sgk: an essential convergence point for peptide and steroid hormone regulation of ENaC-mediated Na\(^{+}\) transport

CARLA J. FALETTI,1 NICOLA PERROTTI,2,3 SIMEON I. TAYLOR,2 AND BONNIE L. BLAZER-YOST1

1Biology Department, Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana 46202; 2Diabetes Branch, Division of Intramural Research, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892; and 3Dipartimento di Medicina Sperimentale e Clinica “G. Salvatore,” Universita di Catanzaro, Catanzaro, 88100 Italy

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The kidneys regulate homeostatic control of extracellular Na\(^{+}\) concentration, extracellular fluid volume, and blood pressure. Located in the apical membrane of the principal cells lining the renal distal tubule, the amiloride-sensitive epithelial Na\(^{+}\) channel (ENaC) is a key component in fluid-electrolyte balance. The expression and activity of this channel is regulated by hormones, mainly aldosterone, insulin, and antidiuretic hormone (ADH). Each of these hormones increases Na\(^{+}\) reabsorption by upregulating the number of active channels in the membrane, lengthening the open probability (\(P_o\)) of ENaC, or a combination of the two mechanisms (10).

The signal transduction pathways linking each of the hormone receptors to ENaC are still under investigation. Activation of protein kinase A (PKA) is necessary for ADH stimulation, whereas phosphoinositide 3-kinase (PtdIns 3-kinase) is required for insulin- and aldosterone-induced Na\(^{+}\) reabsorption (5, 10, 18). The downstream effectors between either the PKA or the PtdIns 3-kinase pathway and ENaC are largely unknown. One of the potential links to the channel may be serum and glucocorticoid-induced kinase (sgk).

sgk is a novel serine/threonine kinase first identified in the Con8.hd6 rat mammary tumor cell line (24). Subject to stimulation by several growth factors and hormones, sgk enzyme activity is regulated by reversible phosphorylation events from multiple signal transduction pathways (6, 13, 16). sgk activity is increased in an additive manner in response to serum and glucocorticoids (16).

Implicated as a modulator in aldosterone-stimulated Na\(^{+}\) transport, sgk is induced in the presence of this mineralocorticoid in the A6 model renal cell line and in cultured cortical collecting duct cells as well as rat kidney and colon (7, 14, 15, 21). In cultured cells, the observed increase in sgk mRNA is evident within 30 min after the addition of aldosterone (7). Coexpression of sgk and ENaC subunits in Xenopus oocytes increases the amiloride-sensitive current from two- to sevenfold over oocytes expressing ENaC subunits alone (7, 15, 21).

sgk is a downstream effector of the PtdIns 3-kinase pathway, which is required for both aldosterone- and insulin-induced increases in Na\(^{+}\) transport (5, 16, 18). We demonstrated previously (5) that aldosterone stimulation results in an increase in PtdIns 3-kinase activity and the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) in the A6 cell line, suggesting...
synthesis of an aldosterone-induced protein mediator at or before the PtdIns 3-kinase step. Thus aldosterone may modulate the synthesis of multiple steps in the phosphoinositide pathway. The effects of the peptide hormone insulin on Na\(^+\) transport have also been found to work through a PtdIns 3-kinase-dependent pathway (18). In the A6 model renal epithelial cell line, PIP\(_3\) is increased within minutes after stimulation with insulin.

PIP\(_3\) is required for the activation of phosphatididylinositol-dependent kinase (PDK)-1 and -2. sgk is activated in response to insulin stimulated by PDK-1 phosphorylation of a threonine residue at position 256. Phosphorylation of this position is accelerated by the phosphorylation of serine-422 by PDK-2, which is activated in response to insulin stimulation (12, 13, 20, 22).

Although both insulin and aldosterone cause an increase in PIP\(_3\), the natriferic effects of the two hormones are additive. In addition, it was demonstrated recently that sgk is phosphorylated in response to both insulin and aldosterone (23).

