Dominant functional role of the novel phosphorylation site S811 in the human renal NaCl cotransporter

Omar A. Z. Tutakhel, Frans Bianchi, Daniël A. Smits, René J. M. Bindels, Joost G. J. Hoenderop, and Jenny van der Wijst

Department of Physiology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands

ABSTRACT: The NaCl cotransporter (NCC) is essential for electrolyte homeostasis and control of blood pressure. The human SLC12A3 gene, which encodes NCC, gives rise to 3 isoforms, of which only the shortest isoform [NaCl cotransporter isoform 3 (NCC3)] has been studied extensively. All NCC isoforms share key phosphorylation sites at T55 and T60 that are essential mediators of NCC function. Recently, a novel phosphorylation site at S811 was identified in isoforms 1 and 2 [NaCl cotransporter splice variant (NCCSV)], which are only present in humans and higher primates. The aim of the current study, therefore, is to investigate the role of S811 phosphorylation in the regulation of NCC by a combination of biochemical and fluorescent microscopy analyses. We demonstrate that hypotonic low-chloride buffer increases S811 phosphorylation, whereas phosphorylation-deficient S811A mutant hinders phosphorylation at T55 and T60 in NCCSV and NCC3. NCCSV S811A impairs NCC activity in a dominant-negative fashion, although it does not affect plasma membrane abundance. This effect may be explained by the heterodimerization of NCCSV with NCC3. Taken together, our study highlights the dominant-negative effect of NCCSV on T55 and T60 phosphorylation and NCC activity. Here, we reveal a new function of NCCSV in humans that broadens the understanding on NCC regulation in blood pressure control.—Tutakhel, O. A. Z., Bianchi, F., Smits, D. A., Bindels, R. J. M., Hoenderop, J. G. J., van der Wijst, J. Dominant functional role of the novel phosphorylation site S811 in the human renal NaCl cotransporter. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: hypertension · kidney · NCC · splice variant

Hypertension (high blood pressure) affects more than 1.3 billion people worldwide and can lead to severe cardiovascular complications, including cerebrovascular disease, heart failure, ischemic heart disease, and renal failure (1–6). This makes hypertension the greatest contributing risk factor to morbidity and mortality worldwide. The cause of hypertension is identified in only ~10% of cases (secondary hypertension) (7, 8). It is of critical importance to increase our understanding of the pathophysiology of hypertension. Kidneys play a key role in the control of blood pressure by regulating sodium (Na+) excretion (9–13). Fine-tuning (5–10%) of Na+ excretion primarily takes place in the distal convoluted tubule (DCT) by Na+ reabsorption via the NaCl cotransporter (NCC) (14–16).

Since the cloning of NCC, 3 splice isoforms of NCC have been identified that are the result of alternative splicing of the SLC12A3 gene (14). Thus far, most research has focused on the shortest NCC isoform, NaCl cotransporter isoform 3 (NCC3), as the other 2 isoforms are not present in rodents (17). NCC isoforms 1 and 2, which result from the alternative splicing of exon 20, are 9 aa longer than NCC3 (17, 18). Subsequent studies have confirmed the presence of both long and short forms of the exon 20—20a and 20b, respectively—which possibly indicates a splicing donor site (19). Compared with NCC isoform 1, isoform 2 lacks 1 aa at glutamine residue 95 within the amino (N)-terminal domain; therefore, NCC isoforms 1 and 2 are practically indistinguishable and, in this study, are collectively referred to as NCC splice variant (NCCSV). Of interest, NCCSV is only present in humans and higher primates. In the human kidney, NCCSV mRNA comprises ~44% of the total NCC content (14).

The importance of NCC in the regulation of blood pressure is illustrated by such diseases as Gitelman syndrome (OMIM: 263800) (20–22) and familial hyperkalemic
hypertension (FHHt; OMIM: 145260) (23–26). Gitelman syndrome is caused by loss-of-function mutations in the SLC12A3 gene that encodes NCC (20–22), which results in renal Na⁺ wasting and hypotension (18, 27). In contrast, FHHt is a form of hypertension that results from the gain of function of NCC as a consequence of mutations in genes that code for key signaling molecules (23, 28). In FHHt, mutations have been identified in WNK1 and WNK4, which encode the with no lysine (WNK) kinases (23), or in KLHL3 and CUL3, which code for Kelch-like 3 and Cullin 3, respectively (28). WNK kinases phosphorylate and activate Ste20-related proline-alanine–rich kinase (SPAK) and oxidative stress response 1, which, in turn, phosphorylate T55 and T60 in NCC (14, 29, 30). Phosphorylation of these sites is essential for NCC activity (16, 29, 31, 32) and plasma membrane abundance (33, 34).

Of interest, Gonzales et al. (17) have described a novel phosphorylation site on S811, which is only present in NCCSV. Our group has previously shown that NCCSV plays a role in physiologic (14) and pathophysiologic (35) processes; however, the importance of NCCSV S811 phosphorylation in NCCSV and NCC3 function remains unknown. Therefore, the aim of the current study was to investigate the role of NCCSV S811 in the phosphorylation and activity of NCCSV and NCC3. We used a combinational approach of immunoblotting with phospho-specific Abs, cell-surface biotinylation, and fluorescence microscopy to examine the influence of S811 on the phosphorylation, membrane abundance, and activity of NCC. In addition, a phospho-specific Ab against S811 was used to assess S811 phosphorylation under hypotonic low-chloride (Cl⁻) condition. Finally, the interaction of NCCSV with NCC3 was assessed by fluorescence lifetime imaging microscopy (FLIM).

