WNK4 Enhances the Degradation of NCC through a Sortilin-Mediated Lysosomal Pathway

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ABSTRACT

WNK kinase is a serine/threonine kinase that plays an important role in electrolyte homeostasis. WNK4 significantly inhibits the surface expression of the sodium chloride co-transporter (NCC) by enhancing the degradation of NCC through a lysosomal pathway, but the mechanisms underlying this trafficking are unknown. Here, we investigated the effect of the lysosomal targeting receptor sortilin on NCC expression and degradation. In Cos-7 cells, we observed that the presence of WNK4 reduced the steady-state amount of NCC by approximately half. Co-transfection with truncated sortilin (a dominant negative mutant) prevented this WNK4-induced reduction in NCC. NCC immunoprecipitated with both wild-type sortilin and, to a lesser extent, truncated sortilin. Immunostaining revealed that WNK4 increased the co-localization of NCC with the lysosomal marker cathepsin D, and NCC co-localized with wild-type sortilin, truncated sortilin, and WNK4 in the perinuclear region. These findings suggest that WNK4 promotes NCC targeting to the lysosome for degradation via a mechanism involving sortilin.

J Am Soc Nephrol 21: 82–92, 2010. doi: 10.1681/ASN.2008121275

WNK (with no lysine [K]) kinase is a subfamily of serine/threonine kinases.1 Mutations in two members of this family, WNK1 and WNK4, result in pseudohypoaldosteronism type II,2 featuring hypertension, hyperkalemia, and metabolic acidosis. Previous studies showed that wild-type (WT) WNK4 inhibits the activity and surface expression of sodium chloride co-transporter (NCC) in Xenopus oocytes.3,4 Interestingly, one study showed that a NCC harboring five different Gitelman-type mutations exhibits low activity that is mainly due to a reduction of functional NCC inserting into the plasma membrane.5 Our previous study6 also indicated that NCC surface expression is regulated by altering its degradation through the lysosomal pathway. These combined studies suggest that alteration of NCC function can result from perturbing its protein synthesis,7,8 glycosylation and processing,9,10 and delivery to the plasma membrane.11 Golbang et al.11 showed that WNK4 also blocks the forward trafficking of NCC; however, the exact molecular basis of interference of WNK4 kinase with NCC forward trafficking and enhancing its degradation through a lysosomal pathway remains to be clarified.

There are two major sorting mechanisms involving targeting lysosomal proteins to lysosomes.12,13 One is the mannose-6-phosphate receptor (M6PR)-mediated mechanism,14–16 and the other...
is mediated by sortilin. The trans-Golgi network (TGN) is the major site of sorting for newly synthesized proteins that are targeted to lysosomes for degradation. These newly synthesized proteins first bind to either M6PR or sortilin in the TGN and subsequently recruit the Golgi-localized, γ-ear-containing, ADP-ribosylation factor–binding proteins (GGAs) clathrin and adaptor protein 1 (AP1) or AP3 to form clathrin-coated vesicles to initiate their trafficking to endosomes, following a secretory pathway, or to lysosomes for degradation.

Sortilin is a newly identified lysosomal targeting receptor that is involved in the alternative sorting of the lysosomal sphingolipid activator protein prosaposin and the GM2 activator protein. It is a homolog of the yeast vacuolar sorting receptor Vps10p and belongs to the type I Vps10p superfamily. The human sortilin gene encodes 833 amino acids. It contains an N-terminal propeptide, a furin cleavage site, a large luminal domain, a single transmembrane region, and a short cytoplasmic tail. The major pool of sortilin accumulates in the TGN and vesicles, whereas 10% of sortilin is present in the plasma membrane. The truncation of the cytoplasmic tail of sortilin (sortilin TRU) leads to a disruption of the lysosomal sorting function, causing a majority of sortilin TRU to be retained in the TGN; however, a small portion of sortilin TRU may leak to the plasma membrane or vesicles. Sortilin TRU can serve as a dominant negative mutant. Sortilin seems to have multiple functions. Sortilin not only binds different ligands such as neurotensin, receptor associated protein, and prosaposin, but also is involved in intracellular sorting, endocytosis, and signal transduction. Studies have shown that sortilin binds the glucose transporter Glut4 and is translocated with Glut4 to the plasma membrane in response to insulin stimulation, suggesting that sortilin may be involved in the regulation of membrane transporters; therefore, we speculated that WNK4 might promote the degradation of NCC through the sortilin-mediated lysosomal pathway. Here, we report that WNK4 downregulates the steady-state protein levels of NCC in Cos-7 cells. Sortilin TRU reverses the degradation of NCC promoted by WNK4. The N-terminus of NCC binds sortilin WT as well as sortilin TRU, to a lesser extent. WNK4 co-localizes with NCC and sortilin and increases NCC co-localization with cathepsin D, a lysosomal marker. These data suggest that WNK4, NCC, and sortilin interact with one another to facilitate the WNK4-promoted degradation of NCC through a lysosomal pathway by a sortilin-mediated targeting mechanism.

