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WNK1 Regulates Phosphorylation of Cation-Chloride-coupled Cotransporters via the STE20-related Kinases, SPAK and OSR1*[S]

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The WNK1 and WNK4 genes have been found to be mutated in some patients with hyperkalemia and hypertension caused by pseudohypoaldosteronism type II. The clue to the pathophysiology of pseudohypoaldosteronism type II was its striking therapeutic response to thiazide diuretics, which are known to block the sodium chloride cotransporter (NCC). Although this suggests a role for WNK1 in hypertension, the precise molecular mechanisms are largely unknown. Here we have shown that WNK1 phosphorylates and regulates the STE20-related kinases, Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). WNK1 was observed to phosphorylate the evolutionary conserved serine residue located outside the kinase domains of SPAK and OSR1, and mutation of the OSR1 serine residue caused enhanced OSR1 kinase activity. In addition, hypotonic stress was shown to activate SPAK and OSR1 and induce phosphorylation of the conserved OSR1 serine residue, suggesting that WNK1 may be an activator of the SPAK and OSR1 kinases. Moreover, SPAK and OSR1 were found to directly phosphorylate the N-terminal regulatory regions of cation-chloride-coupled cotransporters including NKCC1, NKCC2, and NCC. Phosphorylation of NCC was induced by hypotonic stress in cells. These results suggested that WNK1 and SPAK/OSR1 mediate the hypotonic stress signaling pathway to the transporters and may provide insights into the mechanisms by which WNK1 regulates ion balance.

WNK[2] kinases (with no lysine (K)) comprise a family of novel serine/threonine protein kinases conserved among multicellular organisms (1, 2). The kinase domain of this family is unique in that it lacks the conserved lysine residue previously known to be important for ATP binding in the catalytic site. A conserved lysine in subdomain I of the WNK kinases is thought to be essential for their catalytic activity (1, 3). There are four human WNK family members. *WNK1* and *WNK4* were identified as genes mutated in families of patients with pseudohypoaldosteronism type 2 (PHA II) human hypertension (4). The *WNK1* gene mutation consists of a deletion within its first intron, leading to increased expression, whereas mutations in the *WNK4* gene are found in the coding sequence near the coiled-coil domains.

PHA II patients are treated by thiazide diuretics, which function as antagonists of the Na-Cl cotransporter (NCC, also known as thiazide-sensitive cotransporter (TSC) or Na-Cl transporter (NCCT)), suggesting that the activity of NCC could be potentially involved in the development of PHA II. Previous studies using *Xenopus* oocytes have showed that wild-type WNK4 inhibits the surface expression and the activity of NCC, whereas one of the disease-causing mutants of WNK4 attenuated this inhibitory effect (5, 6). However, comparison of wild-type and mutant WNK4 revealed no differences in NCC surface expression in polarized epithelial cells (MDCK II cells), suggesting that the regulation of intracellular NCC localization by WNK4 might be unrelated to the pathogenesis of PHA II (7). WNK4 has also been reported to inhibit surface expression of the secretory potassium channel (ROMK) and Cl^- base exchanger SCL26A6 (CFEX), in addition to NCC, in *Xenopus* oocytes (8, 9). Furthermore, the disease-causing mutant of WNK4 was shown to increase paracellular chloride permeability in MDCK cells (10, 11). In contrast to WNK4, little is known about the functions and regulation of WNK1. WNK1 does not directly affect NCC activity in *Xenopus* oocytes but has been shown to modulate the inhibitory effects of WNK4 on NCC (6). Although WNK1 activates the MEK5-ERK5 pathway and phosphorylates synaptotagmin, there is no direct evidence to link WNK1 and transporter function (12, 13). Moreover, a recent study reported that WNK1 regulates the epithelial sodium channel through glucocorticoid-inducible kinase (SGK1), but the mechanisms of SGK1 activation by WNK1 have not been fully elucidated (14, 15).

NCC contains 12 transmembrane domains and is closely related to the Na-K-2Cl cotransporters, NKCC1 and NKCC2 (16–18). NCC and NKCC2 are expressed in the kidney and function in renal salt reabsorption, whereas NKCC1 is expressed ubiquitously and plays a key role in

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure showing the specificity of anti-SPAK/OSR1 and anti-OSR1-P antibodies.

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[2] The abbreviations used are: WNK, with no lysine (K); NCC, Na-Cl cotransporter; KCC, K-Cl cotransporter; NKCC, Na-K-2Cl cotransporter; SPAK, Ste20-related proline-alanine-rich kinase; OSR1, oxidative stress response 1; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MS, mass spectrometry; LC-MS/MS, liquid chromatography coupled to tandem MS; GST, glutathione *S*-transferase; PHA II, pseudohypoaldosteronism type 2; MDCK, Madin-Darby canine kidney cells.

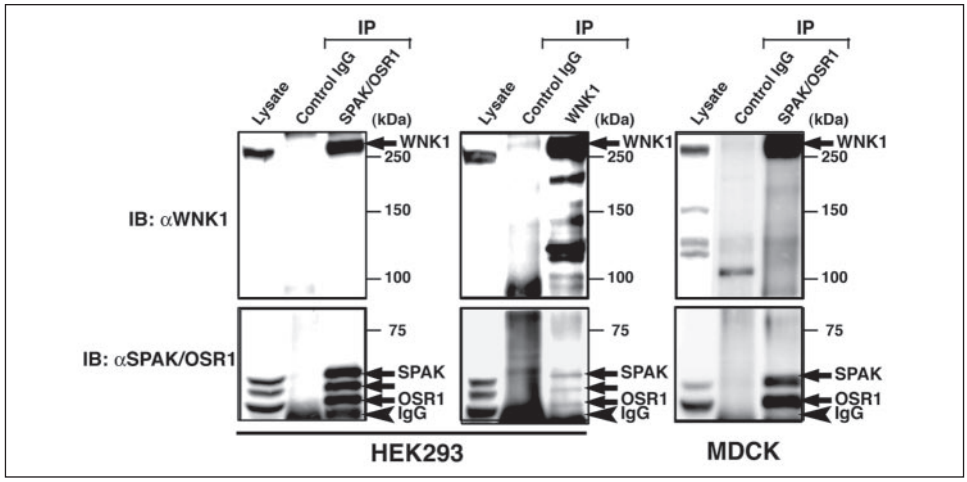

FIGURE 1. **Association of WNK1 with SPAK and OSR1.** SPAK and OSR1 were immunoprecipitated (*IP*) from 0.4 mg of lysates prepared from HEK293 cells (*left*) or MDCK cells (*right*) with 2 μg of SPAK/OSR1 antibody, fractionated by SDS-PAGE, and immunoblotted (*IB*) with the indicated antibodies. WNK1 was immunoprecipitated from 1 mg of HEK293 lysates with 10 μg of the WNK1 antibody (*middle*). As a control, immunoprecipitations were also performed in parallel experiments with rabbit IgG (Chemicon International).

