Self-interacting Domains in the C Terminus of a Cation-Cl⁻ Cotransporter Described for the First Time*

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The first isoform of the Na⁺-K⁺-Cl⁻ cotransporter (NKCC1), a widely distributed member of the cation-Cl⁻ cotransporter superfamily, plays key roles in many physiological processes by regulating the ion and water content of animal cells and by sustaining electrolyte secretion across various epithelia. Indirect studies have led to the prediction that NKCC1 operates as a dimer assembled through binding domains that are distal to the amino portion of the carrier. In this study, evidence is presented that NKCC1 possesses self-interacting properties that result in the formation of a large complex between the proximal and the distal segment of the cytosolic C terminus. Elaborate mapping studies of these segments showed that the contact sites are dispersed along the entire C terminus, and they also led to the identification of a critical interacting residue that belongs to a putative forkhead-associated binding domain. In conjunction with previous findings, our results indicate that the uncovered interacting domains are probably a major determinant of the NKCC1 conformational landscape and assembly into a high order structure. A model is proposed in which the carrier could alternate between monomeric and homo-oligomeric units via chemical- or ligand-dependent changes in conformational dynamics.

Cation-Cl⁻ cotransporters (CCCs) belong to an important family of transport systems that couple the movement of Cl⁻ to that of Na⁺ and/or K⁺ across cell surfaces (1–4). One of the first CCCs identified was the secretory Na⁺-K⁺-Cl⁻ cotransporter (5), also called NKCC1. Since then, six other family members have been identified: the renal Na⁺-K⁺-Cl⁻ cotransporters (6), the Na⁺-Cl⁻ cotransporter (7), and the K⁺-Cl⁻ cotransporters (8–11) called NKCC2, NCC, and KCCs, respectively. The CCC family also includes carrier-like molecules for which no ionic substrates have been identified to date; they are called orphan CCCs (12).

The main function of a CCC is to regulate the ion or volume content of individual cells and to facilitate salt movement across various epithelia (1, 4); CCCs may play accessory roles in acid/base balance or regulation of cell pH by transporting NH₄⁺ at the K⁺ site or by modulating intracellular [Cl⁻] through the activity of these proteins is tightly modulated through interactions with regulatory enzymes (15–20) and with cytoskeletal elements (21, 22). The best example studied thus far is the phosphorylation-dependent activation of NKCC1, a ubiquitous CCC that is expressed basolaterally in polarized cells (1–4, 23).

The CCCs exhibit a common structure, illustrated in Fig. 1A below by a hydropathy plot model of the human (hu) NKCC1. Based on computer-aided analyses from which this model is derived, the CCCs are predicted to have a membrane-associated domain flanked by cytosolic termini. For NKCC1, the membrane localization of the central domain has been confirmed indirectly by the identification of regions that mediate ion transport (24–26) and that behave as transmembrane segments when expressed as fusion proteins in rabbit reticulocyte lysates (27). For this carrier system, likewise, the intracellular localization of both termini has been confirmed indirectly by the identification of epitope sites that are not accessible unless the lipid bilayer is permeabilized (28) and by the identification of phosphoacceptor sites that are involved in transporter regulation (3, 16, 18, 19).

Indirect evidence suggests that CCCs are assembled at the cell surface as homo-oligomeric structures. For example, cross-linking of NKCC1, NKCC2, or NCC in membrane extracts leads to the formation of large complexes, the molecular size of which is twice that of the monomeric forms (29, 30). In agreement with these studies, we and others have observed that non-functional NKCC1 mutants or NKCC2 splice variants can reduce the activity of functional versions of these carriers in heterologous expression systems, implying that they exert a dominant negative effect through direct homo-oligomerization of the carriers or through their association with an accessory protein (31, 32). High-resolution imaging data confirming the interpretation of such findings are unavailable at present.

