Serum and Glucocorticoid-regulated Kinase Modulates Nedd4-2-mediated Inhibition of the Epithelial Na\textsuperscript{+} Channel*

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The epithelial Na\textsuperscript{+} channel (ENaC) forms the pathway for Na\textsuperscript{+} absorption across epithelia, including the kidney collecting duct, where it plays a critical role in Na\textsuperscript{+} homeostasis and blood pressure control. Na\textsuperscript{+} absorption is regulated in part by mechanisms that control the expression of ENaC at the apical cell surface. Nedd4 family members (e.g. Nedd4, Nedd4-2) bind to the channel and decrease its surface expression by catalyzing its ubiquitination and degradation. Conversely, serum and glucocorticoid-regulated kinase (SGK), a downstream ligase (including Nedd4 and Nedd4-2) reduce ENaC surface expression (7–9). They contain multiple WW domains that bind to PY motifs in the C termini of α-, β-, and γENaC (10–12). This interaction facilitates the ubiquitination of ENaC, catalyzed by a ubiquitin ligase domain at the C terminus of Nedd4 family members (7, 8). Ubiquitination reduces ENaC at the cell surface by increasing the rate of channel degradation (7). Liddle’s syndrome is caused by defects in this regulatory pathway; mutations in the ENaC PY motifs disrupt their interaction with Nedd4 family members, resulting in increased expression of ENaC at the cell surface, and hence, excessive Na\textsuperscript{+} absorption (10, 13, 14). Thus, the Nedd4 family of proteins is critically important in reducing Na\textsuperscript{+} absorption.

Conversely, the renin-angiotensin-aldosterone pathway increases renal Na\textsuperscript{+} absorption, in part by increasing the expression of ENaC at the cell surface (15). This pathway plays a key role in responding to Na\textsuperscript{+} depletion and hypovolemia. Moreover, disruption of this pathway underlies several acquired and genetic disorders of blood pressure control, including primary aldosteronism and glucocorticoid-remediable aldosteronism (5). An important downstream mediator of aldosterone is serum and glucocorticoid-regulated kinase (SGK), (16, 17). SGK transcription is induced by aldosterone over a very rapid time course (30–60 min) (16, 17), and it is post-translationally activated in response to insulin and other stimuli by phosphorylation through the phosphoinositide 3-kinase pathway (18). Thus, it has been proposed that SGK integrates a variety of signals that modulate renal Na\textsuperscript{+} absorption (19). SGK increases the expression of ENaC at the cell surface (20), but little is known about the mechanisms involved. Previous work reported that SGK phosphorylates Ser/Thr residues within the sequence RXRXXS/T (18, 21). However, ENaC subunits are not phosphorylated by SGK (19). Thus, it seems likely that SGK phosphorylates one or more proteins involved in controlling ENaC surface expression, although such SGK substrates have not yet been identified.

Two observations suggest the possibility that the Nedd4 family and SGK might converge in a common pathway to regulate ENaC surface expression. First, SGK contains a PY motif (see Fig. 1A), suggesting that it might bind directly to WW domains in Nedd4 or Nedd4-2. Second, Nedd4-2 (but not Nedd4) contains three sequences that fit the consensus for WW domains in Nedd4 or Nedd4-2. Second, Nedd4-2 (but not Nedd4) contains three sequences that fit the consensus for WW domains in Nedd4 or Nedd4-2. Two observations suggest the possibility that the Nedd4 family and SGK might converge in a common pathway to regulate ENaC surface expression. First, SGK contains a PY motif (see Fig. 1A), suggesting that it might bind directly to WW domains in Nedd4 or Nedd4-2. Second, Nedd4-2 (but not Nedd4) contains three sequences that fit the consensus for WW domains in Nedd4 or Nedd4-2.
disrupt binding to the hNedd4-2 WW domains. A FLAG epitope (DYKDDDDK) was introduced at the C terminus of SGK to allow immunodetection. This epitope did not alter ENaC stimulation by SGK.