epithelial salt secretion and cell volume regulation. NKCC1 cotransport activity is controlled by the phosphorylation/dephosphorylation of several threonine and serine residues in response to decreases in cell volume or intracellular [Cl]. Three of the phosphoacceptors in the N terminus of NKCC1 have been identified, and the amino acid sequences surrounding these residues are highly conserved among the members of the cation-chloride-coupled cotransporter family, suggesting that phospho-regulatory mechanisms are conserved among these cotransporters (19). Although several protein kinases, such as SGK1 and c-Jun N-terminal kinase, have been proposed as candidates for the activators of NKCC1, there is no evidence showing that any kinase directly phosphorylates NKCC1 *in vivo* (20, 21). It has been previously reported that the STE20-related kinases, SPAK (also called PASK (proline-alanine-rich Ste-20-related kinase)) and OSR1, bind to the N-terminal regions of the cation-chloride cotransporters KCC3, NKCC1, and NKCC2 (22). Moreover, WNK4 has been identified as a putative SPAK-binding protein by yeast two-hybrid screening (23). Expression of a dominant-negative form of SPAK decreased cotransport activity and phosphorylation of NKCC1 (24). Therefore, SPAK is thought to play an important role in the regulation of NKCC1.

In this study, we have identified SPAK as a WNK1-binding protein and provided evidence that WNK1 acts as a direct activator of SPAK and OSR1. Moreover, we have shown that SPAK and OSR1 directly phosphorylate the N-terminal regulatory regions of NKCC1, NKCC2, and NCC. These results have raised the possibility that WNK1 regulates the activities of a number of transporters through SPAK/OSR1 and that this regulation contributes to the pathogenesis of hypertension.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Plasmid Construction—Human WNK1, human WNK4, rat SPAK, human NKCC1, human NKCC2, mouse NCC, and mouse OSR1 coding regions were amplified by PCR using Marathon-Ready cDNA (Clontech) as templates. GST-PAK3-(65–135) expression plasmid was kindly provided by T. Akiyama (25). To construct mammalian expression vectors, pCMV-FLAG and pCMV-T7, the fragment encoding one copy of the FLAG epitope or the fragment encoding one copy of the T7 epitope was inserted into pCMV vector, respectively. Several mutant cDNAs encoding WNK1, WNK4, SPAK, OSR1, NKCC1-(1–289), NKCC2-(1–181), or NCC were generated by polymerase chain reaction and subcloned into the pCMV-FLAG, pCMV-T7, pGEX4T-1, or pGEX4T-3 as indicated. The accuracy of all clones was verified by DNA sequencing.

Yeast Two-hybrid Screening and MS/MS Analysis—Full-length human WNK1 was fused to the GAL4 DNA-binding domain, and yeast two-hybrid screening was performed as described (26). LC-MS/MS analysis was performed as described previously (27). Briefly, FLAG-WNK1 was expressed in HEK293 cells and immunoprecipitated by anti-FLAG antibody. The immunocomplexes were eluted with a FLAG peptide and then digested with *Achromobacter* protease I, and the resulting peptides were analyzed using a nanoscale LC-MS/MS system.

Antibodies—Antibody to WNK1 was generated with a peptide corresponding to the N-terminal 18 amino acids of human WNK1. Anti-SPAK/OSR1 antibody was prepared by immunizing rabbits with a keyhole limpet hemocyanin-conjugated synthetic peptide (RAKKVRRVPGSSG, amino acids 362–374 of human SPAK and amino acids 314–326 of human OSR1). Anti-phospho OSR1 polyclonal antibody was produced in rabbit by immunizing with a keyhole limpet hemocyanin-conjugated synthetic phosphopeptide corresponding to residues 319–332 of OSR1 (RRVPGS (pS) GRLHKTE). The serum was affinity-purified with phosphopeptide- and the non-phosphopeptide-conjugated cellulose. Monoclonal antibodies against FLAG and T7 were purchased from Sigma and Novagen, respectively.

Immunoprecipitation and Immunoblotting—HEK293 and MDCK cells were cultured in Dulbecco's modified Eagle's medium with standard supplements. HEK293 cells were transfected with the indicated plasmids by the calcium phosphate precipitation method at 50–80% confluence. After 24 h after transfection, cells were lysed in 1% Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM dithiothreitol, 0.27 M sucrose). Protein complexes were immunoprecipitated with the indicated antibodies according to standard procedures. Isolated protein complexes were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Hybond-P, Amersham Biosciences). Blots were probed with the indicated antibodies, and bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences) according to standard procedures. For ^{32}P labeling, transfected cells were incubated for 6 h with [^{32}P]phosphate (1 mCi/ml) and then lysed as described above.

Expression of GST-tagged Fusion Proteins in Escherichia coli—The pGEX constructs were transformed into *E. coli* BL21 cells, and a 0.5-liter culture was grown at 37 °C to an A_{600} of 0.8. Isopropyl-D-galactosidase was added to final 0.2 mM to induce protein expression, and the cells were cultured for another 16 h at 20 °C. Cells were harvested by centrifugation and lysed by freeze-thawing and sonication in 1% Triton X-100 lysis buffer. Glutathione *S*-transferase (GST)-tagged proteins were puri-

