Novel Insights Regarding the Operational Characteristics and Teleological Purpose of the Renal Na⁺-K⁺-Cl⁻ Cotransporter (NKCC2s) Splice Variants

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The absorptive Na⁺-K⁺-Cl⁻ cotransporter (NKCC2) is a polytopic protein that forms homooligomeric complexes in the apical membrane of the thick ascending loop of Henle (TAL). It occurs in at least four splice variants (called B, A, F, and AF) that are identical to one another except for a short region in the membrane-associated domain. Although each of these variants exhibits unique functional properties and distributions along the TAL, their teleological purpose and structural organization remain poorly defined. In the current work, we provide additional insight in these regards by showing in mouse that the administration of either furosemide or an H₂O-rich diet, which are predicted to alter NKCC2 expression in the TAL, exerts differential effects on mRNA levels for the variants, increasing those of A (furosemide) but decreasing those of F and AF (furosemide or H₂O). Based on a yeast two-hybrid mapping analysis, we also show that the formation of homooligomeric complexes is mediated by two self-interacting domains in the COOH terminus (residues 671 to 816 and 910 to 1098), and that these complexes could probably include more than one type of variant. Taken together, the data reported here suggest that A, F, and AF each play unique roles that are adapted to specific physiological needs, and that the accomplishment of such roles is coordinated through the splicing machinery as well as complex NKCC2–NKCC2 interactions.

INTRODUCTION

The second isoform of the Na⁺-K⁺-Cl⁻ cotransporter (NKCC2) is restricted in its localization to the apical membrane of the thick ascending loop of Henle (TAL; see Payne and Forbush, 1994; Kaplan et al., 1996; Yang et al., 1996). It plays a critical role in salt reabsorption along this nephron segment, thereby participating in the maintenance of extracellular fluid volumes and excretion of free H₂O (Greger, 1985; Gimenez and Forbush, 2003; Fernandez-Llama et al., 2005). The physiological importance of NKCC2 in mammals is suggested by the occurrence of salt wasting, uncompensated polyuria, or systemic hypertension when the transporter is functionally impaired (Simon et al., 1996; Bartter et al., 1998; Takahashi et al., 2000; Alvarez-Guerra and Garay, 2002).

NKCC2 belongs to the cation-Cl cotransporter (CCC) family, which is comprised of several isoforms that mediate cell surface Cl⁻-dependent Na⁺ and/or K⁺ cotransport. Members within this family are very homologous to one another, possessing 12 transmembrane domains (tm) flanked by cytosolic termini (see model of NKCC2 in Fig. 1). Previous studies have revealed that a number of CCC isoforms also occur in splice variants (Payne and Forbush, 1994; Igarashi et al., 1995; Race et al., 1999; Plata et al., 2001; Gagnon et al., 2002; Hebert et al., 2004). Among terrestrial vertebrates, e.g., at least four types of NKCC2s have been described: NKCC2F, A, and B, which are identical to one another except for a 96-bp exon that encodes the second tm and part of the following connecting domain. Although each of these variants exhibits unique functional properties and distributions along the TAL, their teleological purpose and structural organization remain poorly defined. In the current work, we provide additional insight in these regards by showing in mouse that the administration of either furosemide or an H₂O-rich diet, which are predicted to alter NKCC2 expression in the TAL, exerts differential effects on mRNA levels for the variants, increasing those of A (furosemide) but decreasing those of F and AF (furosemide or H₂O). Based on a yeast two-hybrid mapping analysis, we also show that the formation of homooligomeric complexes is mediated by two self-interacting domains in the COOH terminus (residues 671 to 816 and 910 to 1098), and that these complexes could probably include more than one type of variant. Taken together, the data reported here suggest that A, F, and AF each play unique roles that are adapted to specific physiological needs, and that the accomplishment of such roles is coordinated through the splicing machinery as well as complex NKCC2–NKCC2 interactions.

Abbreviations used in this paper: CCC, cation-Cl cotransporter; Ct, COOH terminus; ctrl, control; EIR, essential interacting region; hu, human; ms, mouse; NKCC2, Na⁺-K⁺-Cl⁻ cotransporter; OM, outer medulla; RT-PCR, reverse transcriptase PCR; sa, shark; TAL, thick ascending loop of Henle.
could constitute a large fraction of the NKCC2-derived mRNAs in some species (Gagnon et al., 2002).

In mammals, the NKCC2 splice variants differ not only in their distribution and expression levels, but also in their kinetic features (Gimenez et al., 2002; Plata et al., 2002; Gagnon et al., 2003). By way of illustration, F displays the lowest affinity for ions under controlled conditions and B the highest ($K_m$: F > A > B), whereas A displays the highest transport capacity and B the lowest ($V_{max}$: for A ≥ F > B); for convenience, $K_m$ values based on two of these studies are summarized in Table I. Regarding AF, intriguingly, influx studies in the *Xenopus laevis* oocyte expression system have shown that this variant is nonfunctional even if it is able to reach the cell surface (Gagnon et al., 2002).

