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Serum and Glucocorticoid-induced Kinase (SGK) 1 and the Epithelial Sodium Channel Are Regulated by Multiple with No Lysine (WNK) Family Members*[S]

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The four WNK (with no lysine (K)) protein kinases affect ion balance and contain an unusual protein kinase domain due to the unique placement of the active site lysine. Mutations in two WNKs cause a heritable form of ion imbalance culminating in hypertension. WNK1 activates the serum- and glucocorticoid-induced protein kinase SGK1; the mechanism is noncatalytic. SGK1 increases membrane expression of the epithelial sodium channel (ENaC) and sodium reabsorption via phosphorylation and sequestering of the E3 ubiquitin ligase neural precursor cell expressed, developmentally down-regulated 4-2 (Nedd4-2), which otherwise promotes ENaC endocytosis. Questions remain about the intrinsic abilities of WNK family members to regulate this pathway. We find that expression of the N termini of all four WNKs results in modest to strong activation of SGK1. In reconstitution experiments in the same cell line all four WNKs also increase sodium current blocked by the ENaC inhibitor amiloride. The N termini of the WNKs also have the capacity to interact with SGK1. More detailed analysis of activation by WNK4 suggests mechanisms in common with WNK1. Further evidence for the importance of WNK1 in this process comes from the ability of Nedd4-2 to bind to WNK1 and the finding that endogenous SGK1 has reduced activity if WNK1 is knocked down by small interfering RNA.

WNKs[5] (with no lysine (K)) are large protein-serine/threonine kinases found in all multicellular and a few unicellular eukaryotes (1). WNK1, the first member of the family identified in mammals, was found in searches for novel components of protein kinase cascades (2). WNK1 is expressed ubiquitously, consistent with effects on many cell types (2–4). Numerous splice forms, containing from just under 2000 to almost 2400 residues, are known, including one lacking most of the kinase domain (KS-WNK1), which is enriched in kidney (5, 6).

The four WNK family members are distinct from all other protein kinases in that their catalytic lysine is shifted from its usual position buried in the N-terminal part of the kinase core to a more exposed position in the glycine-rich loop (2, 7). The strict conservation of the unique catalytic core structure of the WNK family in organisms such as *Chlamydomonas*, *Phycomyces*, *Arabidopsis*, and mammals suggests conserved properties for these kinases.

Our initial characterization of WNK1 revealed that the kinase activity is sensitive to hypertonic stress (2). The subsequent discovery that WNK1 and WNK4 are genetically linked to a rare type of hypertension, pseudohypoaldosteronism type 2 (PHA2) (4), demonstrated the importance of WNK function in man and an implicit significance of the sensitivity of WNK1 kinase activity to osmotic stress. Further characterization showed that activity is increased in response to increased and decreased ionic strength (8). The consequences of WNK mutations in PHA2 are hyperkalemia, renal tubular acidosis, and eventually hypertension (1, 9–11). WNK1 knock-out mice do not survive, but heterozygotes have low blood pressure (12), consolidating the concept that WNK1 impacts blood pressure directly.

The investigation of the actions of WNKs led to some of the first and most logical connections to ion transporters and channels, *e.g.* the NaCl cotransporter (NCC), NaCl KCl cotransporters (NKCC), the renal outermedullary potassium channel, and the epithelial sodium channel (ENaC) (13–22). Although a connection between WNKs and ion transport proteins is predictable, the mechanisms driving regulation of transporters by WNKs are not.

The biochemical analysis of WNK1 action and the identification of WNKs in genome and kinome screens have suggested mechanisms that are beginning to be linked to transporter regulation. For example, WNKs have been found in screens of kinases important in endocytosis (23). WNK1 and one or more of the other family members are thought to regulate several

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[5] The abbreviations used are: WNK, with no lysine; CHO, Chinese hamster ovary; ENaC, epithelial sodium channel; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; PHA2, pseudohypoaldosteronism type 2; SGK, serum and glucocorticoid-induced kinase; siRNA, small interfering RNA; KS, kidney specific.

protein kinases that modulate ion transport, including the serum and glucocorticoid- inducible protein kinase SGK1, which affects ENaC, and the kinases oxidative stress-responsive 1 (OSR1) and the serine-, proline-, alanine-rich kinase SPAK, which control activities of NaCl KCl cotransporters, the NaCl cotransporter, and some related ion cotransporters (15, 18–20, 24–26).

SGK1 interferes with endocytosis of ENaC by phosphorylating the E3 ubiquitin ligase neural precursor cell expressed, developmentally down-regulated 4-2 (Nedd4-2) (27, 28). Nedd4-2 promotes ENaC endocytosis, reducing sodium reabsorption (29). Phosphorylation by SGK1 results in 14-3-3 binding, which prevents Nedd4-2 function (30). We made the key observations that SGK1 and ENaC are regulated by WNK1 (15). The kinase activity of WNK1 is not required for SGK1 activation; thus, how WNK1 controls SGK1 activity remains unclear (24). Effects of WNKs on ENaC are controversial. Some studies suggest that WNKs may inhibit or have no effect on ENaC (31–33). Because questions remain about the abilities of WNKs to regulate this pathway, in this study we have examined the effects of the four WNK family members on SGK1 and ENaC activities using the same reconstituted system, and we have further explored some of their biochemical properties.