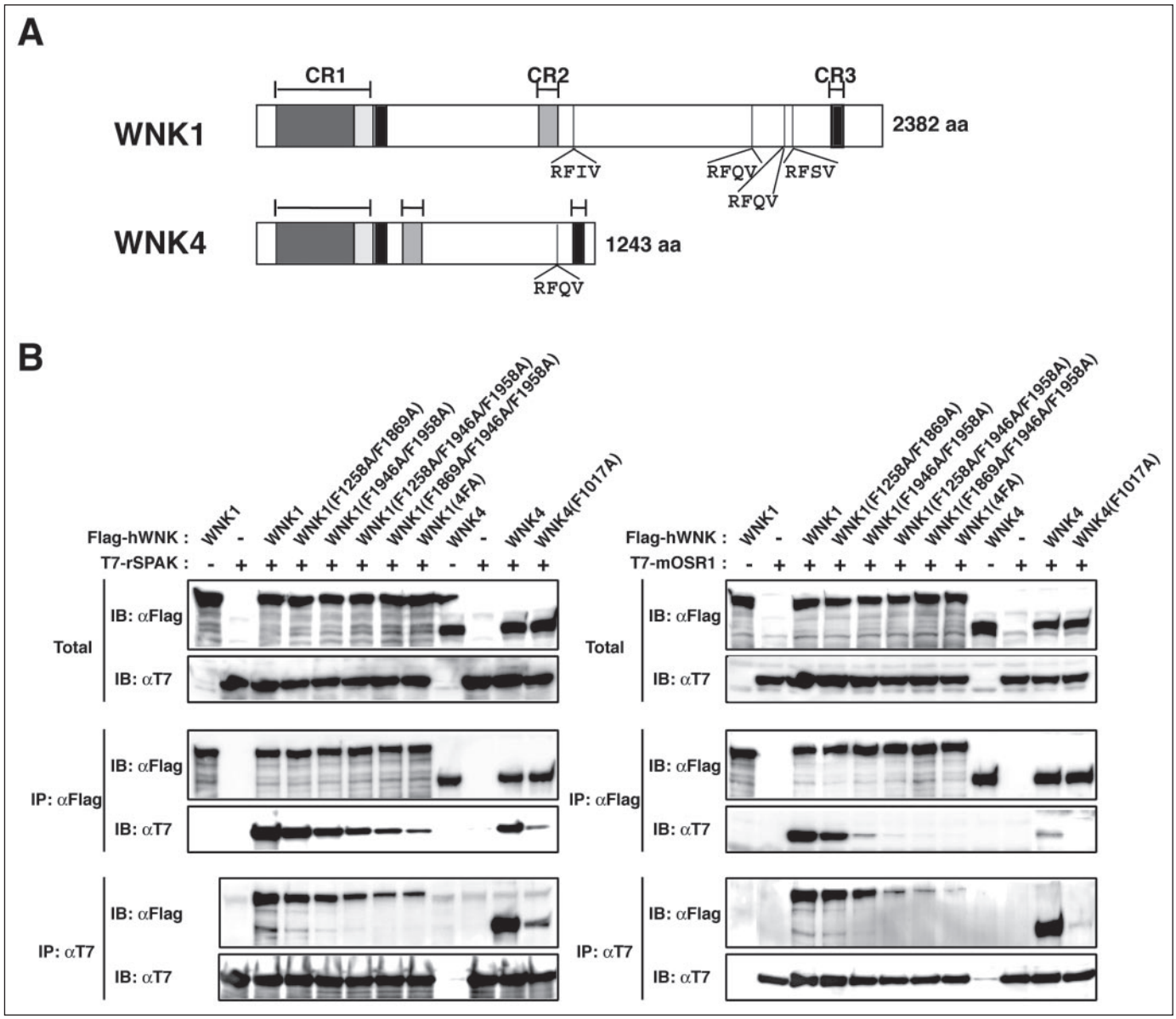

FIGURE 2. **The putative SPAK-binding motifs within WNK kinases are important for binding to SPAK and OSR1.** *A*, schematic of WNK1 and WNK4 domains. The regions of conserved homology, CR1 (kinase domain), CR2, and CR3, are indicated by *horizontal bars* above each protein. The locations of putative SPAK-binding motifs consisting of the consensus sequence (R/K-F-*X*-V/I) are indicated by *vertical bars*. *B*, T7-tagged rat SPAK (*left*) and mouse OSR1 (*right*, *mOSR1*) were co-transfected with various WNK1 and WNK4 mutants as indicated. Protein complexes were co-immunoprecipitated (*IP*) using either T7 antibody (α*T7*) or FLAG antibody (α*FLAG*) and then immunoblotted (*IB*) with αT7 or αFLAG. *hWNK*, human WNK.

fied from the lysates using glutathione-Sepharose and eluted from the resin in 10 mM glutathione.

Kinase Assays—Immunoprecipitated and GST-protein complexes were incubated in a kinase buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP (2 μCi). After incubation for 30 min at 30 °C, the reactions were terminated by the addition of SDS sample buffer, and the proteins were separated by SDS-PAGE. Substrate phosphorylation was analyzed by autoradiography and an image analyzer (Fujix BAS 2500). For determination of phosphorylation sites by MS/MS, GST-SPAK(KM) was incubated with WNK1-(1–665) in a buffer containing 1 mM ATP for 6 h. Solutions used in hypotonic stress experiments were as follows. Basic medium contained 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$/$MgCl_2$, 1 mM Na_2HPO_4/Na2SO4, and 15 mM sodium HEPES, pH 7.4. Low Cl^- hypotonic medium contained 67.5 mM sodium gluconate, 2.5 mM potassium gluconate, 0.5 mM $CaCl_2$/$MgCl_2$, 1 mM Na_2HPO_4/Na_2SO_4, and 7.5 mM sodium HEPES, pH 7.4.

RESULTS

Identification of the STE20-related Kinases SPAK and OSR1 as WNK1-associated Molecules—To identify a protein(s) that physically associates with WNK1, we employed two strategies: yeast two-hybrid screening and FLAG tag immunoprecipitation assays coupled with LC-MS/MS analysis. Several positive clones and putative binding proteins were identified, including STE20-like kinase SPAK. As SPAK was identified by both approaches, we analyzed it further. To investigate whether endogenous SPAK is associated with WNK1 in living cells, we generated antibodies to a peptide of SPAK (the sequence is a 100% match of the corresponding sequence of OSR1, a kinase closely related to SPAK) and a peptide of human WNK1. The anti-SPAK/OSR1 antibody reacted with three bands of 62, 60, and 58 kDa in HEK293 cell extracts and with two bands of 62 and 58 kDa in MDCK cell extracts (Fig. 1). The specificity of anti-SPAK/OSR1 antibody was confirmed by the competition

experiments using the antigen peptide used for immunization (Supplemental data, Fig. S1). The anti-WNK1 antibody reacted with a band of ~250 kDa in several cell lines (Fig. 1). We subjected a lysate from HEK293 cells to immunoprecipitation with anti-WNK1 antibody and then immunoblotted the precipitates with anti-SPAK/OSR1 antibody. SPAK and OSR1 were found to coprecipitate with WNK1 (Fig. 1, *middle*). WNK1 was also detected with immunoprecipitates of SPAK and OSR1 from HEK293 cells or MDCK cells, indicating that these form an endogenous complex in living cells (Fig. 1, *left* and *right*).

SPAK and OSR1 bind to the cation-chloride transporters KCC3, NKCC1, and NKCC2 through a putative binding motif (R/K)F*X*(V/I) within the N-terminal tails of the transporters (22). In a yeast two-hybrid screen, SPAK was found to bind to WNK4, which contains a putative binding motif (23). A search of the amino acid sequences of human WNK1 revealed the presence of four putative SPAK-binding motifs (Fig. 2*A*). To further investigate the role of these motifs in WNK1 binding to STE20-related kinases, we co-expressed various forms of WNK1 with SPAK or OSR1 and performed co-immunoprecipitation experiments. Wild-type WNK1 was found to bind strongly to rat SPAK and mouse OSR1. WNK1(F1258A/F1869A) or WNK1(F1946A/F1958A), in which we mutated two of four SPAK-binding motifs by replacing the Phe residues with Ala, moderately deceased binding to SPAK/OSR1, and the additional mutations to the binding motifs of WNK1 showed gradually weak binding to SPAK/OSR1 depending on the number of mutations (Fig. 2*B*). These results suggested that WNK1 can associate with SPAK/OSR1 through four putative binding motifs.

