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Disease-causing mutations in the acidic motif of WNK4 impair the sensitivity of WNK4 kinase to calcium ions

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Abstract

WNK4 is a serine/threonine protein kinase that is involved in pseudohypoaldosteronism type II (PHAII), a Mendelian form disorder featuring hypertension and hyperkalemia. Most of the PHAII-causing mutations are clustered in an acidic motif rich in negatively charged residues. It is unclear, however, whether these mutations affect the kinase activity in any way. In this study, we isolated kinase domain of WNK4 produced by *E. coli*, and demonstrated its ability to phosphorylate the oxidative stress-responsive kinase-1 (OSR1) and the thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) *in vitro*. Threonine 48 was identified as the WNK4 phosphorylation site at mouse NCC. The phospho-mimicking T48D mutant of mouse NCC increased its protein abundance and Na⁺ uptake, and also enhanced the phosphorylation at the N-terminal region of NCC by OSR1. When the acidic motif was included in the WNK4 kinase construct, the kinase activity of WNK4 exhibited sensitivity to Ca²⁺ ions with the highest activity at Ca²⁺ concentration around 1 μM using kinase-inactive OSR1 as a substrate. All tested PHAII-causing mutations at the acidic motif exhibited impaired Ca²⁺ sensitivity. Our results suggest that these PHAII-causing mutations disrupt a Ca²⁺-sensing mechanism around the acidic motif necessary for the regulation of WNK4 kinase activity by Ca²⁺ ions.

Keywords

WNK4; serine threonine protein kinase; pseudohypoaldosteronism type II; calcium sensing; OSR1; NCC

Introduction

Mutations in with-no-lysine (K) kinase-4 (WNK4) leads to pseudohypoaldosteronism type II (PHAII, also known as familial hyperkalemia and hypertension or Gordon's syndrome), which is characterized by hypertension, hyperkalemia, and metabolic acidosis [17]. The clinical manifestations of PHAII are opposite to those of Gitelman syndrome caused by loss-of-function of the thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC). Thus, it has been suspected that a gain-of-function in NCC is the reason causing PHAII [10]. Indeed, *In vitro* and *in vivo* evidences indicate that NCC is a target of WNK4, and PHAII-causing mutations of WNK4 increase NCC activity [8;18–20]. In addition, WNK4 phosphorylates and

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activates Ste20-type kinases SPAK and OSR1, which in turn phosphorylate and activate NCC [11;15]. However, it is unclear whether NCC could be directly phosphorylated by WNK4. Most PHAII-causing mutations are clustered in an “acidic motif” rich in negatively charged amino-acids downstream the kinase domain [17]. Little is known about the function of the acid motif and how the mutations in the acidic motif alter the biochemical properties of WNK4.

An understanding of the biochemical properties of WNK4 kinase has been limited by the lack of a pure preparation of WNK4 kinase. The kinase domain of WNK4 was either incapable of being produced in *E. coli* [15] or the purified kinase domain did not exhibit kinase activity [16]. A breakthrough was recently made by Ahlstrom and Yu who purified full-length WNK4 from mammalian cells and demonstrated its kinase activity using SPAK and OSR1 as substrates [1]. However, a 40-kDa kinase (termed p40) with unknown identity was co-purified with WNK4 [1]. This co-purified p40 kinase renders further characterization of WNK4 kinase difficult. Furthermore, this also put previously demonstrated WNK4 kinase activity in question because all WNK4 constructs expressed in eukaryotic cells may associate with p40.

In this study, we purified the kinase domain of WNK4 from *E. coli* and demonstrated its activity in phosphorylating OSR1 and NCC. We also found that the kinase activity of WNK4 is regulated by Ca^{2+} ions and this regulation is impaired by PHAII-causing mutations in the acidic motif of WNK4.

Material and Methods

cDNA constructs

The mouse NCC (IMAGE:4237274, GenBank accession number BC038612) cDNA was purchased from Open Biosystems (Huntsville, AL). The human WNK4 cDNA was provided by Drs. Xavier Jeunemaitre and Juliette Hadchouel. The human OSR1 cDNA was cloned from HEK-293 cells by PCR and verified by sequencing.