RESULTS

Effect of Sortilin on WNK4-Mediated Inhibition of NCC Protein Expression

We have shown that WNK4 WT enhances NCC degradation through the lysosomal pathway. To determine whether a lysosomal sorting mechanism such as sortilin is involved in WNK4-promoted degradation, we co-transfected Cos-7 cells with hemagglutinin (HA)-NCC in combination with either green fluorescence protein (GFP)-sortilin WT or sortilin TRU (Figure 1) in the absence (Figure 2, A and B) or the presence of myc-WNK4 WT (Figure 2, C and D). There was no significant change in the steady-state amount of NCC in the absence of WNK4 in both NCC + sortilin WT and NCC + sortilin TRU groups as compared with NCC alone (control) group ($P > 0.05; n = 4$). In the presence of WNK4 WT, NCC protein level was significantly reduced by 52% (47.9 ± 8.7% in NCC + WNK4 WT group) versus 100% in the NCC alone [control group]; $P < 0.001; n = 6$). There was also a significant reduction in NCC expression in NCC + WNK4 + sortilin WT (sortilin WT group, 60.2 ± 5.0%) as compared with the control group ($P < 0.01; n = 6$); however, there was no significant difference in NCC expression between sortilin WT group and WNK4 WT group. In contrast, in NCC + WNK4 + sortilin TRU (sortilin TRU group), NCC protein expression (92.9 ± 6.8%) was significantly increased as compared with either WNK4 WT group ($P < 0.001; n = 6$) or sortilin WT group ($P < 0.05; n = 6$), indicating that the sortilin TRU likely reverses the inhibitory effect of WNK4 on NCC protein expression through its dominant negative effect on endogenous sortilin, because Cos-7 cells express endogenous sortilin protein. These data suggest that WNK4 enhances the degradation of NCC through a lysosomal pathway via sortilin.

Figure 1. Human WNK4, sortilin, and NCC are shown with predicted domains used in the experiment. (A) Myc-tagged WNK4 WT with predicted domains. KD, kinase domain; AI, auto-inhibitory domain; AI, auto-inhibitory domain; Cc, coiled coil domain. (B) GFP-tagged sortilin WT and its truncated mutant sortilin TRU lacking its cytoplasmic tail. Pp, propeptide; LD, luminal domain; T, transmembrane region; Ct, cytoplasmic tail. (C) GST-tagged NCC WT and its amino terminus of NCC (1 through 135) and carboxy terminus of NCC (605 through 1031). NT, amino terminus; T, transmembrane domain, CT, carboxyl terminus.
teracts with sortilin using co-immunoprecipitation (co-IP) experiments in Cos-7 cells. As shown in Figure 3C, A and B, in the cells co-transfected with HA-NCC WT in combination with either GFP-tagged sortilin WT or TRU, the anti-GFP antibody immunoprecipitated both sortilin WT and TRU. In addition, both sortilin WT and TRU co-immunoprecipitated with NCC (samples 1 and 2 in Figure 3, A and B), although sortilin TRU bound to NCC with much less avidity. In contrast, in the cells co-transfected with a GFP vector and HA-NCC WT without sortilin, the anti-GFP antibody did not immunoprecipitate NCC with much less avidity. In contrast, in the cells co-transfected with either GST-NCC (1 through 135) or GST-NCC (605 through 1031) truncation mutants in combination with either GFP-sortilin WT or TRU, NCC (1 through 135) was able to pull down sortilin WT (sample 1) as well as sortilin TRU (sample 2) but to a lesser extent, whereas NCC (605 through 1031) did not pull down either sortilin WT (sample 3) or sortilin TRU (sample 4). These results suggest that the amino terminus of NCC is the site of interaction with sortilin.

