WNK1 activates SGK1 by a Phosphatidylinositol 3-Kinase-dependent and Non-catalytic Mechanism*

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WNK1 (with no lysine [K]) is a protein-serine/threonine kinase with a unique catalytic site organization. Deletions in the first intron of the WNK1 gene were found in a group of hypertensive patients with pseudohypoaldosteronism type II. No changes in coding sequence of WNK1 were found, but its expression was increased severalfold. We have been investigating actions of WNK1 and have found that WNK1 activates the serum- and glucocorticoid-induced protein kinase SGK1, which impacts membrane expression of the epithelial sodium channel. Here we explore the role of WNK1 in SGK1 regulation. Activation of SGK1 by WNK1 is blocked by phosphatidylinositol 3-kinase inhibitors. Neither the catalytic activity nor the kinase domain of WNK1 is required; rather the N-terminal 220 residues of WNK1 are necessary and sufficient to activate SGK1. Phosphorylation of WNK1 on Thr-58 contributes to SGK1 activation. Finally, we show that WNK1 is required for the activation of SGK1 by insulin-like growth factor 1.

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EXPERIMENTAL PROCEDURES

Materials—Constructs were prepared using standard methods (1, 9, 18). Site-directed mutagenesis was achieved using the QuikChange kit (Stratagene). DNAs were provided as follows: SGK1 and p70, by M. E. Greenberg and J. Blenis, respectively (Harvard University); SGK2 and SGK3, PDK1, and Akt, by Orson Moe, E. N. Olson, and M. A. White, respectively (University of Texas Southwestern Medical Center); Nedd4-2 by P. M. Snyder (University of Iowa); and ENaC α, β, and γ subunits by T. R. Kleyman (University of Pittsburgh). Anti-Myc, anti-hemagglutinin, anti-FLAG, and anti-WNK1 (Q256) were as described.

The abbreviations used are: MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEK5, MEK kinase; ENaC, epithelial sodium channel; IGF-1, insulin-like growth factor 1.

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thesis facility. Double-stranded RNA oligonucleotides were obtained from the institutional facility and are as described previously (9).

Protein Expression—GST-Nedd4-2 and WNK1-(1–555) were purified from Escherichia coli strain BL21 using standard protocols.

Tissue Culture, Cell Treatments, and RNA Interference—HEK293 cells were grown using standard conditions as described previously (19). Cells were transfected and harvested in isotonic lysis buffer containing 1% Triton X-100 and phosphatase and protease inhibitors as described previously (19). As indicated in the figure legends, 10 μM U0126 was added to cells 1 h before harvest, 50 nM wortmannin was added to cells 30 min and 20 μM LY294002 1 h prior to harvest, and 20 μM SB203580 was added to cells 15 h before harvest. IGF-1 (50 ng/ml) was added to indicated cells for 30 min. For RNA interference, HEK293 cells were grown to 30% confluence in 6-well plates and transfected with double-stranded RNA oligonucleotides as described previously (9). On the following day, 1 μg of SGK1 was transfected into each well, and cells were harvested 48 h later.

Immunoprecipitation, Immunoblotting, and Protein Kinase Assays—Proteins were immunoprecipitated from cell lysates with an antibody/lyse ratio of 1:100 and collected using protein A-Sepharose beads. Immunoblotting was performed on proteins in cell lysates or immunoprecipitates that had been resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were developed using enhanced chemiluminescence. In vitro kinase assays of immunoprecipitates were performed as described previously using either recombinant Nedd4-2 or crosstide as substrates (12).

Two-electrode Voltage Clamp Recording of ENaC Channels—cRNAs for mouse α, β, and γ subunits of ENaC (20), rat WNK1, mouse SGK1, and human Nedd4-2 were synthesized using a commercial in vitro transcription kit (Ambion). *Xenopus laevis* oocytes (stages V and VI) were prepared and injected with cRNAs for cDNAs at the indicated concentrations as described previously (12, 21). Injected oocytes were incubated in ND96 bath solution containing (in mM) 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, and 5 Hepes (pH 7.4). 48–72 h postinjection, currents were recorded from oocytes in ND96 bath solution using an OC-725C oocyte clamp amplifier, pCLAMP7 software, and Digidata 1200A digitizer. Voltage clamp protocols consist of voltage steps (400 ms) from −100 to 50 mV in 25-mV increments from 0 mV holding potential. To verify that inward currents were carried by sodium ions, ND96 was replaced by a bath solution containing (in mM) 98 KCl, 1 CaCl₂, 1 MgCl₂, and 5 Hepes (pH 7.4). Statistical comparison was made using the unpaired t test.

