Regions in the Cytosolic C-Terminus of the Secretory Na^+-K^+-$2Cl^-$ Cotransporter NKCC1 Are Required for Its Homodimerization†

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ABSTRACT: The "secretory" Na^+-K^+-$2Cl^-$ cotransporter, NKCC1, is a member of a small gene family of electroneutral cation-chloride cotransporters (CCCs) with 9 homologues in vertebrates. A number of these transporters, including NKCC1 itself, have been shown to exist as homodimers in the membrane, suggesting that this may be a common feature of the CCCs. Here we employ chemical cross-linking studies, a novel co-immunoprecipition assay, and NKCC1/CCC chimeras to further explore the basis and significance of NKCC1 dimerization. An N-terminally truncated NKCC1 (nttNKCC1), in which the first 20 kDa of the 28 kDa cytosolic N-terminus are deleted, forms homodimers as well as heterodimers with full-length NKCC1, indicating that this region of N-terminus is not required for dimerization. On the other hand, replacing the 50 kDa NKCC1 C-terminus with that of several other non-NKCC1 homologues results in chimeric proteins that form homodimers but show little or no heterodimerization with NKCC1, demonstrating that the C-terminus of NKCC1 plays an essential role in dimerization and that NKCC1 dimerization exhibits definite homologue-specificity. Using additional chimeras we find that the residues required for dimer formation lie between amino acids 751 and 998 of (rat) NKCC1. We also show that dramatically overexpressing the nonfunctional truncated protein nttNKCC1 relative to the endogenous NKCC1 in the HEK293 cells results in a modest inhibition of fluxes via the endogenous transporter and a change in its sensitivity to the specific inhibitor bumetanide. These latter results indicate that there is a functional interaction between dimer subunits but that nonfunctional subunits do not necessarily have a dominant negative effect as has been previously proposed.

The "secretory" Na^+-K^+-$2Cl^-$ cotransporter, NKCC1, is a member of a small gene family of electroneutral cation-chloride cotransporters (CCCs).[1] In vertebrates these include (*1*) an "absorptive" Na^+-K^+-$2Cl^-$ cotransporter isoform (NKCC2), a Na^+-Cl^- cotransporter (NCC), and four K^+-Cl^- cotransporter isoforms (KCC1, KCC2, KCC3, and KCC4). The function of two other vertebrate homologues, commonly referred to as CIP and CCC9, remains uncertain. NKCC1 is relatively widely expressed in both epithelial and nonepithelial tissues and is known to play important roles in a variety of physiological processes including transepithelial salt and water transport, hearing, olfaction, pain perception, spermatogenesis, and maintenance of blood pressure and vascular tone (*1*−*6*, *6*−*11*).

Structurally NKCC1 has large intracellular N- and C-termini (*12*) on either side of a central hydrophobic transmembrane domain containing 12 membrane spanning segments (*13*, *14*). Experiments carried out in our laboratory have also shown that NKCC1 exists virtually exclusively as a homodimer in the plasma membrane and that this dimer is sufficiently stable that it remains intact after membrane solubilization with mild detergents (*15*). Subsequent studies from other groups have provided evidence that NKCC2 (*16*), NCC (*17*), and the KCCs (*18*, *19*) also occur as homodimers, suggesting that this is a common feature of the CCCs. However, to date the molecular interactions that underlie this dimerization have not been identified and the relationship between dimerization and function is still unclear. In this regard evidence both for and against a dominant negative effect of nonfunctional CCCs on their functional dimer partners has been found (*16*−*18*, *20*, *21*).

It has also been speculated that CCCs may form heterodimers of different CCC gene family members (*18*, *22*). Among close homologues such as the KCCs, all of which behave as electroneutral KCl transporters but with varying ion affinities, inhibitor sensitivities, and regulatory properties, this could result in considerable flexibility in accommodating KCC properties to specific biological tasks. In fact, evidence that recombinant KCCs are capable of heteromerization among themselves and in some cases with NKCC1 has recently been reported by Simard et al. (*19*). Caron et al.

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[1] Abbreviations: NKCC, Na^+-K^+-$2Cl^-$ cotransporter; NCC, Na^+-Cl^- cotransporter; KCC, K^+-Cl^- cotransporter; CCC, cation-chloride cotransporter; nttNKCC1, N-terminally truncated NKCC1; cttNKCC1, C-terminally truncated NKCC1; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride

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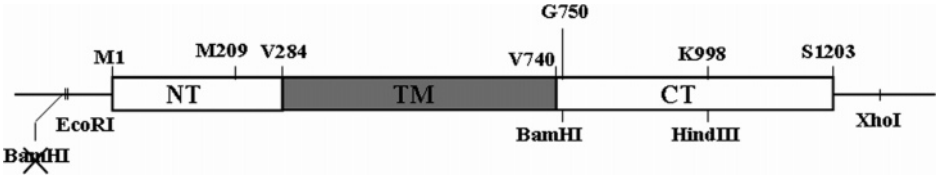

FIGURE 1: The rat NKCC1 insert in the mammalian expression vector pBK-CMVlac$^-$ (pBK$^-$) used in these studies. The locations of the NKCC1 N-terminus (NT), transmembrane domain (TM; shaded), and C-terminus (CT), as well as several relevant restriction sites and amino acid residues, are indicated.

(*22*) have found that the CCC homologue CIP has a dominant negative effect on NKCC1 activity when the two proteins are coexpressed in *Xenopus* oocytes. They were also able to demonstrate co-immunoprecipitation of recombinant CIP and NKCC1 under some circumstances, suggesting that it might modify NKCC1 function via heteromer formation. More generally, however, it should be emphasized that the expression of many of the CCCs is quite cell and/or membrane specific thus precluding the formation of many heterodimers in actual tissues. To date heterodimerization of CCCs has not been demonstrated in a naturally occurring cell and the biological relevance of CCC heterodimerization remains uncertain.