MATERIALS AND METHODS

Mutagenesis and constructs

NCC isoform 1 (accession no. NP_000330.2) was used in all experiments to represent NCCSV. For communo precipitation, enhanced green fluorescent protein (eGFP) C-terminal–coupled human NCC,cv eGFP, NCC3v eGFP, and the phospho-mutants of NCCSV–eGFP—constitutively phosphorylated S811D and non-phosphorylated S811A—in pCMV-SPORT6 were generated as described in Tutakhel et al. (14) and Dimke et al. (16). In addition, human NCCsv, NCCsv, and NCCsv phospho-mutants were cloned into the hemagglutinin (HA) pCINeo-ires-mCherry vector by using standard PCR and restriction cloning. For FLIM and communo precipitation experiments, NCCsv and NCC3 were cloned into the pCINeo vector, and human codon optimized mCitrine and mCherry tags were fused to the C-terminal end separated by a 15-residue-long linker region (3x GGGGS) by using PCR and uracil excision-based cloning (36). For fluorescence microscopy measurements, NCC3v, NCCsv, NCCsv phospho-mutants (S811D and S811A), and NCC3 phospho-mutant (T55A/T60A) were cloned into the pCINeo-ires-eCFP vector by using restriction cloning. The vector that carries halide-sensitive yellow fluorescent protein (YFP) and the mKate gene was described by Vrijgomschild et al. (37) and kindly provided by Dr. Jeffrey M. Beekman (University Medical Center, Utrecht, The Netherlands).

Cell culture and transfection

Cell culture and transfection was performed as described in Tutakhel et al. (14). In brief, human embryonic kidney 293 (HEK293) cells were grown in DMEM medium that was supplemented with 10% v/v fetal bovine serum, 100 μM nonesessential amino acids, and 2 mM l-glutamine. Cells were transiently transfected by using polyethylenimine (1:6: DNA: polyethylenimine; Polysciences, Warrington, PA, USA). Cells that were transfected with empty vectors were used as control (mock). For cotransfection, cells were transfected in a DNA ratio of 1 to 1.

Buffers

The following buffers were used: isotonic control buffer, which contained (mM) 135 NaCl, 5 KCl, 0.5 CaCl₂, 0.5 MgCl₂, 0.5 Na₂HPO₄, 0.5 Na₂SO₄, 15 HEPES/Tris, pH 7.4; hypotonic low Cl⁻ buffer, which contained (mM) 67.5 Na-gluconate, 2.5 K-gluconate, 0.25 CaCl₂, 0.25 MgCl₂, 0.5 Na₂HPO₄, 0.5 Na₂SO₄, 7.5 HEPES/Tris, pH 7.4; dissection buffer, which contained (mM) 300 sucrose, 25 imidazole, 1 EDTA, HCl, pH 7.2, and freshly added protease inhibitors (μM) 50 PMSF, 20 aprotinin, 20 leupeptin, 10 pepstatin A; PBS supplemented with (mM) 1 MgCl₂, 0.5 CaCl₂, NaOH, pH 8.0; lysis buffer, which contained (mM) 50 Tris/HCl, pH 7.5, 1 EDTA, 1 EDTA, 10 Na⁺ β-glycerophosphate, 1 Na-orthovanadate, 50 Na-fluoride, 10 Na-pyrophosphate, 270 sucrose, 150 NaCl, 1% (v/v) Triton X-100, and freshly added protease inhibitors (μM) 50 PMSF, 20 aprotinin, 20 leupeptin, 10 pepstatin A; buffer A, which contained (mM) 50 Tris/HCl, pH 7.5, 1 EDTA, 1× Laemmli sample buffer, which contained (mM) 40 DTT, 12 Tris/HCl, pH 6.8, 0.4% (w/v) SDS, 2% (v/v) glycerol, 0.002% (w/v) bromophenol blue; and Tris-buffered saline and Tween-20 (TBS-T), which contained (mM) 140 NaCl, 20 Tris/HCl, pH 7.5, 0.1% (v/v) Tween-20. NaI solution contained (mM) 140 NaI, 4.2 KCl, 1.4 CaCl₂, 1 MgCl₂, 5.5 v-glucose, 10 HEPES/Tris, pH 7.4, 300 mOsmol/kg H₂O.

Cell treatment

At 48 h post-transfection, HEK293 cells were washed once with PBS. Cells were then incubated for 30 min at 37°C with isotonic control buffer or hypotonic low Cl⁻ buffer. Cells were subsequently used to isolate total cell membrane fractions, cell-surface biotinylation, or for communo precipitation.

Isolation of total cell membrane fractions

Ten volumes of ice-cold dissection buffer were added to HEK293 cells and cells were homogenized by 20 passages through a 21-gauge needle (Eclipse; Becton Dickinson, Franklin Lakes, NJ, USA) that was fitted to a 1-ml syringe (Becton Dickinson). Homogenized cells were centrifuged at 4000 g for 8 min at 4°C, after which the supernatant was centrifuged at 16,000 g for 40 min at 4°C. The pellet of the 16,000 g centrifugation was resuspended in ice-cold dissection buffer and protein concentration was determined by using the Bradford method (MilliporeSigma, Burlington, MA, USA). Proteins were eluted in 1× Laemmli sample buffer. Samples were incubated for 15 min at 65°C before immunoblotting. For immunoblotting of total cell membrane fractions, 20 μg protein was loaded onto gels.

