Calcineurin and Sorting-Related Receptor with A-Type Repeats Interact to Regulate the Renal Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter

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ABSTRACT

The furosemide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) -cotransporter (NKCC2) is crucial for NaCl reabsorption in kidney thick ascending limb (TAL) and drives the urine concentrating mechanism. NKCC2 activity is modulated by N-terminal phosphorylation and dephosphorylation. Serine-threonine kinases that activate NKCC2 have been identified, but less is known about phosphatases that deactivate NKCC2. Inhibition of calcineurin phosphatase has been shown to stimulate transport in the TAL and the distal convoluted tubule. Here, we identified NKCC2 as a target of the calcineurin A\(\beta\) isoform. Short-term cyclosporine administration in mice augmented the abundance of phospho-NKCC2, and treatment of isolated TAL with cyclosporine increased the chloride affinity and transport activity of NKCC2. Because sorting-related receptor with A-type repeats (SORLA) may affect NKCC2 phosphoregulation, we used SORLA-knockout mice to test whether SORLA is involved in calcineurin-dependent modulation of NKCC2. SORLA-deficient mice showed more calcineurin A\(\beta\) in the apical region of TAL cells and less NKCC2 phosphorylation and activity compared with littermate controls. In contrast, overexpression of SORLA in cultured cells reduced the abundance of endogenous calcineurin A\(\beta\). Cyclosporine administration rapidly normalized the abundance of phospho-NKCC2 in SORLA-deficient mice, and a functional interaction between calcineurin A\(\beta\) and SORLA was further corroborated by binding assays in rat kidney extracts. In summary, we have shown that calcineurin A\(\beta\) and SORLA are key components in the phosphoregulation of NKCC2. These results may have clinical implications for immunosuppressive therapy using calcineurin inhibitors.

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The furosemide-sensitive renal Na\(^+\)-K\(^+\)-2Cl\(^-\) -cotransporter (NKCC2) mediates the transepithelial NaCl reabsorption in the thick ascending limb (TAL) and plays an essential role in the urinary concentration and volume regulation.\(^1\) The transport activity of NKCC2 depends on its phosphorylation at several N-terminal, conserved threonine and serine residues, including T96, T101, and T114.\(^2\) These threonines have been identified as targets for the two closely related STE20-like kinases, SPAK (SPS-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1),\(^3\) whereas much less information is known on the identity of the respective phosphatases. Calcineurin, a ubiquitously expressed calcium-/calmodulin-dependent phosphatase, has been shown to stimulate NKCC2 activity in several cell lines.\(^4\) However, the role of calcineurin in the phosphoregulation of NKCC2 in vivo remains to be elucidated. The aim of our study was to identify calcineurin as a key modulator of NKCC2. We found that calcineurin phosphatase is an essential regulator of NKCC2 activity. Our results suggest that calcineurin and SORLA are key components in the phosphoregulation of NKCC2. These results may have clinical implications for immunosuppressive therapy using calcineurin inhibitors.
dependent serine-threonine phosphatase, has been shown to play a role in the context of TAL function.\textsuperscript{6–11} Calcineurin inhibitors are widely used as immunosuppressive drugs and have undisputed benefits in decreasing rejection rates after transplantation, but also have major side effects including hypertension. Their systemic effects on the sympathetic nervous system or the renin-angiotensin-aldosterone axis have been described, but much less information is known about the cellular mechanisms of calcineurin-dependent regulation of renal transporters.\textsuperscript{10–12} We have recently shown that the calcineurin inhibitor tacrolimus caused salt retention and hypertension in mice.\textsuperscript{13} These effects were largely mediated by increased phosphorylation and activity of the renal thiazide-sensitive Na\textsuperscript{+}-Cl\textsuperscript{−}-cotransporter (NCC) of the distal convoluted tubule (DCT).\textsuperscript{13} Calcineurin inhibitors have been also reported to stimulate the function of NKCC2, although the underlying mechanisms remained to be clarified.\textsuperscript{14,15} We therefore hypothesized that calcineurin, similar to its action on NCC, interferes with the phosphorylation of NKCC2. To characterize this process we have made use of mice lacking SORLA, an intracellular receptor involved in sorting and trafficking of diverse proteins.\textsuperscript{16–18} These mice present with marked decrease of baseline NKCC2 phosphorylation that makes them a suitable model to analyze mechanisms that determine phosphorylation of the cotransporter. SORLA modulates the cellular distribution and function of distinct ligands including phosphokinases, but the molecular pathways that link SORLA with NKCC2 function are unclear.\textsuperscript{16–18} Here, we show that calcineurin dephosphorylates and thereby deactivates NKCC2, whereas SORLA modulates this process via its interaction with calcineurin. We have identified SORLA and calcineurin as the components of a novel signaling pathway that determines NKCC2 function. Our results have clinical implications for immunosuppressive therapy using calcineurin inhibitors, as volume and electrolyte disorders often restrict their therapeutic potential.