To determine whether sgk could serve as a possible link between PtdIns 3-kinase- and hormone-regulated effects that increase Na\(^+\) transport through ENaC, we have created clonal cell lines that moderately overexpress wild-type and mutant sgk. The clonal lines were produced by transfecting the A6 model renal cell line. The A6 parental line forms a high-resistance transepithelial monolayer that responds to both steroid and peptide hormones with an increase in Na\(^+\) transport through ENaC, we

**EXPERIMENTAL PROCEDURES**

**Cell culture.** A6 cells derived from the kidney of Xenopus laevis were grown at 27°C in a modified DMEM (no. 91–5055EC; GIBCO BRL, Grand Island, NY) supplemented with 25 U/ml penicillin, 25 \(\mu\)g/ml streptomycin, and 10% calf serum in a humidified 5% CO\(_2\) incubator.

**Creation of stably transfected clones.** The preparation of the sgk-containing expression vectors has been described elsewhere (7). Briefly, clone 645185, containing the complete coding sequence of sgk, was obtained from Research Genetics (Huntsville, AL). A strong Kozak consensus sequence and a myc epitope tag were introduced at the NH\(_2\) terminus by polymerase chain reaction (KlenTaq; Clontech, Palo Alto, CA). The cDNA, flanked by an EcoRI site upstream and a NotI site downstream, was ligated into the PCR cloning vector pCR2.1 plasmid (Invitrogen, Carlsbad, CA) and subcloned into the multiple cloning site of pCI-neo (Promega, Madison, WI).

Three inserts in pCI-neo expression vectors consisting of human sgk, a S422A sgk mutant, or a D222A sgk mutant were transfected into A6 cells. The NH\(_2\) terminus of each gene insert contained a myc tag. The pCI-neo empty vector was also transfected to form a control cell line. For the transfections, the cDNA (4 \(\mu\)g/100-mm\(^2\) dish) was introduced into subconfluent cells using Lipofectamine (GIBCO BRL) in serum-free medium overnight. Serum-containing medium was added the following day, and cells were grown to confluence. Cells were then trypsinized and seeded at either 1.5 (D222A) or 1.10 (sgk, empty vector, S422A) dilution in a toxic concentration (2 mg/ml) of Geneticin (GIBCO BRL). Colonies of 100–200 cells arising from a mother cell were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ). Trypsinized colonies were seeded on 25-cm\(^2\) flasks. Each clonal line was maintained under the selective pressure of 0.5 mg/ml Geneticin.

**Immunopurification.** To isolate and identify sgk, cells were grown to confluence, removed from the tissue culture flasks using trypsinization, and collected as a cell pellet by centrifugation. The cell pellet was washed once with serum-free medium and then twice with phosphate-buffered saline. The cells were resuspended in homogenizing buffer (0.25 M sucrose, 2 mM EDTA, 5 mM Tris, pH 7.5) and disrupted by homogenization using 40 strokes of a Dounce tight-fitting hand-held homogenizer. The suspension was centrifuged (16,000 g) in an Eppendorf microfuge for 2 min. The supernatant was used for sgk immunopurification.

Anti-sgk antisera were obtained by immunizing rabbits with the peptide EVLHKKPYDRTVDW (sgk residues 267–280) Zymed Laboratories, South San Francisco, CA). The cytosolic fractions from each of the cell lines were incubated with anti-sgk antiserum (1:50 dilution) on a rotating wheel at 4°C. After an overnight incubation, the samples were moved to room temperature and incubated for 2 h with protein G bound to a solid support (GammaBind; Sigma) for 2 h. Subsequently, the beads were collected by centrifugation (16,000 g, 10 s) and washed four times with phosphate-buffered saline with 10-min incubations on the rotating wheel between bead collections by centrifugation. After the final wash, the bead pellet was extracted with two-dimensional solubilization buffer (9.5 M urea, 2% Triton, 10 mM dithiothreitol, and 0.2% amphiolines). The immunopurified samples were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

**2D-PAGE.** The immunopurified samples were separated in the first-dimension dimension using immobilized pH gradient gel strips (IPG strips; Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. The first-dimension separation used IPG strips capable of separating proteins having isoelectric points between pH 3 and 10. The estimated isoelectric point of myc-tagged human sgk is 7.9 whereas the estimated isoelectric point for Xenopus sgk is 8.1. For the second dimension, the IPG strips were annealed to a preformed 10% acrylamide SDS slab gel-4% stacking gel to separate proteins having molecular weights between 200,000 and 20,000. After electrophoresis, the separated proteins were electrophoretically transferred to polyvinylidene fluoride blotting membranes and the proteins were visualized using colloidal gold protein stain (Diversified Biotech, Newton Centre, MA).