Cell-surface biotinylation

We performed cell-surface biotinylation as described in De Groot et al. (38). HEK293 cells were subsequently lysed in ice-cold lysis buffer. Cells were homogenized by 20 passages through a 21-gauge needle (Eclipse; Becton Dickinson, Franklin Lakes, NJ, USA) that was fitted to a 1-ml syringe (Becton Dickinson). Homogenized cells were centrifuged at 4000 g for 8 min at 4°C, after which the supernatant was centrifuged at 16,000 g for 40 min at 4°C. The pellet of the 16,000 g centrifugation was resuspended in ice-cold dissection buffer and protein concentration was determined by using the Bradford method (MilliporeSigma, Burlington, MA, USA). Proteins were eluted in 1× Laemmli sample buffer. Samples were incubated for 15 min at 65°C before immunoblotting. For immunoblotting of total cell membrane fractions, 20 μg protein was loaded onto gels.
buffer and centrifuged at 16,000 g at 4°C. Protein concentrations were determined by using the Bradford method (MilliporeSigma). Equal amounts of protein were incubated with neutravidin beads (Pierce, Rockford, IL, USA) for 2 h at 4°C. Beads were washed 3 times with lysis buffer and eluted in 1× Laemmli sample buffer. Samples were incubated for 15 min at 65°C before immunoblotting.

**FLIM**

FLIM images were generated from HEK293 cells at 16 h after transfection with NCC and NCCSV that contained mCitrine and/or mCherry C-terminal fusion plasmids. The total amount of DNA per transfection was 2 μg (1:1 donor acceptor ratio) in WillCo-dish (diameter, 35 mm; WillCo Wells, Amsterdam, The Netherlands) with a cell density of 1.2 × 10^6. Imaging and measurements were performed as described in Verboogen et al. (39). In brief, samples were washed and imaged in live cell imaging solution (HEPES-buffered saline solution; Thermo Fisher Scientific, Waltham, MA, USA). Photon traces in PicoQuant Photon Trace (PT3; PicoQuant, Berlin Germany) format were used to construct FLIM images in image cytometry standard (ICS). Conversion of PT3 files to ICS was performed by using PT32ICS conversion software (39). For FLIM statistics of single cells, photons were pooled for each individual cell and fitted with exponential decay functions that were convoluted with the instrument response function using OriginPro2016 (OriginLab, Northampton, MA, USA). Single pixel-fitted FLIM images were generated for individual cells—without at least 1,000,000 photons per cell—using TRI2 software (v.2.8.6.2; Gray Institute, Oxford, United Kingdom) (40, 41). Settings used for TRI2 were as follows: 7×7-pixel circular binning and thresholding at 15–100% intensity. Lifetime imaging pictures were generated by using Fiji ImageJ (National Institutes of Health, Bethesda, MD, USA; https://imagej.net/Fiji) (42).

**Coimmunoprecipitation**

Transfected HEK293 cells were lysed in ice-cold lysis buffer for 1 h at 4°C. Cell lysate was subsequently collected and centrifuged at 16,000 g at 4°C. Protein concentrations were determined by using the Bradford method. Anti-green fluorescent protein (GFP) or anti-HA Abs were coupled to protein A/G plus agarose using the Bradford method. Anti-NCCSV pS811 Ab [residue 811 of human NCC phosphorylated at S811, RGARP(pS)VSGALD] was raised in sheep and was affinity purified on the appropriate immunogen (PhosphoSolutions, Aurora, CO, USA); anti-mCherry (1:5000); anti-GFP (generated in house, detects eGFP and mCitrine; 1:5000); anti-mCherry (1:1000; Abcam, Cambridge, United Kingdom); and anti-β-actin (1:20,000; MilliporeSigma). Anti-NCCSV pS811Ab [residue 811 of human NCC phosphorylated at S811, R GAR P(pS)VSGALD] was raised in sheep and was affinity purified on the appropriate antigen (MRC-PPU Reagents, Dundee, United Kingdom). Secondary Abs used were as follows: horseradish peroxidase–conjugated secondary Abs that were diluted in 1% w/v nonfat dry milk in TBS-T overnight at 4°C. All PVDF membranes were probed once with indicated primary Abs. Membranes were then incubated with horseradish peroxidase–conjugated secondary Abs that were diluted in 1% w/v nonfat dry milk in TBS-T for 1 h at room temperature and visualized with the imaging system (Chemidoc XR3; Bio-Rad, Hercules, CA, USA) using ECL (Thermo Fisher Scientific). Immunoreactive bands were quantified by using Image Studio Lite software (Li-Cor Biosciences, Lincoln, NE, USA).

**Abs**

The following Abs were used: anti-INCC (1:2000; EMD Millipore); anti-NCC pT55 and anti-NCC pT60 (equivalent mouse NCC pT53 and pT56; 1:2000) as described in Pedersen et al. (43) (PhosphoSolutions, Aurora, CO, USA); anti-HA (1:5000; Cell Signaling Technology, Danvers, MA, USA); anti-GFP (generated in house, detects eGFP and mCitrine; 1:5000); anti-mCherry (1:1000; Abcam, Cambridge, United Kingdom); and anti–β-actin (1:20,000; MilliporeSigma). Anti-NCCSV pS811Ab [residue 811 of human NCC phosphorylated at S811, R GAR P(pS)VSGALD] was raised in sheep and was affinity purified on the appropriate antigen (MRC-PPU Reagents, Dundee, United Kingdom). Secondary Abs used were as follows: horseradish peroxidase–conjugated goat anti-rabbit (1:10,000; MilliporeSigma) and goat anti-mouse (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). All Abs have been validated for their linearity in detection (Supplemental Fig. 1).