### RESULTS

Calcineurin Interacts with NKCC2

Activating phosphorylation of NCC is modulated by the calcineurin Aα isoform (CnAα) located in the DCT.\textsuperscript{13} In view of the structural and functional similarities between NCC and NKCC2 we tested whether calcineurin may determine NKCC2 activity by means of the locally expressed calcineurin Aβ isoform (CnAβ).\textsuperscript{13,19} We could confirm CnAβ localization along

\textbf{Figure 1.} Distribution of CnAβ and its association with NKCC2. (A–C) Immunofluorescence staining of CnAβ and double staining for NKCC2 (A and B) and AQP2 (C). CnAβ shows apical and perinuclear signal in medullary TAL (A; asterisks), apical staining in cortical TAL, no significant staining in DCT (TAL/DCT transition indicated by bars; B), and strong intracellular signal in collecting duct (C); mouse kidney, original magnification, \( \times 400 \). (D) Representative immunoblot of precipitate obtained from immunoprecipitation (IP) of NKCC2 (approximately 160 kDa; D) from rat kidney lysates. Co-immunoprecipitated CnAβ (between 50 and 60 kDa, arrow) is depicted; IgG was used for control immunoprecipitation. (E) Representative immunoblot showing results of GST pull-down assay performed in rat kidney lysate using recombinant N- or C-terminal NKCC2 tails as well as N-terminal NKCC2 mutants mimicking constitutive phosphorylation (T→D) or dephosphorylation (T→A) at relevant residues (T96, T101, and T114) as baits. CnAβ (arrow) interacts with both cytoplasmic tails of NKCC2 and all mutants mimicking its N-terminal phosphorylation but not with mutants mimicking the dephosphorylated transporter. Each experiment was repeated at least three times using kidneys from different animals.
TAL epithelia, with medullary (m) TAL revealing moderate apical and perinuclear signal and cortical (c) TAL showing strong apical signal. DCT segments were virtually negative for CnAβ, whereas collecting duct epithelia showed significant apical staining (Figure 1, A–C). To study the interaction between NKCC2 and CnAβ, co-immunoprecipitation (co-IP) experiments were performed using rat kidney extracts. Substantial association of NKCC2 with CnAβ was observed (Figure 1, D and E). To further characterize this interaction we performed glutathione S-transferase (GST) pull-down assays using recombinant N- or C-terminal NKCC2 tails as well as N-terminal NKCC2 mutants mimicking constitutive phosphorylation or dephosphorylation at functionally important residues (T96, T101, and T114). CnAβ from rat kidney lysate interacted with both cytoplasmic NKCC2 tails and with all NKCC2 mutants mimicking N-terminal phosphorylation. In contrast, CnAβ did not bind with mutants mimicking NKCC2 dephosphorylation (Figure 1, F). These results suggest that CnAβ may be involved in NKCC2 dephosphorylation.

The Calcineurin Inhibitor Cyclosporine Increases Phospho-NKCC2 Abundance

Next, we studied whether pharmacologic inhibition of calcineurin using cyclosporine affects NKCC2 phosphorylation. Effects of cyclosporine are mediated via its high-affinity binding to peptidyl-prolyl isomerases, also termed cyclophilins.20 Immunofluorescence revealed substantial expression of cyclophilin A and cyclophilin B along the mouse TAL (Figure 2, A and B). Immunoblotting and quantitative PCR of isolated mouse nephron segments obtained by microdissection corroborated the presence of both cyclophilins in TAL, suggesting that this nephron segment might be sensitive to cyclosporine (Figure 2, C and D). As the inhibition of calcineurin by cyclosporine at long term may cause systemic effects which may secondarily affect NKCC2 function, we chose a short-term application using cyclosporin A (CsA; 30 mg/kg i.p. for 1 h) in wild-type (WT) mice to obtain more direct information on its local action in TAL. As a result, phosphorylation of NKCC2 was markedly increased at the SPAK/OSR1-dependent threonines (T96, T101; +87%; \( P < 0.05 \)), whereas total NKCC2 abundance was not altered (Figure 3, A and B). Short-term treatment of microdissected TAL tubules with CsA (5 nM for 1 h) also increased the abundance of phospho-NKCC2 compared with vehicle treatment (+80%, \( P < 0.05 \)), which indicates that local calcineurin inhibition in TAL is sufficient to stimulate NKCC2 (Figure 3, C).

As effects of CsA on NKCC2 may be mediated by stimulation of NKCC2-activating kinase pathways involving SPAK and OSR1 we next evaluated phosphorylation of the two homologous kinases within their catalytic (T243 and T185) and regulatory domains (S383 and S325), reflecting their activity.4,21 Immunoblotting and confocal microscopy revealed no significant CsA-induced changes of SPAK/OSR1 phosphorylation within their catalytic or regulatory domains in TAL of WT kidneys or in microdissected TAL segments (Figure 3, C and D, Figure 4, A, B, and F). In contrast, DCT segments showed clear increases of both SPAK-/OSR1-phosphorylation sites by confocal microscopy (+170% at the catalytic and +87% at the regulatory SPAK/OSR1 domain, \( P < 0.05 \)) (Figure 4, C–F). Therefore, CsA-induced increase of phospho-NKCC2 is likely caused by inhibition of calcineurin rather than activation of SPAK/OSR1.
We have previously shown that genetic deletion of SORLA was associated with reduced NKCC2 phosphorylation at the SPAK/OSR1-dependent threonines (T96, T101) in vivo.16 Our present analysis of SORLA-deficient kidneys corroborated this observation (−84% for pT96/pT101-NKCC2, P<0.05; no change of total NKCC2; Figure 5, A and B). Analysis of NKCC2-activating kinases by immunoblotting revealed reduced levels of full-length and truncated SPAK products in medullary kidney lysates from SORLA-deficient mice compared with WT controls which likely reflects changes in TAL. In contrast, OSR1 abundance was not affected by SORLA deficiency (Figure 5, A and B). These data support the notion that SORLA affects the function of SPAK to phosphorylate NKCC2 as proposed previously.16