In this study, we have exploited the yeast two-hybrid system to search for additional partners of NKCC1 using the proximal C terminus as bait, and we have found a sequence that belongs to the distal C terminus of the same protein. We have also found that the association between the two regions involves a large number of residues and that it could also be phosphorylation-dependent. Hence, our results identify the first

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1 The abbreviations used are: CCC, cation-Cl⁻ cotransporter; KCC, K⁺-Cl⁻ cotransporter; NCC, Na⁺-Cl⁻ cotransporter; NKCC, Na⁺-K⁺-Cl⁻ cotransporter; ADH, anti-diuretic hormone; AMPH, ampicillin resistance gene; GAL, truncated; GST, glutathione S-transferase, GS, glutathione-coupled Sepharose; HA, hemagglutinin; hu, human; MICS, multiple cloning site; op, operator; Pₕ, position; Y, EGY48 yeast; Yop, EGY48 yeast transformed with the phop-lacZ reporter plasmid; Ab, antibody; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
time a self-interacting domain in NKCC1 and points toward novel mechanisms by which CCCs regulate their activity.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Reagents, Kits, and Cell Strains—**Unless mentioned otherwise, chemicals, reagents, or kits were from several suppliers. They included the following antibodies (Abs): 1) a mouse anti-hemagglutinin (HA) Ab (Biochem, catalog number MMS-101R; 2) a goat anti-LexA Ab (Roche Applied Science, catalog number 1301977). Vectors and cDNA constructs were propagated in XL1-blue cells (Stratagene). Some of these sequences were also introduced in EGY48 yeast (Clontech) and BL21 *Escherichia coli* cells (Amersham Biosciences) as described below.

**Vectors—**Subcloning, expression of reporter genes, or DNA amplification was carried out with the following (empty or insert-bearing) vectors: 1) pGilda (Clontech), which contains 5' to 3' restriction sites are underlined. They are called C2a, C2b, and C2c/pB42AD. The C759S mutation of C2b was produced with an EcoRI-capped forward primer and an XhoI-capped reverse primer (Table I, and see also Fig. 2). For the other approach, segments in C1t were amplified through 30 cycles of PCR with an EcoRI-capped forward primer and an XhoI-capped reverse primer (Table I, e–h). They were subsequently cloned in pGilda generating C1e, C1f, C1g, and C1h, and C1i/pGilda (Table II, and see also Fig. 2).

**Oligonucleotides used to generate 15 different constructs and to sequence their inserts**

| Insert | Forward | Reverse |
|--------|---------|---------|
| C1t    | tc acc    | cgg cat   |
| C1a    | cgg cta   | cgg cat   |
| C1b    | cc tgg    | cta gag   |
| C1e    | cc tgg    | cta gag   |
| C1f    | gc atg    | tgc atg   |
| C1g    | cc gag    | tgg acc   |
| C1h    | cc gag    | tgg acc   |
| C2a    | gc gaa    | ttc cgt   |
| C2b    | cc gaa    | ttc cgt   |
| C2c    | gc gaa    | ttc cgt   |
| C2d    | gc gaa    | ttc cgt   |
| C2e    | gc gaa    | ttc cgt   |
| C2f    | gc gaa    | ttc cgt   |
| C2g    | gc gaa    | ttc cgt   |
| C2h    | gc gaa    | ttc cgt   |
| C2i    | gc gaa    | ttc cgt   |
| C2j    | gc gaa    | ttc cgt   |
| C2k    | gc gaa    | ttc cgt   |
| C2l    | gc gaa    | ttc cgt   |
| C2m    | gc gaa    | ttc cgt   |
| C2n    | gc gaa    | ttc cgt   |
| C2o    | gc gaa    | ttc cgt   |
| C2p    | gc gaa    | ttc cgt   |

**C1t Constructs—**The bait used for the hybrid screen consists of a 567-bp fragment that encodes the putative cytosolic C terminus of huNKCC1 from residue 9 after the 12th transmembrane domain to residue 197 (see Fig. 1B). It was obtained from an available huNKCC1/pBS (32, 33) through 30 cycles of PCR using the high fidelity PCR master kit (Roche Applied Science) with an Xmal-capped forward primer and an NcoI-capped reverse primer (Table I). The PCR fragment (C1t) was cloned in pGilda generating C1t/pGilda, which should encode a LexA-C1t fusion protein in yeast (Table II, and see also Fig. 2). The insert of C1t/pGilda was also transferred to pGEX as an EcoRI/XhoI fragment generating C1t/pGEX, which should encode a C1t-GST fusion protein in bacteria (Table II, and see also Fig. 2).