EXPERIMENTAL PROCEDURES

Materials—Plasmids encoding mouse SGK1, rat WNK1 and WNK2, and human WNK3 and WNK4 fragments were as described (2, 34, 35). ENaC cDNAs were from mouse, and Nedd4-2 cDNA was from human. Additional fragments were subcloned by standard methods. Site-directed mutagenesis was performed with the QuikChange kit (Stratagene) or by PCR. DNAs were provided by the following: SGK1, from M. E. Greenberg (Harvard); SGTK2 and SGK3, from Orson Moe (University of Texas Southwestern);WNK3, from R. Lifton and K. Kahle (Yale); Nedd4-2, from P. M. Synder (Iowa); and ENaC α, β and γ subunits, from J. D. Stockand (San Antonio). Anti-WNK1 (Gln^{256}) was as described (2). Rabbit anti-SGK1 (U6213) was generated using recombinant SGK1(61–428). Anti-WNK1 phosphothreonine 58 was from PhosphoSolutions. Crosstide (GRPRTSSFAEGRR) was made by the University of Texas Southwestern peptide synthesis facility. Double-stranded RNA oligonucleotides were from Ambion (36).

Cell Culture and Transfection—HEK293, HeLa, and CHO cells were grown using standard conditions (15, 24). Transfection was mediated with either calcium phosphate for kinase assays or FuGENE 6 (Roche Diagnostics) for current measurements. For kinase assays, cells were harvested in isotonic lysis buffer containing 1% Triton X-100 and phosphatase and protease inhibitors as described (37). As indicated, 10 μM U0126 or 50 nM wortmannin was added to cells 1 h or 30 min prior to harvest, respectively. For RNA interference, cells were grown to ~30% confluence in 6-well plates and transfected with double-stranded RNA oligonucleotides using Oligofectamine (Invitrogen) as described (35). On the following day, plasmids were transfected into the cells which were harvested 48 h later. Human siRNA sequences: WNK1 sense, CAGACAGUGCAGUAUUCACTT and antisense, GUGAAUACUGCACUGUCUGTT (Ambion); SGK1 sense, GUCCUUCUCAGCAAAUCAA and antisense, blockUUGAUUUGCUGAGAAGGAC (Santa Cruz); Nedd4-2 strand A sense, GGAAGCUCUUAGAUGGUUU and antisense, AAACCAUCUAAGAGCUUCC; strand B sense, GCAUUUGCCUAACAGACUU and antisense, AAGUCUGUUAGGCAAAUGC; strand C sense, GAAGGCACUUUAUGUACUA and antisense, UAGUACAUAAAGUGCCUUC (Santa Cruz). By PCR, SGK1 mRNA was reduced by 30–40%, and Nedd4-2 mRNA was reduced by 60–70%.

Immunoprecipitation, Immunoblotting, and Protein Kinase Assays—Proteins were immunoprecipitated from cell lysates with an antibody to lysate ratio of 1:100 and collected using protein A-Sepharose beads. Proteins in cell lysates or immunoprecipitates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for immunoblotting. Blots were developed using enhanced chemiluminescence. *In vitro* kinase assays of immunoprecipitates were performed as described using either recombinant Nedd4-2 or crosstide as substrates (15). Recombinant proteins used as substrates or for *in vitro* kinase assays were purified from *Escherichia coli* strain BL21 using standard protocols.

Induction of Endogenous SGK1—SGK1 was induced by treating MDA-MB-231 cells that had been serum-deprived for 24 h with dexamethasone and the proteasome inhibitor MG132 as described by the Conzen laboratory (38).

Whole Cell Patch Clamp Recording of ENaC Channels—HEK293 cells were used in reconstitution experiments as previously described for CHO cells (24). Cells were cotransfected with plasmids encoding α, β, and γ ENaC subunits, fragments of each of WNK isoforms corresponding to WNK1(1–491) (see Fig. 1*A*), enhanced green fluorescent protein, and with or without wild type SGK1, kinase-dead SGK1, wild type Nedd4-2, siRNA for SGK1, siRNA for Nedd4-2, or control oligonucleotides (200 nM) as indicated. In each experiment, the total amount of DNA for transfection was equalized using empty vectors. At 36–48 h after transfection, ruptured whole cell recordings were performed using an Axopatch 200B amplifier. Transfected cells were identified by using epifluorescence microscopy. The pipette and bath solution contained 135 mM potassium methanesulfonate, 10 mM NaCl, 10 mM Hepes (pH 7.2), and 145 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes (pH 7.4), respectively. Capacitance and access resistance were monitored and 75% compensated. The voltage protocol consists of a 0-mV holding potential and 400-ms steps from −100 to 100 mV in 20-mV increments. Amiloride-sensitive currents were determined by subtracting currents in the presence of amiloride (10 μM) from the total currents. Statistical comparison between two groups was made using the two-tailed unpaired *t* test. Multiple comparisons were determined using one-way analysis of variance followed by Tukey's multiple comparison tests. p values < 0.05 and 0.01 were considered significant for single and multiple comparisons, respectively.

