Regulation of the expression of the Na/Cl cotransporter by WNK4 and WNK1: evidence that accelerated dynamin-dependent endocytosis is not involved

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Golbang, Amir P., Georgina Cope, Abbas Hamad, Meena Murthy, Che-Hsiung Liu, Alan W. Cuthbert, and Kevin M. O’Shaughnessy. Regulation of the expression of the Na/Cl cotransporter by WNK4 and WNK1: evidence that accelerated dynamin-dependent endocytosis is not involved. Am J Physiol Renal Physiol 291: F1369–F1376, 2006. First published July 5, 2006; doi:10.1152/ajprenal.00468.2005.—The novel serine/threonine kinases (with no lysine kinases or WNKs), WNK1 and WNK4, are encoded by the disease genes for Gordon syndrome (PRKWN1 and PRKWN4), a rare monogenic syndrome of hypertension and hyperkalemia. These proteins alter the expression of the thiazide-sensitive NaCl cotransporter (NCCT) in Xenopus laevis oocytes, although the details are controversial. We describe here our own experience and confirm that kinase-dead WNK4 (318D>E) is unable to affect Na+ fluxes through the thiazide-sensitive NaCl transporter (NCCT) or its membrane expression as an ECFP-NCCT fusion protein. However, the kinase domain is not sufficient for a functional NCCT since deletion of the acidic motif (a motif unique to WNK family members) completely abolishes functional activity. Indeed, the NH2 terminal of WNK4 (1–620) containing the kinase domain and acidic motif retains full activity, but does not interact directly with NCCT in pull-down assays. Coexpression of WNK1 antagonizes the action of WNK4, and kinase-dead WNK1 (368D>A) or WNK4 carrying a WNK4 disease mutation (565Q>E) behaves in the same way as wild-type WNK1. This suggests kinase activity and charge conservation within the acidic motif are not essential for the WNK1-WNK4 interaction. We also report that WNK4 probably reduces surface expression largely through an effect on forward trafficking. Hence, the effect of WNK4 on NCCT expression is mimicked by dynamin, but the dominant-negative K44A dynamin mutant does not block the action of WNK4 itself. These results further highlight important differences in the mechanism by which WNK kinases affect expression of NCCT vs. other membrane proteins such as ROMK.

The thiazide-sensitive NaCl cotransporter (NCCT) is a typical member of the SLC12 cotransporter family with 12 membrane-spanning domains (5). In the mammalian kidney, expression is confined to the apical membranes of cells lining the distal convoluted tubule (DCT). It transports a small minority of the filtered sodium load (5–8%) in the kidney, but it is a physiologically important fraction with human loss-of-function mutations in NCCT causing hypotension and the monogenic syndrome of Gitelman’s (3). Despite its importance, little was previously known about the regulation of NCCT expression in the DCT, although both aldosterone and chronic treatment with thiazide diuretics have been reported to increase the membrane density of the cotransporter (11, 17). This situation has changed dramatically following the discovery of the With-No-Lysine Kinases (WNK), WNK4 and WNK1, which affect membrane expression of NCCT as well as several other cotransporters, and at least one ion channel, ROMK, within the nephron (7, 15, 20).

The WNKs are a small family of serine/threonine kinases so called because they lack a lysine residue (lysine=K) previously assumed to be essential for kinase activity. They were subsequently identified as the disease genes for another rare monogenic syndrome called Gordon’s syndrome (pseudohypoaldosteronism type II, OMIM #145260). Patients with this syndrome have a characteristic low-renin hypertension and hyperkalemia, i.e., the phenotypic inverse of Gitelman’s syndrome. Their extreme sensitivity to thiazide diuretics also pointed to increased NCCT activity within the nephron (10).