WNK1 Phosphorylates the Evolutionary Conserved Serine Residues of SPAK and OSR1—To achieve specific and efficient phosphorylation of their substrates, many Ser/Thr protein kinases interact with the substrate via sites distinct from the phosphoacceptor sequence (28, 29). We first investigated whether SPAK and OSR1 are substrates of WNK1 kinase. By expression in bacteria, we produced a GST-tagged rat SPAK(KM) in which the Lys residue within subdomain II was replaced with Met. FLAG-tagged WNK1 was isolated from HEK293 cells and added to GST-SPAK(KM), and the mixture was analyzed by an *in vitro* kinase assay in the presence of [γ-^{32}P]ATP. In addition to WNK1 autophosphorylation, we observed that SPAK(KM) was phosphorylated in a time-dependent manner (Fig. 3*A*, *Wild type*). This phosphorylation was dependent on the kinase activity of WNK1 since phosphorylation was barely detectable when a kinase-dead form of WNK1 was used (Fig. 3*A*, *D368A*). To prove that SPAK is directly phosphorylated by WNK1 and not by putative kinases complexed with WNK1, we isolated several bacterially expressed WNK1 fragments tagged with GST. *In vitro* kinase analysis showed that the purified wild-type WNK1-(1–665) directly phosphorylated SPAK, whereas three forms of kinase-dead WNK1, K233M, D368A, or S382A, did not (Fig. 3*B*). Thus, our results indicated that the STE20-like kinase SPAK is a direct substrate of WNK1.

We next performed *in vitro* kinase assays using several deletion mutants of SPAK (Fig. 4*A*). GST-SPAK-(348–553) and GST-SPAK-(369–553), but not GST-SPAK-(400–553), were phosphorylated by WNK1, indicating that the WNK1 phosphorylation site(s) is located in the C-terminal regulatory region (369–400) of SPAK (Fig. 4*B*). Full-length GST-SPAK and GST-SPAK-(1–399) were also phosphorylated by WNK1, but the phosphorylation of these proteins is weaker than that of the C-terminal regulatory domain alone (Fig. 4*B*). It seems likely that the WNK1 phosphorylation site of SPAK is covered by the N-terminal region of SPAK including the kinase domain. There are four Ser/Thr residues, Ser-379, Ser-380, Thr-386, and Ser-394, within the 369–400 fragment of SPAK (Fig. 4*C*). To determine which residues are the site(s) of phosphorylation, various SPAK mutants were tested as substrates.

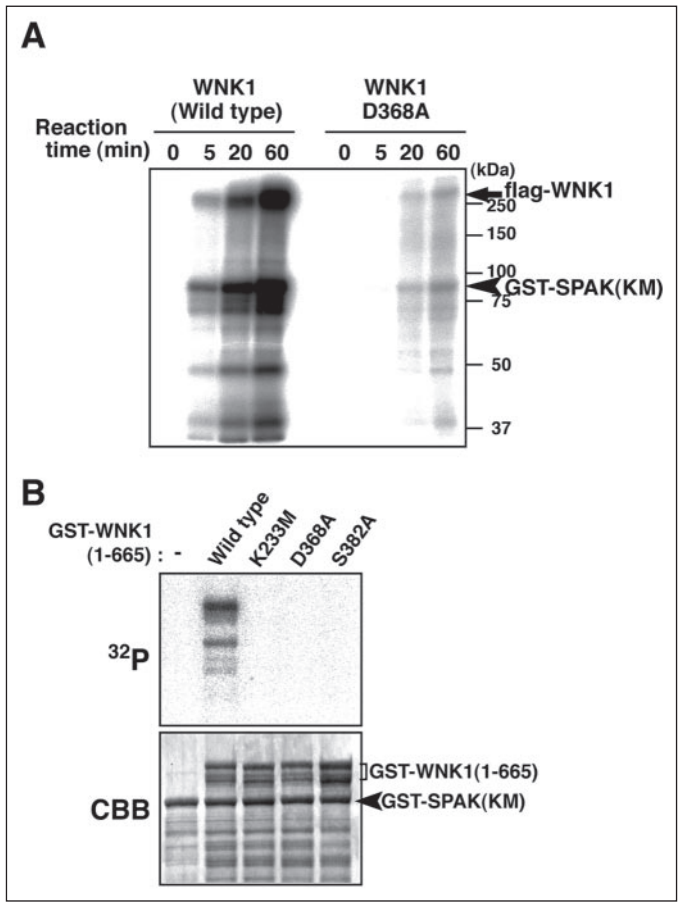

FIGURE 3. **WNK1 directly phosphorylates SPAK protein.** *A*, wild-type WNK1, but not kinase-dead WNK1 (WNK1(D368A)), mediates phosphorylation of SPAK. FLAG-tagged WNK1 and WNK1(D368A) isolated from transfected HEK293 cells were mixed with a GST-tagged kinase-dead form of SPAK (SPAK(KM)) and subjected to an *in vitro* kinase assay. The reaction was stopped at the indicated time points by the addition of SDS sample buffer. The protein complexes were separated by SDS-PAGE, and phosphorylated proteins were visualized by an image analyzer (BAS 2500). *B*, several bacterially produced GST-WNK1 fragments (wild type and three kinase-dead mutants (K233M, D368A and S382A)) and GST-SPAK(KM) were subjected to an *in vitro* kinase assay as indicated. Proteins were separated by SDS-PAGE and then visualized by Coomassie Brilliant Blue staining (*CBB*) and image analyzer (^{32}P).

The fragment 348–553(S380A) was not phosphorylated, indicating that Ser-380 is a major site of WNK1 phosphorylation (Fig. 4*B*). MS/MS analysis of tryptic peptides generated from phosphorylated recombinant SPAK proteins also indicated that Ser-380 is the site of phosphorylation (data not shown). Two small conserved regions were found in the C-terminal regions of SPAK and OSR1 and were named the PF1 and PF2 domains, respectively (Fig. 4*A*). Ser-380 of rat SPAK is located within the PF1 domain, and this Ser residue was highly conserved in other SPAK-related kinases, OSR1 and *Drosophila* Fray (Fig. 4*C*). We mutated the equivalent serine residue (Ser-325) in OSR1 to an Ala residue and examined phosphorylation by the WNK kinases. We found that both FLAG-WNK1 and FLAG-WNK4 were able to phosphorylate the full-length kinase-dead forms of SPAK and OSR1 but that mutation of the Ser residues to Ala of SPAK and OSR1 completely abolished or significantly reduced the phosphorylation (Fig. 4*D*). These data suggested that WNK1 phosphorylates these specific serine residues in SPAK-related kinases.

Ser-325 mutation of OSR1 Causes Its Activation—To examine the role that Ser phosphorylation of SPAK-related kinases plays in their regulation, we generated several mutants of OSR1. It has been recently reported that the N-terminal regulatory domain of p21-activated pro-