**NCC activity measurements using YFP-mKate**

NCC activity was performed as described in Valdez-Flores et al. (22). NCC is able to transport NaI-like NaCl in a hydrochlorothiazide (HCTZ)-sensitive manner (22). In brief, HEK293 cells were cotransfected with the halide-sensitive YFP-mKate construct (37) and the appropriate human NCC pCIneo-IRES-eCFP construct or pCIneo-IRES-eCFP mock. The total amount of DNA per transfection was 2 μg (0.2 μg YFP-mKate + 1.8 μg NCC) in a WillCo-dish with a cell density of 1.2 × 10^6. Nineteen hours after transfection and before measurements, cells were incubated with hypotonic low Cl− buffer for 10 min at 37°C. Cells were then placed in an incubation chamber that contained hypotonic low Cl− buffer (500 μl) and attached to the stage of an inverted microscope (Axiovert 200M; Carl Zeiss, Jena, Germany). NCC-expressing HEK293 cells were identified by detecting eCFP.
fluorescence using a monochromator at 430 nm excitation, and the emitted light was subsequently directed by a 480AF30 dichroic mirror (Omega Optical, Brattleboro, VT, USA). After 30 s, an equal volume of NaCl solution was added to the incubation chamber (final concentration of Na\(^+\) and Cl\(^-\): 105 and 70 mM, respectively). Changes in intracellular halide concentrations were monitored by simultaneous measurements of halide-sensitive YFP fluorescence and halide-insensitive fluorescence of mKate (37). Halide-sensitive YFP was excited at 488 nm by using a monochromator. Fluorescence-emitted light was directed by a 505DRLPXR dichroic mirror (Omega Optical) through a 535AF26 emission filter. mKate was excited at 543 nm, and fluorescence-emitted light was directed by a 560DRL dichroic mirror (Omega Optical) through a 565ALP emission filter onto a Cool-Snap HQ monochrome charge-couple device camera (Photometrics, Tucson, AZ, USA). The integration time of the charge-couple device camera was set at 200 ms for both YFP and mKate with a sampling interval of 3 s. We performed all measurements at 37°C. Quantitative image analysis was performed with Metamorph 6.0 (Molecular Devices, Sunnyvale, CA, USA). For each wavelength, mean fluorescence intensity was monitored in an intracellular region and, for the purpose of background correction, an extracellular region of identical size. After background correction, the fluorescence emission ratio of the green and red fluorescence of YFP and mKate, respectively, was used to determine changes in intracellular halide content as a measure of NCC activity. Signal specificity was assessed by adding 100 \(\mu\)M HCTZ or an equivalent amount of its solvent, DMSO, together with the hypotonic low Cl\(^-\) buffer incubation for 10 min at 37°C. We determined the thiazide-sensitive uptake taking by the difference of the measurements with and without HCTZ.

**Statistics**

Immunoblot data were analyzed by comparing integrated optical densities of bands by 1-way ANOVA with Tukey’s multiple comparisons post hoc test if multiple groups were compared. In FLIM and NCC activity measurements, all depicted data are averages of at least 3 independent experiments, and all statistical analyses were performed by using a 1-way ANOVA with Dunnett’s multiple comparison post hoc test that compared control condition with all other conditions. Depicted values are presented as means ± SEM of at least 3 independent experiments, and values of \(P < 0.05\) were considered statistically significant. All data were analyzed by using Prism 6 (GraphPad Software, La Jolla, CA, USA).

**RESULTS**

**Effect of S811 on NCC\(_{SV}\) phosphorylation at T55 and T60**

To examine the role of phosphorylation at S811 in NCC\(_{SV}\), we generated NCC\(_{SV}\) S811D and S811A plasmids that represent the constitutively phosphorylated and non-phosphorylated forms of S811, respectively. HEK293 cells that were transfected with the respective plasmids were subjected to hypotonic low Cl\(^-\) buffer, which is a known stimulus of NCC phosphorylation at T55 and T60 (Fig. 1A) (29, 44). Of interest, NCC\(_{SV}\) S811A did not demonstrate a significant stimulation of T55 and T60 phosphorylation upon hypotonic low Cl\(^-\) buffer compared with isotonic control buffer (Fig. 1 and Supplemental Fig. 2A). Of note, tNCC density does not differ significantly (Supplemental Fig. 2A). NCC\(_3\), NCC\(_{SV}\), and NCC\(_{SV}\) S811D demonstrated significantly higher phosphorylation at T55 and T60 upon hypotonic low Cl\(^-\) buffer treatment compared with isotonic control buffer (Fig. 1 and Supplemental Fig. 2A). No difference in T55 and T60 phosphorylation was observed between NCC\(_{SV}\) and NCC\(_3\) after treatment with hypotonic low Cl\(^-\) buffer. Of note, isotonic control buffer did not result in T55 and T60 phosphorylation in any of the conditions.
tested (Fig. 1B, C and Supplemental Fig. 2A, black bars). Immunoreactive bands were not observed in cells that were transfected with empty vector (mock) (Fig. 1A).