**Electrophysiology.** Cells were subcultured onto 24-mm Transwell tissue culture treated inserts (Costar, Cambridge, MA) and grown for at least 14 days to achieve confluence as previously described (4, 5, 18). Monolayers of cells were used 14–27 days after seeding. To measure net ion flux, short-circuit current (SCC) techniques were used (3). Electodes were placed on either side of the cell monolayer, which was bathed in serum-free medium maintained at 27°C. A 5% CO\(_2\)-95% O\(_2\) gas lift circulated the medium. Only monolayers with resistances >1,000 \(\Omega\)-cm\(^2\) were used for testing. Transepithelial resistances were monitored by current deflections in response to brief 2-mV pulses. The resistances of the clonal lines were similar to those of the parental line. Specifically, the values were (in \(\Omega\)-cm\(^2\)) as follows: parental line, 1,800 ± 75 (n = 60); sgk, 1,520 ± 94 (n = 45); S422A, 1,520 ± 130 (n =
Hormones were added to the serosal bathing medium after baseline levels of Na\(^+\) transport stabilized (1–3 h). At the conclusion of each experiment, the proportion of SCC due to Na\(^+\) flux was determined by the addition of 10\(^{-5}\) M amiloride to the apical bathing medium. The Na\(^+\) transport component of the SCC was typically >90%.

Values are reported as means ± SE. For statistical comparisons using Student’s t-test, \(P < 0.01\) is considered significant unless otherwise stated.

RESULTS

Sgk is a low-abundance protein in the A6 cell line and is typically difficult to detect in cellular homogenates of unstimulated cells even with the sensitive technique of Western blot detection (23). It is our experience that stably transfected A6 cell lines express proteins that are only severalfold increased over the abundance of the endogenous levels (2). Therefore, immunopurification followed by 2D-PAGE was used to enhance detectability of the native as well as transfected sgk protein. Cytosolic fractions representing equivalent amounts of confluent cells from each of the cell lines were immunoprecipitated with an anti-sgk antibody, and the antibody-bound fraction was separated by 2D-PAGE (Fig. 1). After colloidal gold protein staining, a heterogeneous group of proteins matching the molecular weight (~49,000) and approximate isoelectric point of sgk were detectable in the cells that were transfected with the empty vector. Similar results were obtained in cells of the parental line (data not shown), and this likely represents the endogenous sgk protein. In the transfected cells, these protein isoforms were enhanced in abundance with the D222A kinase-dead mutant consistently expressed at a higher level than the normal sgk or the D422A phosphorylation-defective mutant. Figure 1 is representative of the results of three replicate experiments.

To examine the functional responses of the clonal cell lines, confluent cell monolayers that had been grown on permeable supports were mounted in modified Ussing chambers, incubated in serum-free medium, and voltage-clamped to zero. The resulting SCC was monitored continuously. Basal SCC was determined after the cells had stabilized to an initial baseline level (typically 1–3 h).

The functional effects of each of the gene inserts on basal Na\(^+\) transport are shown in Fig. 2. Clones over-expressing the wild-type sgk demonstrate a 3.5-fold increase in Na\(^+\) transport over the parental A6 cell line. The pCI-neo expression vector alone does not alter basal rates of Na\(^+\) transport because basal Na\(^+\) currents observed in both the empty vector clones and the parental cell line are identical. A point mutation at position 422 that changes a serine to an alanine residue (S422A) prevents phosphorylation and activation...
of sgk by the putative upstream regulator PDK-2. The S422A clone also has a basal Na⁺ transport rate that is the same as the parent cell line. A point mutation at position 222 changes aspartic acid to alanine (D222A) in the kinase domain. The encoded protein has no kinase activity (data not shown). Expression of the D222A kinase-dead sgk mutant causes a significantly depressed level of basal Na⁺ transport in these clones compared with the parent A6 cell line. Each of the four clones maintains stable levels of Na⁺ transport for at least an hour without the addition of hormone (data not shown).