**Binding of SGK to hNedd4 and hNedd4-2**—cDNAs encoding wild-type or mutant (Y298A or K127M) SGK or GFP (negative control) was expressed in COS-7 cells by electroporation, as described previously (13). The cells were lysed and protein solubilized in TBS (150 mM NaCl, 50 mM Tris, pH 7.4) containing 1% Triton X-100 and protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 10 μg/ml pepstatin A). SGK was immunoprecipitated from 100 μl of lysate (1 μg/μl total protein) with anti-FLAG M2 monoclonal antibody (1:1000, Eastman Kodak Co.) and protein A beads (Pierce). hNedd4-2 and hNedd4 (20 μl) were generated and [35S]methionine-labeled by in vitro transcription and translation (TNT kit, Promega) and then incubated with immunoprecipitated SGK, SGK-K298A (or GFP) for 16 h. The beads were washed three times with TBS/1% Triton X-100, separated by SDS-PAGE, and imaged by fluorography.

To detect total SGK, SGK-V298A, or SGK-K127M, 30 μg of lysate was separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and blocked overnight with 5% dry milk in TBS containing 0.1% Triton X-100. The membrane was incubated for 2 h with anti-FLAG M2 antibody (1:1000), 1 h with horseradish peroxidase-coupled sheep anti-mouse IgG (1:50,000, Amersham Biosciences, Inc.), and imaged by chemiluminescence (ECL Plus, Amersham Biosciences, Inc.).

**Expression and Electrophysiology in FRT Epithelia**—FRT cells were grown on permeable filter supports as described (22). The apical and basolateral surfaces were bathed in 135 mM NaCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 100 mM orthovanadate, 2.4 mM K2HPO4, 0.6 mM KH2PO4, 10 mM dextrose, 10 mM -glycerol phosphate, 5 mM EGTA, 1 mM Na orthovandate, 1 mM MgCl2, 20 μM aprotinin, 20 μM leupeptin, and 10 μM pepstatin A. SGK was expressed in COS-7 cells using anti-FLAG M2 antibody (1:1000). The total DNA was held constant by varying the ratio of SGK-K127M, and to immunoprecipitated protein from cells expressing GFP as a negative control (0.02–0.8 μg using TFX 50 (22)). The total DNA was held constant by varying the ratio of hNedd4-2 or SGK to GFP. Expression of GFP did not alter ENaC Na+ currents.

Na+ transport was measured 2–3 days after transfection in modified Ussing chambers (Warner Instrument Corporation). The apical and basolateral surfaces were bathed in 135 mM NaCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 2.4 mM K2HPO4, 0.6 mM KH2PO4, 10 mM dextrose, 10 mM orthovanadate, pH 7.4, at 37°C and bubbled with O2. Amiloride-sensitive short-circuit current was determined as the difference in current with and without amiloride (10 μM) in the apical bathing solution.

**SGK Phosphorylation of hNedd4-2 and hNedd4**—cDNAs encoding hNedd4-2, hNedd4, or GFP (negative control) were expressed in COS-7 cells, solubilized in TBS containing 1% Triton X-100, and 250 μg of the lysates were immunoprecipitated with sheep anti-WW (hNedd4-2 and GFP) or sheep anti-WW2 (hNedd4) (1:1000). Immunoprecipitated protein was suspended in 15 mM MgCl2, 100 μM ATP, 20 mM MOPS, pH 7.2, 2.5 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na orthovanadate, 1 mM MgCl2, 20 μM aprotinin, 20 μM leupeptin, an anti-FLAG M2 antibody (1:1000), and 10 μC of [γ-32P]ATP. The samples were incubated with or without activated SGK (25 ng of SGK1 Δ1–60, S422D, Upstate Biotechnology) for 60 min at 30°C. The beads were washed three times with 500 μl of TBS containing 1% Triton X-100 and proteins detected by SDS-PAGE and fluorography. To detect total hNedd4-2 and hNedd4 protein, COS-7 cells were labeled for 1 h with [35S]methionine, and cell lysates were immunoprecipitated as described above.