**Effect of S811 phosphorylation on phosphorylation of NCC3 at T55 and T60**

As NCCSV and NCC3 are coexpressed at the apical membrane of epithelial cells that line the DCT (14), we hypothesized that S811 phosphorylation could affect phosphorylation at T55 and T60 of NCC3. Coexpression of NCCSV S811A and NCC3 resulted in significantly less phosphorylation at T55 and T60 compared with coexpression of NCCSV and NCC3 when treated with hypotonic low Cl⁻ buffer. Of interest, this decrease was >50% for both T55 and T60 (Fig. 2A, B and Supplemental Fig. 2B, white bars). Coexpression of NCC3 with NCCSV or NCCSV S811D demonstrated significantly higher phosphorylation at T55 and T60 when treated with hypotonic low Cl⁻ buffer compared with isotonic control buffer (Fig. 2 and Supplemental Fig. 2B). Similar to that shown in Fig. 1, NCCSV S811A alone did not demonstrate significant differences in T55 and T60 phosphorylation between isotonic control and hypotonic low Cl⁻ buffer treatment (Fig. 2 and Supplemental Fig. 2B). In isotonic control buffer, there were no differences in the levels of T55 and T60 phosphorylation in any of the conditions tested (Fig. 2B, C and Supplemental Fig. 2B, black bars). Cells that were transfected with mock vector did not display immunoreactive bands (Fig. 2A).

**NCCsv and NCC3 form hetero- and homodimers**

To assess whether the effect of NCCSV S811 phosphorylation on NCC3 T55 and T60 phosphorylation could be exerted via dimerization of NCCSV with NCC3, we used a Förster resonance energy transfer (FRET)–based microscopy approach with C-terminal mCitrine and mCherry fusions of NCCSV and NCC3, respectively. FRET was measured by using FLIM microscopy, which has the advantage that the lifetime (τ) is not dependent on excitation power and probe concentration and is therefore less technically challenging than ratiometric FRET analysis (45). The amount of FRET, which represents the interaction between the donor and acceptor pair, is measured as the lifetime of the mCitrine fluorescent signal. This signal shortens as a result of the energy transfer to the mCherry probe in the case of protein dimerization (39, 46). Coexpression of NCCSV-mCitrine with either NCCSV-mCherry or NCC3-mCherry in HEK293 cells resulted in a significant decrease in lifetime as demonstrated by phasor plots (Fig. 3B) and single-cell analysis (Fig. 3A), which indicated the formation of both hetero- and homodimers of NCCSV with NCCSV and NCC3, respectively. We further assessed the

![Figure 2](https://example.com/figure2.png)

**Figure 2.** NCCSV S811A inhibits phosphorylation at T55 and T60 of NCC3. A) Representative immunoblot of total cell membrane fractions isolated from HEK293 cells that were cotransfected with NCC3 and NCCSV or NCCSV mutants in a 1-to-1 ratio and treated with isotonic control (Co) or hypotonic low Cl⁻ (Hy) buffer. Immunoblots were probed with anti-NCC, anti–phosphorylated T55 (pT55), and T60 (pT60) NCC Ab that depict the immunoreactive band of 130 kDa. β-Actin was used as sample loading control. B, C) Densitometry analysis of the immunoreactive bands of pT55 and pT60 divided by immunoreactive bands of tNCC. We determined the abundance of tNCC and pT55 and T60 upon treatment with hypotonic low Cl⁻ buffer relative to treatment with isotonic control buffer. We compared the relative abundance in hypotonic low Cl⁻ condition of each combination with the relative abundance of NCC3 cotransfected with NCCSV. For each combination, 20 μg protein was loaded on gels. OD, optical density. Values are expressed as means ± SEM of at least 3 independent experiments. *Significance of hypotonic low Cl⁻ buffer compared with its own control; †Significant compared with NCC3 + NCCSV. P < 0.05.
dimerization of NCCsv and NCC3 by performing a competition experiment in which NCCsv-mCitrine was coexpressed with unlabeled NCC3 as a competitor together with NCCsv-mCherry. Competition with unlabeled NCC3 resulted in an increase of the donor lifetime compared with HEK293 cells that coexpressed NCCsv-mCitrine and NCCsv-mCherry (Fig. 3B). In addition, we used SLC41A1 as negative control; this is an Mg2+ transporter that localizes to the apical membrane of the DCT (47, 48), but does not interact with NCCsv. Therefore, NCCsv-mCitrine and SLC41A1-mCherry were coexpressed in HEK293 cells, which did not colocalize (Supplemental Fig. 3A) and demonstrated no significant lifetime decrease—in line with 2 proteins that do not interact (Supplemental Fig. 3A, B). Dimerization, as observed by FRET/FLIM, was further established by coimmunoprecipitation experiments (Fig. 3C and Supplemental Fig. 3C): NCC3 demonstrated an interaction between NCC3 and NCCsv, NCCsv S811D, or NCCsv S811A.

Effect of S811 phosphorylation on NCC function

As NCC activity is regulated by its phosphorylation (29), we used a functional assay to study the effect of S811 phosphorylation on the transport activity of NCCsv and NCC3. The respective plasmids were cotransfected in HEK293 cells with halide-sensitive YFP that was coupled to the halide-insensitive fluorescent red protein, mKate. Before measurements, transfected HEK293 cells were incubated in hypotonic low Cl− buffer to maximally increase NCC phosphorylation and activity (22). After 30 s, NaI was added to the medium, which induced a significant reduction in the YFP-to-mKate ratio in NCC3-expressing cells, which was indicative of NCC transport activity (Fig. 4A, B). Pretreatment with HCTZ (40 min, 100 μM), a well-known inhibitor of NCC, completely abrogated this response (Fig. 4B). As described in Valdez-Flores et al. (22), NCC activity is measured as the difference in the YFP-to-mKate ratio.