In the present paper we employ chemical cross-linking studies, a novel co-immunoprecipition assay, and NKCC1/CCC chimeras to further explore the basis and significance of NKCC1 dimerization. We provide convincing evidence that sequences within the NKCC1 cytosolic C-terminus are essential for NKCC1 dimer formation. Our data also indicate that replacing the NKCC1 C-terminus with that of several other non-NKCC1 homologues results in proteins that can form homodimers but show little or no heterodimerization with NKCC1 itself. Thus the dimerization interaction mediated by the NKCC1 C-terminus exhibits definite homologue specificity. Finally we present evidence that an inactive member of an NKCC1 dimer does not necessarily act in a dominant negative fashion but can modify the function of its active dimer partner.

MATERIALS AND METHODS

Materials. The homobifunctional, water-soluble, amino cross-linking reagent DTSSP (3,3′-dithiobis(sulfosuccinimidylpropionate)) was purchased from Pierce. AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) was from ICN Biomedicals. The human (*23*) and shark (*24*) NKCC1 clones were kindly provided by Dr. Biff Forbush, Yale University. The rat NKCC2 and NCC clones (*25*) were kindly provided by Dr. Gerardo Gamba, Universidad Nacional Autonoma de Mexico. The rat CIP clone was purchased from Open Biosystems.

The antibody α-wCT(r) was raised in rabbits against the recombinant C-terminus of rat NKCC1 (*26*); the antibody α-wCT(g) was raised in goat against the same antigen. The antibody α-wNT(r) was raised in rabbits against the recombinant N-terminus of rat NKCC1 (*7*). The rabbit antipeptide antibody, L320, against rat NKCC2 (*27*) was a generous gift from Dr. Mark Knepper, NHLBI. The antibody 2ndMet was raised in rabbits against amino acids 209−285 of rat NKCC1 expressed as a fusion protein with GST in *E. coli*.

DNA Constructs. The mammalian expression vector pBK-CMVlac$^-$ (pBK$^-$ (*15*)) was used for all expression studies. The full length rat NKCC1 in Bluescript SK (*12*) was cloned between the *Eco*RI and *Xho*I sites of pBK$^-$ in which the *Bam*HI site of the multiple cloning site had been destroyed by blunt end ligation (Figure 1). The N-terminally truncated rat NKCC1 (nttNKCC1) in pBK$^-$ employed here, whose coding sequence begins at M209 (Figure 1), has been described previously (*15*). A C-terminally truncated rat NKCC1 (cttNKCC1) in pBK$^-$ was obtained by inserting a stop codon after S752 (Figure 1). This was done by generating a PCR product encoding the C-terminal serine (S752) followed by a stop codon and 230 base pairs of the rat NKCC1 3′UTR flanked by a 5′ *Bam*HI site and a 3′ *Xho*I site. This PCR product was ligated between the *Bam*HI site (encoding G750 and S751 of NKCC1) and the *Xho*I site of NKCC1 in pBK$^-$ (Figure 1).

Chimeras in which the C-terminus of rat NKCC1 (amino acids 751−1203) was replaced by the C-termini of human NKCC1, shark NKCC1, rat NKCC2, rat NCC, and rat CIP were obtained by ligating suitable PCR products between the *Bam*HI and *Xho*I sites of rat NKCC1 in pBK$^-$ (Figure 1). In all cases except CIP the sequence NWGSS, where GS is coded by *Bam*HI in NKCC1, is conserved among the homologues at the fusion junction. In the CIP fusion protein the sequence across the fusion junction was NWGSVSQA where W749 and G750 are conserved among all homologues and the underlined amino acids are from NKCC1. Additional NKCC1/NKCC2 chimeras in which amino acids 751−998 or 999−1203 of NKCC1 were replaced with the corresponding sequence from NKCC2 were constructed in a similar way from appropriate PCR products ligated between the *Bam*HI and *Hin*dIII, or *Hin*dIII and *Xho*I sites (Figure 1) of NKCC1 or nttNKCC1. The sequence across the *Hin*dIII fusion junction is QKLL in NKCC1 and QKLV in NKCC2 where K998 and L999 are coded by *Hin*dIII in NKCC1.

An alternatively spliced version (*28*) of NKCC1 (alt-NKCC1) was made by deleting amino acids 968−983 of NKCC1 in pBK$^-$ using the Quickchange site-directed mutagenesis kit (Stratagene).

Cell Culture, Transfection, and Stable Cell Lines. HEK293 cells were cultured in Dulbecco's modified essential medium supplemented with 2 mM glutamine, 100 μg/mL each of penicillin and streptomycin (all from Biofluids), and 10% heat inactivated fetal bovine serum (GibcoBRL). Cells were grown in 10 cm plastic dishes in a humidified incubator at 37 °C and 5% CO_2, and subcultured every 3−4 days. Subconfluent (∼80%) HEK293 cells were transiently transfected overnight (19−24 h) with expression vectors using FuGENE 6 (Roche) according to the manufacturer's instructions. Stable transfectants were selected by growth in the presence of 0.8 mg/mL G418 (Invitrogen).

