WNK3-SPAK Interaction is Required for the Modulation of NCC and other Members of the SLC12 Family

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Key Words

Salt transport • Distal tubule • Hypertension • WNK • Thiazide

Abstract

The serine/threonine with no lysine kinase 3 (WNK3) modulates the activity of the electroneutral cation-coupled chloride cotransporters (CCC) to promote Cl⁻ influx and prevent Cl⁻ efflux, thus fitting the profile for a putative “Cl⁻-sensing kinase”. The Ste20-type kinases, SPAK/OSR1, become phosphorylated in response to reduction in intracellular chloride concentration and regulate the activity of NKCC1. Several studies have now shown that WNKs function upstream of SPAK/OSR1. This study was designed to analyze the role of WNK3-SPAK interaction in the regulation of CCCs with particular emphasis on NCC. In this study we used the functional expression system of *Xenopus laevis* oocytes to show that different SPAK binding sites in WNK3 (²⁴¹, ⁸⁷², ¹³³⁶RFxV) are required for the kinase to have effects on CCCs. WNK3-F1337A no longer activated NKCC2, but the effects on NCC, NKCC1, and KCC4 were preserved.

In contrast, the effects of WNK3 on these cotransporters were prevented in WNK3-F242A. The elimination of F873 had no consequence on WNK3 effects. WNK3 promoted NCC phosphorylation at threonine 58, even in the absence of the unique SPAK binding site of NCC, but this effect was abolished in the mutant WNK3-F242A. Thus, our data support the hypothesis that the effects of WNK3 upon NCC and other CCCs require the interaction and activation of the SPAK kinase. The effect is dependent on one of the three binding sites for SPAK that are present in WNK3, but not on the SPAK binding sites on the CCCs, which suggests that WNK3 is capable of binding both SPAK and CCCs to promote their phosphorylation.

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Introduction

The electroneutral cation-coupled chloride cotransporter (CCC) gene family (*SLC12*) is divided in two branches, depending on the cations used for the chloride translocation. The sodium-driven branch

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(Na-[K]-Cl) is made up of three members that exhibit between 50 to 60% identity with each other: the renal thiazide-sensitive Na⁺-Cl⁻ cotransporter NCC and two isoforms of the bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter, NKCC1 and NKCC2. The potassium-driven branch (KCC) contains four different K⁺-Cl⁻ cotransporters, KCC1 to KCC4, which share between 60 and 70% identity. The CCC cotransporters serve many physiological functions that ranging from cellular to organic and complex system physiology. For example, CCCs are critical for cell volume regulation. CCCs work in conjunction to define the intracellular chloride concentration ([Cl⁻]_i), which in neurons is of primary importance to define the type of response to neurotransmitters using chloride channels in the postsynaptic membranes. The activity of CCCs in some epithelial cells and vascular smooth muscle cells is key for regulation of arterial blood pressure. Inactivating mutations in some CCCs are associated with inherited diseases such as Bartter's, Gitelman's and Anderman's disease for NKCC2, NCC, and KCC3, respectively [1]. Several studies demonstrate that CCCs are regulated by cell volume and/or intracellular chloride concentration ([Cl⁻]_i) by means of phosphorylation and dephosphorylation events. It is accepted that phosphorylation activates the sodium branch and inhibits the potassium branch, while dephosphorylation exerts the opposite effects [1-3]. The existence of a kinase/phosphatase pair, the activities of which are modulated by cell volume and/or [Cl⁻]_i, has been proposed to be the modulator of CCC activities in an opposite fashion [4-10]. In this regard, WNK3 increases the activities of the sodium-driven cotransporters NCC, NKCC1, and NKCC2 and decreases the activities of the potassium-driven members KCC1-KCC4 [11-13]. Thus, by promoting Cl⁻ influx and preventing Cl⁻ efflux, WNK3 fits the profile of a putative "Cl⁻-sensing kinase" [14].

The Ste20-type kinases SPAK/OSR1 become phosphorylated in response to decreases in [Cl⁻]_i and regulate the activity of NKCC1 [10, 15-18]. In order to phosphorylate target proteins, SPAK/OSR1 binds to them in a consensus motif containing RFxV/I [16]. Recent evidences show that SPAK/OSR1 are also involved in the regulation of the NKCC2 [19] and NCC [20] cotransporters. Several studies have shown that WNK1 and WNK4 activate SPAK/OSR1, which, in turn, seem to be the final kinases regulating the CCCs [21-23]. In this regard, we have shown that NKCC2 is activated by intracellular chloride depletion, and the signalling pathway required both WNK3 and SPAK [24]. In this

study, however, the mutant WNK3-F1337A, lacking a SPAK binding site, lost its activating properties upon NKCC2, but retained the inhibitory effect towards KCC4. Thus, it is not clear if WNK3-SPAK interaction is required for modulation of other CCCs. This study was designed to define the role of the WNK3-SPAK interaction in the regulation of NCC and other members of the SLC12 family.