RESULTS

Activation of SGK1 and ENaC by WNK Family Members—We compared activation of SGK1 and ENaC by WNK1 in the HEK293 cell line, the same line used for ENaC reconstitution below. Expression of an N-terminal fragment of WNK1(1–220) (Fig. 1*A*) activated SGK1 roughly in proportion to the amount

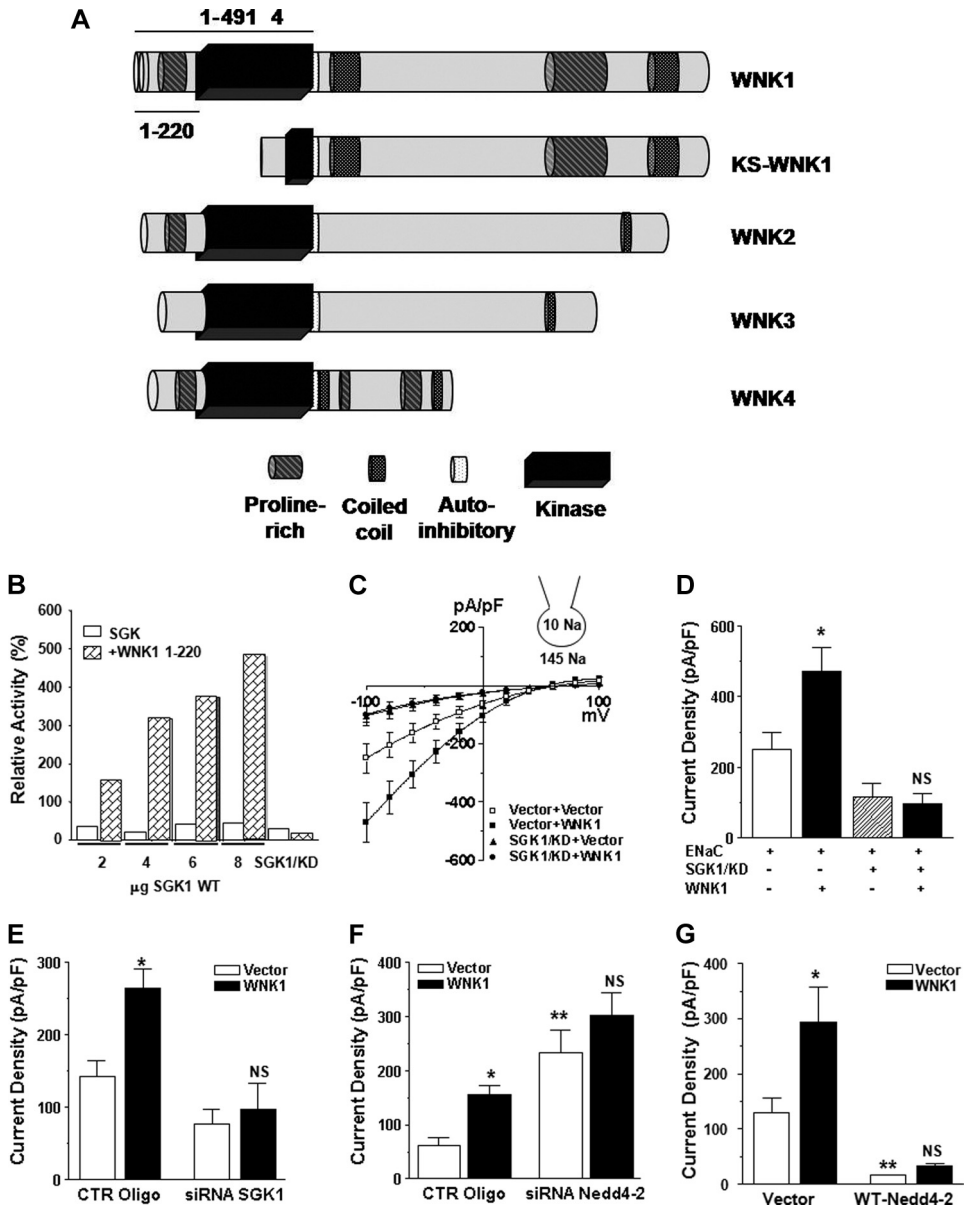

FIGURE 1. **Activation of SGK1 and ENaC by WNK1.** *A*, schematic diagram of WNKs 1–4. *B*, increasing amounts of wild type SGK1 or kinase-dead SGK1 cotransfected with WNK1(1–220) or vector control. The immunoprecipitated SGK1 was assayed with crosstide as substrate. This experiment was repeated twice in triplicate. The activity increase caused by WNK1 was significant for each amount of SGK1. $p < 0.001$ *C*, current-voltage (*I-V*) relationships of currents shown in *D*. Whole cell amiloride-sensitive currents were recorded with the intracellular and extracellular [Na^+] at 10 and 145 mM, respectively. Currents reversed at ~60 mV, indicating ENaC-mediated influx of Na^+ ions. *D*, WNK1(1–491) coexpressed with ENaC comparing effects of kinase-dead SGK1/KD. $n = 6–8$ for each *bar*. Protocol was as in *C*. *E*, experiment as in *D* comparing siRNA with control (*CTR*) or SGK1-targeted oligonucleotides. $n = 5–8$. *F*, experiment as in *D* comparing siRNA with control or Nedd4-2-targeted oligonucleotides. $n = 6–8$. *G*, experiment as in *D* except comparing overexpression of Nedd4-2. $n = 5–7$. In *D–G*, *bars* are inward current density at −100 mV. *, $p < 0.05$ WNK1 *versus* without WNK1. *NS*, not significant WNK1 *versus* without WNK1. *F*, **, $p < 0.01$ vector plus siRNA Nedd4-2 (*3rd column* from *left*) *versus* vector plus control oligonucleotide (*1st column*). *G*, **, $p < 0.01$ vector plus wild type (*WT*)-Nedd4-2 (*3rd column*) *versus* vector alone (*1st column*).

