Hyperosmotic Stress Stimulates Promoter Activity and Regulates Cellular Utilization of the Serum- and Glucocorticoid-inducible Protein Kinase (Sgk) by a p38 MAPK-dependent Pathway*

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We have established that the serum- and glucocorticoid-inducible protein kinase (Sgk) is a new component of the hyperosmotic stress response. Treatment of NMuMG mammary epithelial cells with the organic osmolyte, sorbitol, caused the stable accumulation of Sgk transcripts and protein after an approximately 4-h lag. Transient transfection of a series of sgk-CAT reporter plasmids containing either 5′ deletions or continuous 6-base pair substitutions identified a hyperosmotic stress-regulated element that is GC-rich and is necessary for the sorbitol stimulation of sgk gene promoter activity. Gel shift analysis identified four major DNA-protein complexes in the hyperosmotic stress-regulated element that, by competition with excess consensus wild type and mutant oligonucleotides and by antibody supershifts, contains the Sp1 transcription factor. Several lines of evidence suggest that the p38 MAPK signaling pathway mediates the hyperosmotic stress stimulation of sgk gene expression. Treatment with pharmacological inhibitors of p38 MAPK or with a dominant negative form of MKK3, an upstream regulator of p38 MAPK, significantly reduced or ablated the sorbitol induction of sgk promoter activity or protein production. Using an in vitro peptide transphosphorylation assay, sorbitol treatment activates either endogenous or exogenous Sgk that is localized to the cytoplasmic compartment. Thus, we propose that the stimulated expression of enzymatically active Sgk after sorbitol treatment is a newly defined component of the p38 MAPK-mediated response to hyperosmotic stress.

The extracellular milieu is highly dynamic, and cells adapt to changes in their environment by altering their patterns of gene transcription and protein modification and their cytoskeletal structure. The ability of a cell to sense and appropriately respond to adverse conditions is determined by an integrated signal transduction pathways that trigger adaptive and survival responses or mediate events leading to cell death (1–3). Environmental stresses as divergent as osmotic shifts, contains the Sp1 transcription factor. Several changes in their environment are detected by two different receptors, the histidine kinase receptor Shl1 and the four-pass transmembrane receptor Sho1p. The activation of kinase cascades by these receptors leads to the phosphorylation and activation of the HOG1 proline-directed protein kinase (4, 5). The HOG1 gene is necessary for the maintenance of osmotic gradients in hyperosmolar environments and allows the proliferation of yeast under osmotic stress (6). HOG1 has been shown to phosphorylate various members of the Msn family of transcription factors, resulting in their binding to and transactivation of stress-responsive elements located in the upstream promoter regions of osmotically regulated genes (7–10). One class of osmoregulatory genes targeted by HOG1 encode proteins that have osmoprotective functions, such as the glycerol-3-phosphate dehydrogenase gene that is responsible for the accumulation of the yeast osmolyte, glycerol (10, 11).

In mammalian cells, however, much less is known about the signal transduction pathways that control gene transcription in response to environmental insults. The mammalian homologue of the yeast HOG1 is p38 MAPK, which is a member of the mitogen-activated protein kinase (MAPK) family of serine/threonine protein kinases, has been shown to play a role in transducing stress signals in a variety of cell systems (1, 12–14). Activation of p38 MAPK results in the phosphorylation of a variety of targets, a subset of which regulates transcription of stimulus-specific genes (15). For example, the p38 MAPK phosphorylation of the ATF2, CHOP, Elk1, MEF2C, and SAP1 transcription factors has been shown to induce their DNA binding and transactivation activity (16–19). A number of protein kinases (such as MAPKAP kinase-2, MAPKAP kinase-3, Mnk-1, PRAK, and BSK-B) have also been shown to be targets of p38 MAPK. These studies suggest the existence of additional hitherto unidentified downstream targets of p38 MAPK signaling that may be responsible for mediating transcription of mammalian osmoregulatory genes (20–25). Although, in some cases, p38 MAPK-initiated phosphorylation events can trigger intracellular protein kinase cascades that are generally conserved between metazoans and mammals, culminating in the transcriptional control of specific sets of target genes. The best characterized osmoregulatory signaling pathways have been described in Saccharomyces cerevisiae, in which solute imbalances in the environment are detected by two different receptors, the histidine kinase receptor Shl1 and the four-pass transmembrane receptor Sho1p. The activation of kinase cascades by these receptors leads to the phosphorylation and activation of the HOG1 proline-directed protein kinase (4, 5). The HOG1 gene is necessary for the maintenance of osmotic gradients in hyperosmolar environments and allows the proliferation of yeast under osmotic stress (6). HOG1 has been shown to phosphorylate various members of the Msn family of transcription factors, resulting in their binding to and transactivation of stress-responsive elements located in the upstream promoter regions of osmotically regulated genes (7–10). One class of osmoregulatory genes targeted by HOG1 encode proteins that have osmoprotective functions, such as the glycerol-3-phosphate dehydrogenase gene that is responsible for the accumulation of the yeast osmolyte, glycerol (10, 11).

The abbreviations used are: HOG1, hyperosmolarity glycerol; PI, phosphatidylinositol; PDK1, 3-phosphoinositide-dependent protein kinase-1; PB, phosphate buffered saline, p38 MAPK, 38-kDa mitogen-activated protein kinase; Akt/ PKB, Akt/protein kinase B; Sp1, SV40 early promoter transcription factor-1; EGFl, early growth response gene-1; MKK3, mitogen-activated protein kinase/extracellular signal-regulated protein kinase gene-3; CAT, chloramphenicol acetyltransferase; HA, hemagglutinin; kb, kilobase pair(s); bp, base pair(s).
programmed cell death (26, 27), the functional connections between the immediate targets of p38 MAPK, its downstream effectors, and the cellular response to hypertorsmic stress in mammalian cells have yet to be elucidated.