RESULTS

WNK1 Activates All Three SGK Isoforms—We showed previously that WNK1 activates SGK1 but not the related, kinases p70 S6 kinase or Akt, in transfected cells (12). Because the three isoforms of SGK are differentially expressed in epithelial tissues, we first wanted to know the ability of WNK1 to activate SGK2 and SGK3 using the peptide crosstide as the SGK substrate. We found that these SGK proteins were also stimulated by co-expression with WNK1, although to a more limited extent than SGK1 (Figs. 1 and 2A). The fact that all three SGKs are stimulated indicates that WNK1, which is ubiquitously expressed, can impact events in cells that express any of the three isoforms.

Activation of SGK1 by WNK1 Is Blocked by PI 3-Kinase Inhibitors—In our earlier studies we found that SGK1 is not a substrate of WNK1. To begin to probe the mechanism by which WNK1 increases SGK1 activity, we tested the effects of pharmacological inhibitors of MAPK pathways (Fig. 2, A and B). Co-expression of WNK1 with SGK1 increases SGK1 activity assayed with either recombinant Nedd4-2 or the synthetic peptide crosstide. We found previously that WNK1 stimulates ERK5 activity and has a weak effect on p38 MAPK (9). Both of these pathways have been implicated in SGK1 regulation (22–25). To inhibit the ERK5 pathway, we used U0126. U0126 inhibits the kinases upstream of ERK1/2 as well as ERK5 (26). We found that U0126 had no effect on the activation of SGK1 by WNK1 (Fig. 2A), which is consistent with findings that dominant negative mutants of MEKK2 and -3 did not block SGK1 activation by WNK1 (12). Likewise, the p38 inhibitor SB203580 had no effect on the activation of SGK1 by WNK1 (Fig. 2B). The PI 3-kinase inhibitor wortmannin blocks the activation of SGK1 by a number of hormones including IGF-1 (25). Therefore we tested the effect of wortmannin and found that it substantially reduced the activation of SGK1 by WNK1 (Fig. 2, A and B). In confirmation of an earlier report (27, 28), we also found that the activation of SGK1 by PDK1 is not wortmannin-sensitive (Fig. 2C), indicating that this was not the site of action of PI 3-kinase. Activation of SGK1 by WNK1 was also blocked by another PI 3-kinase inhibitor, LY294002 (Fig. 2D). Activation of SGK1 by WNK1 might be inhibited by wortmannin if the activation of WNK1 itself is PI 3-kinase-dependent. To test this possibility, we examined the effect of wortmannin on the activation of WNK1 by osmotic stress, the only known type of WNK1 stimulus. WNK1 was stimulated 5–10-fold by 0.5 M NaCl (Fig. 2E). There was no effect of wortmannin on its activation. Thus, WNK1 activation apparently was also not a site of PI 3-kinase action.

Activation of SGK1 and ENaC by WNK1 Requires Akt—PDK1 was originally identified as a protein kinase that phosphorylates Akt in a PI 3-kinase-dependent manner. Because SGK1 is phosphorylated by PDK1 independently of PI 3-kinase (27) (Fig. 2C), we tested the possibility that PI 3-kinase exerted an effect required for the activation of SGK1 by WNK1 through the PI 3-kinase effector kinase Akt. A kinase-dead mutant of Akt reduced SGK1 activation by WNK1, whereas a kinase-dead mutant of the related kinase p70 had no effect (Fig. 3A). These data
suggest that activation of SGK1 by WNK1 requires the catalytic activity of Akt.

ENaC subunits were identified by expression cloning in *Xenopus* oocytes (29). Thus, ENaC activity can be measured by reconstitution in oocytes, displaying the appropriate sensitivity to the ENaC-selective inhibitor amiloride. Co-expression of ENaC with WNK1 in oocytes stimulated ENaC activity (Fig. 3B) (12). We have shown previously that stimulation of ENaC by WNK1 occurs via activation of endogenous SGK in oocytes (12). Kinase-dead Akt blocks the effect of WNK1 on ENaC activity (Fig. 3B), consistent with its effect on SGK1 activation by WNK1. Because WNK1 did not activate Akt, Akt most likely acts upstream or in parallel with WNK1.