The mechanism behind this observation came from coexpression studies in Xenopus laevis oocytes showing that WNK4 was able to suppress the surface expression of NCCT in oocytes (20). Significantly, this effect of WNK4 was lost if a mutant WNK4 protein was expressed carrying one of the missense mutations identified in patients with Gordon’s syndrome. These missense mutations do not occur in the kinase domain of WNK4 itself, and all bar one lie within a run of 10 amino acids called the acidic motif (2, 19). This motif consists of residues with predominantly negative charge and although its function is unknown it is a feature unique to all four known WNKs.

Despite WNK4 being an S/T kinase, it is controversial whether its kinase activity is necessary for its interaction with NCCT. The Lifton lab originally reported that it was necessary (19), but a recent publication from the Ellison lab has suggested this is not the case and that WNK4 regulates NCCT expression by protein-protein interactions through its COOH terminal (24). It is also not clear what the reduced NCCT expression represents in terms of trafficking of the protein, since reports to date have looked at expression at single time points. The action of WNK4 could reduce, for example, processing or insertion into the surface membrane; equally it could accelerate removal from the cell surface as has been reported for ROMK (7). To try and address these issues as well as the

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roles of various motifs in WNK1 and WNK4, we have carried out a further study using *X. laevis* oocytes, which is reported here. Our results confirm the importance of the kinase domain of WNK4 in suppression of NCCT expression. However, WNK4 cannot function in the absence of an acidic motif and isolating these domains in an NH2-terminal fragment (1–620) reproduces the full activity of WNK4. WNK1 is functional when coexpressed with WNK4, but blockade of WNK4 is not dependent on its catalytic activity. Finally, we suggest that WNK4 appears to block expression largely through an effect on forward trafficking from the Golgi.

**METHODS**

**Cloning and cRNA synthesis.** Full-length sequences for NCCT and WNK4 were PCR amplified from mouse kidney cDNA and cloned into pcDNA3. The wild-type (WT) sequence of WNK4 was mutated to produce 565Q→E (QE W1) and 318D→A (kinase dead, KD W4) mutants using site-directed mutagenesis. For WNK4 truncations and acidic motif deletions, full-length mWNK4 was used as a template to generate two approximately equal sized NH2- and COOH-terminal fragments (designated 1–620 and 620–CT) to isolate the main motif features; the 30 bp encoding the entire acidic motif (EPEEPEADQH) features; the 30 bp encoding the entire acidic motif (EPEEPEADQH) and 30 bp encoding the entire acidic motif (EPEEPEADQH) of WNK4 in suppression of NCCT expression. However, WNK4 cannot function in the absence of an acidic motif and isolating these domains in an NH2-terminal fragment (1–620) reproduces the full activity of WNK4. WNK1 is functional when coexpressed with WNK4, but blockade of WNK4 is not dependent on its catalytic activity. Finally, we suggest that WNK4 appears to block expression largely through an effect on forward trafficking from the Golgi.

**Expression in *X. laevis* oocytes.** *X. laevis* eggs were harvested and defolliculated as detailed previously (6). The cRNA (10 ng of either NCCT or ECFC-N CCT) was injected in a total volume of 100 nl per oocyte and for coinjections involving WNKs or one of the mutants an additional 10 ng of RNA was added to the injection mix. Water-injected oocytes were used as controls throughout. Oocytes were then incubated in ND96 containing 2 mM sodium pyruvate and gentamicin, 0.1 mg/ml at 18°C for 5 days unless stated otherwise.

For 22Na flux studies, oocytes were placed for 24 h in Cl−-free ND96 solution containing: 96 mM sodium isethionate, 2 mM potassium glutconate, 1.8 mM calcium glutconate, 1 mM magnesium glutconate, 5 mM HEPES, 2.5 mM sodium pyruvate, and 5 mg/ml gentamicin. Thirty minutes before the addition of uptake medium, the oocytes were added to ND96 (Cl− and K+ free) with inhibitors (1 μM ouabain, 100 μM amiloride, and 100 μM bumetanide), according to the protocol of Gamba et al. (5). The oocytes were then transferred to isotonic uptake medium (58 mM NaCl, 38 mM N-methyl-d-glucamine, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, with inhibitors, pH 7.4) containing 22Na+ at a final concentration of 2.5 μCi/ml and incubated in a gently shaking incubator at 30°C for 1 h. The oocytes were then washed five times with 6 ml ice-cold aliquots of isotonic uptake medium and the oocytes were counted individually in a γ-counter (Perkin-Elmer Cobra 5033). Thiazide sensitivity was shown by abolition of 22Na+ uptake with 100 μM hydrochlorothiazide (data not shown).