noblotting in HEK293 cells, its expression was sufficient to support ENaC regulation (Fig. 1, *C–E*). WNK1(1–491)-induced ENaC activity was blocked by expression of kinase-dead SGK1 (Fig. 1*D*) or by knocking down expression of endogenous SGK1 via siRNA (Fig. 1*E*). Further analysis confirmed a role for Nedd4-2 in HEK293 cells. Knocking down Nedd4-2 increased ENaC-dependent sodium current, as expected if ENaC remains on the plasma membrane (Fig. 1*F*), and overexpression of Nedd4-2 suppressed current and blocked stimulation by WNK1 (Fig. 1*G*). These properties reproduce essential aspects of ENaC regulation in other systems that we showed with WNK1 earlier (15, 24).

Using HEK293 cells, we tested the ability of the other WNKs to activate ENaC (Fig. 2, *A* and *B*). The N termini of all four WNK family members increased the amiloride-sensitive sodium current (Fig. 2*B*). The four WNK proteins induced roughly equivalent increases in ENaC activity. We expressed full-length forms of WNK1 and WNK4 and found increases in ENaC activity (Fig. 2, *C* and *D*).

We then determined which other WNK family members, if any, also activated SGK1. Because we had found previously that the extent of SGK1 activation varied widely from experiment to experiment, WNK1 was always included in SGK1 activation experiments as a positive control (15, 24). In HEK293 cells, full-length WNK4 generally activated SGK1 as well as did full-length WNK1 (Fig. 2*E*). Full-length WNK3 expressed relatively poorly, and SGK1 activation by WNK3 was consistently the weakest.

As shown in Fig. 1*B*, the N terminus of WNK1 is sufficient to activate SGK1. Thus, we compared SGK1 activation by WNK fragments that contain the kinase domain and those that contain only the noncatalytic N-terminal segments, comparable with WNK1(1–491) and (1–220) (Fig. 1*A*). These shorter fragments, not only WNKs 1 and 4, but also WNKs 2 and 3, enhanced SGK1 activity (Fig. 3, *A–C*). WNKs 1 and 4 were also found to activate SGK1 in HeLa and CHO cells. We showed previously that the WNK1 kinase domain lacking the

of SGK1 expressed, assayed either with a peptide substrate crosstide (Fig. 1*B*) or with recombinant Nedd4-2 (*e.g.* see Fig. 4*B*). No activity toward either substrate was detected with immunoprecipitated kinase-dead SGK1. ENaC activity was previously examined in *Xenopus* oocytes and CHO cells (15, 24). The low expression of SGK1 in those systems usually required exogenous expression of SGK1 to detect significant ENaC activation. Although SGK1 was not detected by immu-