As our results suggested a role for calcineurin as a candidate phosphatase for NKCC2 dephosphorylation we tested whether SORLA also interferes with the abundance or cellular distribution of CnAβ in TAL. Immunoblotting of mouse kidney extracts using anti-CnAβ antibody produced a band at the predicted size of approximately 60 kDa and a faster migrating band of approximately 53 kDa, likely representing a degradation product.22,23 CnAβ was more abundant in SORLA−/− kidneys, than in WT controls (+112% in medulla and +134% in cortex, respectively; P<0.05; Figure 6, A–D). Confocal evaluation of signals intensities also showed increased apical abundance of CnAβ in SORLA−/− TAL (Figure 6, E and F). By contrast, a difference in CnAβ mRNA levels was not detectable (Figure 6, G). Conversely, transient overexpression of SORLA in human embryonic kidney 293 (HEK293) cells resulted in decreased abundance of endogenous CnAβ (−256%, P<0.05; Figure 7, A and B). These results suggest that SORLA may be involved in cellular trafficking and turnover of CnAβ, which is consistent with its general function as a multi-ligand intracellular sorting receptor.16,17 To corroborate this hypothesis we tested for potential interaction between SORLA and CnAβ. Immunofluorescence showed that SORLA and CnAβ were co-localized in apical and perinuclear sites of TAL (Figure 8, A). For binding assays, renal extracts from rats were employed, rather than extracts from mice, as high IgG background was present in the latter. Co-IP showed an interaction between CnAβ and SORLA (Figure 8, B). Mass spectrometric analysis (matrix-assisted laser desorption/ionization time of flight [MALDI-TOF] mass spectrometry [MS]) confirmed the presence of CnAβ in eluates after SORLA IP (Figure 8, C). To confirm that the co-IP data did not result from nonspecific binding of CnAβ to the extracellular SORLA moiety but rather reflected specific interaction with the cytoplasmic receptor tail, we additionally performed a GST pull-down assay using the whole cytoplasmic to the loading controls. Data are the mean±SD, * P<0.05. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 3. Acute effects of cyclosporin A on NKCC2 phosphorylation. (A) Representative immunoblots showing phospho-NKCC2 and NKCC2 abundances (all approximately 160 kDa) in kidneys from vehicle-treated (veh; n=8) or cyclosporine A-treated (CsA; n=8) mice (30 mg CsA/kg i.p. for 1 hour); GAPDH and β-actin serve as the respective loading controls (both approximately 40 kDa). (B) Densitometric evaluation of signals normalized to the loading controls. (C) Representative immunoblots showing phospho-NKCC2 and phospho-SPAK/OSR1 abundances (between 50 and 60 kDa) in microdissected TAL treated with vehicle (five experiments) or CsA (5 nM for 1 hour; four experiments); β-actin serves as the loading control. (D) Densitometric evaluation of signals normalized to the loading controls. Data are the mean±SD, * P<0.05. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
moiety of SORLA as bait. MS analysis showed significantly higher abundance of CnAβ in the eluates from GST-SORLA compared with GST-control pull-down assays, suggesting that the phosphatase specifically interacts with the intracellular tail of SORLA (Figure 8, D).

Cyclosporine Rescues NKCC2 Phosphorylation in SORLA−/− Mouse Kidneys

Our data so far have suggested that increased calcineurin abundance and activity upon SORLA disruption may, at least in

Figure 4. Acute effects of cyclosporin A on SPAK/OSR1 phosphorylation. (A–D) Representative confocal images of medullary (m) TAL (A and B) and DCT profiles (C and D) from vehicle-treated (n=4) or cyclosporine A-treated (CsA) mice (n=4) labeled for phospho-SPAK/OSR1 (green signals) with antibodies recognizing the phosphorylated catalytic (pT243-SPAK/pT185-OSR1) or regulatory kinase domains (pS383-SPAK/pS325-OSR1), and merge images showing coconitent labeling for NKCC2 or NCC (red signals). (E and F) Quantification of apical phospho-SPAK/OSR1 signals co-localized with NKCC2 or NCC by intensity using ZEN confocal software (Carl Zeiss). Note that phosphorylation of the catalytic SPAK/OSR1 domains was barely detectable in mTAL (A); the quantification was performed only for DCT in this case (E). All values refer to vehicle treatment which was set at 100%. Data are the mean±SD, *P<0.05.

Figure 5. Steady-state abundance of NKCC2, phospho-NKCC2, SPAK, and OSR1 in SORLA−/− mouse kidneys. (A) Representative immunoblots from wild-type (WT) and SORLA−/− mouse kidneys showing SORLA (approximately 250 kDa), NKCC2, phospho-NKCC2 (all approximately 160 kDa), full-length SPAK (FL-SPAK; approximately 60 kDa), its truncated isoforms (SPAK2 and KS-SPAK; between 40 and 60 kDa), and OSR1 (approximately 60 kDa) in medullary extracts from wild type (WT; n=10) and SORLA−/− kidneys (n=10); β-actin or GAPDH bands provide the respective loading controls (approximately 40 kDa). (B) Densitometric evaluation of the immunoreactive signals normalized to loading controls. Note the reduction of pT96/pT101-NKCC2 and SPAK signals in SORLA−/−. Data are the mean±SD; *P<0.05. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
part, be responsible for the decreased level of NKCC2 phosphorylation in SORLA−/− mice. To functionally corroborate this concept, we analyzed the acute effects of CsA on NKCC2 phosphorylation in these mice. As the effects of CsA are mediated by cyclophilins, we first compared their expression in WT and SORLA−/− kidneys by immunoblotting. Cyclophilin A was markedly reduced in SORLA−/− kidneys (−63%, *P<0.05), whereas cyclophilin B was not different (Figure 9, A and B). Nevertheless, CsA induced comparable increases of NKCC2 phosphorylation in WT and SORLA−/− mice (Figure 9, A–D). Calcineurin inhibition, thus, resulted in rescue of the NKCC2-activating phosphorylation in SORLA−/− mice.