Membrane Preparations and Cross-Linking. Crude membranes from HEK293 cells were prepared as follows. The

cells growing in a 10 cm culture dish were detached from the plate by incubation in citrate buffer containing 18.6 mM sodium citrate and 134 mM KCl at 37 °C. The suspended cells were then centrifuged at 1000*g* for 5 min, washed twice in ice-cold PBS, resuspended in 0.5 mL of TEEA (20 mM Tris-HCl, pH 8.0, containing 0.5 mM EDTA, 5 mM EGTA, 10 μM leupeptin, 10 μM pepstatin, 0.57 μM aprotinin and 300 μM AEBSF), and homogenized by passing 4 times through a 25 gauge needle. This material was centrifuged at 1000*g* for 10 min and the supernate saved. The pellet was resuspended in TEEA and rehomogenized and centrifuged as before. The combined supernates from these two homogenization steps were centrifuged at 100000*g* for 30 min, and the resulting membrane pellet was resuspended TEEA at a protein concentration of ~5 mg/mL.

For the cross-linking studies, membranes were diluted to a final protein concentration of 1 mg/mL (measured using the Bio-Rad Protein Assay Kit with bovine IgG as the standard) in PBS and solubilized with 0.3% Triton X-100 on ice for 30 min. This material was centrifuged at 100000*g* for 15 min, and the supernatant, containing solubilized membrane proteins, was saved. DTSSP (1 mM) was then added followed by incubation on ice for 30 min. When cross-linking was carried out in the presence of SDS, SDS was added with Triton X-100. The cross-linking reaction was terminated by the addition of an equal volume of stop solution consisting of PBS containing 100 mM glycine, 0.6% Triton X-100, 10 μM leupeptin, 10 μM pepstatin, 0.57 μM aprotinin and 300 μM AEBSF.

Co-Immunoprecipitation Assay. HEK293 cells in 6 cm dishes were transiently transfected with full length NKCC1 (or with the chimeras indicated) and nttNKCC1 at a plasmid ratio of 1:8 (3 μg total DNA). The following day the cells were washed with PBS and solubilized in 200 μL of PBS containing 0.3% Triton X-100. After 30 min of incubation on ice the solubilized cells were centrifuged at 100000*g* for 30 min and the supernate was saved.

Immunoprecipitation was carried out using the antibody α-wNT(r) which had been preconjugated to protein G beads (Pierce) as follows: Protein G beads (supplied as a 1:1 slurry) were first washed 3 times in PBS containing 1% ovalbumin, and then 1 volume of antiserum was combined with 3 volumes of beads and 17 volumes of PBS plus 1% ovalbumin and incubated at 4 °C for 1 h with constant mixing. The beads were then washed 2 times with PBS containing 1% ovalbumin and 0.3% Triton X-100 and suspended in the same buffer as a 1:1 slurry.

For immunoprecipitation a 185 μL aliquot of solubilized cell supernate was added to 20 μL of preconjugated protein G beads (i.e., 40 μL 1:1 slurry). Following 3 h of incubation at 4 °C with constant mixing, the beads were collected by centrifugation and washed 2 times with PBS containing 0.1% Triton X-100. Next the beads were eluted in 25 μL of PBS containing 0.3% Triton X-100 plus 0.1% SDS for 30 min on ice. The beads were then centrifuged, and the eluate was removed and mixed with 6 μL of 5× SDS−PAGE sample buffer. The material remaining on the beads was also extracted by adding 30 μL of SDS−PAGE sample buffer to the pelleted beads. Samples of the solubilized cell supernate, the material eluted from the beads by 0.1% SDS, and the material remaining on the beads after elution by 0.1% SDS were typically probed by Western blotting to monitor protein expression and recovery. In general the level of expression of all chimeras was similar to that of wild-type NKCC1.

SDS−PAGE and Western Blotting. SDS−PAGE was carried out using 4−12% Tris-glycine gels (Bio-Rad). Immunoblots were incubated with antibodies in 25 mM Tris-HCl, pH 7.4, containing 140 mM NaCl, 4% skim milk powder (BioRad), and 0.04% Tween 20 (Tween 20 was omitted during incubation with the primary antibody). The primary antibodies α-wCT(r), α-wNT(r), 2ndMet, α-wCT-(g), and L320 were used at dilutions of 1:10000, 1:5000, 1:3000, 1:500 and 1:1000, respectively. Horseradish peroxidase-conjugated secondary antibodies were from Pierce. Detection was carried out using the ECL kit from Amersham according to the manufacturer's directions. The positions of Multi Mark colored standards (Invitrogen) are indicated on the blots. Unless otherwise noted the disulfide reducing agent DTT (500 mM) was added to the SDS−PAGE sample buffer.

^{86}Rb Flux Assay. The ^{86}Rb flux assay was carried out as previously described (*29*).

Data Presentation. All experiments were carried out 3 or more times (unless otherwise noted) with similar findings. Results of representative experiments are illustrated. Quantitative results are expressed as means ± SEM.

RESULTS AND DISCUSSION

Chemical Cross-Linking of NKCC1. In Figure 2A we examine the effects of the cross-linking reagent DTSSP on solubilized membranes prepared from HEK293 cells transiently transfected with full-length (rat) NKCC1 or with an N-terminally truncated NKCC1 (nttNKCC1) that begins at M209 of the full length protein (Figure 1). As previously demonstrated in our laboratory with stably transfected cells (*15*), these Western blots illustrate that both of these proteins migrate at molecular weights that are twice their monomeric sizes after DTSSP treatment. These and other previously published results (*15*) provide convincing evidence that NKCC1 exists as a homodimer in the plasma membrane that is stable following membrane solubilization with 0.3% Triton X-100, and further that the N-terminal 208 amino acids of the transporter are not required for dimerization. Note also that the SDS−PAGE used to obtain the Western blots shown in Figure 2A was carried out in the absence of sulfhydryl reducing agents; since NKCC1 runs as a monomer in these gels before DTSSP treatment the NKCC1 dimer does not arise as the result of the formation of disulfide bonds. In our previous work (*15*) we also found that the NKCC1 dimer could be dissociated by treatment with low concentrations of the ionic detergent SDS (<0.1%). In Figure 2B we show the SDS concentration dependence of this effect for NKCC1 and nttNKCC1. In these experiments we find no significant difference in the effect of SDS on the homodimers of the two proteins ($K_{1/2}$ ~ 0.01% in each case), consistent with the hypothesis that the N-terminus of NKCC1 does not play a significant role in dimerization.