Conceivably, important downstream targets of the p38 MAPK stress cascade may themselves be cell-signaling molecules that help trigger and mediate the selectivity of the stress response to environmental cues. We have reported the isolation and characterization of a serine/threonine-serum- and glucocorticoid-inducible protein kinase gene, sgk, by subtractive cloning from a mammary tumor cell cDNA library that is under acute transcriptional control by both serum and glucocorticoids (28, 29). sgk is approximately 45–55% homologous to Akt/ PKB, protein kinase A, protein kinase C, and the rat p70S6K/p85S6K. In serum-treated cells, Sgk is phosphorylated and shuttles between the nucleus and cytoplasm in a cell cycle-dependent manner (30). We have recently shown that Sgk enzymatic activity, phosphorylation and subcellular localization is regulated by PI-3 kinase signaling through PKD1, suggesting a possible role for Sgk in a cell survival pathway (31). Moreover, the physiological stress hormones, glucocorticoids, stimulate sgk promoter activity through a glucocorticoid response element, and sgk is a transcriptional target of the p53 tumor suppressor gene, a known target of genotoxic stress (28, 32). Consistent with a role for Sgk in the cellular stress response, sgk transcripts have been shown to be elevated in response to ischemic injury of the brain, changes in cell volume, and inflammatory disease and in a screen for transcripts involved in wound repair in fibroblasts (33–36). Therefore, to determine whether transcription of the sgk gene is a component of the stress response in mammalian cells, the hypersonal regulation of Sgk was examined in mammary epithelial cells. We demonstrate that an increase in extracellular solute levels stimulates sgk promoter activity and gene expression through the p38 MAPK-dependent pathway.

**EXPERIMENTAL PROCEDURES**

**Cells and Materials—**NMuMg nontransformed mouse mammary epithelial cells were obtained from the National Institutes of Health, Bethesda, Maryland, or derived as described previously from mammary glands of Sprague-Dawley rats. Mammalian cell culture was performed in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10 µg/ml insulin, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cells were propagated at 37 °C in humidified air containing 5% CO₂. Cells were propagated at 37 °C in humidified air containing 5% CO₂.

**Plasmid Constructions**—The construction of the −4.0 sgk-CAT plasmid, which contains sgk promoter sequences (−3560 to +51) cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in the vector pBLCAT3, has been described previously (29). The various sgk promoter-CAT deletions down to −236 sgk CAT were generated utilizing the Erase-a-Base system (Promega, Madison, WI) and are further described elsewhere (32). The −75 sgk-CAT was generated as described (38). The −67 wild-type and mutant sgk-CAT vectors, sense and antisense oligonucleotides of the region between −67 and −35 were synthesized as per usual techniques (Microchemical Facility, Cancer Research Laboratory, University of California, Berkeley), and then 800 pmol of each strand were mixed and annealed. The wild type and mutant double-stranded oligonucleotides were then subcloned into the pCAT Basic vector (Promega) containing the region between −35 and +55 of the sgk promoter. The −67 wild type and mutant vectors were confirmed by DNA sequencing.

**Transfection Methods and CAT Reporter Gene Assays—**NMuMg mammary epithelial cells from 60–70% confluent cultures in 100-mm tissue culture plates were transfected by the calcium phosphate precipitation method as described previously (32). The DNA used in calcium phosphate transfections for CAT assays was held constant at 20 µg, and in appropriate transfections, the total DNA was adjusted to this amount using the empty CAT vector plasmid pBLCAT3 or pCAT Basic. Following transfection for at least 4 h, cells were exposed to a 15% glycerol shock for 3 min at 37 °C. Transfections were post-transfection, the cells were harvested for CAT assays, and the protein content of the cell extracts was estimated with the Bradford procedure (39). A quantitative nonchromatographic assay (40) was used to measure CAT activity in the cell extracts as detailed elsewhere (28).

**Isolation of RNA and Northern Blot Analysis—**NMuMg cells were plated on 10-cm dishes so they were 80–90% confluent on the day of harvest. Cells were scraped on ice using a rubber policeman, washed in 1× PBS, and then pelleted. To each plate, 100 µl of TSM buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 1.5 mM MgCl₂) plus 1 mM diethiothreitol was added, and the cells were incubated for 5 min on ice. Nuclei were pelleted by spinning at 14,000 rpm in an Eppendorf centrifuge at 4 °C. The supernatant was transferred to new tubes, and 100 µl of digestion buffer (0.2 µM Tris-Cl, pH 8.0, 25 mM EDTA, 0.3 M NaCl, 2% SDS) plus 100 µg/ml proteinase K was added. The samples were incubated at 37 °C for 30 min and then extracted twice with 1:1 phenol/chloroform. RNA was precipitated with ethanol overnight at −70 °C, and pellets were resuspended in diethyl pyrocarbonate-treated water plus 0.1% SDS.

**Approximately 10–13 µg of RNA isolated from each sample was separated on a 1.1% agarose/formaldehyde gel run at 3–4 V/cm for approximately 3 h. The RNA was transferred overnight by blotting onto MSI Magnagraph nitrocellulose. The RNA was covalently cross-linked onto the membrane using a Stragatene Stratalinker, and then transfer was confirmed by staining the blot with 0.02% methylene blue dye. The blot was prehybridized for a minimum of 2 h as described previously (28). The blot was then probed with a 32P-labeled fragment of murine sgk cDNA that was labeled by [α-32P]dATP random primed labeling (Roche Molecular Biochemicals). A total of 10 × 10⁶ cpm/ml of probe in hybridization buffer was added to the blot and allowed to incubate at 42 °C overnight. The blot was washed in 2× SSC, 0.1% SDS twice for a total of 1.5 h at room temperature. The blot was then exposed to a Molecular Dynamics PhosphorImager screen for 24 h.