Figure 3. NCCsv and NCC3 form hetero- and homodimers. A) NCCsv-mCitrine was transiently cotransfected with NCCsv-mCherry or NCCsv-mCherry in HEK293 cells. FRET between labels was monitored by fluorescence lifetime imaging. Representative confocal microscopy (left) and convoluted FLIM (right) images of NCCsv-mCitrine (green) with NCCsv-mCherry (red) or NCCsv-mCherry (red). Scale bar, 10 μm. B) Whole-cell apparent fluorescence lifetimes demonstrating hetero- and homodimerization of NCCsv and NCC3 proteins in live cells. C) Coimmunoprecipitation with mCitrine-labeled NCCsv and mCherry-labeled mock, NCCsv, and NCC3. GFP-coupled beads represent the immunoprecipitated NCCsv-mCitrine fraction, and immublots were probed with anti-mCherry and anti-GFP Abs; depicting the immunoreactive NCC band of ~130 kDa. As the GFP Ab binds to mCitrine, it demonstrates that NCCsv-mCitrine (bound to GFP-coupled beads) interacts with both NCCsv and NCCsv-mCherry. As a negative control, NCCsv-mCitrine and mock were coexpressed in HEK293 cells and did not demonstrate an interaction between mCitrine and mCherry. Total cell lysate (input) was probed with anti-mCherry, anti-GFP, or anti-β-actin Abs. β-Actin was used as sample loading control. N ≥ 3; n > 20/condition. *Significant difference between NCCsv-mCitrine + mock and other conditions; # significant differences from NCCsv-mCherry + NCC3. P < 0.05.
(Δ YFP/mKate ratio) between t = 0 and t = 180 s after the addition of NaI. This is plotted as the HCTZ-specific NCC activity: Δ YFP/mKate ratio without HCTZ – Δ YFP/mKate ratio with HCTZ (Fig. 4C). Cells that expressed NCCsv S811A alone or in combination with NCC3 displayed significantly lower NCC activity compared with NCC3 and NCC3 + NCCsv (Fig. 4C). The NCC3 phospho-deficient T55A/T60A mutant (NCC3 T55A/T60A) was included as negative control and demonstrated significantly lower NCC activity compared with wild-type NCC3 and NCC3 + NCCsv (Fig. 4C). Of note, the basal YFP-to-mKate ratio at t = 0 s was not significantly different between the various conditions tested (Fig. 4B).

**Effect of S811 on the abundance of NCC at the plasma membrane**

To determine the effect of S811 phosphorylation on the localization of NCC at the plasma membrane, HEK293 cells that were transfected with mock, NCC3, NCCsv, NCCsv S811D, or S811A mutants were subjected to cell-surface protein biotinylation. NCC protein was demonstrated in total cell lysate (input) and the biotinylated fraction upon both isotonic control and hypotonic low Cl⁻ conditions (Fig. 4D). We corrected the optical density of the biotinylated fraction for the input fraction to assess NCC protein abundance at the plasma membrane. No significant difference was observed between the conditions tested, which indicated an equal amount of NCC protein abundance at the plasma membrane (Fig. 4E). Cells that were transfected with mock vector did not display immunoreactive bands of NCC in the biotinylated fractions or the input (Fig. 4D).

**Generation of specific Abs against NCCsv pS811**

The NCCsv pS811-specific epitope [RGARP(pS)VSGALD] was used to generate a phospho-specific NCCsv pS811 Ab.
We performed immunoprecipitation of the cell lysate of HEK293 cells that were transfected with mock, NCC<sub>3</sub>, or NCC<sub>SV</sub> by using NCC<sub>SV</sub> pS811–conjugated beads (Fig. 5A). We subsequently used a commercially available NCC Ab that detected both NCC<sub>3</sub> and NCC<sub>SV</sub> to determine the total input as well as the specific phosphorylated fraction after immunoprecipitation. Of importance, this tNCC Ab only detected the immunoprecipitated fraction of NCC<sub>SV</sub> under the hypotonic low Cl<sup>−</sup> buffer condition. This indicates that the S811 residue is phosphorylated in hypotonic low Cl<sup>−</sup> buffer. Immunoreactivity was not present in cells that were transfected with mock or NCC<sub>3</sub> (Fig. 5A).

**DISCUSSION**

The current study demonstrates that S811 acts as a dominant regulatory site for the phosphorylation of T55 and T60 in NCC<sub>SV</sub> and NCC<sub>3</sub>. Moreover, our findings demonstrate that the phosphorylation-deficient S811A mutant decreases the transport activity of both NCC<sub>SV</sub> and NCC<sub>3</sub>. The effect of S811 phosphorylation on NCC<sub>3</sub> activity likely occurs via the formation of heterodimers. Together, these findings provide evidence that S811—present only in NCC<sub>SV</sub>—is a crucial new residue involved in NCC regulation.