Purification of GST-fusion proteins

GST-fusion proteins of mNCC 1–135 and 604–1002, WNK4 160–440 and 160–602, and full-length OSR1 were amplified by PCR and subcloned into pGEX-6p-1 vector (GE Healthcare, Piscataway, NJ), individually. Mutations of mNCC, WNK4 and OSR1 were introduced using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacture’s instruction. All cDNAs generated by PCR were sequenced to confirm that no errors were incorporated. GST-fusion proteins were purified following the manufacturer’s direction (GE Healthcare, Piscataway, NJ) with modification as described previously [22]. Removal of GST-tag by PreScission protease was carried out following the manufacturer’s instruction (GE Healthcare, Piscataway, NJ).

Na^+ uptake in *Xenopus laevis* oocytes

The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham. *X. laevis* oocytes preparation, synthesis of capped complementary RNAs (cRNAs), and injection of cRNAs into oocytes were performed as previously described [21;22]. Oocytes were injected with 12.5 ng cRNA/oocyte for HA-tagged mNCC or its mutants or water (as control) two days before Na^+ uptake experiments. Na^+ uptake experiments were performed as described by Sabath *et al.* [12] but without Cl^- free treatment before uptake experiments. The incorporated $^{22}\text{Na}^+$ was determined using a scintillation counter. Na^+ uptake data are presented as means $\pm$ S.E. from 4 experiments with 15 oocytes per group.

***In vitro* kinase assay**

To assess kinase activities of WNK4 and OSR1, 0.1 µg of each kinase was incubated with 2 µg GSTmNCC or 1 µg GST-OSR1 WT or D164A substrates in 25 µl kinase assay buffer containing 0.5 µCi [γ -³²P]ATP (Perkin Elmer, Waltham, MA), 50 mM Tris.HCl (pH 7.5), 10 mM MgCl₂, 2 mM MnCl₂, 1 mM DTT, at 30 °C for 45 min. Then reactions were terminated with 5 µl of 6× electrophoresis sample buffer and resolved by electrophoresis on 10% polyacrylamide gels. After transferred to PVDF, phosphorylation intensities were assessed by autoradiography. Parallel to kinase reaction, equal amount of GST-fusion proteins were resolved by SDS-PAGE and stained by Coomassie Blue for loading control. The activation of OSR1 by WNK4 was performed as described by Vitari *et al* [15].

Western blot analysis

Western blot analyses were performed at 2 days after oocytes injected with HA-mNCC WT, T48A or T48D cRNAs with monoclonal Anti-HA antibody (Sigma-Aldrich, St. Louis, MO, product # H9658, 1:5,000) using a protocol described previously [21;22].

Results and Discussion

The WNK4 kinase domain purified from *E. coli* is kinase active

A “kinase-dead” mutation in WNK4 abolished WNK4-mediated regulation on NCC [18] and TRPV5 [7]. We found that the kinase domain of WNK3, a homologue of WNK4, was able to regulate TRPV5 as was the full-length WNK3 [21]. These observations indicate a critical role of kinase activity in regulating renal transporters. However, due to the difficulty to express WNK4 kinase in *E. coli* or to reconstitute WNK4 kinase activity from WNK4 kinase domain prepared from *E. coli* [15;16], a direct phosphorylation of a transporter by WNK4 has never been shown *in vitro*. As a prokaryotic organism, *E. coli* does not have the same serine-threonine phosphorylation system as eukaryotes [6]. Thus, it is an ideal system to obtain pure kinase activity for WNK4. After several failed attempts, we successfully expressed GST-fusion protein containing amino-acids 160–440 of WNK4, which includes the kinase domain (174–432) of WNK4, using *E. coli* BL21 strain (Fig. 1A). After the GST-tag was cleaved (Fig. 1A), we evaluated its kinase activity. WNK4 160–440 fragment phosphorylated kinase-inactive GST-OSR1 D164A mutant, and the kinase inactive mutation D321A largely abolished the kinase activity (Fig. 1B). The phosphorylation of OSR1 by WNK4 is consistent with previous studies with WNK4 prepared from eukaryotic cells [1;15]. Thus, the WNK4 160–440 segment possesses the kinase activity of WNK4. The removal of the N-terminal region of WNK4 increased the yield and activity of GST-fusion WNK4 kinase domain (not shown). This was an important step to obtain the kinase active WNK4 from *E. coli*.