**Cyclosporine Stimulates Transport Activity in Wild Type But Not in SORLA-deficient TAL**

To assess the effects of CsA on NKCC2 function we evaluated the equivalent short circuit current values (I′sc), a well-established indicator of NKCC2 activity, in isolated perfused TAL from WT and SORLA-deficient mice. As chloride affinity is a rate-limiting step for NKCC2 function, measurements of I′sc were performed under saturating chloride concentration (147 mM Cl−), allowing maximal transport function (Vmax), and under low chloride concentration (30 mM Cl−) to study chloride affinity. Wild-type TALs exhibited high initial I′sc at the saturating chloride concentration (3031 μA/cm²) and approximately 50% of Vmax at the low chloride concentration, suggesting that the latter corresponds to the Km range for NKCC2 transport kinetics. These values markedly decreased after 1 hour of vehicle treatment (1344 μA/cm² at 147 mM Cl− and 22% of Vmax at 30 mM Cl−), suggesting rapid NKCC2 deactivation in this *ex vivo* setting. Basolateral application of CsA for 1 hour resulted in higher I′sc values at both chloride concentrations (1879 μA/cm² at 147 mM Cl− and 31% of Vmax at 30 mM Cl−) compared with vehicle, suggesting that CsA stimulates NKCC2 activity and improves its affinity to chloride (Figure 10, A and B). SORLA deficiency was associated with substantially reduced baseline transport activity at both chloride concentrations (1774 μA/cm² at 147 mM Cl− and 19% of Vmax at 30 mM Cl−). These values did not further decrease after 1 hour of vehicle treatment and did not change in response to CsA which may reflect impaired baseline function of NKCC2-activating kinases in this genotype, notably in the absence of systemic stimuli (Figure 10, A and B). Overall, these results corroborate the functional relevance of calcineurin signaling in TAL.

**dDAVP Increases NKCC2 Phosphorylation in SORLA-deficient Mice**

To explain the lack of major physiologic phenotype in SORLA−/− mice, despite the reduction of baseline NKCC2 phosphorylation and activity, we tested whether the response to vasopressin was impaired by SORLA disruption. Acute administration of the vasopressin V2 receptor agonist dDAVP (1 μg/kg i.p. for 1 hour) to SORLA-deficient mice significantly increased their phospho-NKCC2 levels, although not to the extent observed in dDAVP-treated WT controls (Figure 11, A and B). The fact that SORLA−/− mice were responsive to endocrine stimulation may in part explain the lack of major physiologic phenotype in these mice.

**DISCUSSION**

Phosphorylation of cation-chloride cotransporters (CCC) at conserved N-terminal threonine and serine residues modulates...
their activity through the WNK-SPAK/OSR1 kinase cascade. Commonly, the activity of kinases in the distal nephron is counterbalanced by phosphatases. Based on the present localization studies, binding assays, and functional experiments we suggest that calcineurin phosphatase plays a key role in removing phosphates from NKCC2, thereby suppressing its activity along the TAL. Whereas CnAa is considered to be the major isoform of the renal cortex with predominant expression in DCT, we have confirmed that in TAL, CnAb is the dominant isoform. Co-distribution of CnAb with cyclophilin A and its paralogue, cyclophilin B, suggests that complexes composed of the calcineurin inhibitor CsA and one or both cyclophilin isoforms may form in TAL to locally inhibit CnAb phosphatase activity. Calcineurin inhibitors may of course affect a variety of systemic and renal parameters via activation of the intrarenal renin-angiotensin system and modulation of the release of nitric oxide or endothelin-1. Our experiments, however, clearly demonstrate a local, cell-autonomous action of calcineurin in TAL, since in the setting of isolated TAL tubules, inhibition of its activity by CsA was sufficient to substantially increase NKCC2 phosphorylation and function.

Although we found the CnAb associated with NKCC2 in protein complexes, the transporter lacks known calcineurin binding motifs (PxIxIT- or LxVP). This suggests that CnAb and NKCC2 may interact via a scaffolding mechanism, as shown previously for some substrates of related protein phosphatase 1, such as NKCC1. Nevertheless, the present binding assays pointed to a selective interaction between CnAb and the phosphorylated NKCC2, thus corroborating our functional results. It is, however, still unclear whether

![Figure 7](image1.png)

**Figure 7.** Effects of SORLA overexpression on CnAb abundance. (A) Representative immunoblots showing SORLA and CnAb signals in lysates from HEK293 cells transfected with pCDNA3 vector (control transfection) or pCDNA3 vector containing human SORLA. Mild SORLA signal in the control transfection reflects endogenous protein. CnAb signals appear at approximately 60 kDa; β-actin (approximately 40 kDa) provides the loading control. (B) Densitometric evaluation of CnAb normalized to loading control. Data are the mean±SD, *P<0.05. The experiment was repeated three times.