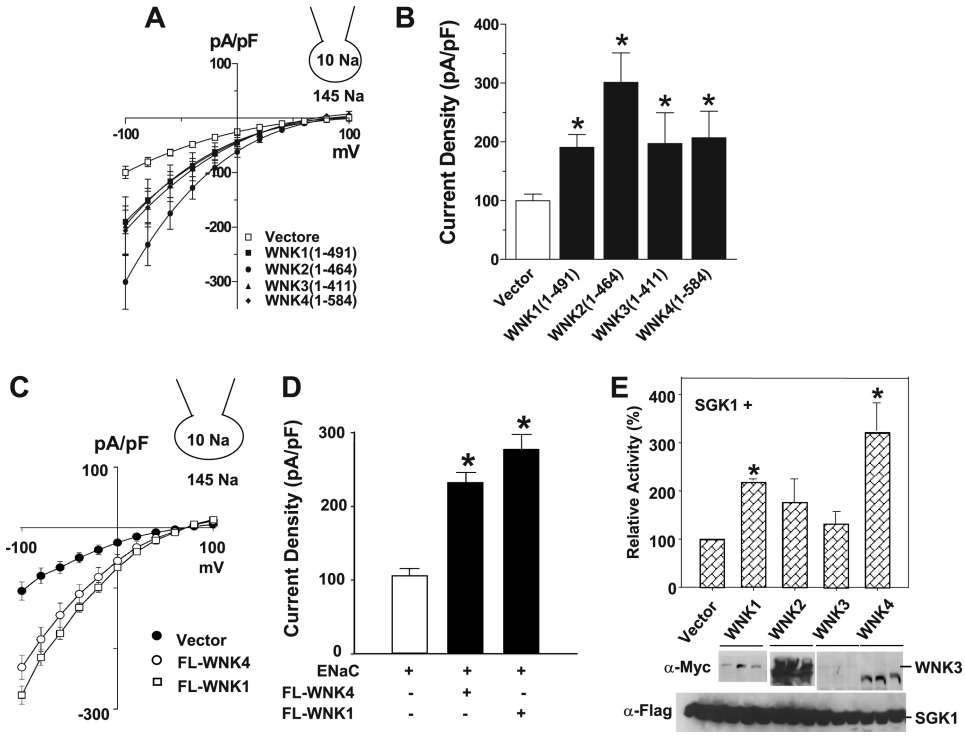

FIGURE 2. **Activation of ENaC and SGK1 by WNKs 1–4.** *A*, current-voltage (*I-V*) relationships of currents shown in *B*. *B*, experiments as in Fig. 1. ENaC activation by the indicated fragments of WNKs 1–4 is shown. *Bars* are inward current density at −100 mV. $n = 6–8$. *, $p < 0.01$ *versus* ENaC alone. *C*, *I-V* relationships of currents shown in *D*. *D*, experiments as in Fig. 1. ENaC activation by full-length WNKs 1 and 4 is shown. *Bars* are inward current density at −100 mV. $n = 6–8$. *, $p < 0.01$ *versus* ENaC alone. *E*, full-length Myc-tagged WNKs 1–4 expressed with full-length FLAG-SGK1. After 24 h, cells were placed overnight in medium lacking serum. SGK1 was assayed using crosstide. WNK1, $n = 6$; WNK2, $n = 18$; WNK3, $n = 12$; WNK4, $n = 6$. *Bottom panels* show expression of Myc-WNKs and FLAG-SGK1 detected by immunoblotting from one of two triplicate experiments. *, $p < 0.05$.

N terminus did not activate SGK1 (24). Consistent with this earlier observation, KS-WNK1, which lacks the WNK1 N terminus (Fig. 1*A*), is unable to activate ENaC (Fig. 3, *D* and *E*). Furthermore, KS-WNK1 inhibits ENaC activation by WNK1.

Activation of SGK1 by WNK4 Has the Same Characteristics as Activation by WNK1—A number of differences have been reported in the actions of WNK1 and WNK4 (14). In view of the diverse N-terminal sequences of the four WNKs, it seemed possible that their actions on SGK1 might take place through different pathways. To characterize the stimulation of SGK1 by WNK4, we compared actions of WNK4 with what is known about activation of SGK1 by WNK1. As is the case for WNK1, the two known sites of activating phosphorylation on SGK1, Thr^{256} and Ser^{422}, must be present for activation of SGK1 by WNK4 (supplemental Fig. S1*A*). Like WNK1, WNK4 had an equivalent ability to activate the other SGK isoforms, SGK2 and SGK3 (supplemental Fig. S1*B*). Inhibition of the phosphoinositide 3-kinase pathway, but not the ERK1/2 and ERK5 mitogen activated-protein kinase pathways, prevented SGK1 activation (supplemental Fig. S1*C*) (24). As is the case with WNK1, both Akt and SGK1 phosphorylated the N terminus of WNK4 (Fig. 4*A*), and mutation of serine 47, the major Akt/SGK1 phosphorylation site on WNK4, reduced its capacity to activate SGK1 (Fig. 4*B*). Thus, despite reported differences in the actions of WNK1 and WNK4, these studies are consistent with the conclusion that WNK1 and WNK4 activate SGK1 by mechanisms that, although not yet biochemically defined, have generally similar characteristics.

WNK1 Binding May Facilitate SGK1 Activation—We showed previously that WNK1(1–491) coimmunoprecipitates with SGK1 from transfected cells. Here, we further defined the interacting region of WNK1 to determine whether the residues required for SGK1 activation are comparable with those required for binding to SGK1. SGK1 bound to both WNK1 residues 1–220 and residues 159–491, suggesting that the binding site may lie at least in part in the region of overlap (Fig. 5*A*). We tested a possible role of the Akt/SGK1 phosphorylation site in the N terminus of WNK1 and found that mutation of the site to alanine (WNK1 T58A) resulted in stronger SGK1 binding, although weaker SGK1 activation (24) (Fig. 4*B* for WNK4). This suggests that binding of WNK1 to SGK1 is not sufficient for its activation. We then compared the binding of SGK1 with the N-terminal regions of the four WNKs and found that binding was detectable with all but WNK3, which was expressed much less well than the others (Fig. 5*B*). No binding was detected with ERK2, and the background in cells expressing empty vector was low. Despite the limited sequence similarities among these WNKs, interaction with SGK1 appears to be a common property.