Materials and Methods

Clones, cloning, sequencing, and antibodies

The following previously characterized clones were used in this study: a wild type human cMyc-WNK3 cDNA clone containing exon 18a without exon 22 (isoform 3) and the catalytically inactive form cMyc-WNK3-D294A [13]; cMyc-WNK3-F1337A in which a SPAK binding site was eliminated [24]; HA-tagged mouse wild-type SPAK cDNA and its catalytically inactive mutant, SPAK-K104R [24, 25]; rat Flag-NCC and NKCC2 cDNAs [26]; rabbit NKCC1 cDNA [12]; and mouse KCC4 cDNA [27]. The following mutations were introduced in corresponding clones using custom primers (SIGMA) and site-directed mutagenesis: NCC-R18A, NCC-F19A, NKCC2-F17A, WNK3-F242A, and WNK3-F873A. To avoid unwanted mutations, the mutant fragment was fully sequenced between two unique restriction sites, digested, and ligated into the original wild-type cDNA.

The protein expression of cMyc-WNK3, HA-SPAK, and Flag-NCC were analyzed using monoclonal antibodies against cMyc, HA, and Flag tags, respectively. Analysis of phospho-NCC at T58 (threonine 58 in rat, corresponding to T60 in humans) or phospho-SPAK at T243 were performed using previously characterized phospho-antibodies [20].

In vitro cRNA translation

To prepare *in vitro* cRNA for microinjection, the wild type or mutant cDNAs were linearized at the 3' end with the appropriate unique restriction enzyme and transcribed with T7 RNA polymerase. The integrity of transcripts was confirmed on agarose gels, and the cRNA concentration was determined by the absorbance reading at 260 nm. The cRNAs were stored in aliquots at -80°C until use.

Preparation of Xenopus laevis oocytes

Adult female *Xenopus laevis* frogs (NASCO, Fort Atkinson, MI) were maintained under controlled light conditions at a water temperature of 18°C. Each frog was housed at least three months after arrival before use. Oocytes were surgically collected under anesthesia by 0.17% tricaine immersion, incubated in Ca²⁺-free ND96 medium (mM: 96 NaCl, 2 KCl, 1 MgCl₂, and 5 HEPES/Tris, pH 7.4), plus 2 mg/ml collagenase A for 1 h, washed four times in regular ND96, defolliculated by hand, and incubated overnight in ND96 at 16°C. The next day, mature oocytes were injected with 50 nl of water or with water containing 0.1–0.4 µg/µl of

cRNA transcribed *in vitro* from the various constructs. Oocytes were incubated in ND96 at 16°C supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml of gentamicin for 2–3 days prior to transport assays or protein extractions for western blotting. The incubation medium was change every 24 hours by daily. Prior to the uptake assay, oocytes were switched to Cl⁻-free ND96 for 2–16 hours or to low-chloride hypotonic stress medium for 16 hours, as previously described [24, 28].

Functional Assays with Cation/Cl⁻ Cotransporters

Xenopus laevis oocytes were injected with cRNA for NCC, NKCC1, NKCC2, or KCC4 alone or in combination with KCC2 cRNA or the wild-type or mutant WNK3 and/or SPAK cRNAs. After 2–3 days of incubation, metolazone-sensitive ²²Na⁺ (for NCC), bumetanide-sensitive ⁸⁶Rb⁺ (for NKCC1 and NKCC2), or Cl⁻-dependent ⁸⁶Rb⁺ uptake (for KCCs) was determined following our standard procedures [11, 24, 27, 29]. Activation of NCC by low chloride hypotonic stress was done as previously described [28]. Depending on the assessed cotransporter, the following inhibitors were used as required: ouabain to inhibit the Na⁺-K⁺-ATPase, amiloride to block the Na⁺ channels, bumetanide to prevent activity of the Na⁺-K⁺-2Cl⁻ cotransporter, and metolazone to inhibit the Na⁺-Cl⁻ cotransporter. The desired concentrations of the inhibitors were present during both the incubation and uptake periods, except when noted otherwise.

All uptake experiments were performed at least three times and included at least 10 oocytes in each experimental group; statistical significance was $p < 0.05$, and results were reported as means $\pm$ S.E. The uptake observed in control groups was taken as 100%, and experimental groups were normalized accordingly. The significance of the differences between groups was tested by one-way ANOVA with multiple comparisons using Bonferroni corrections.