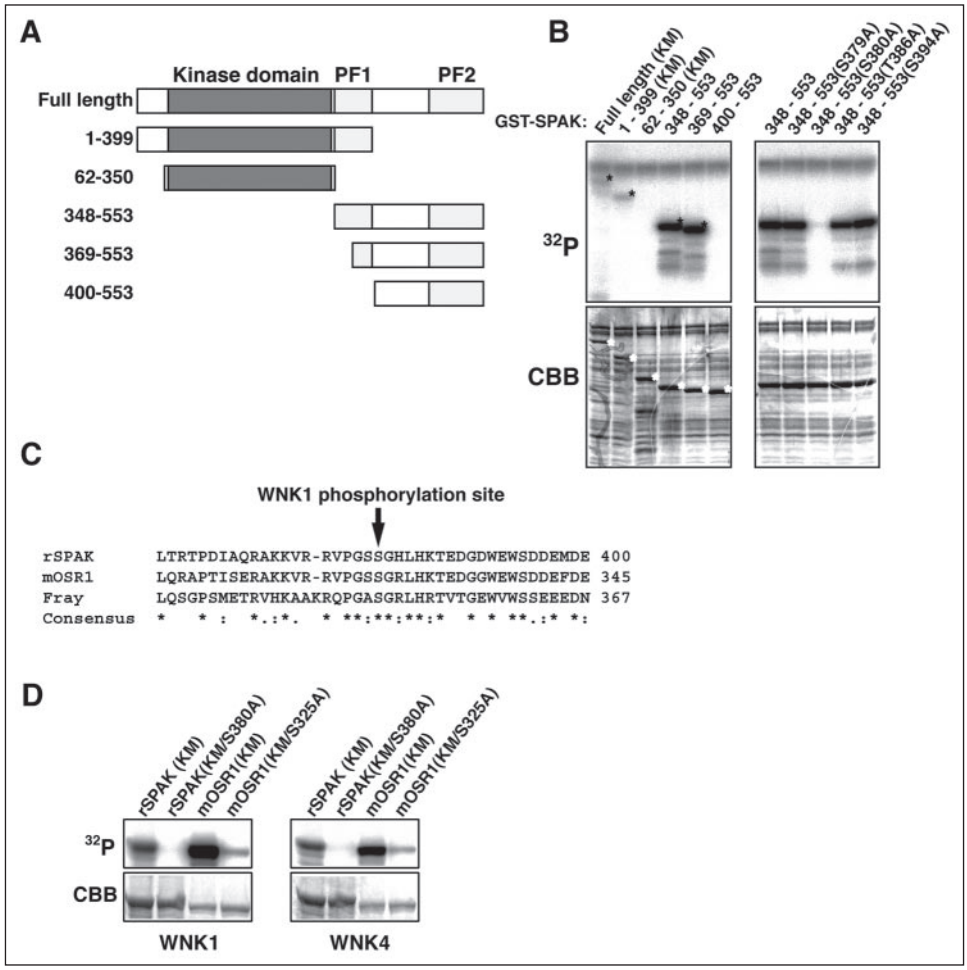

FIGURE 4. **WNK1 phosphorylates the conserved Ser residue in SPAK and OSR1.** *A*, schematic diagram of rat SPAK. Conserved domains among the SPAK-type STE20-related kinases are indicated as follows: kinase domain, PF1 domain (*PF1*), and PF2 domain (*PF2*). Fragments used in this study are also shown. *B*, GST-WNK1-(1–665) *in vitro* kinase assays were performed using the indicated GST-SPAK proteins as substrates and analyzed as in Fig. 3*B*. *Asterisks* indicate the sizes of the substrates. *CBB*, Coomassie Brilliant Blue staining. *C*, the amino acid sequence around the phosphorylation site of rat SPAK (*rSPAK*) is compared with that of mouse OSR1 (*mOSR1*) and *Drosophila* Fray by the ClustalW program. *Asterisks*, identical amino acids; *single* and *double dots*, weakly and strongly similar amino acids, respectively, determined by the criteria of the ClustalW program. *D*, FLAG-WNK1 (*left*) and FLAG-WNK4 (*right*) were isolated from transfected HEK293 cells and subjected to an *in vitro* kinase assay. GST fusion proteins of kinase-inactive SPAK(KM) or OSR1(KM) with or without the Ser to Ala point mutation were used as substrates and analyzed as in *panel B*.

tein kinase (PAK) is a physiological substrate of OSR1 (30). Wild-type GST-OSR1 exhibited a small amount of autophosphorylation, detectable by long exposure of the gel, but GST-OSR1KM showed no detectable activity (Fig. 5*A*). Since mutation of OSR1 Ser-325 to Asp mimics phosphorylation of this site, we generated GST-OSR1(S325D) and tested its activity. GST-OSR1(S325D) showed increased phosphorylation of GST-PAK3-(65–136), relative to wild-type OSR1 (Fig. 5*B*), indicating that the mutation Ser-325 to Asp of OSR1 causes constitutively activation of OSR1. Surprisingly, the mutation of the same site to Ala or Gly, which was expected to abolish phosphorylation, also resulted in causing constitutively activation of OSR1 (Fig. 5*B*, GST-OSR1(S325A), and data not shown). To further investigate the mechanisms of OSR1 activation, we examined several truncated forms of OSR1 (Fig. 5*C*). OSR1-(1–433) and OSR1-(1–344), truncated proteins lacking the PF2 domain, exhibited higher kinase activities than wild-type OSR1. In contrast, OSR1-(1–300), a truncated protein lacking both the PF1 and the PF2 domains, showed no detectable kinase activity (Fig. 5*D*). These results suggested that the PF1 domain of OSR1 is essential for kinase catalytic activity and that the PF2 domain is involved in regulating the catalytic activity.

SPAK and OSR1 Directly Phosphorylate the N-terminal Tail of Cation-Chloride Cotransporters—The activity of NKCC1 is regulated by phosphorylation/dephosphorylation, and examination of phosphorylation sites on NKCC1 revealed that three Thr residues in the N-terminal region, Thr-184, Thr-189, and Thr-202, are necessary for transport activity (19). NKCC2 and NCC are also members of the family of cation-chloride-coupled cotransporters, and the N-terminal regions of both cotransporters are conserved with that of NKCC1 (Fig. 6*A*). To test the possibility that SPAK and OSR1 are responsible for the phosphorylation of NKCC1, and also regulate NKCC2 and NCC, we prepared GST-tagged N-terminal fragments of each of these transporters. Both FLAG-SPAK and FLAG-OSR1 isolated from HEK293 cells were found to phosphorylate GST-NKCC2-(1–181), NKCC1-(1–289) and NCC-(1–138) (Fig. 6*B*). These proteins were also phosphorylated by GST-OSR1(S325D) *in vitro* (Fig. 6*C*), suggesting that phosphorylation was direct. The intensity of each phosphorylated band is comparable with or much higher than that observed using the GST-PAK3-(65–136) substrate, indicating that these cotransporters are good substrates for SPAK and OSR1. We next investigated the phosphorylation site(s) in the thiazide-sensitive transporter NCC using a series of mutations of the Ser/Thr residues that correspond to Thr-184, Thr-189, and Thr-202 of NKCC1. The T53A, T58A, and S71A mutants of NCC showed slightly reduced phosphorylation when compared with wild-type NCC. Moreover, there was no detectable increase in phosphorylation of the triple mutant, T53A/T58A/S71A (Fig. 6*D*). These results suggested that at least *in vitro*, SPAK/OSR1 directly phosphorylates the conserved Ser/Thr residues within the N-terminal regulatory region of NCC corresponding to shark NKCC1.

