Homooligomeric and Heterooligomeric Associations between K\(^{+}\)-Cl\(^{-}\) Cotransporter Isoforms and between K\(^{+}\)-Cl\(^{-}\) and Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) Cotransporters*\(\text{!!}\)

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Little is known regarding the quaternary structure of cation-Cl\(^{-}\) cotransporters (CCCs) except that the Na\(^{+}\)-dependent CCCs can exist as homooligomeric units. Given that each of the CCCs exhibits unique functional properties and that several of these carriers coexist in various cell types, it would be of interest to determine whether the four K\(^{+}\)-Cl\(^{-}\) cotransporter (KCC) isoforms and their splice variants can also assemble into such units and, more importantly, whether they can form heterooligomers by interacting with each other or with the secretory Na\(^{+}\),K\(^{+}\)-Cl\(^{-}\) cotransporter (NKCC1). In the present work, we have addressed these questions by conducting two groups of analyses: 1) yeast two-hybrid and pull-down assays in which CCC-derived protein segments were used as both bait and prey and 2) coimmunoprecipitation and functional studies of intact CCCs coexpressed in Xenopus laevis oocytes. Through a combination of such analyses, we have found that KCC2 and KCC4 could adopt various oligomeric states (in the form of KCC2-KCC2, KCC4-KCC4, KCC2-KCC4, and even KCC4-NKCC1 complexes), that their carboxyl termini were probably involved in carrier assembly, and that the KCC4-NKCC1 oligomers, more specifically, could deploy unique functional features. Through additional coimmunoprecipitation studies, we have also found that KCC1 and KCC3 had the potential of assembling into various types of CCC-CCC oligomers as well, although the interactions uncovered were not characterized as extensively, and the protein segments involved were not identified in yeast two-hybrid assays. Taken together, these findings could change our views on how CCCs operate or are regulated in animal cells by suggesting, in particular, that cation-Cl\(^{-}\) cotransport achieves higher levels of functional diversity than foreseen.

The cation-Cl\(^{-}\) cotransporter (CCC)\(^3\) family includes Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporters (NKCCs) (1–3) and K\(^{+}\)-Cl\(^{-}\) cotransporters (KCCs) (4–8) that are all expressed at the cell surface. The NKCCs exist as two isoforms (1–3), and the KCCs exist as four isoforms and several splice variants (at least five for KCC3 and two for KCC1) (4–11). All of these structures are predicted to contain 12 transmembrane domains flanked by cytoplasmic termini (1, 12–16).

In mammals, NKCC1 as well as KCC1, -3, and -4 have been shown to exhibit wide tissue distributions, whereas KCC2 is apparently confined to the nervous system (4–11, 16–21). They have also been shown to coexist in certain cell types, such as erythrocytes or lens cells, where a number of isoforms/variants (KCC1, KCC3, KCC4) have been identified (10, 11). In certain tissues, localization studies have suggested a more differential distribution (9, 16–21).

Although very homologous to each other, the KCCs display variant affinities for each of the transported ions and for the drug furosemide. In addition, their transport capacity and response to various stimuli are not the same under controlled conditions. In Xenopus laevis oocytes, for example, heterologously expressed KCC2 displays higher \(K_m\) values for Cl\(^{-}\) but lower \(K_m\) values for Rb\(^{+}\) compared with KCC1 and KCC3 (16, 22–25). Along the same line, KCC4 is less active than KCC2 at low levels of intracellular Cl\(^{-}\) but more sensitive to phorbol ester-triggered events (26). Not surprisingly, differences between KCCs and NKCCs are even more pronounced (4, 16, 27).

Several lines of evidence suggest that the NKCCs exist as homooligomers in cells. They are as follows. 1) The size of NKCC1 and NKCC2 has been found to increase by a \(~2\)-fold factor when membranes expressing either protein were treated with cross-linking agents (12, 28). 2) Through GST pull-down assays and yeast two-hybrid mapping analyses, the cytosolic carboxyl terminus (Ct) of the NKCCs was found to harbor two domains that are endowed with self-interacting properties.