Immunoprecipitation and western blotting

Western blots were used to assess the expression of cMyc-WNK3, HA-SPAK, and Flag-NCC protein in cRNA-injected oocytes in different conditions. Immunoblots were performed using monoclonal antibodies against the cMyc, HA, or Flag tags, as previously described [25, 28]. Briefly, groups of 15 oocytes exposed to each condition were homogenized in 4 μ l/oocyte of CellLytic M Cell Lysis Reagent from SIGMA for immunoprecipitation assay or Lysis buffer (50 mM Tris/HCl, pH 7.5, 1mM EGTA, 1mM EDTA, 50 mM sodium fluoride, 5mM sodium pyrophosphate, 1mM 2-mercaptoethanol and protease inhibitors (1tablet per 50 ml)) for immunoblotting using phospho-antibodies, followed by centrifugation at 17,530 g. The supernatants were then recovered, and 50 μ g of total protein was resolved by 8.5% SDS-PAGE and electroblotted onto polyvinylidenedifluoride membranes (PVDF, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked for 1 hr at room temperature in TBS buffer-0.02% Tween-20 plus 5% BSA and exposed to either the mouse anti-Flag peroxidase-conjugated antibody (SIGMA), 1:2000, or the mouse anti-HA peroxidase-conjugated antibody (SIGMA), 1:400, or the rabbit anti-cMyc peroxidase-conjugated antibody (SIGMA), 1:1000 overnight at 4°C.

Polyclonal-actin antibody was used as a control (1:2500). Bands were detected with the ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences, Amersham).

The phospho-antibodies Thr 58-NCC, Thr 243-SPAK (kindly provided by Dario Alessi, Dundee University) were used as previously described [20]. Total protein extraction and SDS-PAGE protocols were the same as those used for the monoclonal antibodies described above.

For immunoprecipitation experiments, 500 μ g of protein extracted from oocytes were incubated with 5 μ g of HA-monoclonal antibody overnight (o/n) at 4°C. Proteins bound to HA-conjugated antibodies were incubated with 50 μ l of protein G-Sepharose (SIGMA) o/n at 4°C with gentle agitation. Immunoprecipitates were washed two times with IP buffer 1X (SIGMA) and with a third wash with IP buffer 0.1X (SIGMA). Bound proteins were heated and eluted in 80 μ l of laemmli sample buffer (Bio-Rad) plus 2% β -mercaptoethanol. For Flag-immunoprecipitation, Anti-FLAG M2-agarose affinity gel was used according to SIGMA protocol. Total immunoprecipitated product was separated by SDS-PAGE (8.5%), transferred onto PVDF membranes and subjected to Western blotting as previously described. The membranes were stripped once according with Amersham ECL Plus Western blotting detection reagents manual and were reprobed with the previously described antibodies.

Assessment of wild-type and mutant WNK3 catalytic activity

HEK293 (human embryonic kidney) cells were cultured on 10-cm-diameter dishes in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. For HEK293 transient transfection, each dish of adherent HEK293 cells was transfected with 20 μ l of 1 mg/ml polyethylenimine (Polysciences) and 5–10 μ g of plasmid DNA in 1 ml of untreated DMEM. The cells were lysed in 0.3 ml ice-cold lysis buffer (50 mM Tris/HCl, pH 7.5, 1mM EGTA, 1mM EDTA, 50 mM sodium fluoride, 5mM sodium pyrophosphate, 1mM 2-mercaptoethanol and protease inhibitors (1tablet per 50 ml)), the lysates were clarified by centrifugation at 26,000 $\times$ g for 15 min at 4°C, and the supernatants were frozen in liquid nitrogen. Protein concentrations were quantified using the Bradford method.

Expression and immunoprecipitation of Flag-WNK3 isoforms

HEK293 cells in 10-cm-diameter dishes were transiently transfected, as described above, with 10 μ g of one of the following N-terminal Flag-tagged WNK3 constructs: Flag-WNK3 WT, Flag-WNK3 D294A or Flag-WNK3 F242A. Thirty-six hours post-transfection, the cells were lysed in ice-cold lysis buffer. The Flag-tagged WNK3 constructs were affinity-purified by incubating 1 mg of the clarified lysate with 5–10 μ l of Flag-agarose for 1 hour at 4°C. The beads were washed three times with lysis buffer containing 150 mM NaCl followed by 2 washes with Buffer A (50 mM Tris-HCl pH7.5, 0.1mM EGTA pH7.5 and 0.1% (v/v) 2-mercaptoethanol). The purified proteins were used for activity measurements.

Purified WNK3 kinase assays

Flag-tagged WNK3 isoforms were purified as described above, and their activity toward SPAK kinase was evaluated. The reaction mixture contained a final volume of 25 μ l of Buffer A containing 2 μ g of recombinant GST-tagged SPAK WT or GST-tagged SPAK KD [D294A], 0.1 mM [32 P]-ATP, and 10 mM MgCl₂. The reaction was performed in a Thermomixer at 30°C for 0, 30 and 60 min, and the reaction was terminated by the addition of 5 μ l of 5X SDS loading buffer. The samples were resolved on a polyacrylamide gel, which was stained with coomassie blue, dried and autoradiographed.