**Figure 2.** The effect of sortilin on NCC protein expression is shown. Two days after transfection of Cos-7 cells with HA-NCC WT and either GFP-sortilin WT or truncated sortilin TRU in the absence or presence of myc-WNK4 WT as indicated, cells were lysed. Lysates were subjected to 5% SDS-PAGE followed by immunoblotting with anti-HA, anti-GFP, and anti-Myc antibodies. (A and C) Representative Western blot results. The top blot shows the steady-state protein level of NCC in both A and C. The bottom blot in A shows actin for protein loading control. The middle and bottom blots in C indicate WNK4 WT and sortilin protein expression, respectively. (B and D) Summary of the results of steady-state NCC protein expression for A (n = 4) and C (n = 6), respectively. Data are presented as the ratio of change from control groups (normalized to 100%) in which NCC+GFP Vect is for A and NCC+CD4+ GFP Vect is for C. There is no statistical difference in NCC protein expression in the absence of WNK4 in either NCC+sortilin WT or NCC+sortilin TRU groups as compared with control group in B. In the presence of WNK4 WT, NCC protein expression is significantly reduced in NCC+WNK4+GFP Vect group as compared with either control group or NCC+WNK4+sortilin TRU group in D (**P < 0.001). NCC protein expression in NCC+WNK4+sortilin WT is also significantly reduced as compared with the control group (*P < 0.01). The level of NCC protein expression in NCC+WNK4+sortilin TRU group was significantly higher as compared with the NCC+WNK4+sortilin WT group (#P < 0.05).

Distribution of Sortilin and Truncated Sortilin

We then examined the distribution of sortilin WT and TRU in Cos-7 cells. Sortilin WT is seen in the perinuclear region, peripheral granular structures, and plasma membrane (Figure 5AA), whereas sortilin TRU is mainly seen in the perinuclear region with some in plasma membrane (Figure 5AD). Sortilin WT co-localizes with TGN 38, a TGN marker, in the perinuclear region (Figure 5AC). Sortilin TRU also co-localizes with sortilin using co-immunoprecipitation (co-IP) experiments in Cos-7 cells. As shown in Figure 3C, A and B, in the cells co-transfected with HA-NCC WT in combination with either GFP-tagged sortilin WT or TRU, the anti-GFP antibody immunoprecipitated both sortilin WT and TRU. In addition, both sortilin WT and TRU co-immunoprecipitated with NCC (samples 1 and 2 in Figure 3, A and B), although sortilin TRU bound to NCC with much less avidity. In contrast, in the cells co-transfected with a GFP vector and HA-NCC WT without sortilin, the anti-GFP antibody did not immunoprecipitate NCC with much less avidity. In contrast, in the cells co-transfected with either GST-NCC (1 through 135) or GST-NCC (605 through 1031) truncation mutants in combination with either GFP-sortilin WT or TRU, NCC (1 through 135) was able to pull down sortilin WT (sample 1) as well as sortilin TRU (sample 2) but to a lesser extent, whereas NCC (605 through 1031) did not pull down either sortilin WT (sample 3) or sortilin TRU (sample 4). These results suggest that the amino terminus of NCC is the site of interaction with sortilin.

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TGN 38 in the perinuclear region (Figure 5A), but, unlike sortilin WT, the expression in the peripheral granular structure is lost. These data indicate that sortilin WT is distributed in the TGN in addition to vesicles and the plasma membrane, and sortilin TRU is mainly retained in the TGN.