WNK1 and SPAK/OSR1 Are Activated by Low Cl^- Hypotonic Stress—Recent reports have shown that activation of shark and human Na-K-Cl cotransporter (NKCC1) by low Cl^- hypotonic stimulation is inhibited in cells expressing a dominant-negative mutant of SPAK and that the phosphorylation state of NKCC1 correlates with that of SPAK (24). Therefore, we tested whether hypotonic and low Cl^- conditions in cells

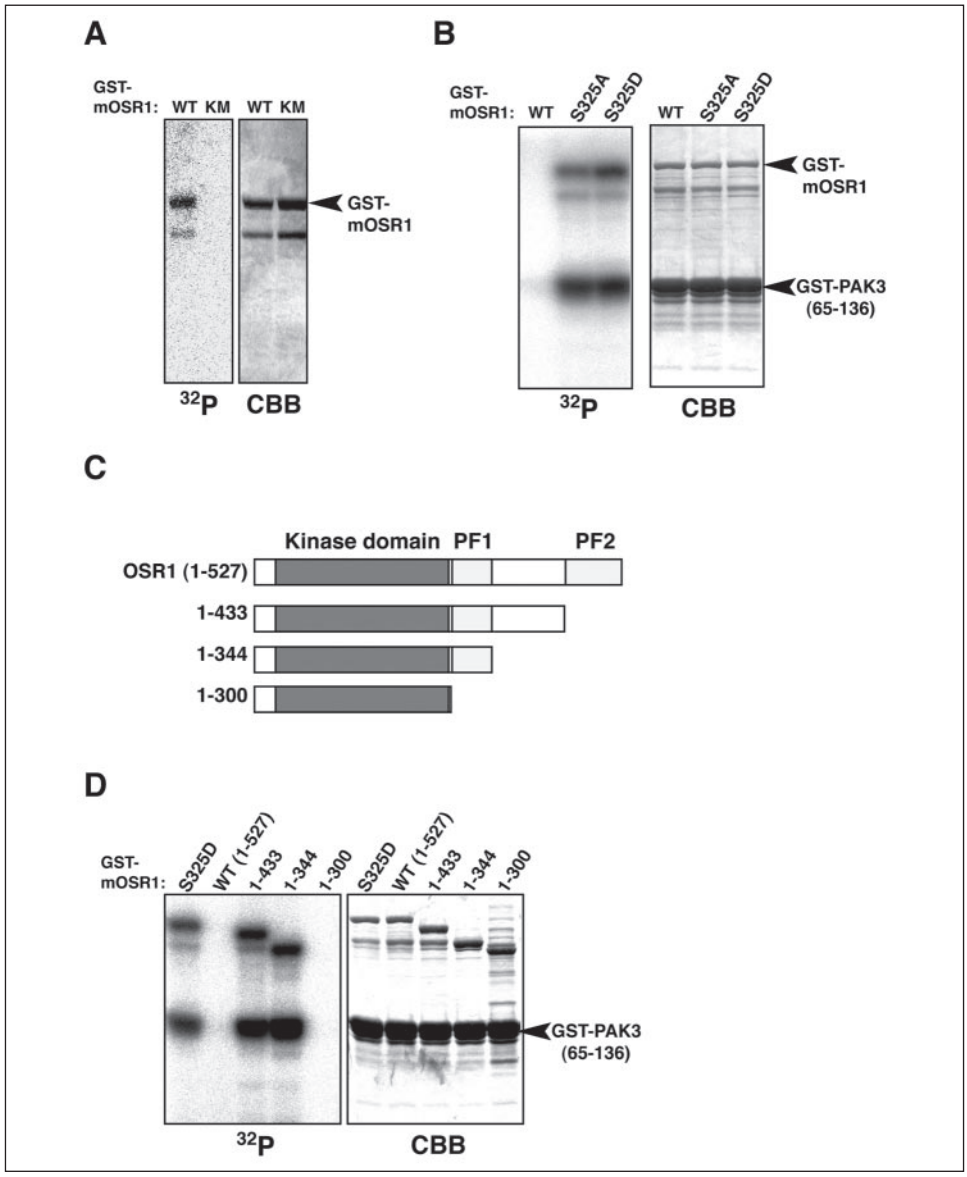

FIGURE 5. **Effects of WNK1 phosphorylation site modification on OSR1 activity *in vitro*.** *A*, kinase assays were performed with equal amounts of GST-tagged wild-type SPAK (*WT*) and kinase-dead form of OSR1(KM). *mOSR1*, mouse OSR1. *B*, GST-OSR1, GST-OSR1(S325A), and GST-OSR1(S325D) proteins were examined for their kinase activity toward GST-PAK3-(65–136). *C*, schematic diagram of mouse OSR1. Fragments used in *panel D* are also shown. *D*, the kinase activities of several OSR1 mutants were measured by *in vitro* kinase assay using GST-PAK3-(65–136) as a substrate. *CBB*, Coomassie Brilliant Blue staining.

lead to the activation of SPAK/OSR1. HEK293 cells were incubated with isotonic or low Cl^- hypotonic buffer, and endogenous WNK1 was immunoprecipitated and subjected to *in vitro* kinase assay using GST-SPAK-(348–553) as a substrate. We found that WNK1 kinase activity increased within 5 min and was sustained for at least 60 min by incubation in low Cl^- hypotonic conditions (Fig. 7*A*). We next examined the effect of low Cl^- hypotonic stimulation on phosphorylation and activation of SPAK/OSR1. When HEK293 cells transfected with an empty vector were incubated with hypotonic and low Cl^- buffer, SPAK/OSR1 autophosphorylation and kinase activity against GST-PAK3-(65–136) were increased (Fig. 7*B*). The phosphorylation of Ser-325 in OSR1 also occurred in cells under hypotonic and low Cl^- conditions. These results, together with those obtained *in vitro*, suggested that WNK1 functions as an activator for SPAK/OSR1 in response to Cl^- hypotonic stress in cells.

To clarify the role of the WNK/SPAK/OSR1 pathway in the phosphorylation of NCC, we performed a ^{32}P labeling experiment. Because we were unable to detect endogenous NCC in HEK293 cells or other cell lines by immunoblotting and immunostaining, T7-tagged mouse NCC was expressed in HEK293 cells. As shown in Fig. 7*C*, NCC was highly phosphorylated under low Cl^- hypotonic conditions. This result agreed well with the *in vitro* phosphorylation data and suggested that activation of the WNK1/SPAK/OSR1 pathway leads to the enhanced phosphorylation of NCC in cells.

DISCUSSION

In this study, we identified the STE20-like kinases, SPAK and OSR1, as targets of WNK1. WNK1 phosphorylates SPAK/OSR1 at a Ser residue within the PF1 domain, which is highly conserved among the mammalian SPAK/OSR1, *Drosophila* Fray, and *Caenorhabditis elegans* Y59A8B.23 gene products. WNK4 and WNK3 were also able to phosphorylate this residue (Fig. 2*F* and data not shown). In addition, *C. elegans* WNK1 phosphorylated the conserved Ser residue of the *C. elegans* SPAK/OSR1 homolog *in vitro*.[3] Thus, phosphorylation of SPAK/OSR1 by WNK kinase may be a common regulatory mechanism among species.

OSR1 mutants having point mutants in their PF1 domain or trunca-

[3] T. Moriguchi and H. Shibuya, unpublished data.