Results and Discussion

The SPAK binding site on NCC is not required for the activation of the cotransporters by WNK3 or intracellular chloride depletion

KCC3a [16] and KCC2a [30] contain a unique SPAK binding site. However, no such a site is present in KCC1, KCC2b, KCC3b, and KCC4. Nevertheless all KCCs are inhibited by WNK3 [11]. Thus, evidently such a site does not seem to be required for WNK3 effects on K⁺:Cl⁻ cotransporters. There is one SPAK binding site in NCC. We first examined the requirement of the SPAK binding site on this cotransporter for the effect of WNK3, particularly because it is known that elimination of the SPAK binding site prevents NCC-SPAK interaction [19, 20]. As shown in Fig. 1A, basal activity of NCC is reduced in the absence of SPAK binding site. However, WNK3 was able to increase the activity of the cotransporter in the absence of the SPAK binding site within NCC. Additionally, the inhibitory effect of the catalytically inactive WNK3-D294A (WNK3-DA) on NCC [13] also occurred in absence of the unique SPAK binding site (Fig. 1B). Therefore, basal activity of the NCC depends on the presence of SPAK binding site on the cotransporter, but the response to the stimulatory and inhibitory effects of wild type WNK3 and kinase dead WNK3, respectively, do not. Similar results were observed for NKCC2 (data not shown). One possibility that could be argued is that the overexpression of WNK3 overcome the absence of a SPAK binding site on the cotransporter. To eliminate this possibility, we assessed the effects of low chloride hypotonic stress that reduce [Cl⁻]_i [24, 28] on NCC activity, with or without the SPAK binding site. Because the biochemical analysis of the NCC-SPAK interaction presented by Richardson et al. [20] was performed by eliminating the arginine instead of the phenylalanine in the SPAK binding site (RFxV), we included both possibilities (NCC-R18A or NCC-F19A). As shown in

Fig. 1. The NCC unique SPAK binding site is not required for activation of the cotransporter by WNK3 (A), inhibition by WNK3-D294A (B), or activation by intracellular chloride depletion (C). After 2–3 days of incubation, metolazone-sensitive 22 Na⁺ uptake was determined in oocytes injected with wild type or mutant NCC cRNA alone or together with 10 ng of cRNA per oocyte of WNK3 (A) or WNK3-D294A (W3-DA) (B). In C, the absence of SPAK binding site on NCC did not preclude the activating response towards intracellular chloride depletion. Oocytes were injected with wild type or mutant NCC (as stated) and incubated for 72 hrs in regular ND96. Then, oocytes were incubated for 16 h in either regular ND96 (210 mosmol/kg H₂O) (white bars) or in Cl⁻-free hypotonic medium (170 mosmol/kg H₂O) (black bars). The next day, metolazone-sensitive 22 Na⁺ uptake was assessed in uptake media with similar osmolarity to which oocytes were exposed the night before. The mean value of wild type NCC injected oocytes was taken as 100%, and the value observed with NCC-F19A or NCC-R18A, in the presence of WNK3, WNK3-D294A, or low chloride hypotonic stress were normalized. Significantly different from the uptake observed in the corresponding control, *p $\leq$ 0.005.

Fig. 1C, wild-type and mutant NCCs were activated by intracellular chloride depletion. Thus, the absence of the SPAK binding site on NCC did not preclude the activating response to intracellular chloride depletion.

Intracellular chloride depletion or WNK3 induce NCC phosphorylation at threonine 58

The effect of intracellular chloride depletion or co-injection with WNK3 on NCC and SPAK phosphorylation was assessed by using specific phospho-antibodies against NCC-T58 or SPAK-T243 (this threonine residue in SPAK is phosphorylated by WNKs [23, 31, 32]). Figure 2 shows representative immunoblots of proteins extracted from oocytes under the diverse conditions described. As expected, phosphorylation of NCC, which was almost undetectable at basal conditions, increased by intracellular chloride depletion maneuvers or by WNK3 co-injection. The intensity of NCC phosphorylation under low Cl⁻ stress was augmented by co-injection of SPAK cRNA. In oocytes exposed to intracellular chloride depletion maneuvers or WNK3 cRNA, faint bands representing endogenous SPAK/OSR1 kinases phosphorylated at T243 were observed. The lower molecular weight of the bands observed with SPAK phospho-antibodies in oocytes not injected with SPAK cRNA is due to the fact that the endogenous kinase in oocytes is OSR1, rather than SPAK; OSR1 lacks a PAPA box in the amino-terminal domain [33]. It is not surprising that SPAK phospho-antibodies cross-react with endogenous OSR1, because kinase domains and phosphorylation sites are conserved among species. Phosphorylation of NCC at T58 or SPAK at T243 by WNK3 was more evident when SPAK cRNA was included in the injection cocktail. As expected, consistent with its inhibitory effect on NCC when coexpressed in oocytes [34], no increased phosphorylation of SPAK was observed in proteins extracted from oocytes co-injected with NCC and WNK4. Thus, although results from Fig. 1 indicates that SPAK binding site on NCC is not required for WNK3-induced activation of the cotransporter, results from Fig. 2 show that WNK3 induces phosphorylation of both SPAK and NCC, suggesting that during activation of NCC, WNK3 interacts with SPAK.