If serum is removed from the medium for 18–24 h, the sgk-transfected cultures exhibit a basal transport rate (SCC = 26.4 ± 1.7 μA/cm²; n = 8) that is only moderately lower (P < 0.02) than that of the sgk-transfected cells maintained in the presence of serum. However, the transport rate of the serum-deprived cultures remains significantly higher (P < 0.000001) than the parental cells that are maintained in serum-containing medium.

To examine the response to the steroid hormone aldosterone, cells were stabilized to an initial baseline current level under continuous voltage-clamp conditions. Aldosterone (10⁻⁶ M) was added to the serosal bathing medium. The cells were continuously monitored for an additional 5 h, at which point amiloride was added to indicate the electrogenic ion transport due to ENaC-mediated Na⁺ transport (Fig. 3). Under these conditions, basal current in the A6 cell line may decrease slightly over time, whereas the aldosterone-treated tissue shows an increase in Na⁺ transport beginning ~60 min after the addition of the steroid hormone (3, 19). The clones expressing the empty vector or the S422A mutant exhibit responses to aldosterone that are virtually identical to that of the parent line. In contrast, the D222A kinase-dead mutant shows no hormone response.

After 5 h, the clones expressing excess sgk exhibit SCC that are 2.5-fold enhanced by aldosterone, but the nature of the response is quite different from that of the parental line. The Na⁺ transport in the non-hormone-treated clones decreases to approximately basal levels over the incubation period. Aldosterone prevents the decline, maintaining the sgk-transfected cells at a level well above what is achievable with aldosterone treatment of the parent line.

Interestingly, despite the increased basal current, overexpression of sgk potentiates the natriferic action of another peptide hormone, ADH. The increase in ADH-stimulated transport is potentiated over threefold in the sgk-expressing clones compared with the parent line. The cells expressing the vector only or the PDK phosphorylation defective mutant S422A respond to ADH stimulation in a manner identical to the parent cell line (Fig. 5). D222A kinase-dead mutants show no response to ADH.

![Fig. 2. Baseline ion transport levels. Short-circuit current (SCC) was monitored as a measure of net ion transport in the A6 parent and stably transfected lines. Bars indicate the means ± SE. The sgk and D222A clones exhibit SCC that are significantly high and lower than the parental line, respectively (P < 0.01). Greater than 90% of the SCC is inhibited by 10⁻⁵ amiloride in all cell lines.](http://ajpcell.physiology.org/)

![Fig. 3. Aldosterone responses in stably transfected A6 cell lines. Cells were maintained under voltage-clamp conditions until a stable baseline current was attained (1–3 h). At time 0, 10⁻⁶ M aldosterone (aldo) was added to the serosal bathing medium of the hormone-treated cells as indicated. The incubations were continued for an additional 5 h, at which point 10⁻⁵ M amiloride was added to the apical bathing medium. Symbols depict means ± SE.](http://ajpcell.physiology.org/)
sensitive transport was measurable within 5 min and reached a maximal level in 10–20 min. Bars indicate the maximal increase in transport from the basal level (ΔSCC). Values are represented as means ± SE. At a P < 0.01 level, only the D222A cell line was significantly different from the parental line.

DISCUSSION

The creation of stably transfected A6 cell clones expressing sgk and sgk mutants S422A and D222A provides epithelial cell models in which to examine the relationship between sgk and ENaC-mediated transport. Previous studies provided evidence that coexpression of sgk in Xenopus oocytes with the α-, β-, and γ-subunits of ENaC significantly increases Na⁺ current (7, 14, 15, 21). However, no analogous studies have been reported in polarized epithelial cells where regulators of the channel as well as the channel itself are naturally present.