In additional experiments (not shown) we have carried out cross-linking studies similar to those shown in Figure 2A on a series of NKCC1 truncation mutants containing the complete NKCC1 N-terminus but with stop codons inserted at various points within the central membrane spanning domain or the intracellular C-terminus of the protein. Our strategy here was to look for regions of the protein that when

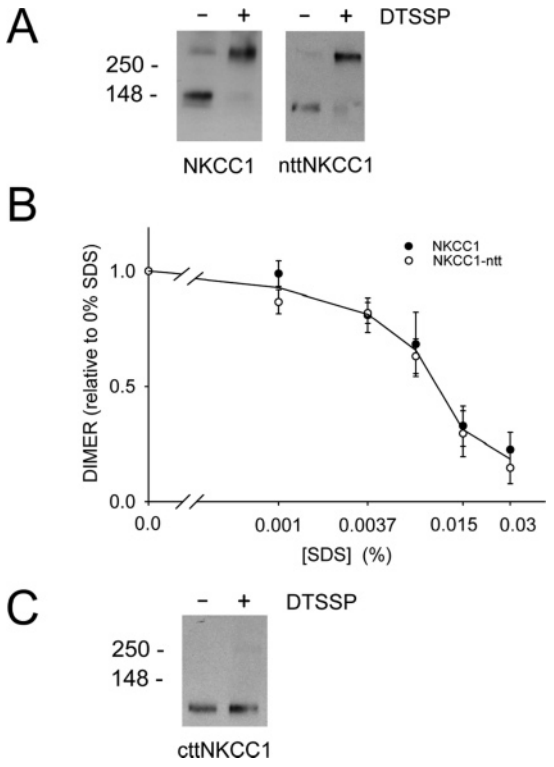

FIGURE 2: Chemical cross-linking of full length NKCC1 and nttNKCC1. A. Membranes from HEK293 cells transiently transfected with NKCC1 or nttNKCC1 were prepared, solubilized, treated with (+) or without (−) the cross-linker DTSSP as indicated, and probed by Western blotting with the antibody α-wCT(r) as described in Materials and Methods. No disulfide reducing agent was added to the SDS−PAGE sample buffer. The weak band at approximately the level of the cross-linked dimer seen in samples not treated with DTSSP is often seen in transiently transfected cells and probably represents a minor amount of aggregated NKCC1 protein. B. Membranes were prepared and treated as in panel A, but cross-linking was carried out in the presence of the concentration of SDS indicted. At each SDS concentration the density of the band representing the cross-linked dimer has been normalized to that observed in the absence of SDS and plotted. Results of 3 independent experiments were averaged to produce the figure. C. Membranes from HEK293 cells stably transfected with cttNKCC1 were prepared and treated as in panel A. The blot was probed with the antibody α-wNT(r).

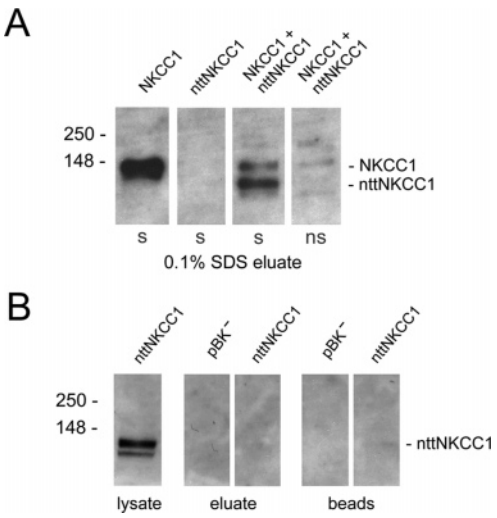

FIGURE 3: Co-immunoprecipiation assay: nttNKCC1 is co-immunoprecipitated with NKCC1 but not with cttNKCC1. A. HEK293 cells were transiently transfected with NKCC1, nttNKCC1, or both NKCC1 and nttNKCC1 as indicated. The next day cells were solubilized and immunoprecipitated with α-wNT(r), a specific (s) antiserum raised in rabbits against the N-terminus of rat NKCC1, or with nonspecific (ns) rabbit serum, as also indicated. The material eluted from the immunoprecipitate by 0.1% SDS was then probed by Western blotting with the antibody α-wCT(g) raised in goat against the NKCC1 C-terminus (all procedures are described in detail in Materials and Methods). The positions of NKCC1 and nttNKCC1 are also indicated on the blot. B. HEK293 cells stably transfected with cttNKCC1 (Figure 2C) were transiently transfected with nttNKCC1 or with empty vector (pBK⁻) as indicated. Immunoprecipitation with α-wNT(r) and Western blotting were carried out as described for panel A. An aliquot of the cell lysate (12 μL), the (total) 0.1% SDS eluate (25 μL plus 6 μL of 5× SDS sample buffer), and the (total) material remaining on the beads (30 μL in SDS sample buffer) are examined (see Materials and Methods). The two bands appearing in the HEK293 lysate represent complex-glycosylated (upper band) and core-glycosylated (lower band) nttNKCC1, respectively. We often observe both complex- and core-glycosylated bands for full length NKCC1 as well (e.g., Figure 4A). The density of the core-glycosylated bands is typically by far the weaker of the two, but its relative intensity varies from experiment to experiment.