Despite the information that has been acquired on the localization, expression, and function of the renal Na$^+$-K$^+$-Cl$^-$ symporters, the physiological basis for the existence of multiple NKCC2 variants remains uncertain at present. Likewise, the mechanisms that underlie the normal operation of the variants have still not been examined in great detail. For example, the hormone ADH and the loop diuretic furosemide have been shown to alter the function and expression of NKCC2 in lagomorphs (Ecelbarger et al., 1996, 2001; Knepper et al., 1999; Gimenez and Forbush, 2003), but whether they exert the same effects on all of the variants is unknown.

Coimmunoprecipitation and cross-linking studies have suggested that NKCC2 is organized at the surface of renal tubular cells as a homooligomer (Ichinose et al., 1999; Moore-Hoon and Turner, 2000; Starremans et al., 2003). The protein domains that lead to the formation of such structures have not been identified but recent two-hybrid mapping analyses using NKCC1 as a model revealed that the COOH terminus (Ct) could play an important role in this regard (Simard et al., 2004a). The mechanisms by which monomers assemble with each other are of particular interest given that nonfunctional NKCCs have been shown to exert dominant-negative effects on their functional counterparts

| $K_m$ values (in mM) | Distribution along the TAL |
|----------------------|---------------------------|
| NKCC2B: 10 Na$^+$, 2 Rb$^+$, 10 Cl$^-$ | Macula densa cells |
| NKCC2A: 10 Na$^+$, 2 Rb$^+$, 25 Cl$^-$ | Cortex and outer stripe of the OM |
| NKCC2F: 60 Na$^+$, 5 Rb$^+$, 100 Cl$^-$ | Inner stripe of the OM |

The $K_m$ values shown represent estimated values based on two independent studies (Gimenez et al., 2002; Gagnon et al., 2003) and the localization data is based on in situ hybridization studies (Igarashi et al., 1995). Here, TAL = thick ascending limb of the loop of Henle, and OM = outer medulla.

**Figure 1.** Hydropathy plot model of huNKCC2. The symbols represent amino acid residues, showing the protein segments N2$\text{huNKCC2}(51-179)$, C1$\text{huNKCC2}(576-836)$, and C2$\text{huNKCC2}(841-1099)$ in gray and the localization of the alternatively spliced exon in white. The nomenclature used to designate these protein segments is explained in MATERIALS AND METHODS and in Table III. The model of huNKCC2 was drawn using the program PLOT (Biff Forbush).

(Isenring et al., 1998b; Jacoby et al., 1999; Caron et al., 2000; Casula et al., 2001).

In this research, studies were performed to elucidate further the functional and structural properties of A, F, and AF. Based on our findings, we propose that the variants each play unique roles in the TAL not only because of differences in their localizations and kinetic features but also because of differences in the mechanisms by which their expression is regulated. We propose, in addition, that the inclusion of different types of NKCC2 variants in the same oligomer could endow AF with unexpectedly important tasks in salt transport along the TAL.

**MATERIALS AND METHODS**

**Overview**

The approaches that were exploited in this research include yeast two-hybrid studies, influx assays, and expression analyses. Chemicals, reagents, and kits were from different suppliers or collaborators, whereas oligonucleotides and *E. coli* strains were from Sigma-Aldrich and Stratagene, respectively.

**NKCC Constructs/pGilda or pB42AD**

These constructs were used for the two-hybrid studies. They consisted of NKCC-derived DNA fragments that were cloned in pGilda (CLONTECH Laboratories, Inc.), a vector that possesses coding sequences for the LexA DNA-binding domain and a His transformation marker (His™), or in pB42AD (CLONTECH Laboratories, Inc.), a vector that possesses coding sequences for the activating domain of a transcription factor, the HA epitope, and a Trp™. The inserts were obtained by reverse transcriptase (RT)-PCR using human kidney RNA and primers.
to which restriction sites were added, or they were generated by bp deletions from existing constructs after creating convenient restriction sites with the Quick Change Mutagenesis Kit (Stratagene) when necessary (see Table II for primers). The constructs were termed according to the NKCC protein segments encoded, using prefixes to designate the specific domains from which the segments are derived (Nt, NH$_2$ terminus; Ct, COOH terminus; 1, proximal portion; 2, distal portion) and numbers in parentheses to designate their position in human (hu) NKCC2. The characteristics of these protein segments are shown in Table III.

**pGilda Constructs**

One such construct was used for a two-hybrid screen; the insert consisted of a 540-bp fragment that encodes the huNKCC2 Ct1 from residue 657 to 836 (see Fig. 1). Based on the nomenclature outlined above, it was termed Ct1$_{NKCC2}(657–836)$. Five other constructs were used for yeast-two-hybrid mapping analyses. They are termed Nt$_2$$_{NKCC2}(51–179)$, Ct$_1$$_{NKCC2}(657–782)$, Ct$_1$$_{NKCC2}(671–836)$, Ct$_1$$_{NKCC1}(834–926)$, and Ct$_1$$_{NKCC1}(759–947)$ (protein segments encoded by two of these constructs are also shown in Fig. 1). Here, the Ct1$_{NKCC1}(759–947)$ and Ct1$_{NKCC1}(834–926)$ protein segments are designated based on their position in huNKCC1. The corresponding protein segments in huNKCC2 would be termed Ct1$_{NKCC2}(652–840)$ and Ct1$_{NKCC2}(727–819)$, respectively. Note that all of the constructs that encode these various NKCC1 protein segments were generated for a recently published study (Simard et al., 2004a).