**Isolation of Nuclear DNA from Calcium Phosphate Transfections**—DNA from calcium phosphate transfections for CAT assays was held constant as described previously (32). The total amount of DNA used in calcium phosphate transfections for CAT assays was held constant as described previously (32). The total amount of DNA by native polyacrylamide gel electrophoresis (8% gel). The labeled DNA was separated from the end-labeled double-stranded DNA by native polyacrylamide gel electrophoresis (8% gel). The labeled DNA was separated from the end-labeled double-stranded DNA by native polyacrylamide gel electrophoresis (8% gel).

**DNA Sequencing and Characterization of a Serine/Threonine Serum- and Glucocorticoid-Inducible Protein Kinase Gene (sgk) in Mammary Epithelial Cells**—The protein contents in the nuclear extracts were determined by the Bradford procedure (39). The sequences of the various oligonucleotides (sense used for DNA binding studies and cold competition experiments are as follows (mutations indicated in boldface type): sgk−67−−35, 5′-GGTCCGCCTGCCGCATCGAGGCTC-3′; −67−35 mutant 1, 5′-GGTCCGCCTGCCGCATCGAGGCCCTC-3′; −67−35 mutant 2, 5′-GGTCCGTTCGCCAACCCTGCCCCTGGAGGCTC-3′; −67−35 mutant 3, 5′-GGTCCGCCTGCCGCTTGGAAACCCTGCCCCTGGAGGCTC-3′; −67−35 mutant 4, 5′-GGTCCGCCTGCCGCTTGGAAACCCTGCCCCTGGAGGCTC-3′; −67−35 mutant 5, 5′-GGTCCGCCTGCCGCTTGGAAACCCTGCCCCTGGAGGCTC-3′. The mutant Sp1-binding sequence is 5′-cagtGCCGGCCGCCCAGT-3′. The mutant CAG-binding sequence is 5′-cagtGCCGGCCGGCTCCAGT-3′. The core CAG nucleic acids have been changed to TTT, and Sp1 is unable to bind to this sequence (42). The EGR1 consensus oligonucleotide sequence is 5′-cagtGCCGGCCGCCCAGT-3′. All oligonucleotides were synthesized by a model 394 synthesizer in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley. Radiolabeling of 5′-ends of the appropriate oligonucleotides was carried out in the presence of equal amounts (10 pmol) of sense and antisense strands, [α-32P]ATP (6000 Ci/mmole; ICN Biomedicals Inc.), and T4 polynucleotide kinase (Roche Molecular Biochemicals) at 37 °C for 30 min, followed by annealing of the labeled strands. The annealing procedure for generating either labeled or unlabeled DNA involved adding 0.1 M NaCl (0.1 volume) to equal amounts of sense and antisense strands, heating for 10 min at 70 °C, and gradually cooling to room temperature. The single-stranded DNA were separated from the end-labeled double-stranded DNA by native polyacrylamide gel electrophoresis (8% gel). The labeled double-stranded oligonucleotide was excised and eluted in 400 µl of TE buffer (10 mM Tris, 1 mM EDTA) and 40 µl of 3 M sodium acetate, pH 5.0, for 3 h, followed by ethanol precipitation, rinsing in 70% ethanol, and resuspension in TE buffer. The radioactive oligonucleotide probes were...
stored at -70 °C. The DNA binding reactions (18 µl) contained nuclear extract proteins (7 µg), 0.5 ng of 32P-labeled (approximately 2 × 106 cpm) DNA probe, poly(dI-dC) (2 µg), and 6 µl of 3× binding buffer (21.6% glycerol, 36 mM Hepes, pH 7.5, 126 mM KCl, 9 mM MgCl₂, 0.9% Nonidet P-40, 180 mM NaCl, 0.9 mM dithiothreitol) and were allowed to incubate from room temperature for 30 min. For competition experiments, a 200-fold excess (unless otherwise noted) of the indicated unlabeled double-stranded oligonucleotides was added prior to the addition of radiolabeled DNA probe. In some cases, the reaction mixtures were incubated at 4 °C with either 5 µl of specific anti-Sp1 polyclonal antibodies (a generous gift from the R. Tjian laboratory, University of California, Berkeley) or an equal volume (equivalent to 100 ng) of anti-p110 PI 3-kinase polyclonal antibody (Santa Cruz Biotechnology, Inc.) for 2 h before the addition of radiolabeled probes. The protein-DNA complexes were resolved on a 4% native polyacrylamide gel (19:1 acrylamide/bisacrylamide) in 1× gel shift running buffer (25 mM Tris base, 40 mM NaCl, 40 mM NaF, 180 mM ZnCl₂, 0.9 mM dithiothreitol) and were allowed to incubate for 1 h at room temperature. The 20% of the filter-bound radioactivity observed with the nonimmune antibodies activity of the transcriptional initiation site of the sgk gene promoter that is responsible for the hyperosmotic stress regulation of sgk gene expression is mediated by one or more preexisting regulatory elements. In the absence of sorbitol, the level of Sgk protein and mRNA remained at a low basal level throughout the entire time course.

To examine whether the sorbitol stimulation of sgk gene expression is a direct response or requires de novo protein synthesis, NMuMg cells were pretreated for 1 h with or without cycloheximide (10 µg/ml), a protein synthesis inhibitor, and then each set of cells was incubated in the presence or absence of 0.3 M sorbitol for 24 h. In the cycloheximide-treated cells, protein synthesis was inhibited by greater than 95% without any significant effects on mRNA synthesis (data not shown). Northern blot analysis revealed that pretreatment of the cells with cycloheximide had no effect on the inducibility of the sgk transcripts in response to sorbitol (Fig. 2). Because the hyperosmotic stress induction of sgk mRNA occurred in the absence of de novo protein synthesis, we suggest that the regulation of sgk gene expression is mediated by one or more preexisting cellular components that are likely to be involved in the transcriptional control of this gene. As we have previously reported (29), exposure to cycloheximide stabilized sgk transcript turnover, which caused both the sorbitol-treated and -untreated cells to produce a higher level of sgk mRNA (Fig. 2). Our results suggest that a hyperosmotic stress-induced signaling pathway stimulates sgk gene transcription, which accounts for the increased production of Sgk protein after sorbitol treatment.