**Binding of hNedd4-2 to ENaC—hNedd4-2, hNedd4, or GFP** were expressed in COS-7 cells were immunoprecipitated, incubated with or without activated SGK (1 mM cold ATP substituted for [γ-32P]ATP) and incubated for 16 h with 20 μl of a6ENaC (transcribed, translated, and [35S]methionine-labeled in vitro). The beads were washed three times with TBS 1% Triton X-100, separated by SDS-PAGE, and imaged by fluorography.

**RESULTS**

**SGK Binds to hNedd4-2 and hNedd4**—The PY motif (PPXY) is a sequence that mediates protein interactions through its binding to type I WW domains (23). SGK contains a sequence that fits the PY motif consensus (PPFY, amino acids 295–298, Fig. 1A). Members of the Nedd4 family contain multiple WW domains, which bind to PY motifs in ENaC. We tested the hypothesis that their WW domains might also bind to SGK. SGK (containing a FLAG epitope at the C terminus) was expressed in COS-7 cells. We detected SGK protein by Western blot (using anti-FLAG M2 antibody) in cells expressing SGK, but not in cells expressing GFP (Fig. 1B). To test for interactions, we incubated immunoprecipitated SGK with hNedd4-2 or hNedd4 (generated and [35S]methionine-labeled by in vitro translation). Both hNedd4-2 and hNedd4 bound to SGK, but not to immunoprecipitated protein from cells expressing GFP (Fig. 1C). The PY motif of SGK mediated these interactions; mutation of a critical residue within the motif (Y298A) abolished SGK binding to hNedd4-2 and hNedd4 (Fig. 1C). This did not result from decreased protein production; the mutant and wild-type constructs generated similar amounts of SGK protein (Fig. 1D).

To investigate the functional role of this interaction, we asked whether the SGK PY motif was required to stimulate ENaC. Expression of α-, β-, and γENaC in FRT epithelial cells generated transepithelial short-circuit Na+ currents that were blocked by amiloride (22). Coexpression of ENaC with SGK increased Na+ current 2.7-fold (compared with ENaC expressed with GFP) (Fig. 1F). In contrast, SGK-V298A did not stimulate ENaC (Fig. 1D), suggesting that the SGK PY motif is required for stimulation.

**SGK Phosphorylates hNedd4-2**—We tested whether SGK kinase activity is required for it to stimulate ENaC in epithelia. In a previous study, mutation of a residue in the ATP binding site (SGK-K127M) abolished the ability of SGK to phosphorylate a peptide substrate (18). We found that this mutation prevented SGK from stimulating ENaC expressed in FRT cells (Fig. 1D), but it did not alter levels of SGK protein (Fig. 1E). This suggests that SGK stimulates ENaC by phosphorylating one or more substrates.

**SGK phosphorylates serine or threonine residues in the context of the sequence RXRXXS/T (18, 21). Interestingly, hNedd4-2 contains three sequences that fit this consensus (Ser221, Thr246, and Ser277), but they are not conserved in hNedd4 (Fig. 2A). We therefore tested the hypothesis that hNedd4-2 is a substrate for SGK phosphorylation. hNedd4-2, hNedd4, or GFP (negative control) were expressed in COS-7 cells, immunoprecipitated, and incubated with [γ-32P]ATP with or without an activated form of SGK (Δ1–60, S422D). We found that SGK phosphorylated hNedd4-2 but not hNedd4 (Fig. 2B). As a control for expression, we labeled cells with [35S]methionine and immunoprecipitated with antibodies against the WW domains. Bands of the appro-
SGK Modulates the Function of hNedd4-2—We tested the hypothesis that phosphorylation alters hNedd4-2 function. ENaC was coexpressed with SGK or GFP (negative control) in FRT cells, along with increasing amounts of hNedd4-2 cDNA. When ENaC was expressed with GFP, hNedd4-2 decreased Na⁺ current in a dose-dependent manner (Fig. 3A). In contrast, hNedd4-2 reduced current to a lesser extent when expressed with SGK (Fig. 3A). However, a kinase inactive mutant (SGK[K127M]) did not decrease inhibition (Fig. 3A), suggesting that phosphorylation was required. Thus, SGK-mediated phosphorylation modulates hNedd4-2 function, decreasing its ability to inhibit ENaC.