**pB42AD Constructs**

One of the interacting proteins identified during the screen is derived from a 777-bp fragment that encodes the huNKCC2 distal Ct; it was called Ct2$_{NKCC2}(841–1099)$. Two other pB42AD-derived constructs, which were employed for the mapping analyses, consisted of Ct2$_{NKCC2}(830–1099)$ and Ct2$_{NKCC1}(970–1212)$. Here, the latter construct was also generated for a recently published study (Simard et al., 2004a), it would encode the protein fragment C2t$_{NKCC2}(848–1099)$ in huNKCC2.

**Shark (sa) NKCC2/Pol1 Constructs**

Three such constructs (saNKCC2A, F, and AF) were used for influx and expression studies. They were already available from previously published work (Gagnon et al., 2002, 2003).
In the vector pGilda

| Name of construct | Length of fused proteins (n residues) | Length of NKCC protein segment (n residues) | Total length of hybrid protein (n residues) | Position in the huNKCC2 Ct (1–457) or Nt (1–183) |
|-------------------|-------------------------------------|-------------------------------------------|------------------------------------------|--------------------------------|
| C1NKCC2(657–836)  | 202                                 | 180                                       | 400                                      | 15–194                      |
| C1NKCC2(657–782)  | 202                                 | 126                                       | 328                                      | 15–140                      |
| C1NKCC2(671–836)  | 202                                 | 166                                       | 368                                      | 29–195                      |
| C1NKCC2(799–947)  | 202                                 | 189                                       | 391                                      | 10–196                      |
| C1NKCC2(814–926)  | 202                                 | 93                                        | 295                                      | 85–177                      |
| Nt2NKCC2(31–179)  | 202                                 | 129                                       | 331                                      | 51–179                      |

In the vector pB42AD

| Name of construct | Length of fused proteins (n residues) | Length of NKCC protein segment (n residues) | Total length of hybrid protein (n residues) | Position in the huNKCC2 Ct (1–457) or Nt (1–183) |
|-------------------|-------------------------------------|-------------------------------------------|------------------------------------------|--------------------------------|
| C2NKCC2(918–1099) | 108                                 | 282                                       | 390                                      | 176–457                      |
| C2NKCC2(870–1099) | 108                                 | 270                                       | 378                                      | 188–457                      |
| C2NKCC2(841–1099) | 108                                 | 257                                       | 365                                      | 201–457                      |
| C2NKCC2(846–1099) | 108                                 | 264                                       | 372                                      | 204–457                      |
| C2NKCC2(867–1099) | 108                                 | 233                                       | 341                                      | 225–457                      |
| C2NKCC2(843–1099) | 108                                 | 257                                       | 365                                      | 250–457                      |
| C2NKCC2(841–1099) | 108                                 | 259                                       | 367                                      | 257–457                      |
| C2NKCC2(918–1099) | 108                                 | 201                                       | 309                                      | 268–457                      |
| C2NKCC2(910–1099) | 108                                 | 149                                       | 257                                      | 206–457                      |

For each construct, the numbers in parentheses indicate the position of the encoded protein segment relative to the huNKCC1 or huNKCC2 amino acid sequence. For the C1NKCC1(759–947), C1NKCC1(834–926), and C2NKCC2(970–1212) protein segments, this position in huNKCC2 is between residue 652 and 838, 727 and 819, and 848 and 1099, respectively.

A search for interactors was performed by transforming YopCiNKCC2(657–856) with a human kidney library cloned in pB42AD and subjecting the transformants to two rounds of selection, first on −Ura−His−Trp (−UHT) plates and subsequently on −Ura−His−Trp−Leu (−UHTL) plates. Resistant colonies were transferred on −UHT+X plates, and those expressing β-gal activity were amplified in a regular yeast medium. Prey plasmids were extracted according to well-established procedures and inserts were analyzed by automated sequencing (Simard et al., 2004a).

Two procedures were performed to confirm the specificity of the identified interactions. First, Yop cells were transformed with a single prey/pB42AD and tested for expression of a hybrid protein by Western blotting after selection of colonies on −Ura−Trp (−UT) plates (see below and examples in lanes 2, 4, and 5 of Fig. 2 B). Second, Yop cells were cotransformed with pairs of plasmids (single prey + empty pGilda versus single prey + LAM/pGilda) and tested for reporter gene activation on −UHT+X plates. Note that LAM, which stands for human lamin C, has been reported not to form complexes or interact with most other proteins (Bartel et al., 1993).