The importance of the SPAK binding site within WNK3 kinase domain on regulation of NCC and other members of the SLC12 family

We have previously shown that NKCC2 activation by intracellular chloride depletion via WNK3-SPAK is

Fig. 2. WNK3 induces phosphorylation at NCC-T58 and SPAK-T243. A representative Western blot analysis performed using the phosphoantibodies pNCC-T58 and pSPAK-T243, Flag-NCC monoclonal antibody, HA-SPAK antibody and β-actin antibody. Total proteins were extracted from *Xenopus laevis* oocytes injected with water or NCC alone or coinjected with SPAK, WNK3, or WNK4 cRNA and exposed to intracellular chloride depletion maneuvers (low Cl⁻ hypotonic stress or coinjection with KCC2 cRNA, as described in).

dependent on the WNK3-SPAK interaction, because elimination of the 1336 RFxV SPAK binding site in WNK3 (by substituting residue F1337 for alanine) reduced the WNK3-SPAK interaction and prevented the positive effect of WNK3 on NKCC2 activity [24]. Thus, we assessed the effect of wild-type WNK3 and mutant WNK3-F1337A on the activity of NKCC1, NKCC2, NCC, and KCC4. As shown in Fig. 3, compared with wild type WNK3, the mutant WNK3-F1337A had no effect on NKCC2, but induced similar activation of NCC and NKCC1, as well as inhibition of KCC4. Thus, the SPAK binding site (¹³³⁶RFxV) within WNK3 is required for the WNK3-induced effects on NKCC2, but not on the other members of the SLC12 family.

WNK3 contains three SPAK binding sites (Fig. 4A). One is located within the kinase domain (²⁴¹RFxV), and the other two are within the long carboxyl-terminal domain (⁸⁷²RFxV and ¹³³⁶RFxV). We eliminated each site to generate the mutants WNK3-F242A, WNK3-F873A, or WNK3-F1337A. The effect of these mutants

Fig. 3. The SPAK binding site (¹³³⁶RFxV) for WNK3 induced effects on NKCC2, but not for NCC and other CCCs. *Xenopus laevis* oocytes were injected with cRNA of NKCC1 (A), NKCC2 (B), NCC (C), or KCC4 (D) alone or together with WNK3 or the mutant WNK3-F1337A, as stated. After 2-3 days of incubation, bumetanide-sensitive ⁸⁶Rb⁺ for NKCC1 and NKCC2, metolazone-sensitive ²²Na⁺ for NCC and Cl⁻-dependent ⁸⁶Rb⁺ uptake for KCC4 were determined following standard procedures. The mean value of each cotransporter alone was taken as 100% and the effect of WNK3 or the mutant F1337A-WNK3 on each cotransporter was normalized to that value. *p<0.0001, Significantly different from the uptake observed in the control group.

Fig. 4. Effect of elimination of single SPAK binding sites within WNK3 on WNK3-induced effects towards CCCs. (A) Schematic representation of SPAK binding sites within WNK3. The SPAK binding motif ²⁴¹RFxV is located at the kinase domain (KD), while ⁸⁷²RFxV and ¹³³⁶RFxV are positioned at the carboxyl terminal domain between coil coiled domains (CC). The phenylalanine residues (F242, F873 and F1337) were substituted with alanine by site directed mutagenesis. Metolazone-sensitive ²²Na⁺ uptake for NCC (B), bumetanide-sensitive ⁸⁶Rb⁺ uptake for NKCC1 (C), and Cl⁻-dependent ⁸⁶Rb⁺ uptake for KCC4 (D) were determined following standard procedures. The mean value of each cotransporter alone was taken as 100% and the effect of coinjection with WNK3, WNK3-F242A, WNK3-F873A, and WNK3-F1337A cRNA was normalized to that value. *p<0.0001, Significantly different from the uptake observed in the control group of NCC, NKCC1, and KCC4 alone, respectively.

on NCC, NKCC1, and KCC4 was subsequently assessed. In all cases, oocytes transfected with a particular cotransporter were co-injected with wild-type

WNK3 or with each of the WNK3 mutant cRNAs to test their effects using exactly the same batch of oocytes as was used with wild-type WNK3. At least