Our electrophysiological results performed on native polarized epithelial cells are consistent with oocyte studies (7, 15, 21). Increased amounts of functional sgk in A6 cells elevates basal levels of Na⁺ transport 3.5-fold over those observed in the parental cell line. We have also extended these studies to examine the effect of point mutations in both a COOH-terminal phosphorylation site and the kinase domain of sgk.

The mutation of S422A prevents phosphorylation of the protein by an upstream regulator, PDK-2 (13, 16). In contrast to the wild type, the S422A mutant does not appear to be active in the unstimulated A6 cells. The cells transfected with the S422A sgk mutant protein have basal transport rates that are the same as those of the parental line or cells transfected with an empty vector. However, expression of the sgk D222A point mutant, which possesses an inactive kinase, not only fails to increase basal current but also inhibits the amount of basal Na⁺ transport compared with the parent line. These findings suggest that the D222A mutant protein competes with endogenous sgk for substrate binding. Functionally, therefore, these mutants act as dominant-negative effectors and serve to under-score the importance of sgk to the maintenance of transepithelial Na⁺ transport.

sgk is activated by multiple phosphorylation events. The serine/threonine kinases PDK-1 and -2 positively regulate enzyme activity (6, 13); PKA has also recently been shown to activate sgk by phosphorylation in response to increases in cAMP (17). It is clear that the phosphorylation cascades that regulate sgk are complex, resulting in multiple phosphorylations, and therefore, the sgk protein might be expected to have a series of isoelectric points. The heterogeneity observed in the isoelectric points of the protein, which is immunoprecipitated by an anti-sgk antibody, is consistent with constitutive levels of phosphorylation at multiple sites (Fig. 1). It is likely that these activating phosphorylation events are necessary for maintaining the basal Na⁺ transport seen in these renal cells maintained under our tissue culture conditions.

The cell lines have been used to explore whether sgk also plays a functional role in hormone-stimulated transport in epithelial cells. Our electrophysiological results are consistent with previous studies indicating that sgk plays a role in the aldosterone-stimulated natriferic pathway (7, 15). Expression of the dominant-negative D222A mutant form of sgk inhibits the ability of the cells to respond to aldosterone. However, our data are not consistent with the contention that sgk synthesis is the rate-limiting step in aldosterone-stimulated Na⁺ transport. The recombinant sgk is driven by the cytomegalovirus promoter and is, therefore, not expected to be regulated by serum or aldosterone. Accordingly, when serum is removed from the medium for 18–24 h, the sgk-transfected cultures maintain an increased transport rate that is significantly elevated over the parental cells that are maintained in serum-containing medium. Assuming that sgk activity is de-

Fig. 4. Insulin responses in the A6 parental and transfected cell lines. Cells were stabilized to a baseline current before the addition of 100 nM insulin to the serosal bathing medium. An increase in amiloride-sensitive transport was measurable within 5 min and reached a maximal level in 10–20 min. Bars indicate the maximal increase in transport from the basal level (ΔSCC). Values are represented as means ± SE. At a P < 0.01 level, only the D222A cell line was significantly different from the parental line.

Fig. 5. Antidiuretic hormone (ADH) responses in the A6 parental and transfected cell lines. Cells were stabilized to a baseline current before the addition of 100 mU/ml ADH to the serosal bathing medium. An increase in amiloride-sensitive transport was detected within 1 min and reached a maximal level in 5–10 min. The bars indicate the maximal increase in transport from the basal level (ΔSCC). Values are represented as means ± SE. At a P < 0.01 level, the D222A and SGK-1 cell lines were significantly different from the parental line.
dependent on phosphorylation of the protein, these findings are consistent with a previous study (17) showing that 32P incorporation into recombinant sgk in COS7 cells was not entirely eliminated after 16 h of serum deprivation. However, when cells are continuously short-circuited in serum-free medium, the basal transport rate in the sgk-transfected cells begins to decline within a few hours, reaching the level of similarly treated control (nontransfected or empty vector transfected) cells within 5–6 h. Aldosterone prevents the decline, maintaining the sgk-transfected cells at a level above what is achievable with aldosterone treatment of the parental line. Thus it appears that in the absence of serum but the presence of additional sgk, aldosterone induces another factor to maintain the increased transport rate. These data are consistent with our previous observation (5) that aldosterone causes an increase in the activity of PtdIns 3-kinase and suggest that this steroid hormone may also induce a rate-limiting step earlier in the phosphoinositide pathway. Cells containing the S422A mutant have a steroid hormone response similar to that of the parent line, providing further evidence that this mutant cannot be activated in the cells.