![Figure 8](image2.png)

**Figure 8.** Interaction between SORLA and CnAb. (A) Immunofluorescence staining for SORLA in a medullary TAL profile of wild-type mouse kidney and double staining for CnAb show apical and perinuclear co-localization of the two products in the merge image. (B) Representative immunoblot of precipitates obtained from immunoprecipitation (IP) of SORLA from rat kidney lysate. Note that CnAb (arrow) co-immunoprecipitates with SORLA; IgG was used for control immunoprecipitation. (C) Mass spectrometric (MS) analysis of IP-eluates confirms the abundant presence of SORLA (broken line arrows) and CnAb (arrows) in eluates from SORLA-IP but not from control-IP. (D) MS analysis shows significantly higher abundance of CnAb (arrows) in eluates from GST-SORLA pull-down assay using GST-fused cytoplasmic SORLA tail as bait (GST-SORLA) compared with GST alone (the CnAb protein score [66] was higher than the threshold for the statistical significance as calculated by the Mascot software [55], *P<0.05). Note that MS-signal of the tryptic peptides of CnAb in the eluates from the GST-SORLA pull-down assay was clearly detectable (black arrows), whereas the corresponding signals in eluates from the control pull-down assay were not detectable or at the detection limit. Each experiment was repeated at least three times using kidneys from different animals.
CnAβ directly dephosphorylates NKCC2 or signals to the transporter via other phosphoenzymes. Some other CCC members such as NKCC1 have been identified as substrates of the protein phosphatase 1 (PP1).\textsuperscript{31–33} Although calcineurin itself may regulate PP1,\textsuperscript{34} NKCC2 does not share the N-terminal PP1-docking motif (RVXF) with NKCC1 and is therefore not a probable substrate for PP1.\textsuperscript{31,35} Furthermore, the present evaluation of NKCC2-phosphorylating kinases SPAK and OSR1 did not point to their significant activation in TAL upon short-term CsA treatment, whereas in DCT clear CsA-induced increases of SPAK/OSR1 phosphorylation were observed suggesting segment-specific mechanisms of CCC activation during acute calcineurin inhibition by CsA. Our data therefore suggest that CnAβ does not modulate SPAK/OSR1 activity in TAL. Since direct biochemical evidence showing that NKCC2 is a substrate of calcineurin is still missing, further studies will be necessary to specify the mechanism of CnAβ-dependent NKCC2 dephosphorylation.

Further support for the role of CnAβ in the regulation of NKCC2 came from the present analysis of SORLA-deficient mice showing nearly complete absence of NKCC2 phosphorylation at T96/T101.\textsuperscript{16} SORLA consists of a large extracellular moiety, a transmembrane domain, and a short cytoplasmic tail. While the extracellular portion of the receptor contains several binding modules enabling its interactions with a number of extracellular proteins, the cytoplasmic tail binds intracellular ligands via adaptor proteins.\textsuperscript{16–18} We have previously identified SORLA as a trafficking factor for SPAK and proposed that missorting of the kinase underlies the impaired phosphorylation of NKCC2 in SORLA-deficient mice.\textsuperscript{16} In contrast to our previous report of similar SPak levels in total kidney lysates from WT and SORLA-deficient mice, the present, separate analysis of medulla and cortex uncovered a moderate decrease of SPAK abundance in the renal medulla of SORLA\textsuperscript{2/2} mice. Since SPAK is highly expressed in the DCT, previous analysis of total kidney lysates may have masked the difference in the medulla. Although SPAK can phosphorylate and activate NKCC2, more recent studies provided several lines of evidence suggesting that OSR1 is the primary kinase activating NKCC2, rather than SPAK.\textsuperscript{36,37} In fact, SPAK along the TAL comprises inhibitory forms (KS-SPAK and SPAK2) which can compete with OSR1 and full-length SPAK for binding to the NKCC2 RFxV/I motif and SORLA\textsuperscript{−/−} mice (n=10); β-actin (approximately 40 kDa) serves as a loading control. (B) Densitometric evaluation of signals normalized to loading controls. (C) Representative confocal images showing immunofluorescence of phosphorylated NKCC2 (pT96/pT101-NKCC2) in medullary TAL profiles of WT (n=4) and SORLA\textsuperscript{−/−} kidneys (n=4) after vehicle or cyclosporine (CsA) administration (30 mg/kg i.p. for 1 hour). (D) Evaluation of the apical phospho-NKCC2 signal intensity using confocal ZEN software shows CsA-induced increases in both strains. All values refer to the WT group on vehicle treatment which was set at 100%. Data are the mean±SD, *P<0.05.
NKCC2 activity in SPAK-deleted mice; §significant differences between WT and SORLA−/− mice treated with vehicle or cyclosporine A (CsA; 5 nM for 1 hour) under saturating chloride concentration (147 mM Cl−) enabling the maximal NKCC2 transport function (Vmax).