WNK1 Interacts with Nedd4-2—Because SGK1 binds to Nedd4-2, we determined whether WNK1 might also bind to the ligase. We coexpressed WNK1 and Nedd4-2 and tested for coimmunoprecipitation. We found that WNK1 was associated with Nedd4-2 as well as with SGK1, the positive control (Fig. 6*A*). To determine whether the interaction occurs through an intermediate or is direct, WNK1 and Nedd4-2 were each expressed in bacteria and tested for binding. As shown in Fig. 6*B*, His-tagged WNK1(1–491) bound to recombinant GST-Nedd4-2. Although SGK1 is not a WNK substrate, WNK1 (Fig. 6*C*) and WNK4 both phosphorylate recombinant Nedd4-2. Mass spectrometry was used to determine the sites of *in vitro* phosphorylation by both WNKs. Two sites were found phosphorylated by both WNK1 and WNK4 (Fig. 6*D*). Two additional sites were also phosphorylated by WNK1, one each in the same two peptides. Because WNKs have very low rates of substrate phosphorylation, we were unable to phosphorylate Nedd4-2 with endogenous, immunoprecipitated WNK1. Also, WNK1 activity toward Nedd4-2 could not be detected in the presence of active SGK1 (*e.g.* supplemental Fig. S1*C*, wortmannin-treated samples, also see Fig. 5 of Ref. 24.

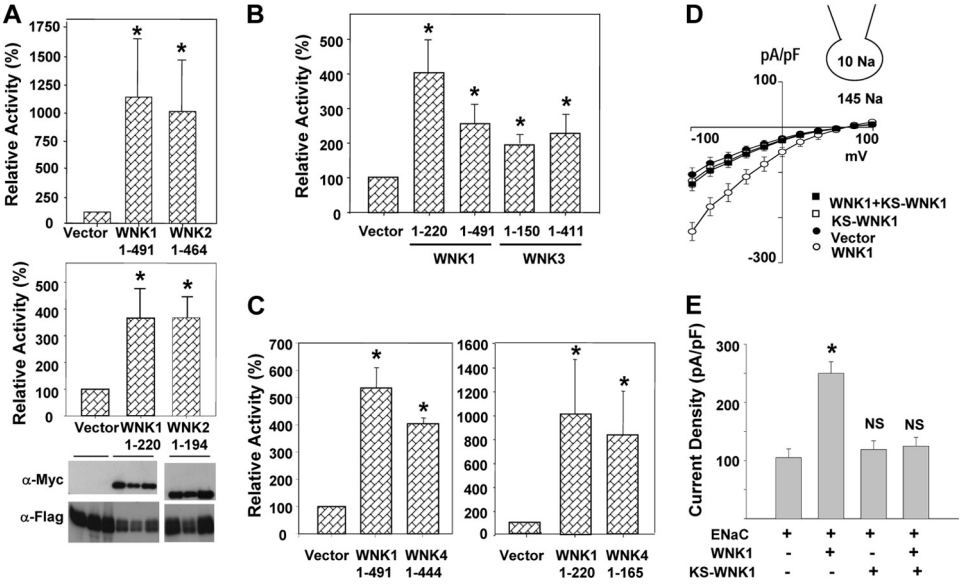

FIGURE 3. **Activation of SGK1 and ENaC by WNK fragments.** *A*, SGK1 activation by the indicated WNK1 and WNK2 N-terminal fragments. SGK1 activity was assayed with crosstide. Experimental protocol was as in Fig. 2*C*. Immunoblots show expression of SGK1 and WNK fragments. One of three similar experiments in triplicate with the longer fragments and of six experiments with the shorter fragments is shown. *Bars* are averages of triplicates from one experiment. *, $p < 0.05$ *versus* SGK1 alone. *B*, activation of SGK1 by the indicated WNK1 and WNK3 N-terminal fragments. One of six similar experiments in triplicate with each fragment is shown. *Bars* are averages of triplicates from one experiment. *, $p < 0.01$ *versus* SGK1 alone. *C*, activation of SGK1 by the indicated WNK1 and WNK4 N-terminal fragments. One of five similar experiments with the kinase-dead fragments and of three similar experiments with the shorter fragments are shown. *Bars* are averages of triplicates from one experiment. *, $p < 0.05$ *versus* SGK1 alone. *D*, current-voltage (*I-V*) relationships of currents shown in *E*. *E*, ENaC activation by WNK1 and effects of KS-WNK1. Experiments are as in Fig. 1. ENaC was coexpressed with KS-WNK1, WNK1(1–491), or both. *Bars* are inward current density at −100 mV. $n = 6–9$. *, $p < 0.01$ *versus* ENaC alone. *NS*, not significant *versus* ENaC alone.

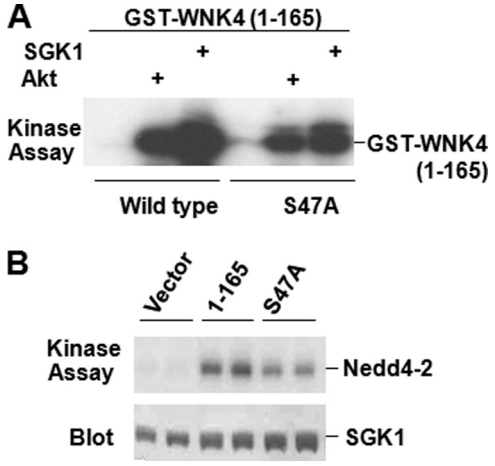

FIGURE 4. **N-terminal phosphorylation site in WNK4 affects SGK1 activation.** *A*, both SGK1 and Akt phosphorylate WNK4 on Ser[47]. GST-WNK4(1–165) wild type or S47A proteins were phosphorylated *in vitro* by either FLAG-SGK1 Δ1–60 (S422D) or hemagglutinin-Akt precipitated from HEK293 cells. An autoradiogram is shown. *B*, WNK4 S47A activates SGK1 more weakly than wild type WNK4. SGK1 activity was assayed with recombinant Nedd4-2. One of two experiments is shown.