NCC Co-localizes with Sortilin WT and Its Mutant Sortilin TRU

We further investigated the cellular location where NCC interacts with sortilin in Cos-7 cells transiently transfected with either GFP-sortilin WT or truncated GFP-sortilin TRU alone or HA-NCC in combination with GFP-sortilin WT or GFP-sortilin TRU as indicated. Forty-eight hours after transfection, cells were lysed, IP and co-IP were performed, and cells were subjected to Western blot analysis. The top blots in the IP section indicate the co-IP results using a polyclonal anti-GFP antibody for IP and probing with a monoclonal anti-HA antibody. The bottom blots in the IP section indicate IP results using an anti-GFP antibody for IP and probing with anti-GFP antibody. The blots in the lysate input section indicate the lysates expressing NCC and sortilin, respectively. In both samples 1 and 2 in A and B, anti-GFP antibody precipitates both sortilin WT and TRU, and both sortilin WT and TRU are able to pull down HA-NCC. In sample 5 in A and sample 3 in B, anti-GFP antibody cannot pull down NCC as the negative control. Samples 3 and 4 in A serve as negative controls as well, because cells were not transfected with HA-NCC. (C) Reciprocal co-IP results. Again, the top blot in the IP section indicates co-IP results using monoclonal anti-HA antibody for IP and probing with a polyclonal anti-GFP antibody. The bottom blot in the IP section indicates IP results using an anti-HA antibody and probing with anti-HA antibody. The blots in the lysate input section indicate the lysates expressing sortilin and NCC, respectively. Again, in samples 1 and 2, anti-HA antibody precipitates HA-NCC, and NCC is able to pull down sortilin WT as well as sortilin TRU, although to a lesser extent. Samples 3 and 4 serve as negative control. --, lysates incubated with preimmune rabbit serum; +, lysates incubated with rabbit anti-GFP antibody.

Sortilin Expresses and Co-localizes with NCC in the Renal Distal Convoluted Tubule

To confirm sortilin expression in native kidney tissue, particularly in the distal convoluted tubule (DCT), where NCC is exclusively expressed, we performed immunohistochemistry, Western blot analysis, and reverse transcriptase–PCR. As shown in Figure 6A, endogenous sortilin was expressed in the cytoplasm and subapical region of the mouse DCT (mDCT) and cortical collecting duct and co-localized with NCC mainly in the subapical region of mDCT. Sortilin mRNA and protein were also expressed in Cos-7, mouse kidney and mDCT cell lines (Figure 6, C and D). In addition, NCC and WNK4 were
expressed in the kidney and mDCT cells (Figure 6, B and D). These data suggest that sortilin, NCC, and WNK4 are coexpressed in native DCT.

**WNK4 Promotes NCC Targeting to Lysosome for Degradation**

We further investigated whether WNK4 increases NCC targeting to the lysosomal compartment by immunofluorescence confocal microscopy. Cos-7 cells were transiently transfected with indicated plasmids and lysed, and the cell lysates were subjected to GST pull-down assay. (Left) GST pull-down results. (Right) Lysate input. GST-NCC (1 through 135) is able to pull down GFP-sortilin WT and GFP-sortilin TRU as indicated in lanes 1 and 2, whereas GST-NCC (605 through 1031) is not able to pull down either GFP-sortilin WT or GFP-sortilin TRU as indicated in lanes 3 and 4. The ability of glutathione to pull down both NCC (1 through 135) and NCC (605 through 1031) is shown in lanes 1 to 4 in the bottom blot. (Right) The lysate inputs for sortilin and NCC are shown in the top and bottom blots, respectively. These findings indicate that the amino terminus of NCC is responsible for its interaction with both sortilin WT and sortilin TRU.

Figure 4. The N-terminal cytoplasmic region of human NCC is responsible for its association with sortilin. Forty-eight hours after transfection, Cos-7 cells transfected with indicated plasmids were lysed, and the cell lysates were subjected to GST pull-down assay. (Left) GST pull-down results. (Right) Lysate input. (Left, top blot) GST-NCC (1 through 135) is able to pull down GFP-sortilin WT and GFP-sortilin TRU as indicated in lanes 1 and 2, whereas GST-NCC (605 through 1031) is not able to pull down either GFP-sortilin WT or GFP-sortilin TRU as indicated in lanes 3 and 4. The ability of glutathione to pull down both NCC (1 through 135) and NCC (605 through 1031) is shown in lanes 1 to 4 in the bottom blot. (Right) The lysate inputs for sortilin and NCC are shown in the top and bottom blots, respectively. These findings indicate that the amino terminus of NCC is responsible for its interaction with both sortilin WT and sortilin TRU.