### Table IV

**Composition of Flux Solutions**

| Solution (in mM) | Na⁺ | Rb⁺ or K⁺ | Cl⁻ | Ca²⁺ | Mg²⁺ | PO₄³⁻ | SO₄²⁻ | HEP | GLU | SUC | OSM |
|-----------------|-----|-----------|-----|------|------|-------|-------|-----|-----|-----|-----|
| A. Regular medium (R) | 87  | 5         | 86  | 2    | 2    | 1     | 1     | 10  | 0   | 0   | 200 |
| B. Modified R (R²) for Na⁺ influxes | 43  | 5         | 86  | 2    | 2    | 1     | 1     | 10  | 0   | 0   | 200 |
| C. Wash         | 19  | 73        | 8   | 2    | 2    | 1     | 1     | 10  | 78  | 0   | 200 |
| D. Barth’s medium | 90.4 | 1         | 90  | 0.7  | 0.8  | 0     | 0     | 10  | 0   | 0   | 200 |

In medium R², NaCl was replaced by an equimolar concentration of NMG-Cl. Barth’s medium contains, in addition, 0.7 mM NO₃⁻ and 2.4 mM HCO₃⁻. All solutions are at pH 7.4. Here, R = regular, GLUC = gluconate, HEP = HEPES, SUC = sucrose, and OSM = osmolality.
this step, the cells were retransformed with another NKCC-bear-
hybrid protein after selection on these ends, Yop cells were transformed with different types of Ct1–Ct1, Ct2–Ct2, or Nt–Ct associations are also possible. To whether they are similar to those of NKCC1, and (c) whether Ct1–Ct2 interacting sites can be identified more precisely, (b)

Yeast Two-hybrid Mapping Analysis

These studies were performed to determine (a) whether the Ct1–Ct2 interacting sites can be identified more precisely, (b) whether they are similar to those of NKCC1, and (c) whether Ct1–Ct1, Ct2–Ct2, or Nt–Ct associations are also possible. To

Expression of saNKCC2s in Oocytes

To obtain synthesis of saNKCC2s in this system, defolliculated stage V–VI oocytes were injected with saNKCC2/PolI-derived cRNA (~2–20 ng/oocyte diluted in H2O) and maintained for 3 d at 18°C in Barth’s (B) medium (see Table IV) + 125 μM furosemide. Groups of oocytes were also injected with H2O alone as controls (cts).

Immunofluorescence Studies of saNKCC2-expressing Oocytes

They were performed as previously described (Caron et al., 2000; Gagnon et al., 2002). The anti-P-NKCC immuno-globulin (Ig), which is specific to the phosphorylated form of various NKCCs (Flemmer et al., 2002) and which was a gift from B. Forbush (Yale University, New Haven, CT), was used as primary antibody, whereas the goat Alexa Fluor 488-conjugated goat anti-rabbit anti-IgG (Invitrogen) was used as secondary antibody.

Flux Studies in saNKCC2-expressing Oocytes

Furosemide was first removed through several rinses in a regular (R) medium (see Table IV). Eggs were then subjected to two consecutive incubation steps: one of 60 min in medium R + 84 mM sucrose (R-SUC) to activate the cotransporter, and another of 45 min in one of two solutions to measure NKCC-mediated accumulation of the isotope. The latter solutions were (a) medium R + 1 μCi/ml 86Rb+ ± 250 μM bumetanide + 10 μM ouabain (for 86Rb+ influxes) or (b) a modified medium R (R*) (in which [Na+] was decreased to 45 mM) + 1 μCi/ml 22Na+ + 10 μM ouabain + 125 μM hydrochlorothiazide + 125 μM amiloride (for 22Na+ influxes). Fluxes were terminated with several washes in a high K+-medium + 250 μM bumetanide + the inhibitors used during the prior incubation, and oocytes were transferred afterwards to 96-well plates (one oocyte/well) pre-filled with 2% SDS and scintillation fluid. 86Rb+ was detected with the TopCountNXT counter (Packard).

Experimental Animals

Male C3H mice (age: 200–400 d, weight: 25–30 g) were kept in the animal care facility of our Center. After a brief stabilization period, they were submitted for 7 d to one of three regimens: (1) a standard diet consisting of Teklad global 2018 mouse (ms) chow (5 g/day) and H2O (10 ml/day); (2) mouse chow (5 g/day) mixed with furosemide (5 mg/day), and H2O (10 ml/day); (3) mouse chow (5 g/day) and H2O (~100 ml/day) supplemented with sucrose (600 mM). At the end of this 7-d period, all mice were killed by neck dislocation.

RNA Studies

Expression levels of A, F, and AF were first determined by RT-PCR. Templates consisted of first cDNA strands that were produced from oligoT-primed human kidney RNA (2 μg/reac-
tions) while primers consisted of sequences that are derived from the 5’ end of exon A or F (sense primers), from the 3’ end of these exons (antisense primers), or from β-actin. The exon-spe-
cific primers were used in three combinations to amplify exon A (sense A + antisense A), F (sense F + antisense F), or AF (sense A + antisense F), whereas the β-actin–specific primers were used as cts. PCR conditions were titrated to generate DNA strands over a linear range of amplification versus time using simple arithmetic plots. All samples were migrated on ethidium bromide–stained agarose gels.

Expression levels of A, F, and AF were also determined by in situ hybridization. For these studies, freshly harvested mouse kidneys were frozen in liquid N and mounted on cryostat supports.

Figure 2. Western analyses. Proteins were extracted from yeast with a lysis solution containing glass beads, 8 M urea, 40 mM Tris-HCl, 0.1 mM EDTA, 125 μM β-mercaptoethanol, 5% SDS, and protease inhibitors (final pH 6.8). In the top panel, the protein extracts were from NKCC/pGilda-transformed cells and the analyses were performed with ~2 mg of bait proteins using an anti-LexA Ab. In the bottom panel, the protein extracts were from NKCC/pB42AD-transformed cells and the analyses were performed with ~0.4 mg of prey proteins using an anti-HA Ab. Here, * indicates that the protein segments expressed by these transformants are currently being studied, 0, that the protein fragment of interest had apparently degraded, and §, that the protein fragment was from another preparation of Ct1NKCC1(759–947)-transformed yeast but that it was run in a distant lane within the same gel.