(A) Measurements of I′sc (low Cl−) % from wild type (WT) and SORLA−/− mice treated with vehicle or CsA at low chloride concentration (30 mM Cl−); the data are expressed as percentage of the respective Vmax values. Note that WT TAL exhibit approximately 50% of their Vmax activity at 30 mM Cl− which suggests that the chosen low chloride concentration corresponds to the Michaelis constant range for the NKCC2 transport kinetics. Data are the mean±SEM; §significant differences between WT and SORLA−/−; *significant differences between the initial I′sc and the values measured after 1 hour of vehicle or CsA treatment; #significant differences between vehicle- and CsA-treated WT or SORLA−/− TAL; 7–26 TAL tubules isolated from at least three different mice were evaluated per group.

Figure 10. Acute effects of cyclosporine on transport activity of isolated perfused wild type and SORLA−/− TAL. (A) Electrophysiologic evaluation of equivalent short circuit currents (I′sc) as indicator of NKCC2 activity in cortical TAL tubules isolated from wild type (WT) and SORLA−/− mice and treated with vehicle or cyclosporine A (CsA; 5 nM for 1 hour) under saturating chloride concentration (147 mM Cl−) enabling the maximal NKCC2 transport function (Vmax).

and thereby reduce NKCC2 activity in a dominant negative fashion.37–39 In line with this idea, SPAK deletion was associated with increased phosphorylation and activity of NKCC2 in vivo.37,40 In contrast, another recent study reported reduced NKCC2 activity in SPAK-deficient TAL ex vivo, suggesting that SPAK-independent NKCC2 phosphorylation may require systemic stimuli such as AVP.37,38,41 Accordingly, work from our and other groups demonstrated that AVP-induced activation of NKCC2 is not impaired by SPAK deficiency.39,41 In view of the current controversy on SPAK function in TAL, functional implications of its reduced abundance in the TAL of SORLA−/− mice remain speculative and, thus, do not satisfactorily explain the strong diminution of phospho-NKCC2 levels. Moreover, the clearly more relevant OSR1 kinase was unaffected by SORLA deficiency, leading us to search for alternative explanations for decreased NKCC2 phosphorylation in SORLA−/− mice. However, SORLA deficiency was also associated with increased renal abundance of CnAβ, especially in the apical compartment of TAL cells in close proximity to NKCC2. These findings suggested that SORLA reduces the cellular abundance of the phosphatase especially at or near the plasma membrane. In line with this idea, overexpression of SORLA in HEK293 cells was associated with decreased abundance of endogenous CnAβ. The apical accumulation of CnAβ in SORLA-deficient TAL could therefore contribute to the decrease of NKCC2 phosphorylation at SPAK/OSR1-dependent phosphoacceptors. The rapid CsA-induced increase of NKCC2 phosphorylation in SORLA−/− mice supported this assumption. Moreover, the present binding assays revealed interaction between the intracellular tail of SORLA and CnAβ, thus, further corroborating the role of SORLA in the regulation of the phosphatase. Consequently, the lack of SORLA likely affects NKCC2 phosphorylation via two distinct pathways, impaired SPAK function and exaggerated calcineurin activity in TAL cells. Apart from these effects, SORLA deficiency was associated with decreased abundance of cyclophilin A which may secondarily attenuate the action of CsA in these mice.

Increase of NKCC2 phosphorylation usually coincides with its activation, although the underlying mechanisms are presently unclear.5,42,43 The present electrophysiologic experiments using isolated TAL provide potential mechanistic links between NKCC2 phosphorylation and function. We propose that NKCC2 phosphorylation facilitates NKCC2’s affinity to chloride which is rate limiting for the transporter’s activity.24,25 Indeed, TAL transport under low chloride condition (30 mM Cl−), measured as the I′sc value, reflected the phosphorylation state of NKCC2. Therefore, the activity was stimulated in CsA-treated WT TAL along with increased phospho-NKCC2 abundance, whereas SORLA-deficient TAL revealed reduced activity and NKCC2 phosphorylation. Moreover, strong reduction of Vmax in SORLA−/− TAL suggests that SORLA substantially facilitates transcellular NaCl transport in TAL. In view of the unaffected total NKCC2 abundance and surface expression in SORLA−/− mice,16 the correlation between decreased NKCC2 phosphorylation and function was therefore evident in this genotype. However, SORLA-deficient TAL still showed substantial residual transport activity which, along with the moderate response of SORLA−/− mice to dDAVP in this study, may explain the absence of a Bartter-syndrome phenotype in these mice. Our data therefore suggest that the maintenance of adequate NKCC2 phosphorylation facilitates its affinity to chloride and hence, transport function. We suggest that this mechanism is impaired in SORLA-deficient mice. Further characterization of the
relationships between NKCC2 phosphorylation, chloride affinity, and transport function should be addressed in future studies using heterologous systems such as frog oocytes.

In summary, we identify CnA\(\beta\) and SORLA as components of a novel signaling pathway involved in the phosphoregulation of NKCC2, as schematized in Figure 12. Our data demonstrate that NKCC2 dephosphorylation depends on calcineurin, although it is unclear at present, whether calcineurin dephosphorylates the transporter directly or signals to NKCC2 via other phosphoenzymes. We further show that SORLA modulates the abundance and intracellular distribution of CnA\(\beta\), thus affecting the NKCC2 phosphorylation and activity. Since calcineurin inhibitor-induced activation of the distal salt transporters has been linked to hypertension and electrolyte disorders,\(^{12,13}\) our results have clinical implications. They further suggest that pharmacologic targeting of SORLA or other proteins regulating calcineurin activity has the potential to derive more specific calcineurin inhibiting drugs, which would retain immunosuppressive activity, with fewer effects on kidney tubule cells. As nephrotoxicity remains the major limiting side effect complicating use of calcineurin inhibitors, such a result would have broad impact.