**Figure 5.** Distribution of sortilin and its co-localization with NCC. Two days after Cos-7 cells were transfected with indicated plasmids, immunostaining and confocal microscopy were performed. (A) In A, sortilin WT in green seems to be distributed in the perinuclear region and peripheral granular structure as well as plasma membrane. In B, TGN 38 is shown in red. C shows the merged picture, indicating the co-localization of sortilin WT with TGN 38 in the perinuclear region in yellow. In D, the truncated sortilin TRU in green is mainly retained in the perinuclear region and some in the plasma membrane, but its expression pattern seems to be different from sortilin WT’s, because the peripheral granular structure is lost. In E, TGN 38 is shown in red. F shows the merged picture, indicating the co-localization of sortilin TRU with TGN 38 mainly in the perinuclear region in yellow. (B) In A and D, sortilin WT and sortilin TRU seem to be distributed in the similar patterns as described in A, respectively. In B, NCC in red is distributed in the perinuclear, cytoplasmic, and plasma membrane regions. C shows the merged picture, indicating the co-localization of NCC with sortilin WT in the perinuclear and cytoplasmic regions in yellow. In E, NCC in red is mainly distributed in the perinuclear region with some in the plasma membrane. F shows the merged picture, indicating that the co-localization of NCC with sortilin TRU in yellow is mainly in the perinuclear region, and its peripheral co-localization with NCC seems to be less. These findings suggest that the truncated sortilin TRU may prevent NCC from WNK4-promoted degradation through the lysosomal pathway by retaining NCC in the perinuclear region (TGN) or diverting NCC to other vesicular compartments other than lysosomes. Bar = 10 μM.
These data suggest that WNK4 promotes NCC accumulation in lysosomal compartment for degradation.

**NCC Co-localizes with Sortilin and WNK4**

We previously showed that WNK4 interacts with NCC.6 We now showed that NCC interacts with sortilin. To examine whether NCC, sortilin, and WNK4 coexpress in the same subcellular compartment, we evaluated the co-distribution of these proteins in Cos-7 cells co-transfected with HA-NCC, GFP-sortilin WT, and myc-WNK4 WT. As shown in Figure 8, NCC, sortilin, and WNK4 are co-localized in the perinuclear region and peripheral granular structures. These combined data suggest that NCC, sortilin, and WNK4 might be associated together and NCC might sequentially bind sortilin and WNK4 that ultimately facilitates the WNK4-promoted NCC targeting to the lysosome for degradation via a sortilin-mediated mechanism.

**DISCUSSION**

Emerging evidence has shown WNK kinase plays an important role in maintaining homeostasis of ion channels and transporters.35–37 Our previous study showed that WNK4 enhances NCC degradation through a lysosomal pathway.6 In this study, we showed that sortilin TRU reverses the inhibitory effect of WNK4 on NCC. We also showed that NCC interacts with sortilin WT as well as TRU, albeit to a lesser extent, via its N-terminus. Sortilin WT is co-localized with NCC throughout the cytoplasm, whereas the sortilin TRU is co-localized with NCC mainly in the perinuclear region. Sortilin is endogenously expressed in the kidney, especially in DCT cells, and co-localize with NCC, indicating that sortilin and NCC may interact each other in vivo.
WNK4 significantly increases NCC accumulation in the lysosomal compartment. These data suggest that WNK4 downregulates NCC by promoting NCC to the lysosome via a sortilin-mediated mechanism, which represents a novel mechanism for the regulation of NCC trafficking by WNK4 kinase.