Yeast Two-hybrid Mapping Analysis

These studies were performed to determine (a) whether the Ct1–Ct2 interacting sites can be identified more precisely, (b) whether they are similar to those of NKCC1, and (c) whether Ct1–Ct1, Ct2–Ct2, or Nt–Ct associations are also possible. To

ing construct and tested for their ability to grow on -UHTL plates or to express β-gal activity on -UHT+X plates.

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From there, they were cut sagittally into 12-μm cryosections, applied directly on glass slides, and fixed in 4% paraformaldehyde for 10 min. The prehybridization and hybridization steps were performed in a Denhardt’s-based solution for 2 and 16 h, respectively. RNA messages were probed with end-labeled oligonucleotides derived from exon 4 (Table II). The hybridization step was followed by several washes in SSC (2×, 1×, 0.5×) at a maximum of 37°C.

**RESULTS**

**Two-hybrid Screen**

Transformation of YopCt1 NKCC2(657–836) with a human kidney library led to the formation of over 4 million UHT-resistant colonies, 41 of which were also UHTL resistant. After the quadruple selection, however, only 32 of the colonies expressed above-background β-gal activity on X-gal plates, and after the confirmation studies, 31 were suspected to enclose Ct1NKCC2 partners.

Detailed DNA analyses revealed that 13 of the colonies identified expressed Ct2 NKCC2 protein segments, 8 of which also differed in amino acid sequence. These distinct prey are illustrated in Fig. 3 A by horizontal bars aligned with the region of Ct to which they correspond. From there, it should be noted that all of the Ct2 NKCC2 prey were characterized exhaustively in this work after noting that they tended to vary in length and could be

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**Western Analyses**

They were performed with yeast protein extracts prepared in a special lysis solution (detailed in Fig. 2 legend) as previously described (Simard et al., 2004a). In brief, extracts were separated on SDS-polyacrylamide tricine gels and transferred by capillarity to Immobilon-P nylon blots (Millipore). Later on, the blots were incubated sequentially with a primary and secondary Ab, and proteins of interest were visualized by chemiluminescence (using the ECL kit, Amershams Biosciences).

**Sequence Analyses, Quantification Methods, Statistics**

DNA characterizations were performed by automated sequencing using plasmid-derived primers (Table II) and restriction analyses. For blast searches, sequence alignments, and structure predictions, we used a combination of programs including PLOT (B. Forbush) and DNASTar (Lasergene). Whole-cell extracts were quantified with the DC protein assay (Bio-Rad Laboratories) and DNA or RNA-specific signals by densitometry. When appropriate, statistical dispersion within datasets was determined by calculating SEMs, and differences between groups of variables were analyzed by Student’s two-tail t tests, rejecting the null hypothesis for \( P < 0.05 \).

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therefore be exploited for the mapping studies described below.

Two-hybrid Mapping Analyses: Expression of the Hybrid Proteins
In these studies, the NKCC-specific regions tested for their ability to support bait–prey interactions in yeast included the distinct Ct2NKCC2 protein segments shown in Fig. 3 (A and D), CtNKCC1 protein segments used in previous studies, and additional NtNKCC2 or CtNKCC2 protein segments. Western analyses, which were performed before the final selection procedures to confirm expression of the hybrid protein, are shown in Fig. 2. They reveal bands of the adequate molecular weight mark for each of the transformants tested, using the anti-LexA Ab to detect bait and the anti-HA Ab to detect prey. Although some bands are fainter than others, we have observed in previous studies that the activation of Leu and LacZ through a bait–prey interaction can occur even if the partners are expressed at low levels. Note that Western analyses in Fig. 2 show only a few examples of the cotransformants that were tested (see lanes 2, 4, and 5 in Fig. 2 B).

Two-hybrid Mapping Analyses: Interaction between Ct1NKCC2(657–836) and the Distinct Preys
These analyses were performed to confirm the bait–Ct2NKCC2 associations identified during the initial two-hybrid screen and results are summarized in Fig. 4 A. As can be seen here, YopCt1NKCC2(657–836) cells transformed with either of the distinct prey uncovered, including the 190-residue NKCC protein segment Ct2NKCC2(910–1099), are able to grow on −UHTL plates (left) and generate strong β-gal activity on −UHT + X plates (columns +X-gal). (A) Each cell expresses a different Ct2NKCC2 prey (among those identified during the initial two-hybrid screen) but the same Ct1NKCC2(657–836) bait. (B) Each cell was cotransformed with a huNKCC1- and a huNKCC2-encoding construct using the Ct1 domain of one isoform and the Ct2 domain of the other isoform. (C) Cells coexpress the protein segments shown in Fig. 3 D along with Ct1NKCC2(657–836) or Ct2NKCC2(830–1099) respectively. All of the cotransformants were incubated at 30°C for 3–6 d.