Fig. 5. The lack of effect of WNK3-F242A upon SLC12 cotransporters is not due to loss of catalytic activity. A-C) *in vitro* kinase assay between wild type WNK3 (A), mutant WNK3-F242A (B) or D294A (C) and SPAK, as stated. Flag-tagged WNK3 wild type and mutant purified from HEK293 transfected cells were incubated for 0, 30 and 60 min in the presence of GST-tagged SPAK and 0.1 mM [32 P]ATP. After incubation the samples were electrophoresed, dried and autoradiographed. WNK3 wild type and mutant WNK3-F242A showed autophosphorylation and migrated around 190 kDa, while phosphorylated SPAK kinase migrated around 60 kDa. A second band around 58 kDa, which seems to be OSR1. No activity was observed using the catalytically inactive form of WNK3 (WNK3-D294A). D-F) *Xenopus laevis* oocytes were injected with cRNA of NCC (D), NKCC1 (E), or KCC4 (F), alone or together with the mutants WNK3-D294A, WNK3-F242A or the double mutant WNK3-D294A/F242A, as stated. After 2-3 days of incubation, metolazone-sensitive $^{22}\text{Na}^+$ for NCC (D), bumetanide-sensitive $^{86}\text{Rb}^+$ for NKCC1 (E), and Cl $^-$ -dependent $^{86}\text{Rb}^+$ uptake for KCC4 (F) were determined following standard procedures. The mean value of basal activity of each cotransporter alone was taken as 100% and the effect of the mutants on each cotransporter was normalized to that value. * $p < 0.0001$, significantly different from the uptake observed in the corresponding control group.

three experiments for each cotransporter were performed. Figures 4B-D show the results for NCC, NKCC1, and KCC4, respectively. Elimination of sites F873 and F1337 had no effect upon the WNK3-mediated activation of NCC and NKCC1, or inhibition of KCC4. In contrast, the elimination of the F242 site completely prevented WNK3-mediated activation of NCC and NKCC1, as well as WNK3-mediated inhibition of KCC4 activity.

Because the F242 site is located within the kinase domain, one potential problem could be that elimination of this SPAK binding site affected the catalytic activity of WNK3. In this regard, it is known that WNK3-mediated effects on CCCs are dependent on its catalytic activity [11-13]. In fact, inactive WNK3 produces the opposite effect. Therefore, we performed an *in vitro* analysis that demonstrated catalytic activity in wild-type WNK3 and WNK3-F242A against itself and against SPAK as a substrate, while no activity was observed using the catalytically inactive mutant WNK3-D294A (Fig. 5A-C). Additionally, the opposite effect induced by the catalytically inactive

WNK3-D294A was not observed using WNK3-F242A. As was previously shown [11-13], WNK3-D294A inhibited NCC and NKCC1, and activated KCC4 (Fig. 5D-F). In contrast, WNK3-F242A showed no effects on the basal activities of these cotransporters. That is, WNK3-F242A had neither a wild type WNK3-like, nor a catalytically inactive WNK3-D294A-like effect. Thus the findings in Fig. 5 supports the hypothesis that the absence of WNK3-F242A effects on the cotransporters, as observed in Fig. 4, was not due to elimination of the catalytic activity in this mutant. Interestingly, Fig. 5 shows that the effect of the catalytically inactive WNK3-D294A was also abolished by the elimination of the WNK3 SPAK binding site F242, suggesting that the opposite effect that are observed when the catalytic activity of WNK3 is prevented (WNK3-D294A), is also dependent on the interaction between WNK3 and SPAK.

As shown in Fig. 4C and 5E, NKCC1 behaved unexpectedly different from the other cotransporters. Although WNK3-F242A no longer activated NKCC1 (Fig. 4C) as wild-type WNK3 or WNK3 mutants F873A

Fig. 6. The SPAK binding site F242 in WNK3 is required for interaction between WNK3, SPAK and NCC. (A) HA-SPAK immunoprecipitation assay was performed using protein extracts of oocytes co-injected with water or cRNA of NCC, WNK3, and SPAK along with wild type or NCC-R18A and WNK3-F242A mutants, as stated. After immunoprecipitation, Western blot analysis against cMyc-WNK3, HA-SPAK, Flag-NCC and β-actin was performed using the corresponding monoclonal antibodies. (B) Flag-NCC immunoprecipitation assay was performed using protein extracts from oocytes injected with NCC cRNA alone or coinjected with WNK3 wild type or mutant (WNK3-D294A, WNK3-F242A) cRNA. Western blot analysis was performed using the corresponding monoclonal antibodies. (C) Flag-NCC immunoprecipitation assay using proteins extracted from oocytes injected with wild type or mutant NCC and WNK3.

and F1337A did, it reduced the activity of NKCC1. There is no clear explanation for this finding, but there is evidence that the activity of NKCC1 against SPAK and its regulators could be different. In contrast to the other members of the SLC12 family, NKCC1 contains two SPAK binding sites in the amino-terminal domain and the cotransporter can be phosphorylated by SPAK, even in the absence of these sites [10, 35]. Additionally, in functional expression experiments in oocytes, the effect of WNK4 on NKCC1 is completely changed from inhibitory (in the absence of SPAK cRNA co-injection [36]) to stimulatory (when SPAK cRNA is co-injected with WNK4 cRNA [37]). This phenomenon does not occur with NCC or any of the KCCs [25, 28, 37].