WNK4 kinase phosphorylates mouse NCC at Thr48 *in vitro*

Using WNK4 160–440 kinase segment, we were able to test whether WNK4 phosphorylates NCC directly. WNK4 160–440 phosphorylated GST-fusion mNCC N-terminal 1–135 segment, whereas no strong phosphorylation was detected in the C-terminal 604–1002 segment (Fig. 1C). Within the N-terminal region, WNK4 phosphorylated mNCC 1–70 but not the 61–135 segment (supplementary Fig. S1A). To further locate the phosphorylation site, we generated four GST-fusion mNCC segments truncated from the N-terminal side (8-, 24-, 42-, and 49–135). All but GST-mNCC 49–135 fragment were strongly phosphorylated by WNK4 (Supplementary Fig. S1B). This indicated that a WNK4 phosphorylation site is present in amino acids 42–48 of mNCC. We mutated the three potential phosphorylation sites Thr44, Ser47, and Thr48 individually into alanine in GST-fusion NCC 1–135. Only when Thr48 was mutated, the phosphorylation of GST-fusion NCC 1–135 was largely

eliminated (Fig. 1D). Thus, Thr48 is a major WNK4 phosphorylation site at mNCC N-terminal region.

Phospho-mimicking mutation at Thr48 increases NCC protein abundance and facilitates the phosphorylation of NCC by OSR1

To assess the effect of phosphorylation of Thr48 on mNCC activity, we introduced phospho-mimicking mutation T48D and non-phosphorylatable mutation T48A into mNCC, respectively. The T48D mutant dramatically enhanced mNCC-mediated Na^+ uptake and increased mNCC protein abundance relative to wild-type (WT) mNCC in *X. laevis* oocytes (Fig. 2A). In contrast, T48A mutant exhibited a decrease in Na^+ uptake with no significant decrease in protein abundance compared to WT mNCC (Fig. 2A).

Because Thr48 is close to SPAK/OSR1 phosphorylation sites Thr53 and Thr58 at mNCC N-terminal region [11], we further determined whether phosphorylation at Thr48 influences OSR1 phosphorylation at mNCC N-terminal region. Phosphorylation of GST-fusion mNCC 1–135 by OSR1 was substantially increased in the presence of the phospho-mimicking T48D mutation than in the presence of the T48A mutation (Fig. 2B). Because phosphorylation of NCC N-terminal region by SPAK/OSR1 activates NCC [11], this direct phosphorylation by WNK4 will facilitate the positive regulation of SPAK/OSR1 on NCC. Thus, WNK4 stimulates NCC in three ways: 1) direct phosphorylation and in turn increasing NCC protein abundance; 2) facilitating the phosphorylation of NCC by SPAK/OSR1 indirectly, and 3) phosphorylating and activating SPAK/OSR1.

Mutations in the acidic motif impair the sensitivity of WNK4 kinase to Ca^{2+} ions

It was unclear whether PHAII-causing mutations affect the kinase activity of WNK4 in anyway. Interestingly, most of the PHAII-causing mutations are located to an “acidic motif” rich in negatively charged amino-acid residues; and all the PHAII-causing mutations result in an alteration in negative charge [5;17]. The negatively charged amino-acid residues in the acidic motif may sense positively charged Ca^{2+} ions, whose concentration may rise by influx from extracellular fluid or by release from Ca^{2+} store. Intriguingly, WNK4 enhanced TRPV5, a Ca^{2+} channel [7], but inhibited transporters for other electrolytes when expressed in *Xenopus* oocytes. This raised a question as whether Ca^{2+} concentration ($[\text{Ca}^{2+}]$) plays a role in regulating kinase activity and alters the direction of WNK4-mediated regulation. Following this line, activation of AngII receptor AT1R also raises intracellular $[\text{Ca}^{2+}]$ through $\text{G}_{q/11}$ /phospholipase C/inositol triphosphate pathway [9]. If Ca^{2+} affects the kinase activity of WNK4, it may be underlying the action of WNK4 on NCC in response to AngII.

To investigate the potential effects of PHAII-causing mutations on the phosphorylation of NCC by WNK4, we included the acidic motif in the GST-fusion WNK4 160–602 construct and introduced three PHAII-causing mutations E562K, D564A, and Q565E [17], individually. The GST-WNK4 160–602 construct was able to phosphorylate OSR1 D164A, as was the GST-WNK4 160–440; in addition, it also gained the new ability to be regulated by $[\text{Ca}^{2+}]$ (Fig. 3A). The kinase activity of WT WNK4 construct 160–602 exhibited $[\text{Ca}^{2+}]$ -dependence with a peak at around 1 μM (Figs. 3B and 3C). In contrast, the three PHAII-mutants exhibited little change in kinase activity at various $[\text{Ca}^{2+}]$ (Figs. 3B and 3C). We were unable to compare the kinase activities between WT and PHAII-causing mutants because of the variation in protein quality and yield from different preparations.