Figure 4. Two-hybrid mapping analyses. Cotransformed yeast were tested for their ability to grow on −UHTL plates (columns +Leu) or generate strong β-gal activity on −UHT + X plates (columns +X-gal). (A) Each cell expresses a different Ct2NKCC2 prey (among those identified during the initial two-hybrid screen) but the same Ct1NKCC2(657–836) bait. (B) Each cell was cotransformed with a huNKCC1- and a huNKCC2-encoding construct using the Ct1 domain of one isoform and the Ct2 domain of the other isoform. (C) Cells coexpress the protein segments shown in Fig. 3 D along with Ct1NKCC2(657–836) or Ct2NKCC2(830–1099) respectively. All of the cotransformants were incubated at 30°C for 3–6 d.
each other if they are highly conserved. As shown in Fig. 4B, the behaviors of the cotransformants suggest that the former scenario is in fact the correct one. For instance, all of the combinations tested support cell growth on \( \text{H11002-UHTL} \) plates (left) and generate strong \( \text{H9252} \)-gal activity on \( \text{H11002-UHTX} \) plates (right).

The bar in Fig. 3C represents a summary of the mapping analyses that were conducted thus far for NKCC1 and 2, assuming that the EIRs of both isoforms are highly homologous. It shows where EIRs in \( \text{huNKCC2} \) are now localized relative to those identified in \( \text{huNKCC1} \) (Fig. 3B). Based on this summary, EIR3 appears to be shorter than initially predicted but, reassuringly, the residues no longer included in this previously defined contact site are in reality poorly matched between NKCC1 and 2. EIR1, conversely, now appears to be at a different position, as suggested by the behaviors of \( \text{Ct1NKCC2(657–836)} \), which was used for the two-hybrid screen in this research, and of \( \text{Ct1NKCC1(834–926)} \), which had been used previously to identify EIR2 in \( \text{huNKCC1} \). Indeed, \( \text{Ct1NKCC2(657–836)} \) interacts with \( \text{Ct2} \) but begins just after the previously defined EIR1, whereas \( \text{Ct1NKCC1(834–926)} \) was not able to interact with \( \text{Ct2NKCC1} \) but corresponds to the longest EIR2-containing protein segment tested to date.

Two-hybrid Mapping Analyses: Interaction between Other NKCC2s

The behaviors of various transformants that coexpress a truncated Ct1 or Ct2 with a full-length Ct2 and Ct1 are shown in Fig. 4C (the protein segments are also illustrated graphically in Fig. 3D). Here, interestingly, it is seen to be more precisely mapped and the localization of EIR1 remains slightly different from that predicted initially (see Fig. 3E).

A number of putative binding sites or residues in \( \text{huNKCC2} \) could account for the EIRs’ properties or play complementary roles in the formation of Ct1–Ct2 complexes; their localization relative to the newly defined EIRs is shown in Fig. 3F. Interestingly, several of these sites/residues (highlighted by an asterisk) are conserved between NKCC1 and 2. As will be discussed later, however, the current analysis has placed a conserved, presumably important FHA-related site outside of one of the newly relocalized EIR.

Two-hybrid Mapping Analyses: a Search for Other Types of Self-interactions

The segments used for these studies, \( \text{Nt2NKCC2(51–179)} \), \( \text{Ct1NKCC2(657–836)} \), and \( \text{Ct2NKCC2(830–1099)} \), are shown in Fig. 1. The only conclusion that could be drawn from the behavior of various cotransformants is that \( \text{Ct1NKCC2} \) does not interact with \( \text{Ct1NKCC2} \) (unpublished data). The coexpression of a bait \( \text{Ct2NKCC2} \) with a prey \( \text{Ct2NKCC2} \) yielded equivocal results, whereas \( \text{Nt2NKCC2(51–179)} \) was found to induce LacZ in the absence of a prey protein and could therefore not be tested (unpublished data).

Flux Studies

Because A, F, and AF are identical in the region of \( \text{huNKCC2} \) that harbors EIRs, and because AF is probably expressed in the same cell types as A or F, we hypothesized that some of the variants could assemble with each other in some regions of the TAL to form heterooligomeric structures in vivo. We have thus determined the potential repercussions of such assemblies by analyzing the functional behavior of A or F coexpressed in oocytes with AF.
The results of these studies are shown in Fig. 5. Quite interestingly, $^8_{\text{Rb}}$ fluxes in oocytes that express both A and AF together are $\sim 50\%$ lower than in oocytes that express A alone, approaching those measured in H$_2$O-injected cts (Fig. 5 A). Similar results are observed when A is replaced by F in these coexpression experiments (Fig. 5 B) or when $^8_{\text{Rb}}$ is replaced by $^{22}\text{Na}$ (Fig. 5 C). In conjunction with our previous findings, these studies suggest that AF exerts a dominant-negative effect on A or F by forming nonfunctional heterodimers with these variants.

**Immunofluorescence**

These studies, which are summarized in Fig. 6, show that all of the variants tested are able to reach the oocytes’ cell surface whether they are expressed individually (Fig. 6, A–C) or in combination (D and E). They also show that the signal obtained is of similar intensity among the variants. For AF, hence, lack of transport activity is apparently not due to lack of cell surface delivery, supporting further the idea of a physical interaction between AF and other variants.