In this work, additional constructs containing shorter C1t inserts (called C1t<sub>DELs</sub>) were produced using two approaches. First, the 3' region of C1t/pGilda was truncated by removing nucleotide segments between a restriction site (Bargl, Clai, Stul, or Xbal) that cuts in the coding sequence of the insert and a downstream restriction site (XhoI) that cuts in the MCS. Bargl and Clai are naturally present in C1t (bps 192–197 and 235–240), whereas Stul and Xbal were created by substitution (bps 281–286 and 501–506) using the QuikChange mutagenesis kit (Stratagene) with mutagenic primers (Table I, b and c). The final constructs (C1a, C1b, C1c, and C1d/pGilda) were generated after excising the 3' end of the insert and ligating the vector-containing fragment pretreated with Klenow in dNTP-supplemented buffer (Table II, and see also Fig. 2). For the other approach, segments in C1t were amplified through 30 cycles of PCR with an EcoRI-capped forward primer and an XhoI-capped reverse primer (Table I, e–h). They were subsequently cloned in pGilda generating C1e, C1f, C1g, C1h, and C1i/pGilda (Table II, and see also Fig. 2).

**C2t Constructs—**One of the interacting proteins identified in the current study (called C2t) is derived from a 729-bp fragment that encodes the distal C terminus of huNKCC1 (Table II, and see also Fig. 1C). It is cloned in the vector pB42AD behind the LexA activating domain and HA-encoding sequence. This insert was also subcloned in another vector as an EcoRI-XhoI fragment. The resulting construct, C2t/pDNA3, should produce a radioactive prey during in vitro translation (Table II, and see also Fig. 2).

Additional constructs containing shorter C2t inserts (called C2t<sub>DELs</sub>) were generated using approaches that are similar to those outlined for C1t<sub>DELs</sub> inserts. Here, the sequences removed at the 3' end of C2t/pB42AD were between a restriction site (Bargl, AccIII, or NcoI) that cuts in the coding sequence of C2t and a downstream restriction site (XhoI) that cuts in the MCS. AccIII and NcoI are naturally present in C2t (bps 329–334 and 484–489), whereas Bargl was created by substitution (bps 117–122) using the QuikChange kit with mutagenic primers (Table II, and see also Fig. 2). As for the PCR-derived constructs, they were produced with an EcoRI-capped forward primer and an XhoI- or BamHI-capped reverse primer (Table I, j–n). They are called C2a, C2b, C2c, and C2d/pB42AD (Table II, and see also Fig. 2).

**The C759S<sub>–</sub>pDNA3 Construct—**To study the C1t-C2t interaction...
with a full-length C2t-bearing or C1t-bearing construct, and they were retransformed. Cells expressing the heterologous protein were retransformed with X-gal, and those expressing \( \text{C}1\text{t/pGilda} \) were transformed once more with C1t/pGilda and seeded on Ura-His-Leu plates. Resistant colonies were subjected to DNA isolation and subjecting the inserts to automated sequencing. 