Phosphorylation of NCC by OSR1 is sensitive to Ca^{2+} in the presence of WNK4

In the presence of GST-WNK4 160–602, WT OSR1 was activated and phosphorylated the N-terminal region of NCC substantially (Fig. 4A). In the presence of WT GST-WNK4 160–602, the phosphorylation of NCC 1–135 by OSR1 was significantly lower at $[\text{Ca}^{2+}]$ of 10

nM than at 1 μ M (Figs. 4B and 4C). In contrast, the differences were not significant between the two levels of $[Ca^{2+}]$ for the PHAII mutants tested, including E562K, D564A, and Q565E. From the data shown in Fig. 4B, it is clear that the OSR1 is more potent than WNK4 kinase domain in phosphorylating NCC, indicating the WNK4-SPAK/OSR1 pathway is dominant in activating NCC over the direct phosphorylation of NCC by WNK4.

It is generally accepted that NCC is a major target of WNK4; however, the direction and mechanism of WNK4 action on NCC have been debated. Evidences from early studies using *Xenopus* oocytes and mammalian cells indicate that WNK4 inhibits NCC and PHAII-causing mutations relieve the inhibition [2;18;19;23]. Later studies also indicate that WNK4 phosphorylates and activates Ste20-type kinases SPAK/OSR1, which in turn phosphorylates and activates NCC [11;15]. More recently, angiotensin II (AngII) was found to turn the negative effect of WNK4 on NCC into a positive one in the presence of AngII receptor AT1R in *Xenopus* oocytes [13]. The PHAII-causing mutations (Q562E and D561A in mouse WNK4), which did not inhibit NCC, also did not respond to AngII. The regulation was enhanced by WT SPAK and was abolished by a dominant-negative SPAK mutant [13]. These observations suggest that WNK4 is inhibitory to NCC at baseline; under conditions such as volume depletion or hyperkalemia, AngII turns the negative effect of WNK4 on NCC into a positive one. The PHAII-causing mutations of WNK4 likely disrupt a process in the AngII signaling pathway and lock WNK4 in a stimulatory mode. Our results suggest that this process involves the Ca^{2+} -sensing mechanism of WNK4. AngII activates AT1R and raises intracellular $[Ca^{2+}]$ [9], this increase in $[Ca^{2+}]$ may be sensed by a putative Ca^{2+} -sensing domain, which involves the acidic motif. The acidic motif may interact with a domain that controls the activity of WNK4 or the kinase domain itself to regulate the kinase activity of WNK4. It is likely the acidic motif acts as a switch to regulate WNK4 kinase activity, which can be turned on by a rise in $[Ca^{2+}]$. The PHAII-causing mutations in the acidic motif all alter the charge in the acidic motif. These mutations may mimic the Ca^{2+} binding states and keep the kinase activity high at baseline.

In summary, our results are in line with a positive effect of WNK4 on NCC and a gain-of-function effect of the PHAII mutations on WNK4. An elevation of $[Ca^{2+}]$ by extracellular cues such as AngII is sensed by a Ca^{2+} -sensing mechanism, which shifts the kinase activity of WNK4 into high gear in response to the rise in $[Ca^{2+}]$. The PHAII-causing mutations in the acidic motif disrupt a Ca^{2+} -sensing mechanism and likely lock WNK4 kinase at a state as if $[Ca^{2+}]$ is elevated, leading to a hyperactive kinase that keeps the activity of NCC and/or other transporters high under baseline. Our results do not exclude a negative regulation of NCC by WNK4 at baseline through forward trafficking [4] and lysosomal degradation pathways [14;23]. However, connections between these pathways and WNK4 kinases activity are yet to be established.

Highlights

- WNK4 kinase domain isolated from *E. coli* exhibits kinase activity.
- WNK4 phosphorylates mouse Na-Cl cotransporter NCC at Thr48 *in vitro*.
- Phospho-mimicking at Thr48 increases NCC abundance and Na^+ uptake activity.
- In the presence of the acidic motif, Ca^{2+} regulates the kinase activity of WNK4.
- PHAII mutations in the acidic motif disrupt a Ca^{2+} -sensing mechanism of WNK4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AngII	angiotensin II
GST	glutathione S-transferase
NCC	Na ⁺ -Cl ⁻ cotransporter
OSR1	oxidative stress-responsive kinase-1
PHAII	pseudohypoaldosteronism type II
WNK4	With-No-lysine (K) kinase-4

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Fig. 1.