Protein trafficking in the eukaryotic systems involves a series of protein–protein interactions between the cargo and protein transport carriers. Cargo proteins are selected for sorting to specific destinations by coat proteins or by receptors that interact with coat proteins. For a cargo protein to exit a sorting compartment, a receptor needs to interact with cytoplasmic coat proteins, such as adaptor proteins and clathrin; then small-coated vesicles are formed from donor membranes in the TGN and traffic the cargo to acceptor membranes such as endosomes and lysosomes. Clathrin is a structural protein that is the main constituent in clathrin-coated vesicles (CCV). These vesicles also include the GGAs and either of two heterotetrameric AP complexes, AP1 or AP3. The GGAs are newly identified monomeric clathrin APs, which have similar function as the AP complexes. The VHS domain of the GGA protein functions as a recognition module for sorting signals. The cytoplasmic tail of M6PR or sortilin binds GGA via its DXXLL motif and subsequently recruits clathrin to form CCVs designated to endosomes and lysosomes. In this study, we showed that NCC interacts with sortilin, most likely in the TGN. It is probable that NCC binds sortilin, subsequently recruiting GGA and clathrin to form CCV.

Figure 7. WNK4 promotes NCC targeting to the lysosomal compartment. Two days after Cos-7 cells transiently co-transfected with GFP-NCC alone or with myc-WNK4 WT, immunostaining and confocal microscopy were performed. Sixteen hours before fixation, Cos-7 cells were pretreated with lysosomal inhibitors leupeptin (30 μM) and E64 (50 μM); therefore, the pool of NCC that would normally be degraded in lysosomes could be visualized and the percentage of NCC co-localized with lysosomal marker cathepsin D was quantified for individual cells. (A) GFP-NCC is seen in green. Cathepsin D is detected by a polyclonal rabbit anti-cathepsin D followed by Cy3-conjugated secondary antibody in red. WNK4 is shown in cyan. DAPI in blue shows the nucleus staining. The merged pictures show the co-localization of NCC with cathepsin D in yellow. In the absence of WNK4, NCC is distributed in plasma membrane, cytoplasmic, and perinuclear regions, and it shows some extent of co-localization with cathepsin D, whereas in the presence of WNK4, NCC is seen mainly in cytoplasmic and perinuclear regions as well as less in plasma membrane, and the degree of its co-localization with cathepsin D seems to be increased. (B) To quantify the percentage of NCC co-localization with cathepsin D in these two groups, the images of 19 cells from three separate experiments in each group were obtained by z axis scanning under confocal microscopy. The percentage of the co-localization of NCC with cathepsin D in yellow was quantified using the manufacturer’s software (Zen; Zeiss). In the presence of WNK4, the percentage of co-localization of NCC with lysosomal marker cathepsin D is significantly higher than that in the absence of WNK4, indicating that WNK4 promotes NCC targeting to lysosomal compartments for degradation.

Figure 8. NCC co-localizes with sortilin and WNK4. Cos-7 cells were co-transfected with myc-WNK4 WT, GFP-sortilin WT, and HA-NCC WT. Forty-eight hours after transfection, immunostaining and confocal microscopy were performed. The distribution pattern for sortilin WT in green seems to be similar to sortilin WT as described in Figure 5A, and NCC expression pattern in red seems to be similar to the NCC pattern as described in Figure 5B. WNK4 in blue is expressed in the perinuclear and cytoplasmic regions. The merged picture shows the co-localization of WNK4, sortilin, and NCC in the perinuclear region in white, indicating that WNK4, sortilin, and NCC might be associated together.
taining NCC may also associate with AP3 to target to the lysosome for degradation. A recent study also showed that WNK4 increases the association of NCC with AP3 to promote NCC delivery to the lysosomal compartment for degradation,\(^4,40\) which is consistent with our conclusion. Our findings further support the notion that NCC is regulated through affecting its forward trafficking in addition to other mechanisms such as altering its abundance,\(^41\)–\(^43\) glycosylation,\(^10\) and phosphorylation.\(^44,45\)