deleted from the sequence resulted in loss of dimer formation. However, in marked contrast to the N-terminally truncated protein nttNKCC1, we found that cross-linking of these C-terminally truncated mutants typically resulted in multiple high molecular weight bands on Western blots that we interpreted as arising from aggregation of these recombinant proteins, possibly as a result of their being misprocessed and/or targeted for destruction by the cell. Accordingly most of these results could not be reliably interpreted. Only one C-terminally truncated NKCC1 mutant was an exception to this pattern; this mutant (cttNKCC1) was truncated after S752 and thus contains the complete N-terminus and membrane spanning domain of NKCC1 but lacks its intracellular 50 kDa C-terminus (Figure 1). When this protein was stably expressed in HEK293 cells, we found no evidence for high molecular weight aggregates. Furthermore, cttNKCC1 could not be cross-linked by DTSSP (Figure 2C), suggesting that the C-terminus of NKCC1 is required for its dimerization.

NKCC1 Co-Immunoprecipitation Assay. To study the involvement of the C-terminus of NKCC1 in dimerization we developed a co-immunoprecipitation assay that takes advantage of the properties of the NKCC1 dimer and utilizes several anti-NKCC1 antibodies produced in our laboratory. Briefly stated (see Materials and Methods for details), we transiently coexpress full-length NKCC1 and nttNKCC1 in HEK293 cells, immunoprecipitate from solubilized cells with the antibody α-wNT(r) raised against the NKCC1 N-terminus, and then treat the immunoprecipitated material with 0.1% SDS to specifically break dimer pairs (Figure 2B). Since the sequence against which the antibody α-wNT(r) was raised is missing from nttNKCC1, we expect this truncated protein to only appear in the immunoprecipitate as a component of full-length NKCC1/nttNKCC1 dimer pairs and to be released by 0.1% SDS. Thus by modifying the C-terminus of the full length protein and probing for nttNKCC1 in the SDS-eluted material we can evaluate the effects of these modifications on dimer formation. A series of experiments designed to test this method are illustrated in Figure 3A. The results shown (from left to right) are from cells transfected with NKCC1 alone, nttNKCC1 alone, and both NKCC1 and nttNKCC1. Immunoprecipitation from these last cells was carried out with both specific (s; i.e., α-wNT(r)) and nonspecific (ns) antibody. In each case the material eluted by 0.1% SDS was probed by Western blotting

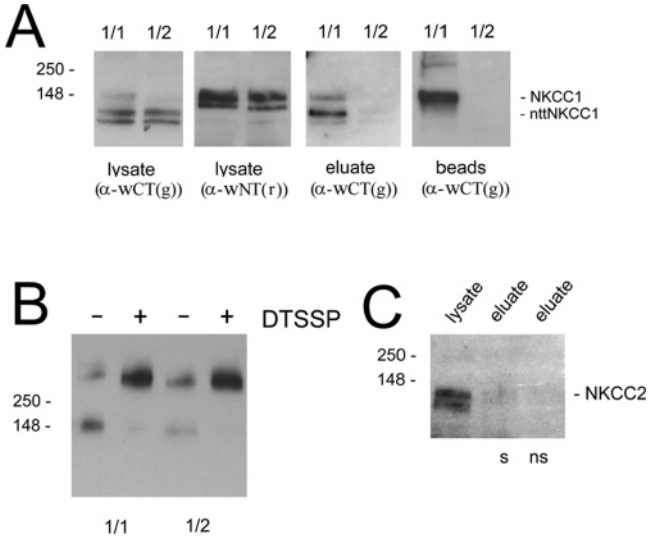

FIGURE 4: Evidence that NKCC1 does not form heterodimers with NKCC2. A. HEK293 cells were transiently transfected with nttNKCC1 and either wild-type NKCC1 (1/1) or an NKCC1/NKCC2 chimera (1/2) in which the C-terminus of NKCC1 was replaced with that of NKCC2 (see Materials and Methods), as indicated. The next day cells were solubilized and immunoprecipitated with α-wNT(r) as described in Materials and Methods. Aliquots of the cell lysates, the total 0.1% SDS eluates, and the total material remaining on the beads (volumes as in Figure 3B) were probed by Western blotting with the antibodies indicated (the second panel from the left is not from the same experiment as the others but shows a typical result). B. Membranes from HEK293 cells transiently transfected with 1/1 or 1/2 were prepared, solubilized, treated with (+) or without (−) the cross-linker DTSSP, and probed by Western blotting with the antibody α-wNT(r) as described in Materials and Methods and in the caption of Figure 2. C. HEK293 cells were transiently transfected with NKCC1 and NKCC2. The next day cells were solubilized and immunoprecipitated with α-wNT(r) or with nonspecific (ns) rabbit serum, as indicated. The cell lysate and the total material eluted from the immunoprecipitate by 0.1% SDS (volumes as in Figure 3B) were then probed by Western blotting with an antibody against NKCC2 (see Materials and Methods).

with the antibody α-wCT(g) against the NKCC1 C-terminus, which recognizes both NKCC1 and nttNKCC1. As predicted, the results in Figure 3A illustrate that nttNKCC1 is only present in the 0.1% SDS eluate from cells where it has been coexpressed with NKCC1 and when immunoprecipitation from these cells is carried out with an NKCC1-specific antibody.

In the experiment illustrated in Figure 3B we employ this assay to test whether nttNKCC1 is co-immunoprecipitated with cttNKCC1. Here HEK293 cells expressing cttNKCC1 were transiently transfected with nttNKCC1 or empty vector (pBK^-), as indicated, and immunoprecipitation was carried out with the antibody α-wNT(r). In these studies we see no evidence for co-immunoprecipitation of nttNKCC1 with cttNKCC1, providing further support for the hypothesis that the NKCC1 C-terminus is required for dimerization.