Loss of WNK1 Reduces Activation of Endogenous SGK1—We have explored reconstitution of SGK1 activation *in vitro* using recombinant SGK1 and WNK1 fragments. However, we have been unable to induce activation. To gain evidence for a role of WNK1 in activation of endogenous SGK1, we knocked WNK1 down with siRNA oligonucleotides and induced expression of endogenous SGK1 in MDA-MB-231 cells with a combination of dexamethasone and the proteasome inhibitor MG132 (38). SGK1 immunoreactivity in the induced compared with vehicle-treated cells is shown in the *left panel* in Fig. 7. SGK1 was then immunoprecipitated and assayed with recombinant Nedd4-2 as substrate (*right panel*). Reduced WNK1 expression resulted in a substantial loss of SGK1 activity, supporting a role for WNK1 in activation of endogenous SGK1.

DISCUSSION

Because the actions of WNK1 and other family members on ENaC are in dispute, we examined the abilities of the four WNKs to regulate ENaC as well as SGK1. All four WNKs have the capacity to activate SGK1 and sodium transport through ENaC in the same reconstituted cell system. Also supporting the relevance of WNKs in SGK regulation, we have now demonstrated that WNK1 is required for activity of induced, endogenous SGK1 in a breast cancer cell line. Thus, we suggest that apparent differences in actions of particular WNK family members reported in other systems are likely to be due to cell type or tissue-specific expression of proteins, including WNKs themselves, which selectively modify their actions.

How might this occur? The relative expression of splice forms offers one possibility. The KS-WNK1 splice variant lacks the first 463 residues of the ubiquitously expressed form and is unable to activate ENaC presumably because it lacks the requisite N-terminal segment. KS-WNK1 is thought to form complexes with WNK1 and possibly other WNKs through common autoinhibitory and coiled-coiled motifs. Depending on its relative expression and effectiveness in interacting with each WNK family member, this kidney-expressed variant may modulate the effects of WNK1 or other WNKs by binding directly to them and perhaps altering their localization or otherwise affecting their access to relevant proteins. This idea is supported by the finding that KS-WNK1 blocks the ability of the WNK1 N terminus to activate ENaC.

The mutations in WNK1 found in two of the initial PHA2 kindreds are intronic deletions that lead to WNK1 overexpression, based on analysis of lymphocytes from affected individuals. The relative impact of these intron deletions on expression of specific splice forms, *e.g.* the ubiquitous and KS-WNK1 variants, in patient kidneys, is not yet clear. Based on our studies, overexpression of the ubiquitous WNK1 isoform should cause increased ENaC activity, and overexpression of KS-WNK1 in the absence of other WNK proteins should have no effect on ENaC. However, KS-WNK1 may instead reduce ENaC activity

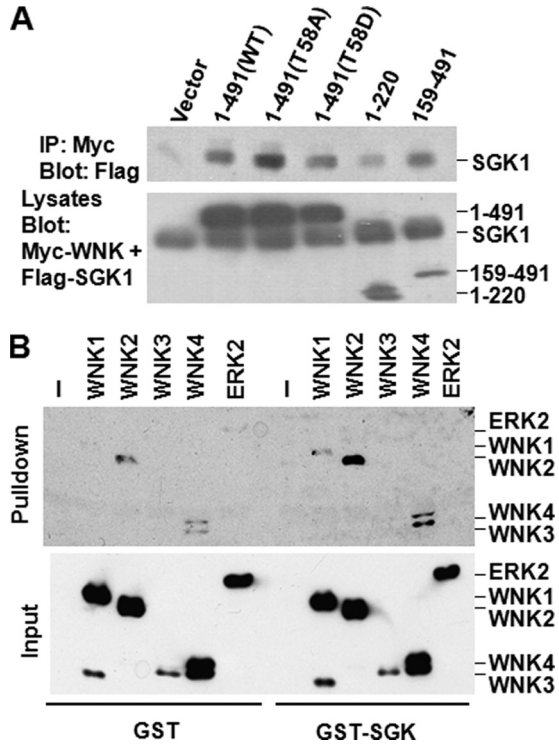

FIGURE 5. **N-terminal fragments of WNKs bind to SGK1.** *A*, the indicated WNK1 N-terminal fragments were immunoprecipitated (*IP*) from cells coexpressing SGK1. Precipitates were immunoblotted with anti-FLAG to detect SGK1. Immunoblots of expressed fragments of one of three experiments are shown. *B*, the N terminus of WNKs 1, 2, and 4 bind to SGK1. Recombinant WNK fragments or ERK2 was added to lysates of cells transfected with GST-SGK1. Complexes were collected on glutathione beads. Immunoblots of one of two experiments are shown.