Phosphorylation Alters hNedd4-2 Binding to ENaC—For hNedd4-2 and hNedd4 to inhibit Na⁺ current, their WW domains must bind to PY motifs in ENaC (7–9). Interestingly, the SGK consensus sites are located between the WW domains of hNedd4-2, suggesting that phosphorylation might alter its binding to ENaC. To test this hypothesis, we immunoprecipitated hNedd4-2 or hNedd4 from COS-7 cells, followed by incubation with one of the ENaC subunits (αENaC, translated in vitro and labeled with [35S]methionine). We chose to use the α subunit, since previous work suggested that the three ENaC subunits are equivalent in their binding to WW domains (9, 11, 12, 24). αENaC bound to hNedd4-2 and hNedd4, but not to immunoprecipitated lysates from cells expressing GFP (Fig. 3B). Phosphorylation of hNedd4-2 by SGK decreased the binding of hNedd4-2 to αENaC (Fig. 3B). In contrast, SGK caused minimal change in the binding of hNedd4 to αENaC (Fig. 3B), consistent with our finding that SGK did not phosphorylate hNedd4.

The data suggest that SGK might increase Na⁺ current in part by decreasing the binding of hNedd4-2 to ENaC. Such a mechanism should therefore be disrupted by mutation of the ENaC PY motifs, which are required for this interaction. To test this hypothesis, we expressed ENaC (wild-type or PY motif mutations) with SGK (or GFP as negative control) in FRT cells and measured amiloride-sensitive short-circuit Na⁺ currents. SGK increased Na⁺ current in cells expressing wild-type ENaC (167%) but not when the PY motifs were mutated in α-, β-, and γENaC (Fig. 3C). Thus, in epithelial cells, stimulation by SGK was dependent on the PY motifs of ENaC.

DISCUSSION

The regulation of ENaC is critically important to maintain Na⁺ homeostasis. Disruption of this regulation causes genetic and acquired forms of hypertension and hypotension (5). Two important regulators of ENaC are aldosterone and its downstream mediator SGK, and the Nedd4 family of ubiquitin protein ligases, which modulate ENaC surface expression in a reciprocal manner. Our data suggest that these two pathways intersect, regulating ENaC in part through a common pathway.

The data support a model in which SGK regulates Na⁺ absorption in part by modulating the inhibition of ENaC by hNedd4-2 (Fig. 4). Under basal conditions (low aldosterone), hNedd4-2 is unphosphorylated and represses epithelial Na⁺ transport; its WW domains bind to ENaC PY motifs, resulting in ubiquitination, endocytosis, and degradation of the channel (Fig. 4, left panel). As a result, hNedd4-2 decreases Na⁺ current by reducing the expression of ENaC at the cell surface. In response to salt deprivation or in pathophysiological states, aldosterone releases this repression of Na⁺ absorption. Aldosterone stimulates the transcription of SGK (16, 17), which binds to hNedd4-2 and phosphorylates one or more Ser/Thr consensus sites (Fig. 4, right panel). The binding of SGK to hNedd4-2 appears to be essential, since mutation of the SGK PY motif prevented it from stimulating ENaC. Phosphorylation of hNedd4-2 reduces its binding to ENaC, resulting in increased ENaC at the cell surface, and hence, increased Na⁺ absorption.