NKCC1 Does Not Dimerize with NKCC2. In Figure 4A we compare the co-immunoprecipitation of nttNKCC1 with either wild-type NKCC1 (1/1) or an NKCC1/NKCC2 chimera (1/2) in which the C-terminus of NKCC1 has been replaced with that of NKCC2 (see Materials and Methods). In the two left-hand panels of Figure 4A we have probed the lysates from HEK293 cells transfected with nttNKCC1 and either 1/1 or 1/2 with α-wCT(g), which recognizes nttNKCC1 (and 1/1), or with α-wNT(r), which recognizes both 1/1 and 1/2 (antibodies used are indicated below the panels). These blots demonstrate that similar levels of nttNKCC1 (first panel on left), and similar levels of 1/1 and 1/2 (second panel on left), respectively, are expressed under both experimental conditions. In the third panel from the left we see that a strong nttNKCC1 signal is seen in the 0.1% SDS eluate from immunoprecipitated wild-type NKCC1 (1/1) as expected. However, there is little if any detectable nttNKCC1 signal in the eluate from immunoprecipitated 1/2; nor is there any nttNKCC1 remaining on the extracted beads (right-hand panel). Thus this experiment provides strong evidence that replacing the C-terminus of NKCC1 with that of NKCC2 abolishes its ability to dimerize with nttNKCC1.

The above result is consistent with the hypothesis that dimer formation arises as a result of interactions between the C-termini of the dimer subunits and is NKCC isoform-specific. However, it is also possible that the chimera 1/2 is simply incapable of forming dimers, for example, because it is misprocessed by the cell or because it requires additional missing NKCC2 sequence. Evidence against this possibility is provided in Figure 4B. Here we show that treatment of the chimeric protein 1/2 with the cross-linker DTSSP results in the appearance of higher molecular weight species similar to the cross-linked homodimers seen with NKCC1 (1/1), thus indicating that 1/2 is capable of forming homodimers with itself. This result is consistent with previous observations that NKCC2 and other CCCs exist as homodimers in the cell membrane and also indicates that the NKCC2 C-terminus is capable of dimer formation as a part of the 1/2 chimera. In additional experiments presented later in this paper we also show that this protein is functionally active and thus properly processed by the cell to the plasma membrane. Taken together the results presented thus far provide strong evidence that an interaction between the C-termini of the two dimer subunits is essential for NKCC1 dimerization and that the C-termini of NKCC1 and NKCC2 are not capable of this interaction with each other.

Finally, in the experiment shown in Figure 4C we confirm for completeness that full-length NKCC2 likewise does not co-immunoprecipitate with NKCC1 when HEK293 cells are cotransfected with these two proteins. Here the lysate from these cells was immunoprecipitated with α-wNT(r), a specific (s) antibody against the NKCC1 N-terminus which does not recognize NKCC2, or with nonspecific (ns) rabbit serum, as indicated. The cell lysate and the total material eluted from the immunoprecipitate by 0.1% SDS were then probed by Western blotting with an antibody against NKCC2. No NKCC2 signal is detectable in either eluate.

Other Replacements of the NKCC1 C-Terminus. To further explore the role of the NKCC1 C-terminus in dimer formation we examined the effects of replacing it with other sequences. Specifically we have studied chimeras in which the C-terminus of rat NKCC1 was replaced with that of shark NKCC1 (1/s), human NKCC1 (1/h), rat NCC (1/ncc), rat CIP (1/cip), and an alternatively spliced (*28*) form (see Materials and Methods) of rat NKCC1 (1/alt). In cross-linking studies (not shown) we have confirmed that all of these chimeric proteins form homodimers consistent with the hypothesis that this is a common characteristic of the CCCs and that the C-termini of these chimeric proteins are capable of this interaction. In co-immunoprecipitation experiments

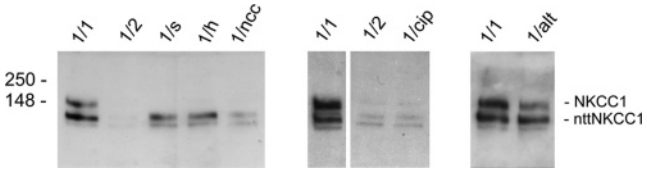

FIGURE 5: Evidence against CCC heterodimers. HEK293 cells were transiently transfected with nttNKCC1 and either rat NKCC1 (1/1) or an NKCC1 chimera in which the C-terminus of rat NKCC1 was replaced with that of rat NKCC2 (1/2), shark NKCC1 (1/s), human NKCC1 (1/h), rat NCC (1/ncc), rat CIP (1/cip), or an alternatively spliced form of rat NKCC1 (1/alt); the construction of these chimeras is described in Materials and Methods. The next day cells were solubilized and immunoprecipitated with α-wNT(r) and aliquots of the 0.1% SDS eluates (25 μL plus 6 μL 5xSDS sample buffer) were probed by Western blotting with the antibody α-wCT(g). The three panels represent results from three separate experiments; each includes a positive control (1/1), and two include negative controls (1/2). Note that in some experiments a very weak nttNKCC1 signal was observed in coprecipitations with 1/2. We suspect that this signal is due to coprecipitation of nttNKCC1 with the endogenous NKCC1 found in HEK293 cells rather than with 1/2 and thus represents a background signal. Why this background signal is not seen in all experiments is not clear to us.