Membrane surface expression measurements were carried out 5 days (ECFP-N CCT) after injection except in the case of the time course experiments. Membrane surface expression of ECFP-N CCT was determined by laser-scanning confocal microscopy with a Leica DMRXA confocal microscope. All images were captured in real time from intact live oocytes using an equatorial section through each oocyte. Images were collected using a ×10 objective lens with brightness and contrast settings kept constant for all oocytes in each injection series. The fluorescent signal in the membrane was quantified using Leica confocal software (version 2.61 of LCS lite) with sampling made at 16 equi-spaced points on the circumference and averaged to give mean total fluorescence intensity in arbitrary fluorescence units (AFUs).

**Western blotting and pull-downs.** Oocytes were placed in lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 μl of protease inhibitor cocktail set III from Calbiochem) and left for 10 min on ice before vigorous pipetting and vortexing. Oocytes were then sonicated, and the yolk and cellular debris were pelleted by centrifugation for 10 min at 10,000 rpm at 4°C. The supernatant was removed and 5 volumes of acetone were added and the eppendorf tube was left on ice for 10 min, before being centrifuged at 10,000 rpm for 5 min to remove any final yolk and debris. Oocyte homogenates were dialyzed at 4°C until required.

Thawed samples (from 10 oocytes) were solubilized in sample buffer (100 mM Tris·HCl, pH 7.6, 5% glycerol, 2% SDS, and 5% β-mercaptoethanol, 0.02% Bromophenol blue) by heating at 90°C for 3 min before loading onto an 8% PAGE gel. The proteins were then separated by SDS-PAGE gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane, blocked (5% nonfat dried milk in PBS with 0.1% Tween 20) and incubated with the appropriate polyclonal rabbit anti-NCCT, anti-WNK4 or anti-WNK1 antibody (all from Alpha Diagnostic International, San Antonio, TX) at 1:800 dilution in PBS. The blot was then washed with PBS with 0.1% Tween 20 and probed with a goat anti-rabbit HRP-conjugated secondary antibody (1 in 1,500 dilution, Amersham Bioscience). The protein was finally visualized with ECL Plus reagents (Amersham Bioscience) according to manufacturer’s instructions.

For pull-down assays, 20 ng of WNK4 fusion protein (1–620 and 620–2000 fragments) was adsorbed onto His-Tag agarose (Novagen), and incubated with the appropriate polyclonal rabbit anti-NCCT, anti-WNK4 or anti-WNK1 antibody (all from Alpha Diagnostic International, San Antonio, TX) at 1:800 dilution in PBS. The blots were then washed with PBS with 0.1% Tween 20 and probed with a goat anti-rabbit HRP-conjugated secondary antibody (1 in 1,500 dilution, Amersham Bioscience). The protein was finally visualized with ECL Plus reagents (Amersham Bioscience) according to manufacturer’s instructions.

The blots were then washed with PBS with 0.1% Tween 20 and probed with a goat anti-rabbit HRP-conjugated secondary antibody (1 in 1,500 dilution, Amersham Bioscience). The protein was finally visualized with ECL Plus reagents (Amersham Bioscience) according to manufacturer’s instructions.