**Identification of a Hyperosmotic Stress-responsive Region of the sgk Promoter**—To functionally dissect the region within the sgk gene promoter that is responsible for the hyperosmotic stress regulation of sgk expression, NMuMg cells were transfected with a series of sgk-CAT reporter genes containing a series of 5′-progressive deletions starting at -1350 bp up-stream of the sgk gene and terminating at +55 bp downstream of the transcriptional initiation site of the sgk gene. Cells were then treated with or without 0.3 M sorbitol, and cell lysates were analyzed for CAT activity 24 h after the hyperosmotic stress. As shown in Fig. 3, sorbitol stimulated transcriptional activity of sgk-CAT constructs containing the three largest promoter regions with deletions ending at -1350 bp, -236 bp, and -78 bp. Maximum stimulation of sgk-CAT activity by hyperosmotic stress (5-8-fold) was observed with these constructs. Sorbitol inducibility of the sgk promoter was lost in deletions beyond -55 bp, indicating that the promoter fragment between nucleotides -78 and -55 bp of the upstream region of the sgk gene is a hyperosmolar stress-responsive}

**RESULTS**

**Stimulation of sgk Transcript and Protein Levels in Response to Hyperosmotic Stress**—To initially test whether sgk gene expression is targeted by the mammalian hyperosmotic stress pathway, the levels of Sgk protein and mRNA were determined in NMuMg (normal murine mammary gland) cells at various times after treatment with 0.3 M of the organic osmolyte sorbitol to induce a hyperosmotic stress. Western blot analysis of whole cell extracts using affinity-purified anti-Sgk polyclonal antibodies (30) revealed that after an approximately 4-h time lag, sorbitol treatment induced a significant elevation in Sgk protein levels (Fig. 1A). A high level of Sgk protein was still maintained 24 h after the hyperosmotic stress. Northern blot analysis of total cellular mRNA of the same time course using a α-32P-labeled murine sgk cDNA probe demonstrated a striking sorbitol stimulation in expression of the 2.2-kb sgk transcript (Fig. 1B). Consistent with the protein data, sgk transcripts were not detected until 4 h after sorbitol treatment, and sgk mRNA levels continued to accumulate 24 h following treatment. In the absence of sorbitol, the level of Sgk protein and mRNA remained at a low basal level throughout the entire time course.

**Identification of a Hyperosmotic Stress-responsive Region of the sgk Promoter**—To functionally dissect the region within the sgk gene promoter that is responsible for the hyperosmotic stress regulation of sgk expression, NMuMg cells were transfected with a series of sgk-CAT reporter genes containing a series of 5′-progressive deletions starting at -1350 bp upstream of the sgk gene and terminating at +55 bp downstream of the transcriptional initiation site of the sgk gene. Cells were then treated with or without 0.3 M sorbitol, and cell lysates were analyzed for CAT activity 24 h after the hyperosmotic stress. As shown in Fig. 3, sorbitol stimulated transcriptional activity of sgk-CAT constructs containing the three largest promoter regions with deletions ending at -1350 bp, -236 bp, and -78 bp. Maximum stimulation of sgk-CAT activity by hyperosmotic stress (5-8-fold) was observed with these constructs. Sorbitol inducibility of the sgk promoter was lost in deletions beyond -55 bp, indicating that the promoter fragment between nucleotides -78 and -55 bp of the upstream region of the sgk gene is a hyperosmolar stress-responsive
region. In additional experiments, this hyperosmotic regulated region was further narrowed to between −67 and −35 base pairs (data not shown, and see below). These results suggest that the region between −67 and −35 of the sgk promoter contains transcription factor binding sites involved in the stimulation of sgk gene transcription in response to hyperosmotic stress.

Functional Analysis of the Hyperosmolar Stress-responsive Region of the sgk Promoter—To test whether the hyperosmolar stress-responsive region defined by the deletion analysis is sufficient to mediate the sorbitol responsiveness of the sgk gene, an oligonucleotide corresponding to the wild type −67 to −35 bp fragment was cloned into pCAT-Basic that already contained the −34 to +55 region of the sgk promoter (38) and linked to the bacterial CAT gene. NMuMg cells were transiently transfected with −67/−35 wild type CAT reporter plasmid, and CAT activity was monitored in cell extracts isolated from cells treated with or without sorbitol. As shown in Fig. 4, sorbitol induced CAT expression by 3.7-fold compared with the unstressed mammary cells. Reporter gene activity in cells transfected with the minimal promoter tkCAT alone was low and unaffected by sorbitol treatment (data not shown). This result establishes that the hyperosmolar stress responsive region of the sgk promoter in transfected mammary epithelial cells is located between −67 and −35 bp upstream of the RNA start site.

Analysis of the hyperosmotic stress-regulated region of the sgk promoter using transcription factor consensus binding site algorithms such as TRANSFAC and MatInspector revealed the presence of a highly conserved GC-rich region (43, 44). Therefore, to further characterize the promoter region necessary for the sorbitol induction of sgk gene expression, five mutants of this region were synthesized in which each mutant contained a different set of six base pairs that were mutated to the same sequence, TTCGAA, which is a sense for the indicated five mutants (M1–M5) of the −67/−35 region of the sgk promoter were cloned into the same pCAT-Basic vector used to generate the wild type CAT reporter plasmid. The CAT reporter activities were monitored in transiently transfected NMuMg cells treated with or without sorbitol. As shown in Fig. 4, the M3, M2, and M5 mutants were fully inducible by hyperosmolar stress at a level comparable with the wild type sgk promoter fragment. In contrast, the M3 and M4 were unable to respond to sorbitol treatment. The M3 and M4 mutations overlapped with and ablated the GC-rich region of the sgk promoter, suggesting that the corresponding transcription factor is a target of the hyperosmotic stress response in mammary epithelial cells.