Our findings demonstrate that S811 has a dominant-negative effect on T55 and T60 phosphorylation. Richardson et al. (44) previously showed that mutation of T60 to alanine in NCC<sub>3</sub> markedly inhibited the phosphorylation of neighboring residues, such as T55, induced by hypotonic low Cl<sup>−</sup> treatment. In addition, the T60A mutant strongly inhibited NCC activity, whereas this was less prominent for the T55A mutant (44). This implies that T60 can exert a dominant-negative effect over other phosphorylation sites in NCC<sub>3</sub> (44). Several other studies have demonstrated that T60 is a key phosphorylation site for NCC activity (29, 34, 44). This has been highlighted by a loss of function mutation at T60 in NCC that is linked to Gitelman syndrome (34, 49). It has also been previously shown that defective T60 phosphorylation corrects the phenotype of FHHt in mice (34). Our study adds a new important site that is solely present in NCC<sub>SV</sub> at S811. Of importance, this site could have a regulatory effect on NCC, as the S811A mutant prevented T55 and T60 phosphorylation and inhibited activity in both NCC<sub>SV</sub> and NCC<sub>3</sub>.

**Figure 5.** Increased NCC<sub>SV</sub> pS811 upon intracellular Cl<sup>−</sup> depletion and schematic model for NCC<sub>SV</sub> S811 regulation. A) Immunoprecipitation of pS811-conjugated beads with cell lysate from HEK293 cells that were transfected with mock, NCC<sub>3</sub>, or NCC<sub>SV</sub>. Indicated NCC isoforms were treated with isotonic control (Co) or hypotonic low Cl<sup>−</sup> (Hy) buffer. pS811-coupled beads represent the immunoprecipitated NCC<sub>SV</sub> pS811 fraction, and immublots were probed with anti-tNCC Ab. Representative immunoblot depicts the immunoreactive NCC<sub>SV</sub> pS811 band of ~130 kDa. B, C) NCC<sub>SV</sub> S811A mutant not only abolishes the hypotonic low Cl<sup>−</sup>-induced phosphorylation at T55 and T60 in NCC<sub>SV</sub>, but also prevents phosphorylation at T55 and T60 in NCC<sub>3</sub>. B) WNK kinases are autophosphorylated (activated) when the intracellular Cl<sup>−</sup> concentration decreases. WNKs can phosphorylate and activate SPAK and oxidative stress response 1 (OSR1), which, in turn, phosphorylate T55 and T60 in NCC. Hypotonic low Cl<sup>−</sup> buffer increases S811 phosphorylation, most likely via the WNK-SPAK/OSR1 signaling pathway. C) The dominant-negative effect of NCC<sub>SV</sub> S811A on the phosphorylation of T55 and T60 in NCC<sub>SV</sub> and NCC<sub>3</sub> may occur via the formation of heterodimers between NCC<sub>SV</sub> and NCC<sub>3</sub>. IP, immunoprecipitation.
and NCC3 (Fig. 5). The inhibitory effect of S811A on NCC function is in line with our previous data that show that Xenopus laevis oocytes that expressed NCCSV S811A exhibited decreased $^{22}$Na$^+$ uptake compared with oocytes that expressed wild-type NCCSV (14). Furthermore, it was shown that WNK4 inhibits both NCC3 and NCCSV function independent of S811, as the NCCSV S811D mutant was not protected from the inhibitory effects of WNK4 (14). Of interest, our results now demonstrate that the S811 residue is phosphorylated upon intracellular Cl$^-$ depletion (Fig. 5A). It is known that T55 and T60 are phosphorylated by the activation of the WNK-SPAK signaling pathway upon Cl$^-$ depletion (29, 30); therefore, it is likely that S811 phosphorylation upon intracellular Cl$^-$ depletion (Fig. 5A) is also mediated by the activation of the WNK-SPAK signaling pathway. However, prediction software suggested that the S811 phosphorylation site is highly similar to a PKA consensus site. Therefore, hormones that regulate the PKA pathway could potentially be involved in the regulation of NCCSV-mediated Na$^+$ transport in DCT. There is a growing body of evidence that demonstrates that NCC abundance and phosphorylation is affected by PKC or PKA downstream of angiotensin II or vasopressin signaling, respectively (43, 50–52).

It has previously been shown that NCC mRNA in the human kidney comprises 55% of NCC3 and 45% of NCCSV (14). To mimic this physiologic distribution, we performed a cotransfection experiment with NCCSV S811A and NCC3 that demonstrated significantly less phosphorylation at T55 and T60 compared with cells that were cotransfected with NCCSV and NCC3. We have demonstrated that NCC3 and NCCSV can dimerize, which results in a mixture of hetero- and homodimers of the 2 variants. Of interest, the observed decrease in the levels of phosphorylation was >50% for both T55 and T60, which suggests that NCCSV S811A not only affected NCCSV, but also blocked T55 and T60 phosphorylation in NCC3 (Fig. 5). Furthermore, cells that coexpressed NCCSV S811A and NCC3 demonstrated significantly impaired NCC activity, which confirmed the dominant-negative effect of NCCSV S811 on NCC3 function. Observations that NCCSV is significantly expressed in the kidney (14), together with the fact that S811 can affect T55 and T60 phosphorylation and function in NCCSV and NCC3, implies a potential key role for S811 in NCC-mediated renal salt handling.