**RNA Studies**

The effect of an H$_2$O-rich diet (mean ingestion $\sim 100$ ml/d versus $\sim 10$ ml/d in the cts) is presented in the top row of Fig. 7. Here, interestingly, PCR analyses illustrated by representative gels in Fig. 7 A, and by densitometry measurements in B, show that the expression levels of A are unchanged following the administration of the H$_2$O-rich diet, whereas those of F and AF decrease substantially (by a factor of 1.8 based on Fig. 7 B, $P < 0.01$, $n = 2–6$). Results obtained by in situ hybridization (Fig. 7 C) are consistent with these findings, showing an apparent decrease in msNKCC2-specific signals with both oligoprobes.

The effect of furosemide is presented in the bottom row. Here again, PCR analyses show that the variants are affected differentially by this maneuver. As illustrated in Fig. 7 (A and B), e.g., the expression levels of A are seen to increase substantially (by a factor of 2.3, $P < 0.01$, $n = 3$) following the administration of furosemide, whereas those of F and AF are seen to decrease (by a factor of 2.4 and 1.5, respectively, $P < 0.01$, $n = 5$). As for the in situ hybridization studies (Fig. 7 C), re-
RESULTS are once more consistent with those obtained by PCR, showing an expected rise in msNKCC2-specific signals with the A oligoprobe but a decrease in these signals with the F oligoprobe.

DISCUSSION

In this work, yeast two-hybrid studies led to the identification of separate domains in huNKCC2 that can interact with each other; one is in the proximal portion of the COOH terminus (Ct1) and the other in the distal portion (Ct2). They revealed, moreover, that the corresponding domains in huNKCC1, which were recently shown to exhibit similar properties (Simard et al., 2004a), can also interact with those of huNKCC2. In conjunction with previous studies demonstrating that the structural unit of both NKCC1 and 2 is oligomeric (Ichinose et al., 1999; Moore-Hoon and Turner, 2000; Starremans et al., 2003), and that the Ct1NKCC1–Ct2NKCC1 interaction can be reproduced in pull down assays (Simard et al., 2004a), the current data strongly suggest that the identified self-interacting domains mediate NKCC2 assembly at the cell surface.

An alternative role that could be played by Ct1 and Ct2 is to promote the formation of molecular interactions within the Ct of one monomer instead of between the Cts of two monomers. Although such a possibility cannot be ruled out with certainty, we still favor the hypothesis that the primary purpose of Ct1 and Ct2 is to support intermonomeric associations. Indeed, the organization of a protein into a tertiary structure, that is, the mechanism by which intramolecular folding occurs, is to a large extent mediated by the action of chaperones, foldases, or isomerases (Fink, 1999), whereas the organization of a protein into a homooligomeric structure generally relies on the presence of self-interacting domains as those described here (Fink, 1999; Engelman et al., 2003).

A significant outcome of the mapping analyses that were conducted in this study is the identification of <200 candidate residues that may underlie the Ct1–Ct2 association. As depicted in Fig. 4 E, these candidate
residues are regrouped into four clusters (called EIRs) that are dispersed along the COOH terminus; EIR1 corresponds to Ct2NKCC2(671-726), EIR2 to Ct2NKCC2(783-816), EIR3 to Ct2NKCC2(910-959), and EIR4 to Ct2NKCC2(1059-1098). This level of resolution was obtained by showing that the Ct1–Ct2 domains, which had already been mapped partially in huNKCC1, are functionally conserved between the isoforms, by sequencing all of the huNKCC2 prey identified in the library, and by analyzing the behavior of yeast expressing truncated versions of Ct1 or Ct2 protein segments.

The importance of mapping the oligomerization domains of NKCC2 stems from the possibility of exploiting certain interacting residues as molecular targets for future structure function studies, and from the insight that could be gained into the molecular principles of oligomerization for this transport system. By way of illustration, it may become possible through various interventions, e.g., site-directed mutagenesis or peptide interference (Bjornson et al., 2003; Polo et al., 2004), to alter carrier assembly as a means of determining whether the monomeric structure is functional or correctly targeted at the cell membrane and whether its kinetic features differ from those of the oligomer. Along the same line, the mapping analyses performed here could lead to the identification of novel motifs or mechanisms that are involved in protein–protein interactions or carrier assembly.

A potential shortcoming of the method employed to map the oligomerization domains of huNKCC2 comes from the widely dispersed localization of the candidate interacting residues. In fact, the specific manner in which these residues are disposed suggests that some level of tertiary organization may be required for Ct1–Ct2 associations to occur in cells and implies that some of the unmapped regions (e.g., those represented by light gray boxes in Fig. 4) will include long stretches of residues flanked by EIRs on both sides. Hence, certain deletions could result in false negatives by generating conformational artifacts and erroneous interpretations of where EIRs reside, or will have to include one of the EIRs. The relocation of EIR1 based on the current analysis may represent a good illustration of these potential limitations.

Left alone, the finding that the COOH terminus of NKCC2 can interact with that of NKCC1 is of disputable physiological importance given that these isoforms are not expressed in the same cell types. However, it does point toward the possibility that certain carriers within the same CCC subgroup or from different CCC subgroups could associate with one another in vivo. Indeed, several of these transport systems share similar amino acid sequences, tissue distributions, and subcellular localizations (Haas and Forbush, 1998 and 2000; Russell, 2000; Hebert et al., 2004). Heterooligomeric assemblies between various CCC isoforms would carry the advantage of broadening functional diversity within the family.