In the kidney, NCC is exclusively expressed in DCT and responsible for 5 to 7% of sodium reabsorption.\(^46\) It is likely that, under normal physiologic conditions, NCC constantly traffics to both the plasma membrane \(via\) a secretory pathway and the lysosome for degradation to maintain its basal steady protein level, which is subject to regulation by many mediators.\(^3,6,10,41–45\) Renal DCT cells contain endogenous WNK4 kinase.\(^2\) In the presence of WNK4, the rate of NCC targeting to the lysosomal compartment is much higher than that of NCC without WNK4. We also showed that WNK4, NCC, and sortilin co-localize together in the perinuclear region, indicating that they might be associated together and/or interact in the same vesicles, traffic together, and facilitate the WNK4-promoted NCC targeting to the lysosome for degradation through a sortilin receptor–mediated mechanism; however, because we used overexpression system, overall protein degradation will inevitably be increased to cope with the excess protein, which is a potential limitation in our study. We believe that, overall, increased protein degradation would not necessarily alter the effect of WNK4 on the regulation of NCC. Taken together, our data suggest that WNK4 kinase could play a role in redirecting NCC’s destination. The exact mechanism of how WNK4 alters NCC trafficking needs to be further explored.

Sortilin not only is involved in sorting of lysosomal hydrolases\(^13\) but also is engaged in intracellular sorting of Glut4,\(^32,34,47\) endocytosis,\(^48\) and signal transduction.\(^49,50\) In this study, we showed that NCC interacts with sortilin WT as well as TRU, to a lesser extent. Sortilin TRU, which lacks the cytoplasmic tail, is still able to bind NCC, indicating that NCC interacts with the luminal domain of sortilin. Because sortilin TRU has low binding affinity to NCC, other potential binding sites in the cytoplasmic tail of sortilin cannot be excluded. The exact reason that sortilin TRU has a low affinity for NCC needs to be clarified. We also found that the N-terminus of NCC is responsible for its interaction with sortilin, but we do not know which sequence or motif in the N-terminus is involved in this interaction. Previous studies demonstrated that WNK4 inhibits NCC surface expression and its function in \(Xenopus\) oocytes\(^3,4\) and mammalian cells\(^6,11\) by a kinase-dependent mechanism. Because the N-terminus of NCC is responsible for interaction with sortilin, it is speculated that WNK4 might phosphorylate the N-terminus of NCC directly or indirectly and alter its interaction with sortilin. Although the mechanism remains to be clarified, it is clear that WNK4 affects NCC sorting direction and targeting. Further experiments will be needed to examine whether the phosphorylation status at the N-terminus of NCC affects its trafficking or final sorting destination. Unlike WNK4, WNK3 stimulates NCC activity in \(Xenopus\) oocytes.\(^51,52\) WNK3 and WNK4 share a high degree of amino acid identity within the kinase domain but a much lower degree of amino acid identity in the amino and carboxyl terminal domains. San-Cristobal et al.\(^53\) reported that amino terminus of WNK3 or WNK4 plays an important role in regulation of NCC activity. How the amino terminal domain of WNK4 affects NCC function or trafficking, affects the interaction with sortilin and NCC, and ultimately changes the direction of NCC sorting remains unknown. Elucidating these molecular mechanisms will definitely provide a novel view on NCC regulation by WNK kinase.

**CONCISE METHODS**

**Plasmids and Constructs**

Human WNK4 WT and human NCC WT were amplified by PCR from a human kidney cDNA library and then subcloned into pCMVTaq 3B vector (Stratagene, La Jolla, CA) and pCMV-HA vector (Clontech, Palo Alto, CA), respectively, as described previously.\(^6\) The GST-tagged full-length NCC, N-terminal intracellular NCC (1 through 135), and C-terminal intracellular NCC (605 through 1031) constructs were generated by PCR amplification using pCMV-HA-NCC as template and by insertion of respective cDNA into modified PRK5-GST vectors. The pEGFP-sortilin WT and its truncated sortilin lacking cytoplasmic tail (pEGFP-sortilin TRU), a dominant negative mutant, were provided by Dr. Carlos R. Morales (McGill University, Hamilton, Ontario, Canada).\(^27\) All constructs, shown in Figure 1, were confirmed by sequence analysis. These constructs have been successfully expressed in African green monkey kidney cells (Cos-7) and confirmed by Western blot analysis.