WNK4 segment 160–440 purified from *E. coli* was kinase active. **A.** WNK4 160–440 segment purified from *E. coli* BL21 before (lane 2) and after (lane 3) the GST-tag was cleaved by GST-PreScission Protease. CBS, Coomassie Blue staining. **B.** Wild-type (WT) WNK4 160–440 phosphorylated GST-OSR1 D164A but kinase-inactive WNK4 mutant (D321A) did not. Shown are autoradiography (autorad) of the phosphorylated GST-OSR1 D164A after *in vitro* kinase assay (left panel), Coomassie Blue staining (CBS) of the GST-OSR1 D164A substrates (middle panel), and inputs of WT and D321A WNK4 160–440 segments (right panel). GST-OSR1 doublet bands represent the full-length GST-OSR1 protein and a proteolytic fragment lacking the C-terminal region [3;15]. **C.** GST-mNCC 1–135 but not GST-mNCC 604–1002 was phosphorylated by WNK4 160–440. **D.** Phosphorylation of GST-mNCC 1–135 by WNK4 160–440 was largely eliminated by T48A mutation.

Fig. 2.

WNK4 kinase activity may positively regulate mNCC. **A.** Phospho-mimicking T48D mutant of HA-tagged mNCC increased Na^+ uptake (*left panel*) and protein abundance assessed by Western blot with HA antibody (*right panel*) compared to non-phosphorylatable T48A mutant or wild-type (WT) mNCC in *X. laevis* oocytes. Data are derived from 4 independent experiments. **B.** In the presence of phosphor-mimicking T48D mutation, phosphorylation of GST-mNCC 1–135 by GST-OSR1 T185E (activated OSR1) was markedly enhanced relative to WT or T48A mutation (*upper panel*). Autorad, autoradiography; CBS, Coomassie Blue staining. A summary of the bands intensities (normalized to those of WT) from 3

experiments is shown in the *right panel*. AU, arbitrary unit. * and # indicate $P < 0.01$ vs. WT and T48A, respectively.

Fig. 3.

Ca²⁺-dependence of WNK4 kinase activity. **A.** Phosphorylation of GST-OSR1 D164A by GST-WNK4 160–602 but not by GST-WNK4 160–440 was elevated by high Ca²⁺ concentration ([Ca²⁺]) in the kinase reaction mixture. Low (L) or high (H) [Ca²⁺] was 10 nM or 1 μM. **B.** Representative autoradiographies of GST-OSR1 D164A phosphorylated by GST-WNK4 160–602 wild-type (WT) or PHAII-causing mutants E562K, D564A, and Q565E at various [Ca²⁺]. **C.** Summary of bands intensities of phosphorylated GST-OSR1 D164A by GST-WNK4 160–602 (WT or PHAII mutants) at various [Ca²⁺] from 3–5 independent experiments. The band intensities of each group were normalized to those at nominal Ca²⁺ free condition in which values are set as 1.0 in each group. AU, arbitrary unit. * indicates $P < 0.01$ vs. 1 nM or 10 nM [Ca²⁺] group.

Fig. 4.

Phosphorylation of mNCC by GST-OSR1 was regulated by Ca²⁺ ions via GST-WNK4 160–602. **A.** Wild-type (WT) but not the kinase-inactive D164A mutant GST-OSR1 phosphorylated GST-mNCC 1–135. Phosphorylation of GST-mNCC 1–135 by GST-OSR1 was only detectable after pre-incubation of GST-OSR1 with GST-WNK4 160–602. The phosphorylation of GST-mNCC 1–135 by GST-WNK4 160–602 was too weak to be detectable when exposed under the same condition. **B.** GST-WNK4 160–602 WT construct or construct containing E562K, D564A or Q565E mutation was evaluated for their ability to activate GST-OSR1 at low (L, 10 nM) or high (H, 1 μM) [Ca²⁺]. Phosphorylation of GST-mNCC 1–135 was used as the measure of GST-OSR1 kinase activity. Shown are representative autoradiographies of phosphorylated GST-mNCC 1–135 of *in vitro* kinase assay results. **C.** Summary of bands intensities of GST-mNCC 1–135 phosphorylation from **B** in the presence of WT GST-OSR1 after treatment with GST-WNK4 160–602 constructs at low or high [Ca²⁺] from 4–8 independent experiments. The bands intensities of each group are normalized to the mean value at high [Ca²⁺] condition. AU, arbitrary unit. * indicates $P < 0.01$.