WNK3 SPAK binding site F242 is required for the interaction between WNK3, SPAK, and NCC

The functional observations gathered suggest that the effect of WNK3 on NCC requires binding between

WNK3 and SPAK by means of the binding site located within the kinase domain (²⁴¹RFxV). Additionally, our data suggest that interaction between WNK3-SPAK-NCC occurs, but that the SPAK binding site on NCC is not required, because NCC can be activated by WNK3 in the absence of the unique site (Fig. 1). We immunoprecipitated HA-SPAK or Flag-NCC with corresponding antibodies followed by Western blot analysis for cMyc-WNK3, HA-SPAK, and Flag-NCC. Immunoprecipitation with anti-HA is shown in Fig. 6A. Similarly to previous report [20], a positive band for Flag-NCC was observed in oocytes co-injected with wild-type NCC and SPAK cRNA, but not in those injected with NCC-R18A and SPAK cRNA. This is consistent with the observation that the basal activity of NCC was significantly reduced when the unique SPAK binding site on NCC was eliminated (Fig. 1).

In proteins extracted from oocytes co-injected with NCC-R18A plus WNK3 and SPAK, a band corresponding

to NCC was detected using an anti-Flag antibody, as was a band corresponding to WNK3 using an anti-cMyc antibody. This observation suggests that a protein complex was formed between WNK3, SPAK, and NCC-R18A in the absence of the SPAK binding site on NCC. The amount of NCC detected was higher when co-injection was performed with wild-type NCC, suggesting that binding between NCC and SPAK improves the formation of the complex. This finding is consistent with the observation that WNK3 is able to activate both wild-type NCC and NCC-F19A or NCC-R18A (Figs. 1, 7).

The last two columns of Fig. 6A show the consequence of the WNK3-F242A mutation. The intensity of the Flag-NCC band detected when WNK3-F242A cRNA was co-injected was similar to that observed in the absence of WNK3 (NCC+SPAK group), suggesting that the presence of Flag-NCC in the protein complex in this group is due to its interaction with SPAK. Moreover, when both WNK3-F242A and NCC-R18A cRNAs were injected, Flag-NCC was no longer in the protein complex, consistent with the absence of a functional effect of WNK3-F242A on NCC (Fig. 4).

These observations together suggest that WNK3 is capable of interacting not only with SPAK, but also with NCC; thus, it is possible that because WNK3 binds both, SPAK and NCC, the binding between SPAK and NCC is not strictly required to form the functional WNK3-SPAK-NCC complex, explaining the ability of WNK3 to activate NCC even in the absence of the SPAK binding site within NCC. To support this conclusion, Fig. 6B shows that WNK3 and NCC do, in fact, interact with each other. In this experiment, Flag-NCC was immunoprecipitated, and proteins were immunoblotted with anti-Flag and anti-cMyc antibodies. The analysis revealed that NCC and WNK3 formed a protein complex that was still present when either the catalytically inactive WNK3-D294A or the non-SPAK-binding WNK3-F242A cRNA constructs were used. It is unlikely that endogenous SPAK within the oocytes could link WNK3 and NCC, because WNK3-F242A also coimmunoprecipitated with NCC. However, to completely eliminate this possibility, we immunoprecipitated wild type Flag-NCC or the mutant Flag-NCC-R18A and probed for WNK3 in the protein complex in the absence of the NCC-SPAK interaction. As shown in Fig. 6C, even in the absence of SPAK binding site, WNK3 and NCC-R18A formed a protein complex, suggesting that it is due to direct interaction between

Fig. 7. Phosphorylation of NCC at T-58 by WNK3-SPAK is prevented by elimination of the F242 SPAK binding site in WNK3. (A) A representative experiment of NCC thiazide-sensitive $^{22}\text{Na}^+$ uptake assessed in a pool of oocytes injected with NCC (white bars) or NCC-R18A (black bars) alone or together with SPAK, WNK3 and/or WNK3-F242A cRNA, as stated. The mean value of NCC thiazide-sensitive $^{22}\text{Na}^+$ uptake in the wild type NCC group was taken as 100% and the rest of the groups were normalized accordingly. * $p < 0.001$, Significantly different from the uptake observed in wild-type NCC group. ** $p < 0.001$, Significantly different from the uptake observed in the control NCC-R18A group. (B) A representative Western blot analysis on protein extracts from the same batch of oocytes, as stated. Monoclonal antibodies were used to detect the presence of cMyc-WNK3, Flag-NCC, HA-SPAK and β -actin. The phospho-antibodies p-NCC-T58 and p-SPAK-T243 were used to recognize phosphorylation of Threonine 58 on NCC and 243 on SPAK, respectively.