Electrophoretic Mobility Shift Assay Analysis of the Transcriptionally Responsive Region of the sgk Promoter—A competitive gel shift analysis was employed to characterize the DNA-binding protein complexes associated with the hyperosmolar stress responsive region of the sgk promoter. The double-stranded wild type −67/−35 oligonucleotide was radiolabeled and incubated with nuclear extracts that were prepared from 4-h sorbitol-treated or control NMuMg cells along with an excess of one of the mutant oligonucleotides (M1–M5). The gel shift pattern of the protein-DNA complexes was visualized by electrophoretic fractionation in a 6% SDS-polyacrylamide gel. As shown in Fig. 5, without any competing oligonucleotides, the −67/−35 region of the sgk promoter forms the same four major protein-DNA complexes in the presence of cell extracts from untreated control or sorbitol treated cells (defined as complexes A, B, C, and D). Incubation with a 200-fold excess of unlabeled M1, M2, or M5 mutant oligonucleotides, which are fully sorbitol-responsive, quantitatively competed with the wild type oligonucleotide for formation of each of the protein-DNA complexes using extracts from either the control or hyperosmolar stressed cells (Fig. 5). A similar complete competition was observed with the wild type oligonucleotide (data not shown). In contrast, mutant oligonucleotides M3 and M4, which effectively ablate the GC-rich sequence and are not sorbitol-responsive, were ineffective as competitors in this gel shift assay (Fig. 5). These results suggest that the GC-rich sequence located at −55 to −46 bp mediates the hyperosmotic stress regulation of the sgk promoter through the function of the corresponding protein-DNA complex in this region of the promoter.

Identification of the Sp1 Transcription Factor as a Component of the Protein-DNA Complex That Binds to the Hyperosmolar Stress-regulated Region of the sgk Promoter—It is well
are the mean and S.D. from three independent sets of experiments.
of the other protein-DNA complexes (data not shown). These results, along with the functional studies described above, implicate Sp1 as a target or a member of a multiprotein complex that is involved in hyperosmotic stress regulation of the sgk promoter.

**Hyperosmotic Stress Regulation of sgk Transcript and Protein Expression Is Mediated by the p38 MAPK Pathway**—It is well documented that p38 MAPK is phosphorylated and activated by the dual specificity MKK3/6 kinases at a TGY motif in response to various forms of stress, including hyperosmotic stress (6, 16, 46). To test whether p38 MAPK is activated in NMuMg mammary cells in response to sorbitol, a Western blot of extracts from cells treated at various times with or without 0.3 M sorbitol was probed with anti-phospho-p38 MAPK antibodies. The phosphorylated form of p38 MAPK was detected in NMuMg mammary cells in response to sorbitol, a Western blot analysis of Sgk protein levels revealed a striking reduction in the amount of Sgk protein induced in hyperosmolar stressed cells treated with either p38 MAPK inhibitor (Fig. 7B).

The p38 MAPK pathway targets and phosphorylates several transcription factors in response to hyperosmotic stress (16), and the attenuation of Sgk protein levels in the presence of p38 pharmacological inhibitors suggested a functional link between p38 MAPK function and the stimulation of sgk promoter activity. To determine whether the p38 MAPK cascade is involved in the hyperosmotic stress stimulation of the sgk promoter, NMuMg cells transfected with the −1.3 sgk-CAT reporter plasmid were pretreated with or without the p38 MAPK inhibitor SB202190 and then incubated in the presence or absence 0.3 M sorbitol to induce the hyperosmolar stress. In the absence of SB202190, the −1.3 sgk-CAT construct was induced on average 4-fold in response to sorbitol treatment (Fig. 7C), and in cells pretreated with SB202190 the hyperosmolar stress induction of sgk promoter was abrogated. There are several p38 MAPK gene family members, and based on the selectivity of the SB203580 and SB202190 pharmacological inhibitors, our results suggest that the p38-α- or p38-β-mediated signaling pathway (47) con-

![Fig. 5. Competitive gel shift analysis of the −67/−35 hyperosmotic stress-regulated region using the M1–M5 mutant oligonucleotides.](image)

![Fig. 6. Evidence for the presence of Sp1 activity in the gel-shifted complex formed with the hyperosmotic stress-regulated region of the sgk promoter.](image)
tributes to the hyperosmotic stress stimulation of sgk promoter activity and protein production.

One of the upstream kinases that activates p38 MAPK in response to cellular stress is MKK3 (16, 46). As a complementary approach to demonstrate the involvement of the p38 MAPK pathway in the hyperosmolar stress stimulation of the sgk promoter, cells were cotransfected with the −236 sgk-CAT reporter construct and with expression plasmids for either the wild type MKK3 kinase (MKK3) or the kinase-dead form of MKK3 (MKK3ΔL) that has been shown to act as a dominant negative inhibitor of the p38 MAPK pathway (48). As shown in Fig. 8, co-transfection with the wild type MKK3 leads to a high level of sorbitol-stimulated reporter activity, whereas the co-transfection of the dominant negative acting MKK3ΔL plasmid abolished the sorbitol induction of sgk promoter activity. These results show that the hyperosmotic stress stimulation of sgk promoter activity requires the MKK3-mediated activation of p38 MAPK kinase. Because treatment with either of the p38 MAPK pharmacological inhibitors fails to completely dampen the hyperosmotic stress stimulation of sgk promoter activity, MKK3 may have one or more as yet unidentified targets in addition to the p38-α or p38-β MAPK isoforms that play a role in the sgk transcriptional response.

Hyperosmotic Stress Induces a Cytoplasmically Localized and Enzymatically Active Sgk Protein—We have previously established that the nuclear/cytoplasmic subcellular distribution of Sgk can be regulated in a stimulus-dependent manner (30). For example, in serum-treated cells Sgk shuttles between the nucleus and cytoplasm in synchrony with the cell cycle, whereas in glucocorticoid-treated cells Sgk is exclusively localized to the cytoplasmic compartment. Therefore, to characterize the effects of hyperosmotic stress on the subcellular distribution of Sgk, NMuMg cells were grown to confluency and treated with 0.3 M sorbitol for 4 h, and the fixed cells were permeabilized and stained with anti-Sgk polyclonal antibody followed by fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody. Indirect immunofluorescence revealed that hyperosmotic stress stimulates the production of a cytoplasmic localized Sgk (Fig. 9).