**Cell Culture, Transfection, and Animals**

Cos-7 cells obtained from American Type Culture Collection (Manassas, VA) were maintained in DMEM supplemented with 10% FCS, 1-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 \(\mu\)g/ml). MDCT cells were a gift of Peter Friedman (University of Pittsburgh, Pittsburgh, PA).\(^43,54\) MDCT cells were maintained in DMEM/F-12 medium containing 5% FCS, 1% penicillin, 1% streptomycin, and 1% neomycin. All other media and components were purchased from Invitrogen (Carlsbad, CA). Cells were transiently transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were used for Western blot analysis, IP, or immunostaining. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee. The kidneys from adult male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) and

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CS7BL/6J mice (Jackson Laboratories, Bar Harbor, ME), were harvested for Western blot analysis and immunohistochemistry.

**Western Blotting Analysis**

Cos-7 cells were harvested and processed as described previously. In brief, cells transiently transfected with various DNA constructs as indicated were lysed in lyss buffer containing 20 mM HEPES (pH 7.5), 120 mM NaCl, 5.0 mM EDTA, 1.0% Triton X-100, 0.5 mM dithiothreiotol, 1.0 mM PMSF, and complete protease inhibitor (Roche Diagnostics, Mannheim, Germany; 1 tablet per 50 ml of solution). The lysates were spun at 6000 × g for 5 min to pellet the insoluble material, and the proteins from supernatant were quantified using a Pierce BCA Protein Assay kit (Pierce, Rockford, IL). After mixing in Laemmli buffer (Bio-Rad, Hercules, CA) and incubating at 37°C for 30 min, the protein sample was separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ) for Western blot. The probing with specific antibodies and subsequent detection membranes (Amersham Biosciences) or Super signal (Pierce) were performed according to standard procedures as described previously.5,55

**IP and Co-IP**

IP and co-IP were preformed as described previously. In brief, Cos-7 cells were transfected with plasmids as indicated. Forty-eight hours after transfection, the cells were lysed. The cell lysates were incubated with primary antibodies for 2 h, then Protein G/A Sepharose beads were added, and the lysates were further mixed and incubated overnight at 4°C. After washing with lysis buffer twice and PBS once, beads were then eluted with Laemmli buffer (Bio-Rad). The eluted proteins were separated by SDS-PAGE, transferred onto PVDF membrane, and probed with appropriate antibodies. For co-IP experiments, blots were first probed with an antibody to the first binding partner. The PVDF membranes were then stripped and reprobed with an antibody to the second binding partner. Reciprocal IP was also performed with respective antibodies.

**GST Pull-Down Assay**

GST pull-down assay was modified and carried out as described previously. In brief, Cos-7 cells were co-transfected with GST-tagged N-terminus or C-terminus of NCC in combination with either sortilin WT or sortilin TRU. Forty-eight hours after transfection, cells were lysed. The cell lysates were incubated with primary antibodies for 2 h, then Protein G/A Sepharose beads were added, and the lysates were further mixed and incubated overnight at 4°C. After washing with lysis buffer twice and PBS once, beads were then eluted with Laemmli buffer (Bio-Rad). The eluted proteins were separated by SDS-PAGE, transferred onto PVDF membrane, and probed with appropriate antibodies. For co-IP experiments, blots were first probed with an antibody to the first binding partner. The PVDF membranes were then stripped and reprobed with an antibody to the second binding partner. Reciprocal IP was also performed with respective antibodies.

**Statistical Analysis**

The data are presented as means ± SE. Statistical significance was determined by t test when two groups were compared or by one-way ANOVA, followed by Bonferroni post hoc tests when multiple groups were compared. We assigned significance at P < 0.05.

**ACKNOWLEDGMENTS**

This work is supported by National Institutes of Health grants DK068226 (H.C.) and DK R01 32753 (W.B.G.).

Part of this work was presented annual meeting of the American Society of Nephrology; San Francisco, CA; November 2, 2007.

We thank Dr. Carlos R. Morales (McGill University, Hamilton, Ontario, Canada) for kindly providing sortilin plasmid. We thank Drs. Janet D. Klein and Misti A. Blount for helpful suggestions and critical reading of the manuscript. We thank Dr. Young-Hee Kim for help in immunohistochemistry. We also thank Dr. William McClellan for help with the statistical analysis.

**DISCLOSURES**

None.
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