**Table I**

| Name of the construct | Length of the fused proteins (in residues) | Length of NKCC1 fragments (in residues) | \( P_n \) in NKCC1 | Total length (in residues) | \( P_n \) in C1t or C2t |
|-----------------------|------------------------------------------|----------------------------------------|------------------|----------------------------|------------------|
| C1tpGilda             | n                                       | 202                                    | 189              | 759–947                    | 391              | 1–189            |
| C1spGilda             | 202                                      | 165                                    | 759–923          | 367                        | 1–165            |
| C1hpGilda             | 202                                      | 94                                     | 759–852          | 296                        | 1–94             |
| C1dpGilda             | 202                                      | 79                                     | 759–837          | 281                        | 1–79             |
| C1hpGilda             | 202                                      | 65                                     | 759–823          | 267                        | 1–65             |
| C1fpGilda             | 202                                      | 93                                     | 834–896          | 294                        | 76–188           |
| C1gpGilda             | 202                                      | 86                                     | 800–885          | 288                        | 42–127           |
| C1hpGilda             | 202                                      | 52                                     | 834–885          | 254                        | 76–127           |
| C1hpGilda             | 202                                      | 70                                     | 813–882          | 272                        | 55–124           |
| C1tpGilda             | 202                                      | 165                                    | 763–927          | 367                        | 5–189            |
| C2tpB42AD             | 202                                      | 243                                    | 970–1212         | 351                        | 1–243            |
| C2spB42AD             | 108                                      | 162                                    | 970–1131         | 310                        | 1–162            |
| C2hpB42AD             | 108                                      | 111                                    | 970–1080         | 219                        | 1–111            |
| C2spB42AD             | 108                                      | 43                                     | 970–1012         | 151                        | 1–43             |
| C2tpB42AD             | 108                                      | 85                                     | 1128–1212        | 193                        | 159–243          |
| C2spB42AD             | 108                                      | 84                                     | 1089–1172        | 192                        | 120–203          |
| C2hpB42AD             | 108                                      | 36                                     | 1113–1148        | 144                        | 143–178          |
| C2spB42AD             | 108                                      | 118                                    | 1031–1148        | 226                        | 62–178           |
| C2tpB42AD             | 108                                      | 218                                    | 994–1211         | 326                        | 25–242           |
| C1tGEX                | 227                                      | 189                                    | 759–947          | 416                        | 1–189            |
| C2tpCDNA              |                                          |                                          | \( \approx 243 \) | 970–1212                   | \( \approx 243 \) |
| C970–1212c from first codon | 0                                      | \approx 243                            | 970–1212         | \( \approx 1–243 \) |
| C1072–1212c from first Met | 0                                      | 141                                    | 1072–1212        | 141                        | 103–243          |
| C795–1212c from pDNA3  |                                          |                                          | \approx 454      | 759–1212                   | \( \approx 1–189\) |
| C795–1212c from first codon | 0                                      | 419                                    | 794–1212         | 419                        | 36–189\(\text{in C1t}) |

**RESULTS**

The cell strain used for this experiment is called YopC1t and consists of pSop-lacZ-transformed EGY48 yeast (Yop) retransformed with a bait-bearing plasmid (C1t/pGilda), which encodes the proximal region of the huNKCC1

in the context of an intact domain, a nucleotide segment that encodes the huNKCC1 cystolic C terminus (residues 759–1212 corresponding to bps 2275–3634 of the coding sequence) was amplified from huNKCC1/pBS through 30 cycles of PCR with an EcoRI-capped forward primer and an XhoI-capped reverse primer (Table I). The resulting fragment (C759–1212) was subsequently cloned in pDNA3 (Table II, and see also Fig. 2).

**Yeast Two-hybrid Screen**—EGY48 yeasts (Y) were first transformed with the construct pSop-lacZ and seeded on Ura plates generating the strain Yop. These cells were transformed once more with C1t/pGilda and seeded on Ura–His–Leu plates generating YopC1t. The latter strain was subsequently tested for expression of the hybrid protein by Western blotting (see below and see Fig. 3A) and tested for autonomous reporter activation on Leu plates containing 80 \( \mu \text{g/mL} \) X-galactosidase (X-gal). A search for interactors was performed by transforming YopC1t cells with a human heart library cloned in pB42AD and subjecting the transformants to two rounds of selection, first on Ura–His–Trp plates and subsequently on Ura–His–Trp–Leu plates. Resistant colonies were transfected on Ura–His–Trp–Leu plates containing X-gal, and those expressing \( \beta \)-galactosidase activity were amplified in regular yeast medium. Prey plasmids were extracted according to well-established procedures, and inserts were analyzed by automated sequencing.

Two procedures were carried out 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 (see below) after selection of colonies on Ura–His–Trp–Leu plates, respectively. Cells expressing the heterologous protein were retransformed with a full-length C2t-bearing or C1t-bearing construct, and they were tested for their ability to grow on Ura–His–Trp–Leu plates or to express \( \beta \)-galactosidase activity on Ura–His–Trp + X-gal plates.