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\(\text{The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 51 and Figs. 51 and 52.}\)

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The abbreviations used are: CCC, cation-Cl\(^{-}\) cotransporter; Ct, carboxy terminus; HA, hemagglutinin; KCC, K\(^{+}\)-Cl\(^{-}\) cotransporter; Nt, amino terminus; NKCC, Na\(^{-}\)-K\(^{-}\)-Cl\(^{-}\) cotransporter; TM, transmembrane; Glu, glutamic acid; KCC4, KCC2-KCC4, and even KCC4-NKCC1 complexes), that their carboxyl termini were probably involved in carrier assembly, and that the KCC4-NKCC1 oligomers, more specifically, could deploy unique functional features. Through additional coimmunoprecipitation studies, we have also found that KCC1 and KCC3 had the potential of assembling into various types of CCC-CCC oligomers as well, although the interactions uncovered were not characterized as extensively, and the protein segments involved were not identified in yeast two-hybrid assays. Taken together, these findings could change our views on how CCCs operate or are regulated in animal cells by suggesting, in particular, that cation-Cl\(^{-}\) cotransport achieves higher levels of functional diversity than foreseen.

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implying that these carriers could assemble into pairs of monomers (14, 15). 3) In heterologous expression systems, NKCC2 has been shown to coimmunoprecipitate with NKCC2 and NCC with NCC (29, 30).

The structural unit of the Na\(^+\)-independent CCCs is currently unknown. Based on the oligomeric configuration of the NKCCs and a recent study by Blaesse et al. (31),\(^4\) one might predict that each of the KCCs can also exist as homooligomeric units assembled through the distal portion of single monomers. At the same time, it is unclear that heterooligomeric associations between isoforms would be supported by the Ct given that the KCCs, although quite homologous to one another, have still diverged to some degree from a common ancestor.

In this work, various studies were conducted to define the high order structure of the KCCs. In particular, we found that several of these carriers can assemble not only into homooligomers but also into functionally relevant heterooligomers enclosing different CCCs. Such findings bear great implications as to how CCCs actually operate in native tissues or as to how their inactivation in various disorders leads to specific phenotypes. They are also crucial from the perspective of drug development for individual CCC members.

**EXPERIMENTAL PROCEDURES**

**Supplies**—Chemicals, reagents, or kits were from different companies and included monoclonal antibodies (Roche Applied Science and Amersham Biosciences), oligonucleotides (Sigma), enzymes/buffers used for cDNA constructions (New England Biolabs, Fermentas, and Invitrogen), the TNT rabbit reticulocyte lysate system (Promega), the GST SpinTrap Purification Module (Amersham Biosciences), and the Yeast Matchmaker Two-hybrid System (Clontech). All cDNAs were propagated in XL1 blue cells (Stratagene).

**Constructs**—All of the cDNA sequences and oligonucleotides used are listed as supplemental material in Table 1S. Several of these sequences (rabbit KCC1, rat KCC2, human KCC3A and KCC3B, mouse KCC4, and human NKCC1) were already cloned in the appropriate vector from previous work (13–15, 25, 26).

**CCTs in pGilda or pB42AD**—Several CCC-encoding cDNAs were used for the yeast studies. They were designed with the premise that their localization is cytosolic based on previous hydrophathy analyses (4–8, 14, 15, 25, 26, 36) and that the KCCs might also form oligomeric units by interacting through their Cts as shown for NKCC1 (14, 15). Regions minimally required for the NKCC1-NKCC1 interaction to occur are shown in Fig. 1S (provided in this work as supplemental material), where they are aligned as *scaled horizontal bars* with the various CCT-derived protein segments used.

Based on these alignments, corresponding regions in the KCCs were tested in yeast to determine whether they were also endowed with interacting properties. After we realized that they were not (see below), additional protein segments from the cytosolic Cts and amino terminus (Nt) were tested. In particular, the Ct-derived protein segments (some of which are also illustrated in Fig. 1S as *scaled horizontal bars*) were all longer than the deduced interacting regions except for one, and all belonged to a large domain that extends from transmembrane domain 12 to the end of the Ct.

pGilda and pB42AD are the yeast vectors in which inserts were cloned; one has coding sequences for the LexA DNA-binding domain and a His transformation marker (TM), and the other has sequences for the activating domain of a transcription factor, the hemagglutinin (HA) tag, and a Trp\(^\text{TM}\). The CCC inserts were obtained from several constructs by PCR through appropriate primers to which restriction sites were added. Final constructs were generated by cloning inserts in empty or insert-enclosing vectors through one or several ligation step(s).