**CONCISE METHODS**

**Animals, Tissues, Treatments**

Adult (10–12 wk) male WT and SORLA\(^{-/-}\) mice and Sprague-Dawley (SD) rats were kept on standard diet and tap water. 12 WT and 12 SORLA\(^{-/-}\) mice were used for steady state evaluation (\(n=4\) for morphologic and \(n=8\) for biochemical evaluation for each genotype). For evaluation of short-term effects of calcineurin inhibition WT or SORLA\(^{-/-}\) mice were divided into groups receiving vehicle (cremophor) or cyclosporine A (30 mg/kg body wt) for 1 hour by intraperitoneal injection (at least \(n=6\) in each group). For morphologic evaluation, mice were anesthetized and perfusion-fixed retrogradely via the aorta using 3% paraformaldehyde.\(^{37}\) For biochemical analysis, mice were sacrificed and the kidneys removed. For co-IP and GST pull-down assays six adult SD rats were sacrificed and kidney removed. All experiments were approved by the Berlin Senate (permission G0220/12).
Cell Culture, Treatments
HEK293 cells were cultivated in 75 cm² cell culture flasks or on glass cover slips in DMEM medium (PAN-Biotech) with 5% calf serum and 1% penicillin/streptavidin at 37°C, 95% humidity, and 5% CO₂. To evaluate effects of SORLA overexpression on endogenously expressed CnAβ, HEK293 cells (70% confluence) were transiently transfected with full-length human SORLA in pcDNA3.1 plasmid or with empty pcDNA3.1 (Life Technologies, Carlsbad, CA) using JetPEI transfection reagent (Polyplus), incubated for 48 hours at 37°C, and evaluated by immunoblotting.

Primary Antibodies
Antibodies recognizing NKCC2 and phospho-(p)-T96/T101-NKCC2 (SPAK/OSR1-dependent phosphorylation) were generated in our laboratory and described previously. Antibody against NCC was generated in the laboratory of David Ellison. Antibody against SORLA was kindly provided by C. M. Petersen (Aarhus University). Antibodies against cyclophilin B (Abcam, Inc.), aquaporin 2 (Santa Cruz Biotechnology, Dallas, TX), CnAβ (EMD Millipore, Billerica, MA or Santa Cruz Biotechnology), SPAK (Cell Signaling Technology, Danvers, MA), β-actin (Sigma-Aldrich, St. Louis, MO), or glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology) were purchased from commercial sources. Antibodies to ORS1 and phosphorylated SPAK/OSR1 species (pT243-SPAK/pT185-OSR1 [T-loop] and pS383-SPAK/pS325-OSR1 [S-motif]) were obtained from the University of Dundee.

Immunofluorescence
Cryostat sections from mouse and rat kidneys (7 μm) were incubated with blocking medium (30 min), followed by primary antibody diluted in blocking medium (1 hour or overnight). For multiple staining, antibodies were sequentially applied, separated by washing step. For detection of phosphorylated kinases and transporters the antibodies were preabsorbed with corresponding non-phosphorylated peptides in 10-fold excess for 1 hour before the application on kidney sections. Fluorescent Cy2-, Cy3- or Cy5-conjugated antibodies (Dianova) were applied for detection. Sections were evaluated in a Carl Zeiss confocal microscope (LSM 5 Exciter). For confocal evaluation of phospho-NKCC2, phospho-SPAK/OSR1, or CnAβ signals, kidney sections were double-stained for NKCC2 or NCC to identify TAL or DCT, respectively. At least 15 similar tubular profiles were evaluated per individual animal. Intensities of the confocal fluorescence signals were scored across each profile using ZEN 2008 software (Carl Zeiss GmbH), and mean values of the phospho-NKCC2, phospho-SPAK/OSR1, or CnAβ signals co-localized with NKCC2 or NCC signals were obtained. Background fluorescence levels were determined over cell nuclei and subtracted from the signal.

Immunoblotting and Co-immunoprecipitation
For immunoblotting, whole kidneys or excised renal medullas and cortices were homogenized in buffer containing 250 mM sucrose, 10 mM triethanolamine, protease inhibitors (Complete; Roche Diagnostics, Indianapolis, IN), and nuclei removed by centrifugation (1000 × g for 10 min). Supernatants (postnuclear homogenates) were separated in 10% polyacrylamide minigels, electrophoretically transferred to polyvinylidene fluoride membranes, and detected using respective primary antibodies, HRP-conjugated secondary antibodies (Dako), and chemiluminescence exposure of X-ray films (Fujifilm). Films were evaluated densitometrically (Alpha Imager, BioZym).

GST Pull-down
GST pull-down assays were performed with rat kidney lysates using recombinant GST-fused cytoplasmic SOLRA tail, the total N- or C-terminus of NKCC2, or mutants mimicking its constitutive N-terminal phosphorylation (T→D) or dephosphorylation (T→A) at functionally relevant residues (T96, T101, or T114) as baits. The respective NKCC2-DNA constructs were described previously. The cytoplasmatic tail of SORLA was amplified by PCR from a pcDNA3.1 vector containing complete human SORLA cDNA using Phusion DNA polymerase (forward primer: 5'-tatatagaaattctctgctggttgctgctgtgg-3'; reverse primer: 5'-tatatactcagagtgtgtctgttttgagttg-3'), predicted PCR product size 280 bp) and cloned into pGEX-6P1 vector using EcoRI and Xhol restriction sites. The resulting pGEX-SORLA-GST plasmid was verified by DNA sequencing. GST-fused proteins or GST (control) were fixed on glutathione magnet beads (Invitrogen) and incubated with rat kidney lysates in GST-buffer (50 mM HEPES, 200 mM NaCl, 10 mM CaCl₂, 1× complete protease inhibitor; pH 7.5) overnight at 4°C with agitation. After this process, the beads were washed with GST-buffer, boiled in 1× Laemmli buffer, and the eluates were analyzed by immunoblotting or MALDI MS.