**Protein Analyses**—Radioactive prey bound to GS beads were eluted with 10 \( \mu \text{g/mL} \) of rabbit reticulocyte lysate premixed with amino acids, and 0.4 mCi/mL \( [\text{35S}]\text{Met or 20 \muCi}} \text{cold Met. A fraction of the proteins synthesized were also treated with a phosphorylation mix using the following reaction solution (in 100 \muL): radioactive prey (1.5 dilution), protein kinase A or protein kinase C mixture (2 \muL cold diluted in appropriate buffers), ATP (100 \muM), and for some of the reactions, [\gamma-\text{32P}]ATP (10 \muCi/mL). The pull-down assay as such was carried out by incubating \( \approx 2 \mug \) of proteins immobilized on GS beads with 10 \muL of labeled prey for 16 h at 4 °C in a binding buffer (100–200 mM NaCl, 1 mM EDTA, 0.5–2.0% Triton X-100, and 20 mM Tris-HCl, pH 6–8) followed by several washes in the same buffer.

**Sequence Analyses**—The bp content of cloned inserts was determined by automated sequencing using plasmid-derived primers (Table I). Blast searches, sequence alignments, and structure predictions were carried out with DNAStar (Lasergene), the PLOT program (Biff Forsbus), and online programs available through NCBI, EMBL-EBI, and the BCM Search Launcher web pages.

**RESULTS**

The Yeast Two-hybrid Screen—The cell strain used for this experiment is called YopC1t and consists of pSop-lacZ-transformed EGY48 yeast (Yop) retransformed with a bait-bearing plasmid (C1t/pGilda), which encodes the proximal region of the huNKCC1
cytosolic C terminus. Transformation of YopC1t with a human heart library/pB42AD and selection of these cells on –Ura–His–Trp plates led to the formation of over 1 million colonies. Of these colonies, 41 grew on –Ura–His–Trp–Leu, and 32 expressed above background β-galactosidase activity on –Ura–His–Trp–Leu + X-gal.

Confirmation studies in the yeast expression system revealed that the identified interactions were specific (results not shown). Interestingly, inserts found in the prey plasmids of β-galactosidase-positive –Leu-resistant colonies corresponded to the coding regions of seven different proteins. One of the inserts was a 729-nucleotide sequence that encodes the distal C terminus to which they correspond. Here, black is used to indicate that the protein segments induced strong β-galactosidase activity, and white is used to indicate that they induced no (or only weak) activity. As will be reported later in greater detail, additional protein segments were produced by in vitro translation and tested for their ability to interact with C1t in pull-down assays. For this reason, they are included in the mapping analysis described in Fig. 2B. Here again, black is used to indicate that an interaction occurred between bait and prey.

Results of the mapping analysis are summarized in Fig. 2B1. The various protein segments that were produced in yeast (19 total) are represented by horizontal bars aligned with regions of the C terminus to which they correspond. Here, black is used to indicate that the protein segments induced strong β-galactosidase activity, and white is used to indicate that they induced no (or only weak) activity. As will be reported later in greater detail, additional protein segments were produced by in vitro translation and tested for their ability to interact with C1t in pull-down assays. For this reason, they are included in the mapping analysis described in Fig. 2B. Here again, black is used to indicate that an interaction occurred between bait and prey.

An interpretation of the mapping studies is provided schematically in Figs. 2B3 (bottom bar) and 4A, highlighting regions that are minimally required for the interaction in black, highlighting the regions that are not required in white, and highlighting the regions that may or may not be required in gray. These figures show that the essential interacting regions are dispersed over a long distance in the C terminus. In C1t, they correspond to C759–762 and C854–923 (subscribed characters indicate the residue sequence in huNKCC1), and in C2t, they correspond to C994–1072 and C1132–1211. The figures also show that an interaction occurred between bait and prey will not occur unless two essential interacting regions are present in each of the partners.

Sequence analyses of the essential interacting regions identified in this work are summarized in Fig. 4B. The upper panel depicts the amino acid content of each essential interacting region, and the lower panel depicts the position (Pn) of these regions (represented as boxes) relative to the entire C terminus and to various protein domains (represented as a long horizontal line and shorter horizontal lines, respectively). The lower panel also shows the percentage of homologies between the essential interacting regions of NKCC1, NKCC2, and NCC (dark gray, ~90% homology; medium gray, 75–80% homology; white, ~50% homology) and the Pn’s of positively or negatively charged residues in the C terminus (vertical lines).