**Biotinylation.** Oocytes injected with appropriate cRNA were incubated for either 15 or 120 h in ND96 with 2 mM pyruvate and 0.1 mg/ml gentamicin. Oocytes in batches of 20 were washed three times in ND96 then incubated for 1 h in 2 mg ml EZ-Link Sulfo-NHS-SS biotin (Pierce, Rockfort, IL) in ND96 solution at 4°C. The coupling reaction was quenched by the addition of quenching solution (Pinpoint Cell Surface Protein Isolation Kit, Pierce), and incubated for a further 5 min at 4°C. After being rinsed twice with ND96, they were transferred to an appropriate volume of cell lysis buffer + protease inhibitors (Protease Inhibitor cocktail II, Calbiochem) and lysed as detailed above. The protein lysate was diluted via the addition of 4X volume of lysis buffer + protease inhibitors and added to columns containing half the supernatant volume of NeutrAvidin Gel (Pierce), columns were capped and incubated at 4°C on end to end rotating stage. Columns were then centrifuged at 3,000 rpm for 1 min at 4°C and sequentially washed three times with one column volume of wash solution + protease inhibitors. Columns were finally centrifuged at 3,000 rpm for 1 min between each wash and flow-through discarded. Biotinylated proteins retained on the NeutrAvidin Gel were recovered by the addition of 60 μl of 1X SDS-PAGE buffer +DTT and incubated for 1 h at 4°C then centrifuged at 3,000 rpm for 2 min.
Protein concentration was determined by BCA total protein assay (Pierce); 20 μg were then electrophoresed by SDS-PAGE and Western blotted as described above. Band densitometry was estimated using version 1.36b of the ImageJ software (http://rsb.info.nih.gov/ij/).

Data analysis. For all oocyte experiments, at least 10–15 oocytes were injected for each cRNA used. Differences between groups were compared by one-way ANOVA with post hoc comparison by t-testing. Figures show representative experiments that were replicated using at least four different batches of oocytes from different donor animals. The SPSS statistical package (software version 11) was used throughout with significance defined as P < 0.05.

RESULTS

Effect of WT and kinase-deficient WNK4 on NCCT-mediated 22Na uptake. To confirm the previously reported inhibition of NCCT activity by WNK4, we co-injected their cRNA into oocytes together with cRNA for NCCT and measured 22Na uptake. WT WNK4 produces substantial inhibition of activity (Fig. 1A). However, expression of the kinase-deficient (KD) mutant WNK4 (318D>A) showed no effect on 22Na uptake. This suggests that the effect of WNK4 on NCCT is indeed dependent on intact kinase activity.

Exploration of the antagonistic interplay of WNK4 and WNK1. The expected inhibition of NCCT by WNK4 is completely suppressed when WT WNK1 is coexpressed in the oocytes (Fig. 1B). To explore the importance of WNK1 kinase activity for this effect, we coexpressed the kinase-dead KD WNK1 (368D>A) and showed that it was as effective as WT WNK1 in blocking WNK4 inhibition. We also looked at the possible role of the acidic motif in WNK1 by coexpressing a mutant form of WNK1 (637Q>E WNK1) that carries a charge-changing mutation in its acidic motif. Again 637Q>E WNK1 behaved similarly to WT WNK1 and blocked the effect of WNK4 on NCCT activity. None of the WNK1 proteins affected 22Na uptake in the absence of WNK4 (Fig. 1B).

Effect of WNK4 on ECFP-NCCT membrane expression and trafficking. To show that the changes in 22Na uptake were paralleled by changes in expression of NCCT in the oocyte surface membrane, we expressed NCCT with an NH2-terminal ECFP tag producing a blue fluorescent fusion protein, ECFP-NCCT (Fig. 2). Expression of ECFP-NCCT by the oocytes rose steadily following cRNA injection with peak fluorescence seen 5 days after injection (Fig. 2). Coexpressing WNK4 reduces membrane expression of ECFP-NCCT. There is no effect on initial expression within the first 24 h of coinjection, but by 40 h the ECFP-NCCT expression in WT WNK4-injected oocytes is significantly reduced. This separation increases at later time points, so that by day 5 there is a substantial reduction in blue fluorescence from the coinjected oocytes (Fig. 2).