We have recently established that Sgk protein kinase activity is activated in response to serum growth factors through a phosphatidylinositol 3-kinase-mediated pathway (31). Furthermore, other groups have shown that sorbitol inhibits other PI 3-kinase downstream targets, such as Akt/PKB and p70 S6 kinase (49–51). Interestingly, this inhibition is not due to the inhibition of PI 3-kinase or PDK1 activity but instead through the activity of the PP2A phosphatase. Therefore, to determine whether the sorbitol induced the slow migrating phosphorylated form of Sgk as a result of signaling through PI 3-kinase, cells were pretreated with or without the pharmacological inhibitor for PI 3-kinase, LY294002. This drug renders the PI 3-kinase molecule inactive by preventing ATP binding (52). As shown in Fig. 10, in the absence of LY294002, a Sgk protein doublet (49 and 51 kDa) is induced in response to hyperosmotic stress. Our previous work has demonstrated that these two protein species represent hyperphosphorylated and hypophosphorylated forms of Sgk (30). We also previously documented that an inhibition of PI 3-kinase activity prevented the appearance of the hyperphosphorylated form of Sgk (31). Consistent with these results, LY294002 ablated production of the slower migrating (and hyperphosphorylated) form of Sgk (Fig. 10B), suggesting that the Sgk protein, which is produced after sorbitol treatment, is targeted by the PI 3-kinase signaling pathway.

To biochemically assess whether hyperosmotic stress had any effect on endogenous Sgk protein kinase activity, NMuMg cells were treated with or without sorbitol for 8 h, and the isolated cell extracts were incubated with affinity-purified anti-Sgk antibody or nonimmune antibodies as a negative control. As a positive control for this assay, Sgk was immunoprecipitated from serum-treated and -untreated cells. The Sgktide peptide (KKRRNRRLSVA) was utilized as a substrate in an in vitro transphosphorylation assay, and the Sgk-specific activity was assayed by quantification of the conversion of [3H]acetyl-CoA into [3H]acetyl-chloramphenicol by the two-phase fluor density assay described under “Experimental Procedures.” The resulting CAT activities were normalized to protein levels to determine the CAT-specific activity, and the -fold induction was calculated as a ratio of the CAT-specific activity observed in sorbitol-treated cells to that observed in control cells. Three independent assays (data points) were performed in triplicate, and the bar graphs show the mean from these experiments.

Fig. 7. Inhibitors of the p38 MAPK suppresses the hyperosmotic stress stimulation of Sgk protein and promoter activity. A, NMuMg cells were treated with (+) or without (−) 0.3 M sorbitol in serum-free media and at the indicated time points. Cell extracts were electrophoretically fractionated in SDS-polyacrylamide gels. Western blots were probed with phospho-p38 MAPK-specific antibodies to analyze the production of active p38 MAPK. B, cells were treated with the p38 MAPK pharmacological inhibitors SB203580 (20 μM) or SB202190 (10 μM) for 30 min, and then the cells were treated with (+) or without (−) 0.3 M sorbitol in serum-free media for 6 h. Total cell extracts were electrophoretically fractionated in SDS-polyacrylamide gels and Western blots probed for the production of Sgk as described under “Experimental Procedures.” C, cells were transfected with the −1.3 sgk-CAT reporter plasmid, as described in the legend to Fig. 3. 24 h post-transfection, the cells were pretreated for 30 min with or without SB202190 (10 μM). The cells were then treated with or without 0.3 M sorbitol in serum-free media for 24 h, harvested, and lysed, and CAT activity was assayed by quantification of the conversion of [3H]acetyl-CoA into [3H]acetyl-chloramphenicol by the two-phase fluor density assay described under “Experimental Procedures.” The resulting CAT activities were normalized to protein levels to determine the CAT-specific activity, and the -fold induction was calculated as a ratio of the CAT-specific activity observed in sorbitol-treated cells to that observed in control cells. Three independent assays (data points) were performed in triplicate, and the bar graphs show the mean from these experiments.
Sgk protein, human embryo kidney 293 cells (HEK 293) were transfected with the pCDNA3 HA-sgk expression vector and then treated with either isosmotic or hyperosmotic media. The immunoprecipitated HA-Sgk protein, using antibodies directed against the HA epitope tag, was assayed for transphosphorylation activity using the Sgktide substrate. As shown in Fig. 11, hyperosmotic stress stimulated the kinase activity of exogenously expressed Sgk protein approximately 3-fold over isosmotic treatment. After the peak production of active Sgk within the first 5–15 min after sorbitol treatment, the level of Sgk transphosphorylation activity returned to basal levels during the next 60 min. Although activation of Sgk is transient, this result suggests that hyperosmotic stress regulates Sgk expression at the transcriptional level and also posttranslationally regulates Sgk enzymatic activity.

**DISCUSSION**

The evolutionarily conserved ability of an organism to adapt to extreme changes in nutrient levels, temperature, or osmolarity is crucial to the survival of unicellular prokaryotes as well as the eukaryotic cells that constitute mammalian tissues. Several signaling cascades have been shown to play a necessary role in the detection and response to environmental stress. One such evolutionarily conserved pathway employs the activation of the p38 MAPK/HOG1 family of protein kinases (14), which can be triggered by osmotic imbalances, UV light radiation, heat shock, DNA-damaging agents, FAS-induced cell death, and exposure to the protein synthesis inhibitor anisomycin (46, 48, 53). Several transcription factors and protein kinases have been identified as cellular targets of p38 MAPK in mammalian cells; however, relatively little is known about their respective gene targets and protein substrates. We and others have shown that an important cellular feature of sgk is its acute transcriptional control in rodent and human cell lines (28, 29, 32, 33, 38, 54–55), and our study shows for the first time that the transcriptional regulation of sgk can be directly linked to a specific stress signaling pathway involving p38 MAPK in mammalian cells.