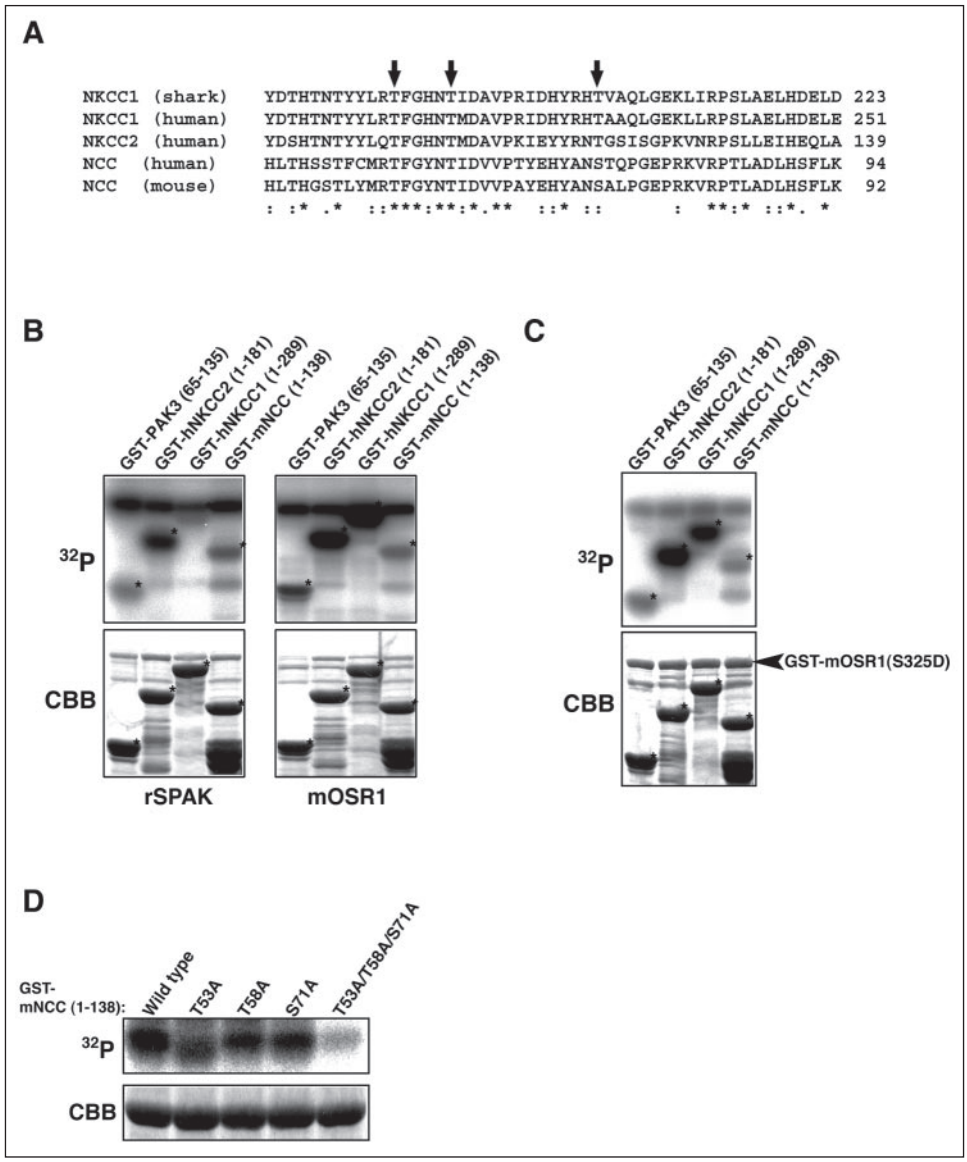

FIGURE 6. **N-terminal regulatory tail of cation-chloride-coupled cotransporter is phosphorylated by SPAK and OSR1.** *A*, alignment of the amino acid sequences of the regulatory regions in the N terminus of cation-chloride-coupled cotransporter, shark NKCC1, human NKCC1, human NKCC2, human NCC, and mouse NCC (*mNCC*). *Asterisks*, identical amino acids; *single* and *double dots*, weakly and strongly similar amino acids, respectively, were determined by the criteria of ClustalW program. *B*, T7-SAPK (*left*) or T7-OSR1 (*right*) isolated from HEK293 cells were assayed with GST-PAK3-(65–136), GST-hNKCC2-(1–181), GST-hNKCC1-(1–289), and GST-mNCC-(1–138) as substrates. Proteins were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue (*CBB*) staining and an image analyzer (BAS 2500). *Asterisks* indicate the sizes of the substrates. *mOSR1*, mouse OSR1. *C*, GST-OSR1(S325D) was assayed for kinase activity as in *panel B*. *D*, GST-fusion proteins of NCC N-terminal fragments with the indicated Ser/Thr to Ala point mutations were used as substrates in GST-OSR1(S325D) kinase assays and analyzed as in *panel B*.

tion mutants lacking the PF2 domain exhibited higher kinase activities than wild-type OSR1 (Fig. 5). Moreover, a truncated mutant lacking the PF1 domain of OSR1 displayed no detectable kinase activity (Fig. 5*D*). It has been reported that many STE20-related kinases contain autoinhibitory domains and that removal of these regulatory domains results in a significant increase in kinase activity (31). Therefore, our results suggested that mutation of Ser-325 may cause constitutive activation of kinase activity due to conformational changes resulting from removal of the autoinhibitory PF2 domain rather than by an effect of negative charge. In the case of OSR1, the PF1 domain appeared to play an essential role in kinase catalytic activity, whereas the PF2 domain might be involved in regulating catalytic activity.

Mutation of the site in OSR1 that is phosphorylated by WNK1 resulted in enhanced OSR1 kinase activity, indicating that WNK1 plays an important role in the activation of SPAK and OSR1. However, *in vitro* phosphorylation of recombinant SPAK and OSR1 proteins by WNK1 or co-expression of SPAK and OSR1 with WNK1 in cells resulted in only weak activation of SPAK and OSR1 (data not shown). Therefore, WNK1 might not be the sole activator of SPAK and OSR1. The phosphorylation of multiple sites by several kinases has been shown to be required for the full activation of some kinases. For example, Akt is activated by phosphorylation on two residues, one in the activation loop of the kinase domain and the other located C-terminal to the catalytic domain (32). Phosphorylation of these sites in Akt is catalyzed by two kinases, 3-phosphoinositide-dependent kinase-1 (PDK1) and another tentatively called PDK2. Further studies will be needed to fully identify the kinase(s) that phosphorylates and activates SPAK/OSR1.