these proteins. Thus, WNK3 and NCC interact with each other, even in the absence of catalytic activity or the $^{241}\text{RFxV}$ SPAK binding site in WNK3. In this regard, interaction between other WNKs and NCC have been documented [34, 38].

WNK3 SPAK binding site F242 is required for WNK3 induced SPAK and NCC phosphorylation

The activation of NCC by intracellular chloride depletion or by WNK3 is associated with increased phosphorylation of the cotransporter at T58 (corresponding to T60 in human NCC) (Fig. 2) [28]. To define the requirement of the SPAK binding site on NCC and WNK3 in this process, we assessed the phosphorylation status of wild-type NCC or mutant NCC-R18A when coexpressed with SPAK, wild-type WNK3, or mutant WNK3-F242A. Figure 7 shows a representative experiment, with the corresponding Western blots generated using protein extracts from the same batch of oocytes as those used for the functional analyses. Figure 7A reproduces what we observed in Figs. 1-4. This is, (i) basal activity of NCC-R18A was lower than wild-type NCC; (ii) SPAK by itself had no effect upon NCC basal activity; (iii) WNK3 was able to activate both, the wild-type and the mutant NCC-R18A; and (iv) WNK3-F242A was not able to activate, neither NCC, nor NCC-R18A.

Results from Fig. 7B are consistent with the functional observations. The Flag-NCC panel shows that NCC was similarly expressed in all groups. The use of specific phosphoantibodies against NCC-T58 revealed that phosphorylation of NCC was very low to undetectable in basal conditions, but increased by co-expression of NCC with wild-type WNK3 alone or together with SPAK (also shown in Fig. 2). NCC-R18A, T58 phosphorylation also increased by coexpression with WNK3 or WNK3 together with SPAK. However, consistent with its lack of functional activation of NCC, the phosphorylation of the cotransporter was not increased by WNK3-F242A. Using the p-T243-SPAK antibodies, we observed no phosphorylation of endogenous SPAK/OSR1 in oocytes injected with wild type or mutant NCC-R18A, but a slight band was detected when WNK3 was included in the injection cocktail (similar to Fig. 2). SPAK T-243 phosphorylation was more evident when SPAK cRNA was coinjected with WNK3, together with NCC or NCC-R18A. However, it was completely absent in the presence of WNK3-F242A.

Our results are consistent with previous observations that NCC and SPAK interact with each other by means of NCC binding site located in the amino-terminal domain [20] (Fig. 8A). This interaction is clearly required to sustain basal tonic activity of NCC and NKCC2, as elimination of the unique SPAK binding site

Fig. 8. Proposed model for WNK3 and SPAK interaction in the regulation of NCC. (A) SPAK and NCC interact by the SPAK binding motif on the cotransporter. (B) WNK3 is able to bind both SPAK and NCC and induces phosphorylation of SPAK, which in turns phosphorylates NCC [20, 19]. (C) The binding of SPAK to NCC is not strictly necessary for the activation of the cotransporter by WNK3, because WNK3 interacts with both SPAK and NCC.

on each cotransporter reduced basal activity (Fig. 1). However, our data also support the assertion that the effects of WNK3 on NCC and other CCCs require interaction with, and activation of, the SPAK kinase. The effects are dependent on one of the three binding sites for SPAK that are present in WNK3. Interestingly, for NKCC1, NCC, and KCC4, a key SPAK binding site within WNK3 is the one located in the kinase domain (²⁴¹RFxV), while for NKCC2, it is one of the binding sites present in the carboxy-terminal domain [24]. Although the SPAK binding site on NCC is necessary to sustain the protein's basal activity, it is not required for WNK3 activation of the cotransporter, since neither NCC-R18A nor NCC-F19A are activated by WNK3 (Fig. 1). The fact that no KCC isoforms, with the exception of KCC3a, contain SPAK binding sites, but WNK3 nevertheless inhibits the activity of all of them [11], supports this hypothesis. Thus, as shown in Fig. 8B and 8C, we suggest that WNK3 is capable of binding both SPAK and NCC (or other CCCs), forming a protein complex that can be created regardless of a direct interaction between SPAK and NCC (Fig. 8C). In conclusion, our study shows that WNK3-induced effects on NCC and other members of the SLC12 family are dependent on the WNK3-SPAK interaction.

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