As shown in Fig. 12, we propose that expression of mouse NMuMg mammary epithelial cells to hyperosmotic medium activates the p38 MAPK cascade, which in turn stimulates sgk transcription by targeting a GC-rich hyperosmotic stress-regulated element in the sgk promoter that is recognized by the Sp1 transcription factor. Activation of this pathway induces an increase in the production of an active Sgk protein kinase that maintains its dependence on the PI 3-kinase pathway for its phosphorylation and activity. Several lines of evidence demonstrated a role for p38 MAPK in the stimulation of sgk transcription. The robust stimulation of sgk transcription and protein production can be disrupted by treatment with p38 MAPK-specific pharmacological inhibitors or by exogenous expression of dominant negative MKK3, the immediate upstream regulator of p38 MAPK. Moreover, ectopic expression of wild type MKK3 further stimulated the magnitude of the sorbitol-induced sgk promoter activity. Our results also suggest that the activated p38 MAPK cascade either directly or indirectly targets a Sp1 transcription factor protein complex on the sgk promoter to stimulate sgk transcription (Fig. 12). Sp1 is a Cys-His zinc-finger-binding protein that has been shown to bind specifically to the GC-rich GCCCGGC CCC sequence (42, 45) that we defined as the hyperosmotic stress-regulated ele-

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**FIG. 8. Effects of wild type and dominant negative MKK3 on the hyperosmotic stress stimulation of sgk promoter activity.** NMuMg mammary epithelial cells were transiently co-transfected with the p3CDNA3 HA-sgk reporter plasmid along with either the wild type (MKK3) or kinase dead dominant negative MKK3 (MKK3AL) expression plasmid. Another set of cells were transfected only with the p3CDNA3 HA-sgk reporter plasmid. The cells were then treated with (+) or without (–) 0.3 M sorbitol for 24 h, and the CAT-specific activity was determined in the isolated cell extracts as described in Fig. 7. Each set of assays was performed in triplicate, and the reported values are the mean and S.D. from three independent sets of experiments.

**FIG. 9. Hyperosmotic stress induction of the endogenous Sgk protein localizes to the cytoplasmic compartment.** NMuMg mammary cells were treated with (+) or without (–) 0.3 M sorbitol in serum-free media for 4 h. The subcellular distribution of Sgk was examined by indirect immunofluorescence microscopy using affinity-purified rabbit polyclonal antibodies to Sgk followed by fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibodies. No immunofluorescence staining is observed when preimmune antiserum is used as the primary antibodies (data not shown).
ment in the sgk promoter. Our studies are generally consistent with previous observations in the yeast S. cerevisiae in which the Msn family of transcription factors are targeted by the yeast p38 MAPK homologue HOG1 and bind to a CCCCT DNA sequence that is similar to the Sp1 binding core sequence (7, 8, 10). In earlier studies, Sp1 was viewed as a component of the basal transcriptional machinery. However, recent studies have shown that Sp1 transactivation activity is also subject to regulation by several different extracellular cues including oxidative and shear stress (58, 59). Our results now implicate the Sp1 transcription factor as a component in the hyperosmotic stress response that leads to the stimulated expression of sgk.

A deletion analysis initially identified a hyperosmotic stress-responsive region between −67 and −35 bp of the sgk promoter that accounts for the striking sorbitol induction of sgk gene expression. Fine mapping of this 32-bp region defined a GCCGCGCCC sequence at −54 to −44 bp as being necessary for the hyperosmotic shock induction. Competitive electrophoretic mobility shift assays and mutational analyses demonstrated the presence of Sp1 in the transcription factor complex that interacts with the hyperosmotic stress-responsive region and thereby implicates the Sp1 transcriptional regulator as a necessary factor for this response. For example, inclusion of Sp1-specific antibodies in the gel shift reaction caused a specific loss of one of the predominant protein-DNA complex bands with a corresponding increase in a new lower molecular weight protein-DNA complex. Also, the consensus Sp1 oligonucleotide strongly competed for formation of each of the four major protein-DNA complexes observed with the −67 to −35 hyperosmotic stress-regulated region of the sgk promoter. In contrast, a mutant Sp1 oligonucleotide, which is incapable of binding Sp1, and a wild type consensus oligonucleotide corresponding to the EGR1 transcription factor, which also binds to a GC-rich DNA site, were unable to compete in this assay. It is likely that the Sp1 transcription factor functions in the hyperosmotic stress response through an interaction with another unidentified nuclear factor, because transfection of a reporter plasmid containing the consensus Sp1 binding site with or without an expression plasmid for Sp1 (60) failed to be sorbitol-responsive (data not shown). Consistent with this concept, we observed multiple protein-DNA complexes in an electrophoretic mobility gel shift assay using an oligonucleotide corresponding to the −67 to −35 hyperosmotic stress-responsive region. We are currently examining whether one or more of the protein factors in these DNA-protein complexes may be transcriptional targets of the hyperosmotic stress cascade that interact with Sp1.

