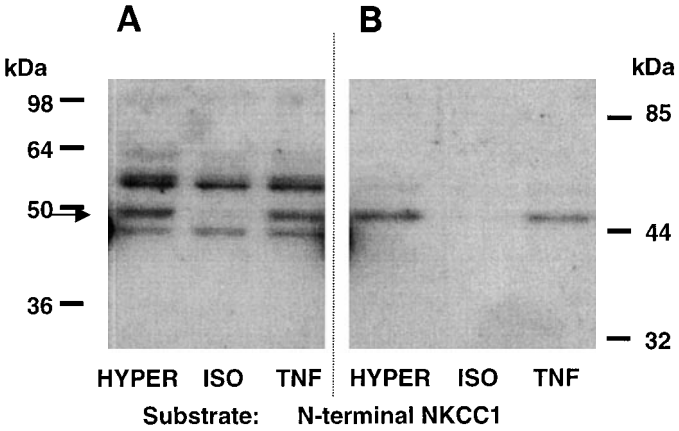

Fig. 9. Volume-sensitive phosphorylation of NKCC1 by JNK. In-the-gel kinase assays of extracts (*A*) and JNK1 immunoprecipitates (*B*) from hypertonically shrunken (Hyper), normal (Iso), and TNF-treated (TNF) cells, using NH_2-terminal NKCC1 fusion protein as substrate. Treatment with hypertonic medium or TNF (2.8 nM) was 30 min. Identical results were obtained in 1 additional experiment.

shrinkage. Our results provide the first demonstration that JNK is activated by cell shrinkage in the absence of hyperosmolarity or increased intracellular ionic strength. This explains why JNK is not activated by the permeant solute urea (24). Hyperosmolar activation of other members of the MAPK family has also been observed (10, 20), but whether this is a response to cell shrinkage and has a functional role is unknown. No activation of ERK1 or ERK 2 was detected in hypertonically shrunken endothelial cells. Another family of kinases, the Src tyrosine kinases, have been implicated in neutrophils, where $p59^{fgr}$ and $p56/59^{hck}$ are phosphorylated and activated both by hypertonic and isosmotic shrinkage (14). Tyrosine kinase inhibition prevented phosphorylation and activation of these kinases and blocked volume-sensitive activation of NHE1, but a specific role for these kinases has not been demonstrated. No hypertonic activation of ERK1 or ERK2 was observed in these cells as well.

The results of this study demonstrate that JNK is capable of phosphorylating NKCC1 in vitro, but this does not necessarily indicate that JNK phosphorylates NKCC1 in vivo. However, several findings are consistent with in vivo phosphorylation. The kinase was activated by both hypertonic and isosmotic shrinkage and did not phosphorylate a variety of other proteins, indicating some degree of specificity for NKCC1. No other volume-sensitive kinases capable of phosphorylating NKCC1 were consistently detected, although we cannot rule out other kinases that do not survive the extraction procedure. Both the NH_2-terminal and COOH-terminal putative cytoplasmic domains are phosphorylated, which is consistent with recent phosphopeptide analysis of endogenously phosphorylated NKCC1 from duck red blood cells (15). Although it is not possible to quantitate phosphorylation in our assay, volume-sensitive phosphorylation was more robust with the NH_2-terminal fusion protein as substrate, suggesting that it is preferentially phosphorylated. This would be consistent with the recent demonstration that activation of shark NKCC1 requires phosphorylation on the NH_2 terminus (1).

The mechanism by which cell shrinkage activates JNK is unknown. The fact that activation survives denaturing conditions indicates covalent, rather than allosteric, modification, which is consistent with the activation of JNK by phosphorylation (3). This indicates the existence of a kinase cascade whereby JNK, which may not be inherently volume sensitive, is phosphorylated and activated by an upstream, volume-sensitive protein kinase. Candidates include several kinases that are known to phosphorylate and activate JNK (3). This study thus provides evidence for a volume-sensitive protein kinase cascade that results in activation of JNK and subsequent phosphorylation of the Na-K-2Cl cotransporter. Although further studies are needed to demonstrate that JNK phosphorylates NKCC1 in vivo, this is the first demonstration of a volume-sensitive protein kinase that can phosphorylate a volume-regulating target. This kinase cascade may play an important role in the regulation of cell volume and provide clues about initial volume-sensing mechanisms. Because JNK is activated by other stresses, the results suggest an important link between cellular stress responses and the regulation of cell volume.

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