Isolation of Nephron Segments and Renal Tubule Perfusion
For isolation of renal tubules, kidneys from WT mice were digested with the type II collagenase (306 U/ml; PAN-Biotech) for 15 minutes at 37°C, nephron segments were isolated and used for immunoblotting, quantitative PCR, or perfusion. Perfusion and transepithelial measurements in freshly isolated mouse cortical TAL segments were performed as described. Briefly, isolated TALs were incubated with CsA (5 mM) or vehicle (0.006% DMSO) for 1 hour in incubation buffer (140 mM NaCl, 0.4 mM KH₂PO₄, 1.6 mM K₂HPO₄, 1 mM MgCl₂, 10 mM Na-acetate, 1 mM α-ketoglutarate, 1.3 mM Ca-glucanate, 3.75 mg/ml glycine, 0.48 mg/ml trypsin inhibitor, 0.25 mg/ml DNase I, 5 mg/ml albumin, pH 7.4) at 30°C. Tubules were then transferred to the bath on a heated microscope stage and perfused using a concentric glass pipette system with a double-barreled perfusion pipette. One barrel
was used for voltage measurement and perfusion with control solution containing 147 mM Cl\(^-\) (145 mM NaCl, 0.4 mM KH\(_2\)PO\(_4\), 1.6 mM K\(_2\)HPO\(_4\), 1 mM MgCl\(_2\), 5 mM glucose, 1.3 mM Ca-glucuronate, pH 7.4) at a rate of 10–20 nl/min. The other barrel was used for constant current induction (13 nA) and fluid exchange to luminal low chloride solution containing 30 mM Cl\(^-\) (28 mM NaCl, 0.4 mM KH\(_2\)PO\(_4\), 1.6 mM K\(_2\)HPO\(_4\), 62 mM Na\(_2\)SO\(_4\), 1 mM MgCl\(_2\), 1.6 mM Ca-glucuronate, 5 mM glucose, 51 mM mannitol, pH7.4). Measurements were performed both during symmetric luminal and basolateral perfusion with control solution and after subsequent change of the luminal and basolateral perfusates to low chloride solution. Cable equations were used to calculate transepithelial resistance \(R_{te}\). Equivalent short circuit current \(I_{sc}\) was calculated from \(R_{te}\) and \(V_{te}\) according to Ohms law.

MALDI Mass Spectrometry
The co-IP or GST pull-down samples were washed/equilibrated with ammonium bicarbonate in acetone/tritium and digested with 0.02 μg trypsin at 37°C for 24 hours. Then peptides were desalted and concentrated utilizing the ZipTipC18 (EMD Millipore, Billerica, MA) technology and eluted directly onto the MALDI target (Bruker Daltonik, Bremen, Germany) using a-cyano-4-hydroxycinnamic acid as matrix. The subsequent MS analyses were carried out using MALDI-TOF MS (Bruker Daltonik). Calibrated and annotated spectra were subjected to a database search (Swiss-Prot, Zürich, Switzerland) utilizing Bruker Bio-Tool 3.2 and the Mascot 2.2 search engine, which compares the experimental MALDI-TOF MS data set with the calculated peptide masses for each entry in the sequence database and use of empirically determined factors to assign a statistical weight to each individual peptide match. Quantification of relative abundances of the tryptic peptides corresponding to CNAB in eluates was performed as described previously\(^{47}\); statistical significance was calculated by the Mascot software.

Quantitative Real-time PCR
Samples of six WT and six SORLA \(^{-/-}\) kidneys or isolated TAL tubules were homogenized, and total RNA was prepared using the RNeasy total RNA kit (Qiagen). Genomic DNA was digested by DNase, and cDNA was synthesized by reverse transcription of 1 μg total RNA. Three sets of primers were designed (CnA\(_B\): 5’-gcaaccatgaatgcagacacc-3’ and 5’-ctggaccaaacacaaacggt-3’; CypA: 5’-cttgaccaacaaacacggt-3’ and 5’-tgccttctttcaccttccca-3’; CypB: 5’-gcaaggaggaagagcct-3’ and 5’-tgcaggagtgtttcctttc-3’). Amplification was performed using a real-time PCR TaqMan Fast 7500 (Applied Biosystems) and the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). Threshold cycle (\(C_\text{T}\)) values were set in the linear phase of exponential amplification. The expression levels of each gene were normalized to glyceraldehyde-3-phosphate dehydrogenase expression level using the \(\Delta\Delta C_\text{T}\) method.

Statistical Analysis
Results were evaluated using routine parametric statistics. Groups were compared by means of the Student’s \(t\)-test or, if the data violated a normal distribution, the nonparametric Mann–Whitney test. A probability level of \(P<0.05\) was accepted as significant. All results are expressed as the mean±SD.

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DISCLOSURES
None.

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