The time course suggests that WNK4 may be increasing removal of NCCT from the surface. However, in oocytes incubated with brefeldin-A to block forward trafficking from the Golgi (17), the rate of endocytosis was not significantly affected by coexpression of WNK4 (Fig. 3). The reduction in ECFP-NCCT fluorescence in the presence of brefeldin corresponded to a rate of 1.0 (0.5–1.4%, 95% CI) %/h in the absence of WNK4 and 0.8 (0.2–1.4) %/h in its presence.

To confirm that the KD WNK4 (318D>A) mutant is non-functional against ECFP-NCCT fluorescence as well as 22Na uptake, it was expressed instead of WT protein with the results shown Fig. 4. It behaves like the WNK4 565Q>E mutant, which is a loss-of-function disease mutation used here as a further negative control (Fig. 4A). Expressed as either WT or the QE mutant, WNK1 affected ECFP-NCCT fluorescence in a parallel fashion to its effect on 22Na uptake (Fig. 4B).

Surface biotinylation. Because the oolema is narrow compared with the spacial resolution of the confocal microscope, we confirmed surface expression of ECFP-NCCT using biotinylation to capture surface proteins on intact oocytes. Figure 5 shows that blottable NCCT protein is present at the surface 15 h after cRNA injection and the signal is not affected by coexpression of WNK4. The signal is much stronger after 120 h where it is obvious that there is less blottable surface NCCT protein in oocytes coexpressing WNK4 in keeping with the time course of confocal fluorescence shown in Fig. 3. Coexpression of either the QE or delAM mutants of WNK4 produces the same level of surface expression as NCCT injected alone, which is in keeping with their lack of effect on ECFP-NCCT fluorescence (Fig. 4).

Immunoblotting. To confirm expression of mutant WNK proteins and exclude an inhibitory effect on ECFP-NCCT cRNA translation, total protein lysate of appropriately injected oocytes were Western blotted for NCCT and WNK proteins (Fig. 6). A full-length WNK1 is expressed following injection of WT, QE or KD WNK1 cRNA and the level of WT WNK1
is not affected by coexpression of WNK4. Similarly WNK4 protein expression is not affected by the WNK4 cRNA injected (WT or KD) or coexpression of WNK1. Expression of ECFP-NCCT was similar in all oocytes regardless of coinjection of WNK4 or WNK1 cRNAs (Fig. 6). The abundance of the higher MW bands (presumably representing various glycosylation states of NCCT) on the NCCT blots did vary between experiments and different batches of eggs. We cannot easily explain this finding, but there was no correlation between the different intensities of these bands and the actual confocal fluorescence recorded from ECFP-NCCT in the oocytes (Figs. 4 and 6).

Effect of WNK4 truncation and deletion of the acidic motif of WNK4 on ECFP-NCCT membrane expression. The WT WNK4 protein was expressed as an NH2-terminal truncation (1–620) which included the kinase domain and acidic motif. This fragment showed similar activity to the full-length WT WNK4 suppressing the ECFP-NCCT fluorescent signal (Fig. 7A). However, injection of cRNA for the reciprocal COOH-terminal fragment (620-CT) produced no effect on ECFP-NCCT expression. This suggests that only the NH2-terminal
half of the WNK4 protein is necessary for its functional inhibition of NCCT trafficking. To look further at the importance of the acidic motif, cRNA for a mutant WNK4 lacking the 10 amino acid residues making up the AM motif (delAM W4) was coinjected with ECFP-NCCT. This mutant which retains the kinase domain behaved in the same way as the QE W4 mutant and failed to significantly block ECFP-NCCT expression (Fig. 7B).

To look for evidence that the WNK4 fragments are able to interact directly with NCCT we attempted pull downs using oocyte lysates expressing NCCT and either of the WNK4 fragments. The WNK4 fragments are clearly expressed in the lysates, but neither was able to pull down NCCT suggesting they do not interact directly (Fig. 8).