How might phosphorylation alter the binding of hNedd4-2 to ENaC? Although we do not yet have direct evidence to support a mechanism, it is interesting to note that the potential phosphorylation sites are located in segments between WW domains. For example, Ser221 and Thr246 are located between WW domains 1 and 2, and Ser227 is located between WW domains 2 and 3 (Fig. 2A). Perhaps phosphorylation induces a conformational change in hNedd4-2, altering the accessibility of the WW domains to bind ENaC. Interestingly, GenBank™ contains at least three different hNedd4-2 splice forms, differing in the number of potential SGK phosphorylation sites. The splice form we studied (Nedd4La, also known as Nedd18) corresponds most closely to mouse Nedd4-2 and has three potential phosphorylation sites. A second form, KIAA0439, lacks a 20-amino acid segment between WW domains 1 and 2, which deletes one of the SGK sites (Thr246). A third variant, DKKzip234p2422, lacks the second and third SGK sites, as well as WW domain 2. Importantly, each splice form appears to be
endocytosis/degradation. In this way, SGK could increase Na\(^+\) transport in epithelia, despite its ability to inhibit ENaC expressed in collecting duct epithelia, and all three inhibit ENaC (Fig. 3 and Ref. 25). Thus, it seems possible that the splice forms could be differentially phosphorylated, and thus, differentially regulated by SGK. Alternatively, perhaps only the site present in all three forms is phosphorylated (Ser\(^{251}\)).

What is the function of the interaction between SGK and hNedd4-2? A recurring theme is that many signaling molecules assemble into complexes to target their activity to specific subcellular domains. Interactions between signaling molecules can occur through adapter proteins, such as protein kinase A anchoring proteins (26). Interactions can also be direct; phosphorylation of the N-methyl-D-aspartic acid receptor by calcium- and calmodulin-dependent protein kinase II is mediated by a direct interaction (27). Thus, perhaps SGK binding targets its kinase activity to hNedd4-2. Binding can also be required to activate a kinase; N-methyl-D-aspartic acid receptor binding activates calcium- and calmodulin-dependent protein kinase II, a mechanism thought to be important for learning and memory (28). Similarly, SGK might be activated through its binding to hNedd4-2.

In addition to hNedd4-2, the function of ENaC may also be regulated by other ubiquitin protein-ligases. Ned4 is expressed in the renal collecting duct (29) and inhibits ENaC (7, 8, 30). However, SGK consensuses phosphorylation sites are not present in Ned4, suggesting that SGK does not modulate the activity of this ubiquitin protein-ligase. Consistent with such a hypothesis, we found that SGK did not phosphorylate hNedd4 or alter its binding to αENaC. This raises several possibilities. Perhaps Ned4 inhibits ENaC in a constitutive manner. More likely, the inhibition of ENaC by Ned4 might be regulated by different mechanisms. In this regard, it has been reported that cystolic Ca\(^{2+}\) alters the localization of Ned4, resulting in its translocation to the cell surface (31). Alternatively, Ned4 might be phosphorylated by a different kinase or might be transcriptionally regulated. Finally, it is possible that Ned4 does not regulate Na\(^+\) transport in epithelia, despite its ability to inhibit ENaC expression in heterologous cells.

Could SGK modulate ENaC function by additional mechanisms? Several observations raise this possibility. For example, it is possible that the SGK PY motif competes with ENaC for binding to the WW domains of hNedd4-2 (or other ubiquitin protein-ligases). In this way, SGK could increase Na\(^+\) current by directly blocking the binding of hNedd4-2 to ENaC. However, competition for hNedd4-2 binding does not appear to be the sole mechanism, since SGK kinase activity was also required for ENaC stimulation and for SGK to modulate hNedd4-2 function. In addition, SGK might stimulate ENaC through mechanisms independent of hNedd4-2. In support of this possibility, in Xenopus oocytes, mutation of the ENaC PY motifs did not prevent SGK from increasing Na\(^+\) current (20), in contrast to our data in epithelia. It was also reported that SGK could bind directly to the C terminus of α- and βENaC (19). However, SGK did not phosphorylate ENaC, and the functional role and the sequences that mediate this interaction are not yet known.

Our data suggest that aldosterone, SGK, and hNedd4-2 converge into a common pathway to regulate Na\(^+\) absorption. Within this pathway, hNedd4-2 and related members of the Nedd4 family might form a central point of convergence to regulate ENaC surface expression, and hence, to maintain Na\(^+\) homeostasis.

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