We also demonstrated that SPAK and OSR1 directly phosphorylate not only NKCC1 but also NKCC2 and NCC. These cation-chloride-cotransporters contain 12 transmembrane domains that are flanked by hydrophilic N- and C-terminal domains. It has been previously shown that three phosphorylation sites on the N terminus of shark NKCC1, Thr-189, Thr-184, and Thr-202, are necessary for full activation of transport activity (19). The sites of OSR1 phosphorylation in NCC include the three conserved Thr residues within the N-terminal regulatory region of the cation-chloride-coupled cotransporter family (Fig. 5), suggesting that WNK1 and SPAK/OSR1 could contribute to the regulation of transport activity. In fact, this hypothesis is supported by the recent finding that expression of both SPAK and WNK4 with NKCC1 in *Xenopus* oocytes results in a significant increase in NKCC1 activity (33). NCC, the mammalian thiazide-sensitive Na-Cl transporter, is expressed at the apical membrane of the distal convoluted

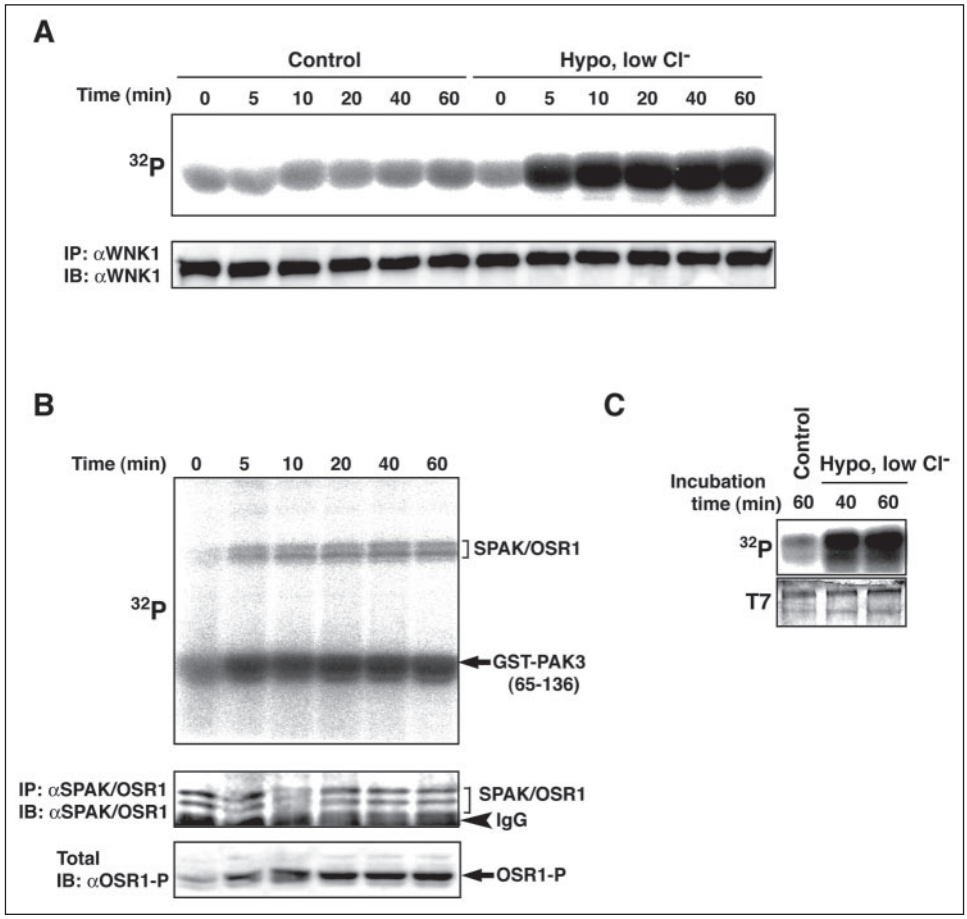

FIGURE 7. **Activation of WNK1 and SPAK/OSR1 by low Cl^- hypotonic stress.** *A*, HEK293 cells were incubated in isotonic buffer (*Control*) or low Cl^- hypotonic buffer (*Hypo, low Cl^-*) for the indicated times. The kinase activity of endogenous WNK1 was measured by an immune complex kinase assay using GST-SPAK-(348–553) as a substrate. The amount of immunoprecipitated WNK1 was detected by immunoblotting with the WNK1 antibody (immunoprecipitation (*IP*), αWNK1; immunoblotting (*IB*), αWNK1), and the phosphorylated GST-SPAK-(348–553) was detected by an image analyzer (BAS 2500) (*^{32}P*). *B*, HEK293 cells were incubated in low Cl^- hypotonic buffer for the indicated times. Endogenous SPAK/OSR1 was immunoprecipitated with the SPAK/OSR1 antibody, and subjected to an immune complex kinase assay using GST-PAK3-(65–136) as a substrate. The amount of SPAK/OSR1 in each immune complex was determined by immunoblotting (immunoprecipitation, αSPAK/OSR1; immunoblotting, αSPAK/OSR1). To monitor the Ser phosphorylation state of OSR1, lysates prepared from transfected cells were subjected to immunoblotting with the phospho-OSR1 antibody. Similar results were obtained in three different experiments. *C*, phosphorylation of T7-tagged NCC in HEK293cells. HEK293 cells were transfected with T7-NCC, metabolically labeled with [^{32}P]phosphate for 6 h, and then placed with isotonic buffer (*Control*) or low Cl^- hypotonic buffer for the indicated times prior to lysing. T7-NCC was immunoprecipitated using αT7 antibody.

tubule. Loss-of-function mutations in NCC have been shown to cause Gitelman syndrome, a disease characterized by salt wasting, hypokalemic metabolic alkalosis, and hypocalciuria. These clinical symptoms are the opposite of the symptoms observed in PHA II patients. The mutations in WNK1 associated with PHA II are intron deletions that cause increased expression of WNK1. Our findings supported the hypothesis that WNK1 phosphorylates and activates NCC, and this may provide a good explanation for pathogenesis of PHA II. However, the physiological relevance of these phosphorylation events to hypertension must be further evaluated by examining the regulation of NCC transport activity. In contrast to NCC, NKCC2, the bumetanide-sensitive cotransporter, is expressed in the apical membrane of the thick ascending limb of Henle's loop. Disruption of the *NKCC2* gene causes Bartter syndrome, an autosomal recessive disease characterized by metabolic alkalosis, hypokalemia, and hypercalciuria accompanied by a reduction in arterial blood pressure. Thus, it might be possible that activation of NKCC2 could account for hyperkalemia and hypertension in patients harboring *WNK1* mutations.

Tissue distribution reveals that WNK1 is widely expressed (1, 2). *WNK1*-deficient mice exhibit embryonic lethality, which indicates that WNK1 has important functions in many tissues, in addition to the kidney (34). NKCC1, SPAK, and OSR1 are also ubiquitously expressed and have multiple functions, such as regulation of cell volume, modulation of neuron excitability, AP-1-dependent gene expression, and regulation of the actin cytoskeleton (17, 30, 35). In this study, we identified a signaling pathway consisting of the PHA II disease-associated kinase WNK1 and the STE20-related kinases SPAK and OSR1, which culminates in the phosphorylation of several cotransporters. We hope that these findings will contribute to our understanding of the biological function for WNK1, not only in the pathogenesis of hypertension but also in other processes.

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Addendum—While this report was in preparation, a study describing WNK1/WNK4 phosphorylation of the same key serine residue, but also identifying an additional threonine residue within the activation loop, was published (36).

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