**Effect of dynamin on ECFP-NCCT expression.** Expression of ROMK in oocytes is blocked by WNK4 coexpression. The mechanism is thought to involve accelerated endocytosis through clathrin-coated vesicles, which is blocked by a dominant-negative form of dynamin, K44A (7). To see whether dynamin has a similar role in NCCT trafficking, we first studied the effect of injecting dynamin cRNA alone. This mimicked the effect of WNK4 by producing substantial inhibition of membrane fluorescence from ECFP-NCCT, but coexpressing WNK4 and dynamin together produced no further inhibition of membrane expression (Fig. 9). The GTPase-defective form of dynamin (16), K44A, was used to explore the role of dynamin further. Expressed in *X. laevis* oocytes, this mutant exerts a profound dominant-negative effect on dynamin-dependent endocytosis (7, 18). Injection of the dominant-negative K44A mutant did not reverse the effect of WNK4 (Fig. 9).

**DISCUSSION**

The literature on the role of WNK4 kinase activity in its interaction with NCCT is divided. Two previous studies used the kinase-dead (318D/H11022A) mutant WNK4 protein that lacks a key aspartic acid within the highly conserved kinase domain of the WNK kinases, but have reported opposite findings (20, 24). In our hands, the KD (318D>A) mutant is not able to reduce either 22Na uptake or the membrane expression of our ECFP-NCCT fusion protein. Hence, it behaves as a loss-of-function mutant as originally reported by the Lifton group (20). The 318 aspartate residue of WNK4 binds Mg2+ is highly conserved in the domain of all WNKs and mutagenesis (D>A) renders it catalytically inactive (21). It is not clear then why its behavior is discrepant, but it is noticeable that the WNK4 protein used by us and the Lifton lab is a full-length protein. The study reporting negative results with the 318D>A mutant actually used a protein some 56 residues shorter at the NH2-terminal that includes the kinase domain (23). Selective inhibitors do not yet exist for WNK kinases, so it is not possible to address the catalytic importance of WNK4 pharmacologically; although they may become necessary to explore the role of WNKs in cell culture and intact animals.

Our results with the WNK4 truncation experiments showed that only the NH2-terminal of WNK4 (residues 1–620) is necessary to suppress NCCT expression. This again contrasts with a previous report that it is the COOH-terminal 222 amino acid residues that are crucial for suppression of NCCT expression (24). In our hands, the COOH-terminal fragment (620-CT) of WNK4, which contains the coil-coil domain, had no effect
on ECFP-NCCT expression. We were also not able to show interaction of our NH$_2$-terminal fragment with NCCT despite repeated attempts at a pull down. However, using the same approach we have been able to show interaction of WNK1 and ROMK in oocyte lysates (1). Hence, we can find no evidence that direct interaction of WNK4 and NCCT is necessary for WNK4 to affect NCCT expression. Our results with deleting the acidic motif (delAM W4) also suggest that the acidic motif within the fragment has a crucial role. It is absolutely conserved in all WNKs and the missense mutations identified in subjects with Gordon’s syndrome all change charge of amino acid residues within it (6, 9). We speculate that this motif may perform some crucial protein-protein interaction perhaps docking the WNK4 kinase domain with its phosphorylation target.

Previous studies reporting an effect of WNK4 on NCCT membrane localization have only reported expression at a single time point. Hence, it has not been obvious how the trafficking of the NCCT cotransporter is interrupted by WNK4. From the time course of the membrane fluorescence signal, the early appearance of ECFP-NCCT was unaffected by WNK4 coexpression. However, when trafficking from the Golgi was stopped in oocytes maximally expressing ECFP-NCCT (at 120 h) with brefeldin-A the rate of endocytosis was the same regardless of whether or not WNK4 was coexpressed. This suggests that the predominant action of WNK4 is to block forward trafficking. There are no previous reports on the rate of endocytosis of NCCT in oocytes, but our data suggest it is a much slower process than the ROMK channel which undergoes comparatively rapid endocytosis with a half-life of $<4$ h (12).