(Figure 5), we observed strong interactions of nttNKCC1 with the human, shark, and alternatively spliced NKCC1 chimeras, a much weaker signal with the NCC chimera, and no significant signal (relative to the 1/2 chimera) with the CIP chimera. Thus these experiments indicate that, at least in the case of NKCC1, dimer formation displays significant homologue specificity. Although our results suggest that NKCC1 and NCC may be capable of (weak) heterodimer formation, this phenomenon is unlikely to be of any biological significance because NCC is only found in the apical membrane of the renal distal convoluted tubule (*1*) in cells that do not express NKCC1.

Self-Interactions in the NKCC1 C-Terminus. Using the yeast two-hybrid system Simard et al. (*30*) found evidence for two interacting domains within the C-terminus of NKCC1. In subsequent studies using the same methodology this group also identified corresponding interacting regions in the C-terminus of NKCC2 (*31*). By systematically screening fragments of various lengths from NKCC1 and NKCC2 they were able to localize these interacting sequences to regions corresponding to residues 769−914 and 1015−1203 in the proximal and distal C-terminus, respectively, of (rat) NKCC1 (the corresponding residues in human NKCC2 are residues 671−816 and 910−1098, respectively). They suggested (*30*) that the interactions between these regions might be involved either in intramolecular interactions within the NKCC1 C-terminus (i.e., in C-terminal folding) or, more interestingly, in intermolecular interactions associated with dimer formation. In the latter case dimer bonding could result from the corresponding residues in the proximal C-terminus of each dimer subunit interacting with those in the distal C-terminus of the other subunit (Figure 6A). Since our results indicate that an interaction between C-termini is essential for NKCC1 dimerization, we wanted to test the possibility that these interactions identified by Simard et al. might underlie this phenomenon. To do this we constructed the chimeras 1/[2.1] and ntt1/[1.2]; the former contains the proximal interacting region from the C-terminus of (rat) NKCC2 (corresponding to residues 751−998 of rat NKCC1) and the distal interacting region from NKCC1

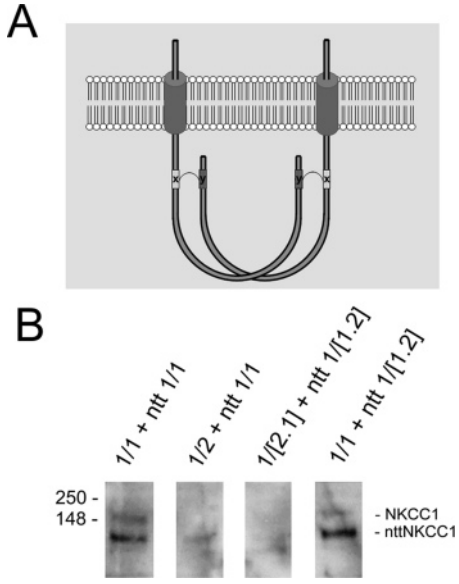

FIGURE 6: Evidence that previously identified interactions within the NKCC C-terminus are not involved in dimer formation. A. A schematic representation of hypothetical dimer formation via interacting regions in the proximal and distal C-terminus of two NKCC1 molecules. A region x (shaded light gray) in the proximal C-terminus of each dimer subunit interacts with a region y (shaded dark gray) in the distal C-terminus of the other subunit. B. HEK293 cells were transiently transfected with NKCC1 (1/1), with nttNKCC1 (ntt1/1), or with the chimeras indicated. In the protein 1/2 the C-terminus of NKCC1 was replaced with that of NKCC2; in the protein 1/[2.1] the amino acids between the *Bam*HI and *Hin*dIII sites of NKCC1 (residues 751−998; Figure 1) were replaced with the corresponding residues from NKCC2; in the protein ntt 1/[1.2] the amino acids between the *Hin*dIII site and stop codon of nttNKCC1 (residues 999−1203 of NKCC1; Figure 1) were replaced with the corresponding residues from NKCC2. The day following transfection cells were solubilized and immunoprecipitated with α-wNT(r) and aliquots of the 0.1% SDS eluates (25 μL plus 6 μL of 5× SDS sample buffer) were probed by Western blotting with the antibody α-wCT(g).

(residues 999−1203 of rat NKCC1) while this arrangement is reversed in the latter (see Materials and Methods and Figure 6 caption). These two chimeras would be expected to dimerize with each other according to the scheme illustrated in Figure 6A via one NKCC1-type interaction (the distal C-terminus of 1/[2.1] with the proximal C-terminus of ntt1/[1.2]), and one NKCC2-type interaction (the proximal C-terminus of 1/[2.1] with the distal C-terminus of ntt1/[1.2]). But as shown in Figure 6B no detectable ntt1/[1.2] is coprecipitated with 1/[2.1]. On the other hand, ntt1/[1.2] is coprecipitated with 1/1 (Figure 6B) and in cross-linking experiments (not shown) we have confirmed that the chimeras 1/[2.1] and ntt1/[1.2] form homodimers. Thus the chimeras 1/[2.1] and ntt1/[1.2] are fully capable of dimer formation but not with each other. We conclude therefore that the C-terminal interactions identified by Simard et al. are likely to be involved in intramolecular folding within individual C-termini rather than in reciprocal interactions associated with dimer formation. Furthermore, since ntt1/[1.2] dimerizes with 1/1 we also conclude that the residues in the NKCC1 C-terminus that are required for dimer formation must lie between amino acids 751 and 998.