Insulin stimulation in the A6 cell line results in a predictable increase in Na+ transport (7–10 µA/cm2) that is independent of the basal starting current (8). We also recently demonstrated (2) that this arithmetic increase is maintained in A6 cells that were stably transfected with ENaC subunits, indicating that, in stark contrast to the basal transport, insulin-stimulated Na+ flux is independent of the quantity of functional α- and β-subunits.

With insulin stimulation, A6 cells expressing the transfected wild-type sgk demonstrate the same magnitude change in Na+ transport observed in the parental cell line. Thus the expression of additional sgk does not potentiate the natriuretic response, indicating that this is not a rate-limiting step in insulin action. sgk is, however, a necessary component in insulin-induced signal transduction cascade as evidenced by the lack of an insulin response in cells expressing the kinase-dead mutant, D222A.

We showed previously (18) that PtdIns 3-kinase activity is increased in response to insulin in the A6 cell line. In a manner analogous to the aldosterone response, these data indicate the presence of an insulin-sensitive rate-limiting step between PtdIns 3-kinase and sgk that controls the magnitude of the response to this peptide hormone.

In contrast to the insulin results, our studies indicate that altering the levels of sgk in A6 cells affects the rate of ADH-induced Na+ reabsorption. The sgk clones exhibit a potentiated response to ADH. It is well documented that ADH stimulation of Na+ transport is mediated via adenylate cyclase/PKA signaling. One possible explanation for the potentiated response to ADH is provided by the recent demonstration that PKA can phosphorylate and activate sgk (17). However, if the sgk protein cannot be activated by PDK-2 (S422A mutant), the presumptive PKA phosphorylation does not activate Na+ transport. These data are consistent with recent studies in COS7 cells in which the enzyme activity of the S422A mutant could not be stimulated by insulin or 8-(4-chlorophenythio)adenosine 3’,5’-cyclic monophosphate (17). Thus it appears that phosphorylation of the permissive PDK-2 site is required for activation due to phosphorylation at other sites on the protein.

The functional mechanisms of sgk activation of ENaC are unknown. Recently, it was reported that in rat distal nephron segments ENaC traffics toward the apical cellular pole coincidentally with the induction of sgk (14). The mechanism of ENaC stimulation by sgk has also been investigated in oocytes coexpressing sgk and ENaC. Alvarez de la Rosa and colleagues (1) used patch-clamp analysis to demonstrate that the P0 is not altered by sgk. In addition, these authors showed that the kinase alters the abundance of ENaC in the plasma membrane (1). These results are consistent with recent results regarding functional changes in epithelial cells in response to hormonal stimuli. Blocker-induced noise analysis has been used to assess changes in channel activity in epithelial cells in response to insulin, aldosterone, and ADH (4, 9, 11). An increase in functional channel density with no increase in P0 was found in response to each hormone. In addition, using labeled channel subunits, we recently demonstrated (2) that ADH stimulates the insertion of ENaC into the apical membrane from a subapical pool of channels. Thus the hormones regulate functional channel density, and sgk appears to be a required factor in this process. Although it is unlikely that sgk is the rate-limiting step in either insulin- or aldosterone-stimulated Na+ transport, sgk activity is an essential component of basal as well as hormone-stimulated Na+ transport in the A6 cell line. Expression of a dominant-negative mutant of sgk inhibits all natriuretic activity.

The clonal lines that have been created as part of these studies will be useful to examine in detail the complex phosphorylation events regulated by hormonal stimulation. A complete elucidation of the role of sgk is important for developing additional therapeutic targets for hypertension and other diseases involving regulation of salt and fluid homeostasis.

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Present address of S. I. Taylor: Lilly Research Laboratories, Mail Drop 0424, Indianapolis, IN 46285.

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