Since WNK4 increases ROMK endocytosis through a dynamin-dependent pathway (8), we hypothesized that a similar mechanism may operate for NCCT. Coexpression of dynamin does mimic the effect of WNK4 in X. laevis, but the dominant-negative dynamin (K44A) is not able to block the action of WNK4. This might suggest that the action of WNK4 itself is dynamin-independent (25). However, if WNK4 is blocking forward trafficking of NCCT rather than accelerating, its endocytosis the lack of effect of dynamin K44A is perhaps not unexpected. In parallel (unpublished studies) with ROMK and WNK4, we showed that our dynamin K44A protein is functional in oocytes, so its lack of effect here indeed suggests that WNK4 affects trafficking of ROMK and NCCT through different mechanisms.

Expressed in the absence of WNK4, WNK1 has no effect on $^{22}$Na uptake through NCCT or localization of ECFP-NCCT in the oocyte membrane. However, coexpression of WNK4 and WNK1 completely abolished the inhibitory effect of WNK4 on NCCT expression. This has been reported previously, but our results differ significantly in the detail of this interaction (23, 24). First, we found the catalytically inactive 368D>A WNK1 mutant to be as effective as WT WNK1 suggesting that WNK1 inhibition of WNK4 does not rely on phosphorylation through WNK1 kinase activity. This is physiologically relevant because the predominant WNK1 isoform in the distal nephron is an NH$_2$-terminal truncation (KS-WNK1) that deletes the entire kinase domain (4). It has recently been reported that this truncated form is inactive in oocytes unless expressed in the presence of the full-length WNK1, when it exerts a dominant-negative effect (13). Our results suggest that this behavior of the KS-WNK1 isoform may not be explained simply on its lack...
of catalytic activity. Second, we looked at the effect of a typical charge-changing mutation within the acidic motif of WNK1 that is known to abolish the ability of WNK4 to regulate NCCT expression (6, 19). In vitro, these mutations show loss-of-function in the interaction of WNK4 with NCCT and a gain-of-function in the WNK4 interaction with ROMK (6, 7, 20). Nevertheless, we found that 637Q/H11022 WNK1 behaved like WT WNK1 and inhibited WNK4. This suggests that charge-conservation within the acidic motif of WNK1 is not critical to WNK1-WNK4 interaction in the same way that it is for WNK4 regulation of NCCT (and ROMK). WNK1 is a large protein with other motifs that might control the putative hetero-oligomerization of WNK1 with WNK4. Of course this assumes there is direct protein-protein interaction despite the limited evidence that the two proteins do interact. Yang et al. (24) for example showed that an NH2-terminal fragment of WNK1 (1–555) will immunoprecipitate a WNK4 fragment containing the kinase domain in oocytes. But at least one other group has recently reported that WNK1 and WNK4 do not stably interact in HEK 293 cells over expressing full-length WNK proteins.
nor do they interact in a yeast 2-hybrid system (22). Hence, it is possible that the functional blockade of WNK4 by WNK1 in the oocyte may not involve their direct interaction at all. WNK1 may be, in fact, part of a cascade regulating WNK4 and hence act indirectly.

Before the discovery of the WNK kinases, NCCT was seen as a rather static transporter protein in the apical membrane of cells lining the DCT. It now appears that it may be a very dynamic transporter with its trafficking from the membrane regulated by the interplay of WNK4 and WNK1. The nature of this pathway remains to be identified, but it is distinct from the dynamin-dependence shown by WNK4 in regulating ROMK expression. The reason for this mechanistic complexity in adjacent nephron segments is not clear and further work is needed to identify the factors regulating expression of WNK4 and WNK1 in the kidney.

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