Fluxes via NKCC1 Constructs. In Figure 7 we examine the bumetanide-sensitive (i.e., NKCC-specific) component of ^{86}Rb influx in HEK293 cells transiently transfected with

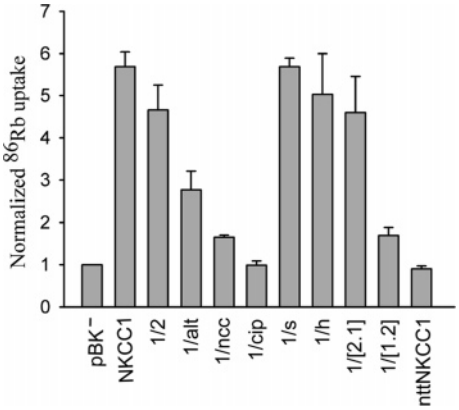

FIGURE 7: Bumetanide-sensitive ^{86}Rb fluxes via NKCC1 constructs. ^{86}Rb fluxes were measured as previously described (*29*) in HEK293 cells transiently transfected with the NKCC1 constructs indicted. Flux was measured in the presence and absence of 250 μM bumetanide. For each construct the bumetanide-sensitive component of ^{86}Rb flux was calculated and normalized to that measured in cells transfected with pBK$^-$ on the same day. Uptake in the presence of 250 μM bumetanide was approximately 6% of that measured in its absence in cells transfected with pBK$^-$ and was similar in magnitude for all constructs. Construct nomenclature is as in Figures 5 and 6. For NKCC1, 1/2, 1/alt, and 1/ncc, $n = 3$; for nttNKCC1, $n = 5$; for all others, $n = 2$.

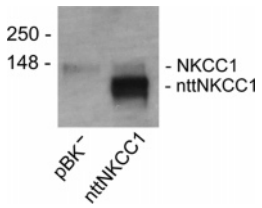

FIGURE 8: Stable expression of nttNKCC1 in HEK293 cells. Membranes from HEK293 cells stably transfected with pBK$^-$ or nttNKCC1 (in pBK$^-$) were probed with the antibody 2ndMet via Western blotting (see Materials and Methods for details). 2ndMet was raised against a sequence which is identical in human and rat NKCC1 (residues 209−285 of rat NKCC1) and therefore should detect the endogenous (human) NKCC1 in the HEK293 cells and (rat) nttNKCC1 with equal effectiveness. The blot illustrates that nttNKCC1 is expressed at dramatically higher levels than the endogenous NKCC1 in the HEK293 cells; similar results are seen with HEK293 cells transiently transfected with nttNKCC1 (not shown).

the various NKCC1 constructs studied in this paper. Transfection with NKCC1 itself yields a ~6-fold increase in ^{86}Rb uptake over the basal level observed with the empty vector pBK$^-$; this basal flux is known to be due to the endogenous NKCC1 found in the HEK293 cells (*29*). Figure 7 demonstrates that all of the constructs studied here, with the exceptions of 1/cip and nttNKCC1, show at least some functional activity. As already noted we have confirmed that all of these proteins are capable of homodimer formation.

Since nttNKCC1 readily forms dimers with NKCC1 but is apparently itself nonfunctional, we carried out several experiments designed to examine its effects on the endogenous NKCC1 found in the HEK293 cells. Figure 8 shows a Western blot of membranes prepared from HEK293 cells stably transfected with the empty vector pBK$^-$ or with nttNKCC1, as indicated. This blot illustrates that nttNKCC1 is expressed at dramatically higher levels than the endogenous NKCC1 in the HEK293 cells. Accordingly it is reasonable to assume that most, if not all, of the full length NKCC1 in these cells is present in heterodimers with nttNKCC1. In ^{86}Rb uptake studies we found fluxes of 5.83 ± 0.20 nmol/mg of protein/min ($n = 6$) in cells stably transfected with pBK$^-$, and 3.87 ± 0.21 nmol/mg of protein/min ($n = 5$) in cells stably transfected with nttNKCC1. This modest inhibition (~30%) of flux via the endogenous NKCC1 by nttNKCC1, also seen in cells transiently transfected with nttNKCC1 (Figure 7), is not consistent with a dominant negative effect although it does suggest that there may be some functional coupling between dimer subunits. To explore this further we examined the effect of bumetanide on ^{86}Rb fluxes into cells transfected with pBK$^-$ or nttNKCC1. In these experiments we found that 0.5 μM bumetanide inhibited $50 \pm 3\%$ and $57 \pm 3\%$ of the ^{86}Rb flux into cells transfected with pBK$^-$ and nttNKCC1, respectively ($n = 5$, experimental conditions were as in Figure 7; results from stably and transiently transfected cells were combined). Analysis of these data using a paired *t*-test yielded a *p* value of 0.02 indicating that bumetanide is significantly less effective in inhibiting ^{86}Rb fluxes in cells overexpressing nttNKCC1. These effects of nttNKCC1 expression on fluxes via the endogenous NKCC1 of HEK293 cells indicate the existence of a functional interaction between dimer subunits.

Concluding Remarks. Our results indicate that amino acids 751−998 within the cytosolic C-terminus of (rat) NKCC1 contain one or more regions that are essential for dimer formation. We find no evidence for NKCC1 dimer formation with NKCC2 or CIP and only a weak interaction with NCC. As already mentioned, Caron et al. (*22*) have found that the CCC homologue CIP has a dominant negative effect on NKCC1 activity when these two proteins are coexpressed in *Xenopus* oocytes. Based on our data we would suggest that this effect of CIP occurs via some mechanism other than NKCC1/CIP heterodimer formation. We should also stress that our results do not address the possibility that other CCC homologues may heterodimerize amongst themselves or with NKCC1. Indeed Simard et al. (*19*) have recently presented evidence that the KCCs heterodimerize and that KCC1 and KCC4 form heteromers with NKCC1. Additional experimentation will be required to determine the biological, structural, and functional significance of these interactions.

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