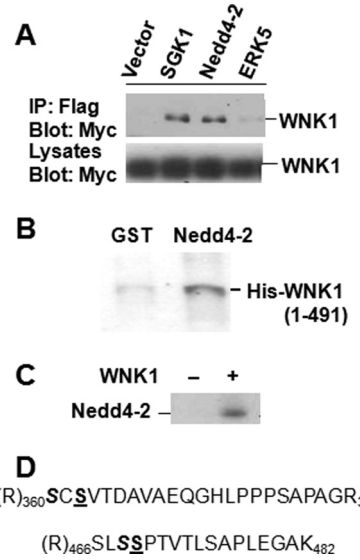

FIGURE 6. **WNK1 binds to and phosphorylates Nedd4-2.** *A*, Myc-WNK1 and FLAG-Nedd4-2, FLAG-SGK1, or FLAG-ERK5 were coexpressed. FLAG immunoprecipitates were immunoblotted with anti-Myc to detect associated WNK1. One of two experiments is shown. *B*, purified His_6-WNK1 (1–491) and GST-Nedd4-2 expressed in bacteria were mixed, and GST fusion proteins were harvested on glutathione-agarose. The associated WNK1 (1–491) was detected with anti-His_6. One of two experiments is shown. *C*, Nedd4-2 was phosphorylated *in vitro* by WNK1. An autoradiogram is shown. *D*, sites phosphorylated in Nedd4-2 by WNK1 and WNK4 determined by mass spectrometry are shown. Coverage of Nedd4-2 was ~80%. Two sites were phosphorylated in common by both WNK1 and WNK4 and are shown in *bold* and *underlined*. Two additional sites phosphorylated by WNK1 are *bold* and *italicized*.

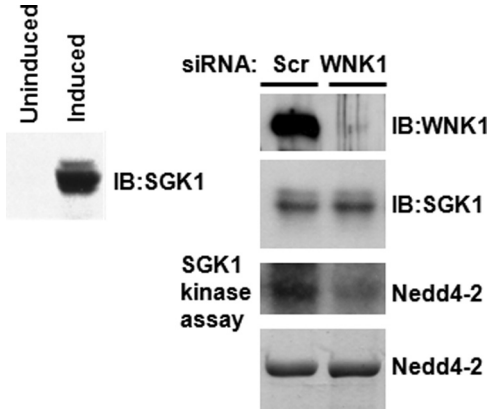

FIGURE 7. **WNK1 is required for activation of endogenous SGK1.** SGK1 was induced with dexamethasone as described under "Experimental Procedures" in MDA-MB-231 cells previously transfected with control scrambled (*Scr*) or WNK1 siRNA. SGK1 was immunoprecipitated and assayed for kinase activity using recombinant Nedd4-2. *Left*, SGK1 expression with and without induction. *Right*, WNK1 and SGK1 in the lysates were immunoblotted (*IB*). Nedd4-2 in the assay was also blotted, and an autoradiogram of phosphorylation reflects kinase activity. One of two experiments is shown.

by interfering with the stimulatory action of full-length WNK1 or other WNK family members.

Four other PHA2 families contained mutations in WNK4, all of which were in a coiled-coil segment, a site of protein-protein interactions, just C-terminal to the kinase and autoinhibitory domains. Again, the effects of these mutations on biochemical properties of WNK4 have yet to be mechanistically defined, raising questions about how to interpret data from experiments with these mutants. Studies in transgenic animals have not uniformly compensated for altered gene dosage, also making them difficult to interpret.

The original WNK1 knock-out mouse was created using a strategy that inserted β-galactosidase after intron 1. We have found that both heterozygotes and homozygotes express a small but significant amount of a ~45-kDa protein that cross-reacts with antibodies to the WNK1 N terminus, consistent with expression of an N-terminal fragment of WNK1. Thus, some of the studies with these animals may be confounded by expression of a WNK1 fragment with some biological activity. Because this fragment presumably includes the sequences necessary for SGK1/ENaC regulation, conclusions about phenotype must be interpreted cautiously.

The kinase activity of WNK1 is not required for SGK1 activation; thus, how WNK1 controls SGK1 activity remains unclear (24). There are few conserved features within the N termini of WNKs 1–4. Earlier work had shown that WNK1 contains an N-terminal site for phosphorylation by Akt and/or SGK1 (24, 39, 40). A comparable site is present in WNK4, but we have been unable to demonstrate phosphorylation of the N termini of WNKs 2 or 3 by SGK1. Yet, at least three WNKs interact with SGK1 in pulldown assays. Despite low sequence identity, SGK1 binding is a property apparently common to WNKs.

A recent study demonstrated that single nucleotide polymorphisms in WNK1 are linked to hypertension and hyperkalemia as well as increased risk for essential hypertension (41). These polymorphisms span the WNK1 gene, including the promoter and 3′ regions, with some of those most highly symptom-cor-

related localized to the intronic region of WNK1 deleted in PHA2 patients. On the other hand, polymorphisms in the control group appeared to decrease hypertension risk, lending credence to the suggestion that the impact of WNKs on blood pressure is complex, and an understanding of the interplay between WNKs themselves, WNKs, and transporters such as ENaC, NaCl cotransporter, and renal outermedullary potassium channel, and these channels themselves is yet to be realized.

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