Through these depictions, it is seen that the essential interacting regions are highly conserved among the Na+-dependent CCCs except for the proximal portion of essential-interacting-region-3. These regions are also seen to harbor only 1 cysteine residue (Pn 59 in essential-interacting-region-3) and to be par-
particularly rich in neutral and hydrophobic residues when compared with other regions in the C terminus. Remarkably, a putative protein kinase A type 2 (PKA-2) site is found at the beginning of essential-interacting-region-4, and a PP-1 site is found just after essential-interacting-region-3; as will be discussed later, kinases and phosphatases are believed to play a key role in NKCC1 regulation by interacting with the N terminus of the carrier (5, 15, 16, 34) and its C terminus as well (5, 23, 36). Other consensus sites involved in protein-protein interactions include four sites belonging to forkhead-associated binding domains (Fig. 4B) and several sites belonging to Src homology and PDZ class III domains (not shown). It is noteworthy that the C1i protein segment, which is very similar to C1t except for lacking a critical residue belonging to a forkhead-associated binding domain consensus site (TQAL), is not able to support growth of C2t-transformed yeast on −Ura−His−Trp−Leu plates (Fig. 2B1).

Pull-down Assays—These studies were undertaken to confirm the C1t-C2t interaction outside of the yeast system. GST or C1t-GST fusion proteins were generated in E. coli and transformed with an empty pGEX vector or with C1t/pGEX, and they were immobilized on GS beads. Prey were generated by in vitro translation from C759−1212/pcDNA3 or C2t/pcDNA3. Their predicted length (419 and 141 residues) is shorter than expected (454 and 243 residues) based on the Pn of the first AUGs downstream of the T7 promoter (Fig. 1, B and C); for this reason, they will be called C794−1212 and C1072−1212. For the pull-down assays as such, GST-GS beads or C1t-GST-GS beads were incubated with equivalent amounts of prey and electrophoresed on SDS gels alongside with an aliquot of in vitro translated proteins not preincubated with the bait. Results of these studies are detailed below.

In Fig. 5A, Coomassie Blue staining of the protein gels as such shows that GS beads are highly enriched in plasmid-derived proteins. Based on an internal control, the quantity of bound C1t-GST in lane 4 is 1.5 μg, and the quantity of bound GST in lane 5 is 3.0 μg. In Fig. 5B, autoradiograms of similar gels reveal that in vitro translation of C759−1212 and C2t produces two major bands in each case (lanes 1 and 4). The lower 46-kDa band in lane 1 probably corre-
**DISCUSSION**

In this study, we have exploited the yeast two-hybrid system to uncover novel partners of NKCC1. We have used the proximal region of the cytosolic C terminus (C1t) as bait and have identified a candidate interactor that corresponds to the distal region of the same domain (C2t). Control studies and pull-down assays confirmed that the association between C1t and C2t is specific, demonstrating for the first time that the NKCC1 C terminus possesses self-interacting properties.

Additional analyses in which the two-hybrid system was used as a mapping tool to narrow the interacting regions further revealed the existence of several contact sites between C1t and C2t. In fact, it was possible to deduce from such analyses that a minimum of four regions (called C759–762, C854–923, C954–1072, and C1132–1212) are required for the C1t-C2t interaction to occur in vitro. Quite remarkably, these sites occur at remote Pns along the C terminus separated from one another by over 59 residues. Although further mutagenic studies will be required to identify the interacting residues more definitively, it can already be presumed at this stage of the mapping analysis that the C1t-C2t association is complex and that it probably relies on proper folding of the C terminus.

A potential shortcoming of the approach used to identify C1t-C2t-interacting sites is that it does not elucidate the role played by residues outside of essential interacting regions. For example, residues critically involved in the formation of C1t-C2t complexes could be missed if they occur in a protein segment that is also devoid of such essential interacting regions. In addition, improper folding of the C terminus resulting from a truncation in NKCC1 could have precluded associations that normally occur in vivo when the carrier is intact, leading to incorrect interpretations of where the essential interacting regions are localized. One possible example that illustrates these limitations comes from the identification of a C1t-binding site that belongs to a forkhead-associated domain (TQAL) but that is partly outside of an essential interacting region. Indeed, when a single residue is removed from this site (as in C1i), the C2t-derived high molecular weight protein segment (eluted from an unstained gel) confirmed that this was the case (results not shown).