The four major protein-DNA complexes that form on the hyperosmotic stress-regulated element in the sgk promoter remained unaffected by sorbitol treatment. There are several precedents for promoters being activated through a p38 MAPK cascade that do not show obvious changes in protein-DNA complexes. For example, the lipopolysaccharide induction of a NF-κB consensus binding site reporter construct was inhibited by the p38 MAPK pharmacological inhibitor SB203580 and by co-expression of a dominant negative p38 MAPK under conditions that did not change the observed gel-shifted DNA-protein complexes (61). We speculate that sgk transcription may be stimulated by the recruitment of specific coactivators or altered interactions with components of the basal transcriptional machinery that can potentially be phosphorylated by p38 MAPK in response to hyperosmotic stress. For example, the TFIID component of the basal transcription complex has been shown

**Fig. 10.** Effect of hyperosmotic stress on the phosphorylation state and enzymatic activity of endogenous Sgk protein. A, affinity-purified anti-Sgk antibodies (Sgk) or nonimmune antibodies (NI) were added to total cell extracts from cells either treated with (+) or without (−) 0.3 M sorbitol for 8 h. As a positive control for Sgk enzymatic activity, Sgk was immunoprecipitated from 48-h serum-starved cells that had been incubated with (+) or without (−) 10% serum for 24 h. Transphosphorylation activity in the immunoprecipitated protein was monitored by quantifying the formation of [32P]-labeled Sgktide peptide in each reaction. Sgk-specific transphosphorylation activity was determined by subtracting the filter-bound radioactivity observed with the nonimmune antibodies from that observed with the anti-Sgk antibodies. B, NMuMg cells were pretreated with either 50 mM LY294002 or Me2SO carrier for 16 h and then incubated with (+) or without (−) 0.3 M sorbitol in serum-free media. After 8 h, cells were harvested, and Western blots of electrophoretically fractionated total cell extracts were probed for the expression of Sgk protein.

**Fig. 11.** Effect of hyperosmotic stress on the enzymatic activity of exogenous Sgk protein. HEK 293 cells overexpressing HA epitope-tagged Sgk (HA-Sgk) were treated with either isosmotic (Iso) or hyperosmotic (0.3 M sorbitol) media for the indicated times. HA-Sgk was immunoprecipitated from each cell extract using anti-HA-specific antibodies and assayed for kinase activity using Sgktide as the substrate. The anti-HA-Sgk immunoblot analysis is shown in the lower panel.
to be phosphorylated by p38 MAPK and also to interact with Sp1 in other cell systems (61, 62). Conceivably, other cellular components that are known to be phosphorylated by p38 MAPK, such as transcription factors and protein kinases (16–18, 20–22, 25, 63, 64), could potentially interact with Sp1 in a p38 MAPK-dependent manner and play a role in the hyperosmotic stress response.

We propose that the Sgk mediates certain cellular responses to osmolar imbalances, because after hyperosmotic stress of mammary epithelial cells, Sgk protein levels are robustly induced and maintained at a high level throughout the time course of sorbitol treatment. As predicted by the promoter studies, pretreatment of cells with the p38 MAPK inhibitors SB203580 and SB202190 resulted in the significant attenuation of Sgk protein levels. Although one other study has reported that anisosmotic changes regulate sgk transcript levels (34), our study is the first to define the sgk promoter elements that mediate the hyperosmotic stress stimulation of transcripts, to uncover a connection between Sgk and the p38 MAPK stress signaling cascade, and to demonstrate an increase in the production of endogenous Sgk protein and Sgk-specific serine/threonine protein kinase activity. In response to hyperosmotic stress, the Sgk protein is localized throughout the cytoplasm that presumably includes the cytoplasmic surface of the plasma membrane. One potential role of Sgk in mediating the cellular hyperosmotic stress response may be to regulate cell volume through activation of the epithelial sodium channel (55, 56). Our results suggest that the enzymatic activity of Sgk is important for this response, and we are attempting to define the membrane interactions between endogenous Sgk and the epithelial sodium channel that are necessary for channel activity that probably account for the increased channel activity that is observed in response to hyperosmotic stress (65).

Following treatment of cells with serum and/or growth factors, PI 3-kinase activity is induced, which initiates a PDK-1 signaling pathway (66). These events are responsible for the activation of the downstream kinases Akt/PKB, p70 S6 kinase, and Sgk (31, 67–70). A well documented consequence of the activation of Akt/PKB is the phosphorylation of the proapoptotic protein Bad, which regulates the cell survival response (71–73). Both Sgk and Akt/PKB are downstream targets of the PI 3-kinase-dependent activation of PDK1. Treatment of 3T3L1 preadipocytes with sorbitol has been shown to inhibit Akt/PKB as well as p70 S6 kinase activity (49–51). It has been demonstrated that PI 3-kinase and PDK1 are active under this hyperosmolar condition, and consequently the decrease in the cellular pool of phospho-Akt/PKB is attributable to the PP2A phosphatase (49, 50). We observed that sorbitol induced a phosphorylated and kinase-active form of endogenous Sgk that is sensitive to the LY294002 PI 3-kinase inhibitor. It is therefore possible that in the mammary epithelial cells used in our study, the activity of the PP2A phosphatase remains low enough to maintain the activity of endogenous Sgk. In this regard, ectopic expression of sgk in the human embryo kidney 293 cells (HEK 293) only transiently produced an active Sgk enzyme, suggesting that the constitutive PP2A phosphatase activity plays a counter regulatory role on Sgk in these cells. We have previously shown that a lack of phosphorylation of Sgk at both the critical Thr256 and Ser422 residues results in an inactive Sgk kinase (31). Therefore, as an alternative explanation, it is possible that in the presence of sorbitol, Sgk is only phosphorylated on one of these residues and as a result cannot sustain its activity in the HEK 293 cells.

Several groups have reported that perturbations of the PI 3-kinase signaling pathway can affect insulin-dependent glucose uptake by the GLUT4 transporter and other insulin responses (74–78). Conceivably, Sgk, which is expressed in the kidney (55, 56) and expressed at high levels in the kidney glomeruli and distal tubules, may be involved in the hyperosmotic response to high plasma glucose levels in which p38 MAPK activity has been shown to be induced and Akt/PKB activity inhibited (49, 50, 79–81). Moreover, one other report suggests that Sgk may also play a role in the nephropathy that is associated with the diabetic disease state (82). Further stud-
ies are in progress to identify the physiological substrates of Sgk under high osmolarity conditions, which will add greatly to the understanding of the role of Sgk in cellular stress signaling pathways.

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