Activation of SGK1 by WNK1 Is Increased by Phosphorylation of WNK1 Thr-58—WNK1 Thr-58 has been shown to be an IGF-1-stimulated site of Akt phosphorylation (30, 31) (Fig. 4A). We found that Akt as well as SGK1 itself phosphorylated WNK1 in vitro (Fig. 4B). Mutation of WNK1 Thr-58 to alanine reduced phosphorylation by Akt substantially and also reduced phosphorylation by SGK1, suggesting that SGK1 phosphorylates WNK1 not only on Thr-58 but also on additional sites. With an antibody generated against phospho-Thr-58 of WNK1, we found that WNK1-(1–491) was constitutively phosphorylated on Thr-58 in HEK293 cells and wortmannin reduced its phosphorylation (Fig. 4C). WNK1-(1–491) T58A and T58D were defective in activating SGK1 compared with wild type WNK1 (Fig. 4D), although partial activation of SGK1 was observed. These results indicate that phosphorylation of Thr-58 by either endogenous Akt or other kinases such as SGK

**FIGURE 2.** Activation of SGK1 by WNK1 is sensitive to inhibitors of PI 3-kinase. A, cells were treated with 10 μM U0126 for 1 h or 50 nM wortmannin for 30 min prior to harvest. SGK1 activity was assayed with crosstide as substrate. B, cells were treated with 20 μM SB203580 overnight or with 50 nM wortmannin (WM) for 30 min prior to harvest. SGK1 activity was assayed with recombinant Neddy-2 as substrate. C, SGK1 activation by PDK1 is insensitive to wortmannin (WM). D, cells were treated with 20 μM LY294002 for 1 h prior to harvest. SGK1 activity was assayed with crosstide as substrate. E, activation of WNK1 by osmotic stress is insensitive to wortmannin (WM). The activity of endogenous WNK1 was measured in immunoprecipitates as autophosphorylation. Experiments were repeated two to four times.

**FIGURE 3.** Akt is required for activation of SGK1 and ENaC by WNK1. A, kinase-dead mutant of Akt blocks SGK1 activation by WNK1. The two panels are from the same gel. Represented is one of five experiments. B, co-expression of kinase-dead Akt (Akt-KD) prevents WNK1 stimulation of ENaC. *, p < 0.05 versus ENaC + WNK1.
isoforms enhance the activation of SGK1 by WNK1. Because the activation of Akt requires PI 3-kinase, the activation of SGK1 by WNK1 apparently requires PI 3-kinase activity to promote WNK1 Thr-58 phosphorylation by Akt. The phosphorylation of WNK1 by SGK1 may reflect positive feedback.

WNK1 Activates SGK1 and ENaC by a Mechanism Independent of Its Kinase Activity—Because mutation of Thr-58 reduces the ability of WNK1 to activate SGK1, we determined whether the N-terminal region of WNK1 containing Thr-58 is required for SGK1 activation. We found that not only was the catalytic activity of WNK1 dispensable for its ability to activate SGK1 but the entire catalytic domain was also dispensable (Fig. 5, A and B). Instead, WNK1-(1–220) alone, which contains Thr-58 and additional residues preceding the kinase domain, was sufficient to activate SGK1 (Fig. 5B). We evaluated the relationship of these biochemical findings to increased ENaC-dependent sodium current and found that WNK1-(1–220) caused a similar activation of ENaC, whereas the kinase domain lacking the N-terminal residues had no effect (Fig. 6A). Consistent with results using longer fragments, mutation of Thr-58 reduced activation of SGK1 by WNK1-(1–220) (Fig. 5C). Likewise, mutation of Thr-58 greatly reduced the ability of WNK1-(1–491) and -(1–220) to activate ENaC (Fig. 6B). These findings clearly demonstrate that the N terminus of WNK1, when phosphorylated on Thr-58, is capable of activating SGK1 and ENaC independently of WNK1 catalytic activity.

WNK1 Is Required for Activation of SGK1 by IGF-1—Thus far, we have shown that WNK1 is sufficient to activate SGK1. To address whether WNK1 is necessary for SGK1 activation by hormones, we knocked down WNK1 expression by small interfering RNA and examined SGK1 activity (Fig. 7A). In the presence of WNK1, IGF-1 treatment
led to 3–4-fold increase in SGK1 activity. When WNK1 expression was reduced by using double-stranded RNA oligonucleotides, IGF-1 failed to activate SGK1 significantly. This is strong evidence that WNK1 is required for SGK1 activation by IGF-1. As a control to demonstrate that IGF-1 action had not been generally disrupted, we also examined the activation state of Akt in the same cells. As shown in Fig. 7B, Akt activation, as indicated by anti-phospho-Thr-308 and anti-phospho-Ser-473 immunoblots, was not altered by the depletion of WNK1.