Results of the pull-down assays are also shown in Fig. 5B. As anticipated, the quantities of radioactive prey that associate with GS beads vary as functions of the type of prey (C759–1212 versus C794–1212 versus C1072–1212 versus C970–1212) and type of beads (GST-GS versus C1t-GST-GS). For C759–1212, C794–1212, and C970–1212, these quantities are much higher with C1t-GST-GS when compared with GST-GS (lane 3 versus lane 2 and lane 6 versus lane 5). Quantification of prey-derived signals (Fig. 5C) reveals that the differences are 35-fold for C759–1212 (specific binding 2.5%), 20-fold for C794–1212 (specific binding 0.7%), and 10-fold for C970–1212 (specific binding 3.0%). For C1072–1212, on the other hand, specific binding was low regardless of the buffer used during incubation. These results confirm that C1t can interact with C2t in vitro, and they also suggest that a 102-residue segment in C2t is required for the association.

Phosphorylation of the prey with protein kinase A or a mixture of protein kinase Cs did not affect the relative quantities of radioactive proteins that can associate with GS beads (results not shown). In these studies, however, [32P] phosphorylation of prey synthesized with cold Met could not be detected by autoradiography (results also not shown). Thus, C1t or C2t may not be phosphorylatable in this in vitro system, or the quantity of [32P] incorporated in the prey may have been below detection threshold.
protein segment is no longer able to induce strong β-galactosidase activity in yeast when coexpressed with C2t.

Left alone, the identification of a TQAL site in a self-interacting region of NKCC1 is an intriguing finding because it points toward novel mechanisms by which CCCs assemble with one another. Indeed, forkhead-associated binding domains have been shown to act as phosphopeptide-binding modules that enable phosphorylation-dependent protein-protein interactions (36). Based on the presence of a TQAL site in essential-interacting-region-1 and of a putative PKA-2 site in essential-interacting-region-4, it is tempting to postulate that C2t is the phosphopeptide-containing epitope of C1t. Unfortunately, the \textit{in vitro} system used in this work was not adequate to address such a possibility. In addition, the involvement of the C terminus in phosphoregulation of NKCC1 has not been examined in depth thus far. It will therefore be a challenging task in future studies to determine whether self-interactions within the NKCC1 C terminus are phosphorylation-dependent.

The uncovering of large NKCC1 segments that behave as binding partners \textit{in vitro} and that belong to distinct segments of the C terminus allows us to make speculations regarding the various conformational states that could be assumed by this region of the transporter. These speculations are illustrated in Fig. 6 through simplified representations of the cytosolic C terminus, assuming that the C\textsubscript{759–923} region corresponds to one binding site and the C\textsubscript{994–1211} region to another binding site. The models proposed in this figure are also based on structural predictions derived from hydrophaty plot analyses of huNKCC1 (3, 5, 39), and thus, they presuppose that the interacting segments are both on the cytosolic side of the lipid bilayer and interrupted by a segment that is also cytosolic.

The first type of structural configuration that could be supported by a C1t-C2t interaction involves the formation of intramolecular contacts between C\textsubscript{759–923} and C\textsubscript{994–1211} (Fig. 6A). As a result of such contacts, it is predicted that the C terminus would acquire a globular conformation with its distal end lying relatively close to the central hydrophobic domain. It is also predicted that this configuration would be important in defining accessibility of the C terminus to other proteins. For example, the acquisition of intramolecular bonds between C\textsubscript{759–923} and C\textsubscript{994–1211} could prevent the formation of intermolecular bonds between the same regions and force the cotransporter to adopt a monomeric state or interact differently with its usual regulatory partners.

The formation of intermolecular contacts between C\textsubscript{759–923} and C\textsubscript{994–1211} is at the basis of another type of configuration that could be supported by the cytosolic C terminus. In fact, the occurrence of such contacts would signify that NKCC1 is organized as a homo-oligomeric structure in the cellular environment. Here, in addition, it is predicted that different types of high order structures could be formed, taking again into consideration the presence of two interconnecting sites that belong to distinct domains of the C terminus. For one such type (Fig. 6B), the C terminus of two monomers would be interlocked through a pair of C\textsubscript{759–923}–C\textsubscript{994–1211} bonds, and for another type (Fig. 6C), the C terminus of four monomeric subunits would be assembled through two pairs of C\textsubscript{759–923}–C\textsubscript{994–1211} bonds. In the latter scenario, however, each monomer would supposedly interact with more than one monomer.