Although the NCCSV-to-NCC3 mRNA ratio in the human kidney is 45%, on average, the ratio of NCCSV to NCC3 expression varied from 19 to 63% in 7 human kidneys (14). The large difference in the NCCSV-to-NCC3 ratio suggests that the splicing of NCC is highly regulated in humans (19). In humans, it has been shown that ~95% of genes are alternatively spliced (53). The production of alternatively spliced mRNA is diversely regulated by a variety of external stimuli or/and genetic makeup. Between human individuals, altered splicing can lead to differences in alternative spliced mRNAs that can impact the abundance or function of a protein (54, 55). Mechanisms of alternative splicing machinery are highly variable and not well understood (54). In several well-documented examples, such natural variation of alternative splicing has indeed been demonstrated to contribute to the development of various diseases, such as cancer and diabetes (56–60). Moreover, altered splicing of genes is suggested to influence drug response (61); therefore, unraveling the mechanism behind the splicing ratio of NCCSV to NCC3 in the kidney might revise our understanding of salt transport and the response to diuretics in the distal part of the nephron.

To explore the molecular mechanism by which NCCSV S811 exerts a dominant-negative effect on NCC3, we conducted FLIM and coimmunoprecipitation experiments. Here, we have demonstrated that both the constitutively phosphorylated and nonphosphorylated NCCSV S811 mutants form heterodimers with NCC3, which indicates that the effect of S811 on the phosphorylation of NCC3 can occur via interaction between the 2 isoforms. Phosphorylation status of S811 did not affect the interaction between NCCSV and NCC3. Of importance, our findings show, for the first time to our knowledge, that NCCSV and NCC3 form hetero- and homodimers in living cells and that NCCSV competes with NCC3 on the functional level. Homodimerization of 2 NCC3 has been proposed in previous studies that used immunoblotting (22, 62–64), and it has also been found that NCC is present at the plasma membrane in a dimerized form (63, 64). Whereas it has been demonstrated for other proteins that dimerization occurs at the surface of the plasma membrane (65), our data now suggest that the dimerization of NCC already occurs in the cytosol, as a decreased lifetime of NCC3 was observed in organellar membranes. Dimerization of transmembrane proteins is a common mechanism that precedes advanced functional regulation of a protein. For example, in the tyrosine kinase pathway, Erb2/Erb3 cross-phosphorylate each other after dimerization (66, 67). From its dimeric nature, NCC resembles the NaKCl cotransporter (64, 68), which suggests that other members of the family of electroneutral cation-chloride cotransporters form similar oligomeric structures. Of interest, it has recently been demonstrated that NCC can interact with the epithelial Na$^+$ channel (ENaC) (69). Moreover, inhibition of NCC also affected the function of ENaC, likely via the direct interaction of both proteins (70). As NCC and ENaC are coexpressed along the later part of the DCT, it has been suggested that this interaction can regulate Na$^+$ reabsorption in the DCT (70, 71). Heterodimerization of NCCSV with NCC3 suggests that NCCSV phosphorylation adds an additional regulatory mechanism by affecting the phosphorylation of N-terminal T55 and T60 of NCC3 via dimerization.

NCC abundance at the plasma membrane is a powerful mechanism with which Na$^+$ reabsorption in the kidney is modulated. In the current study, cell-surface biotinylation of NCC-transfected cells revealed that a change in S811 phosphorylation of NCCSV does not
affect the abundance of NCC$_{SV}$ at the plasma membrane, where NCC is functionally active. Moreover, there is no change in the plasma membrane abundance of NCC in any of the NCC isoforms or mutants when cells are exposed to hypotonic low Cl$^-$ stress. Our findings indicate that, upon hypotonic low Cl$^-$ stress, translocation of NCC to the plasma membrane is not affected. Rather, intrinsic T55 and T60 phosphorylation of NCC at the plasma membrane is increased. Our findings are in agreement with previous studies that have demonstrated that NCC surface expression is not affected by the phosphorylation status of T60 or S811 in X. laevis oocytes (14, 29). Similarly, the triple mutation of T55, T60, and Ser73 to alanine did not significantly change NCC abundance at the plasma membrane (33). Previously, a relationship between T60 phosphorylation and the membrane stability of NCC has been suggested (34). However, several studies have demonstrated a decreased abundance of NCC$_3$ T60A in both the biotinylated fractions and input fraction, which suggests an unaltered abundance at the plasma membrane (33, 34).

Taken together, our results demonstrate that S811 of NCC$_{SV}$ plays a crucial role in NCC function and could therefore be a key factor in renal salt handling. Additional experiments are needed to find the kinases that regulate S811 phosphorylation, which will provide more insight into the influence of S811 on NCC activity and might be crucial in elucidating the pathogenesis of essential hypertension.

ACKNOWLEDGMENTS

The authors thank Marco A. Valdez-Flores (Department of Physiology, Radboud Institute for Molecular Life Sciences), Sjoerd Verkaart (Department of Physiology, Radboud Institute for Molecular Life Sciences), Geert v. d. Bogaart (Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences), and Danielle R. J. Verboogen (Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences) for technical assistance and advice. This work was supported by the Netherlands Organization for Scientific Research (VENI 863.13.010 and VICI 016.130.668) and the Dutch Kidney Foundation (PHD12.14 and 16O104). The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

O. A. Z. Tutakhel, F. Bianchi, R. J. M. Bindels, J. G. J. Hoenderop, and J. van der Wijst conceived and designed the research; O. A. Z. Tutakhel, F. Bianchi, D. A. Smits, and J. van der Wijst performed the research; O. A. Z. Tutakhel, F. Bianchi, and D. A. Smits analyzed the data; O. A. Z. Tutakhel, F. Bianchi, D. A. Smits, and J. van der Wijst drafted the manuscript; and all authors read and approved the final manuscript.

REFERENCES


DOMINANT ROLE OF S811 IN NCC FUNCTION