FIGURE 7. Activation of SGK1 by IGF-1 requires WNK1. Cells were serum-deprived for 24 h and then either untreated or treated with 50 ng/ml IGF-1 for 30 min. A, top, SGK1 activity (siRNA, small interfering RNA); middle, anti-FLAG immunoblot to detect SGK1; bottom, WNK1 immunoblot. B, Akt activity in the same experiment as in A detected with anti-Akt pT308 (top) and pS473 (middle) antibodies; bottom, immunoblot of total lysate Akt. Represented is one of three experiments.

DISCUSSION

WNK1 activates the protein kinase SGK1 in a manner that leads to increased ENaC-mediated sodium transport (12). Over-expression of WNK1 is sufficient to activate SGK1, and expression of endogenous WNK1 is required for the stimulation of SGK1 activity by IGF-1.

Activation by WNK1 requires phosphorylation of SGK1. Although WNK1 binds SGK1 directly, WNK1 does not phosphorylate it. Thus, we have evaluated other potential mechanisms of SGK1 activation. Experiments thus far indicate no relationship to MAPK cascades. WNK1 has some similarities to the Ste20p family, many of which activate MAPK kinase kinases either by phosphorylation or through association. WNK1, like Ste20ps, has some capacity to activate the MAPK kinase MEKK3. We showed previously, however, that MEKK3 is not involved in SGK1 activation (12). p38 and ERK5, both of which can be activated by MEKK3, have been implicated in the regulation of SGK1 in certain contexts (22–24). We find, however, that neither of these nor ERK1/2 MAPKs are involved in SGK1 activation by WNK1.

Our results suggest that the PI 3-kinase pathway is essential for WNK1 action. PI 3-kinase activity is not required to activate the kinase activity of WNK1 because inhibition of PI 3-kinase does not interfere
with the activation of WNK1 by osmotic stress. However, Akt does phosphorylate WNK1 on Thr-58, a site that has little or no effect on the kinase activity of WNK1 (30, 31). We propose that phosphorylation of WNK1 Thr-58 by Akt is the PI 3-kinase-dependent step in the mechanism underlying the activation of SGK1.

Previous work on SGK1 has shown that, like Akt, it is activated by phosphorylation on the two well described activating phosphorylation sites, a residue in the activation loop Thr-256 of SGK1 and a second site in a C-terminal hydrophobic motif, SGK1 Ser-422 (28). We found that activation of SGK1 by WNK1 required phosphorylation of these two sites. PDK1 is thought to be the activation loop kinase based on biochemical analysis (27, 28). The kinase(s) responsible for phosphorylating the other SGK1 site is unknown, and WNK1 may not have a major effect on Ser-422 phosphorylation; although the S422D mutant of SGK1 was slightly more active than the wild type enzyme, it retained the ability to be activated by co-expression with WNK1 (12). This suggests that WNK1 is involved in promoting phosphorylation of the SGK1 activation loop site. On the basis of our results, we propose that WNK1 and PDK1 cooperate to activate SGK1 (Fig. 8). This mechanism is unique to SGK family members and is not involved in regulating related kinases such as p70 S6 kinase. A PI 3-kinase-dependent step is involved not at the level of PDK1 but because of a requirement for phosphorylation of WNK1 on Thr-58. In this model, IGF-1 induces SGK1 activation by stimulating WNK1 phosphorylation on this site. In our model, the role of Thr-58 does not preclude the possibility that there are additional PI-3-kinase-sensitive components required for SGK1 activation.

How Thr-58 phosphorylation of WNK1 promotes SGK1 activation is not apparent. Because WNK1 binds to SGK1, WNK1 may serve as a scaffold to assemble a protein complex required for efficient SGK1 activation. Phospho-Thr-58 may create a binding site for proteins involved in SGK1 activation or induce a conformational change in SGK1 that is permissive for its activation. To date, however, we have been unable to induce SGK1 activation in vitro or in extracts by adding WNK1 under phosphorylating conditions. Thus, it seems likely that additional components are necessary to mediate SGK1 activation.

A screen of consensus phosphorylation sites indicates that WNK1 Thr-58 may also be a substrate for cAMP-dependent protein kinase, calcium- and calmodulin-dependent protein kinase II, several protein kinase C species (scaitsite.mit.edu), and probably additional AGC family members. These enzymes most likely account for the residual phosphorylation on WNK1 Thr-58 in cells treated with wortmannin to inhibit PI 3-kinase and may allow other hormonal systems to potentiate SGK1 activation and the subsequent activation of ENaC. SGK1 itself may provide a feed forward mechanism to enhance its activation by further phosphorylating WNK1 on Thr-58.

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