A role for C1t and C2t in intermolecular assembly can be implied more readily if the indication exists that NKCC1 homo-oligomerizes at the cell surface. Although we have not verified that this was the case, two lines of evidence favor the idea that NKCC1 does operate as a high order structure composed of more than one ion-carrying subunit. The first line of evidence is based on a general predilection shared by most ion transport systems to form homomultimers in the lipid bilayers of cells (40–42), and the second line of evidence, is based on experimental data from which a hypothetical structure definition of CCC proteins has emerged in recent years (29, 30).

The common tendency of ion transport systems to assemble as high order structures has been revealed in part by x-ray crystallography or NMR spectroscopy. For example, high resolution imaging studies have demonstrated that Shaker channels in lipid bilayers exist primarily as homodimeric and homotetrameric structures, the assemblies of which are critically driven by a conserved self-interacting domain in the N terminus (43). For another ion transport system, band 3, such studies have also revealed a clear preference for homodimerization and homotetramerization at the cell surface, but unlike Shaker channels, assembly of HCO\textsubscript{3}/Cl\textsuperscript{−} exchangers is supported by C-terminally located binding arms (44). It is now recognized that several other solute carriers, e.g. the Na\textsuperscript{+}/H\textsuperscript{+} exchangers (40), the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (45), etc., are organized as homo-oligomeric structures.

Evidence showing that members of the CCC family are also defined structurally as homo-oligomeric units has come mainly from cross-linking studies. As mentioned earlier, Moore-Hoon and Turner (30) have observed that the molecular size of NKCC1 could change by a 2-fold factor when membranes ex-
pressing this carrier were treated with cross-linking agents; of importance, these chemically induced complexes were found to contain no other proteins besides NKCC1. In the course of such studies, Moore-Hoon and Turner (30) also observed that the behavior of a mutant rat NKCC1 lacking most of the cytosolic N terminus (NKCC1_{1212-1205}) was similar to that of the wild type carrier. Taken together, these findings favor the idea that C1t and C2t are involved in intermonomeric associations.

In the absence of crystallographic data, it is not possible to determine with certainty whether NKCC1 has a preference for the monomeric or multimeric state, and thus, whether C1t and C2t are involved in intra- or intermolecular interactive processes. Based on the properties generally ascribed to self-interacting domains, however, it appears unlikely that the sole purpose of C1t and C2t would be to assist NKCC1 in intramolecular folding. Indeed, whereas the latter process is primarily driven by the action of various enzymes including chaperones, foldases, and isomerases (46), higher levels of structural organization such as homo-oligomeric associations have been shown to rely essentially on the existence of correctly folded self-interacting domains (46, 47). As reported for band 3 (42), thus, it is likely that C1t and C2t allow NKCC1 to adopt a variety of configurations at the cell surface by underlying homo-oligomerization of the carrier, perhaps in a regulated fashion.

The cytosolic C terminus of CCC family members is highly conserved, especially among the KCC isoforms (9, 48). Several of these members, in addition, have been shown to coexist in the same cell types (1–4, 48, 49). Thus, the findings reported here raise the interesting question of whether C1t and C2t could also play a role in hetero-oligomeric associations between different CCC members. Such associations have been shown to bring about diversity in the pharmacologic and kinetic properties of various ion channels (50). For the CCCs, similarly, hetero-oligomerization could potentially lead to the formation of structures with unique functional characteristics differing from those of the parent homo-oligomers. At the same time, this possibility raises additional questions regarding the true functional signature of individual CCCs based on previous characterizations.

In conclusion, the identification of self-interacting domains in the C terminus of NKCC1 represents a very important finding in the research area of cation-Cl\textsuperscript{-} cotransport, providing new information in relation to the tertiary and quaternary structure of CCC proteins and leading the way to future inves-
tigations aimed at determining the mechanisms and specificity of molecular interactions for these transport systems.

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Self-interacting Domains in the C Terminus of a Cation-Cl⁻ Cotransporter Described for the First Time
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