The existence of conserved oligomerization domains among CCC family members suggests that certain NKCC2 variants could also assemble as heterooligomeric complexes. Two lines of evidence are in fact consistent with this possibility. (1) Ct1 domains appear to interact specifically with Ct2 domains (and vice versa) throughout a region of huNKCC2 that is identical among the variants (Fig. 3 B). (2) When expressed in X. laevis oocytes, AF can exert dominant-negative effects on A or F (Fig. 6). This variant, which probably constitutes a substantial fraction of NKCC2-derived transcripts in the mammalian TAL based on the current PCR data, and which has been isolated from the renal medulla where A and F are coexpressed, could thus play a similar role in vivo (Payne and Forbush, 1994). Collectively, these observations also suggest that the inclusion of both the A and F exons in the same protein is not simply the result of splicing artifacts as previously suspected.

If AF does play an important role in vivo, transcriptional or posttranscriptional activity for this variant should be affected by a number of stimuli or conditions that act upon NKCC2-expressing cells. According to Western analyses performed in previous studies, these stimuli or conditions could include a long-term increase in circulating ADH levels or the administration of furosemide; indeed, both maneuvers have been shown to up-regulate NKCC2 synthesis in lagomorphs (Ecelbarger et al., 1996, 2001; Knepper et al., 1999). In the present work, we showed that the expression of AF in mouse was also affected by such maneuvers, that is, the number of AF transcripts decreased after 7-d regimens of H2O-rich or furosemide-enriched diets. The data reported here thus point toward a potential role for AF in adaptation responses, which may necessitate diverse levels of NKCC2 activity along the TAL.

The potential involvement of AF in overall salt reabsorption by the TAL is perhaps easier to decipher if the effect of different maneuvers on transcriptional and posttranscriptional activities for the other variants are also known. To this effect, our studies in mouse showed that the observed modifications in expression levels were not always matched between AF and the other variants. Such behaviors imply that the number of non-functional AF-containing oligomers could vary substantially under certain conditions and, accordingly, that changes in expression levels and transport activities for A or for F could also be poorly matched. In this setting, the regulated production of AF variants could represent a means by which separate NKCC2-expressing nephron segments alter their transport capacities dif-
ference in response to the same transcriptional stimulus.

Growing evidence suggests that the optional inclusion or excision of specific exons in single gene-derived transcripts is a highly regulated process that involves specific components of the splicing machinery (Beyersmann, 2000; Xie and Black, 2001). Hence, unmatched changes in the expression levels of A, F, or AF may imply that this machinery plays an important role during NKCC2-dependent adaptation responses. For instance, if a spliceosome becomes less active at the 3′ splice site of exon F in A-expressing cells or at the 5′ end of exon A in F-expressing cells, the number of AF transcripts produced would be expected to decrease and that of A or F to increase in parallel. Changes in expression levels through regulation of alternative splicing could then constitute an important posttranscriptional mechanism by which NKCC2-expressing cells coordinate their action appropriately in response to distinct sets of environmental cues.

Although we have not explored the mechanisms by which the H2O-rich diet led to changes in transcriptional posttranscriptional activities for some of the variants, the large quantity of low salt fluid ingested by the mice and the pattern of responses observed suggest that these mechanisms are related, at least in part, to ADH suppression. In lagomorphs, for example, this hormone has been shown to increase NKCC2 expression, whereas the H2O diet administered here produced the opposite effect in that it decreased the expression of F and AF by >1.5-fold while leaving that of A relatively unchanged (Ecelbarger et al., 1996, 2001; Knepper et al., 1999). Such behaviors may also imply that A-mediated ion transport is more affected than F-mediated transport by changes in circulating ADH levels.

In this work, the mechanisms underlying the effect of loop diuresis were also not explored. It is nonetheless tempting to postulate that furosemide led to the observed changes by decreasing [Cl−] in TAL cells. Indeed, several studies have shown that the activity of both NKCC1 and NKCC2 increases when intracellular Cl− (Cl−) is reduced nonpharmacologically (Lytle and Forbush, 1992; Haas and Forbush, 1998, 2000; Isenring et al., 1998a; Russell, 2000; Isenring and Forbush, 2001; Darman and Forbush, 2002; Gagnon et al., 2002, 2003; Simard et al., 2004b). Although regulatory events that affect the transporter at posttranslational steps could play an important role in this response (Lytle and Forbush, 1992; Darman and Forbush, 2002), such mechanisms do not exclude the possibility of Cl−-dependent regulatory events acting at earlier steps, especially in the setting of long term adaptation responses. In this regard, we showed that furosemide also exerted differential effects on variant expression, increasing that of A substantially, but reducing that of F and AF. These behaviors support the idea of a Cl−-dependent pretranslational regulatory step, and imply once more that A-mediated ion transport is more affected than F-mediated transport by changes in Cl−.

In conclusion, we have identified novel features regarding the modus operandi of the NKCC2 splice variants. These features may endow NKCC2-expressing cells with much greater functional diversity than expected for both the normal operations and the adaptation responses. Further studies are required to understand how the NKCC2-dependent splicing machinery is regulated and determine the extent of its contribution to changes in A, F, or AF expression along TAL.

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