**KCC4-(921–1057)/pGEX4T3**—To generate a bait protein for GST pull-down assays, the insert of KCC4/pPol1 was cut with restriction enzymes (SacI at bp 1965 and Xhol at bp 3175), and one of the fragments (length 1206 bp) was transferred to the vector pGEX4T3 (between restriction sites Smal and Xhol after blunting SacI with Klenow and dNTPs). The resulting construct was recut at two Smal sites (to exclude a 2758-bp fragment encoding most of KCC4), and the vector-containing fragment, which encodes the distal Ct of KCC4 between residues 921 and 1057, was recircularized to generate KCC4-(921–1057)/pGEX4T3 (see bottom bar of Fig. 1S).

**CCTs in pCDNA3**—To generate prey proteins for GST pull-down assays and to express KCC4 in HEK-293 cells for protein studies, two additional constructs were used. One of these constructs, termed NKCC1-(759–1212)/pCDNA3 and available from previous work (14), encloses a fragment that encodes residue stretch 759–1212 in the carrier’s Ct. The other construct, termed KCC4/pCDNA3, was produced by transferring the insert of KCC4/pPol1 (previously tagged NH\(_2\)-terminally with c-myc; see Ref. 26) as a 3312-bp HindIII-Smal fragment between the EcoRV sites of pCDNA3, blunting HindIII with Klenow and dNTPs before the ligation.

**CCTs in Oocyte Vectors**—Several constructs were used for protein analyses and functional studies in *X. laevis* oocytes. They were available from previous work (25, 26) or engineered *de novo* for the current work. The inserts correspond to CCT-encoding sequences (cloned in pPol1 or pGEM-HE) and to the human Na\(^+\)-glucose transporter type 1 (SGLT1)-encoding sequence (transferred for this work from PBS to Pol1 as an EcoRI fragment). The vectors, on the other hand, correspond to modified pGEMs from which stable cDNA-derived cRNAs can be produced in *X. laevis* oocytes. They contain a T7 bacterial promoter, a cloning site flanked by the *X. laevis* \(\beta\)-globin untranslated regions, a poly(A) tract, and an Nhel linearizing site.

A c-Myc epitope tag (MEQKLISEEDL) or HA epitope tag (MYPYDVPDYA) was added in front of several coding sequences through the following sequential steps: 1) by cutting these sequences (in their vector) at a restriction site “x” just before the first ATG and at a nearby downstream restriction site “y”; 2) by ligating the resulting fragments to a pair of complementary oligonucleotides designed to encode the tag in frame and to possess \(x\)- and \(y\)-compatible single-stranded
cDNA ends once hybridized. The 5′–3′ restriction sites used for rabbit KCC1, rat KCC2, human KCC3A, and human SGLT1 were, respectively, XbaI-HincII, Xhol-NarI, XbaI-NsiI, and Xmal-EcoRI. Note that tagged mouse KCC4/PoI and tagged human NKCC1/PoI were already available from previous studies (26).

**Yeast Two-hybrid Mapping Studies**—Cotransformants were generated and analyzed as previously described (14, 15). In brief, the following steps were pursued. 1) EGY48 yeast (Ys) were transformed with the p8op-lacZ UraTM-enclosing vector (which can encode LeuTM and lacZ through built in LexA-dependent operators) and were seeded on −Ura (−U) plates to generate YOULHs. 2) YOULHs were transformed with a CCC/pGilda and were seeded on −Ura−His (−UH) plates to generate YOULHs. 3) YOULHs were transformed with a CCC/pB42AD to generate YOULHS, after which they were tested for their ability to grow on −Ura−His−Leu−Trp (−UHLT) plates or express β-galactosidase activity on −UHT plus X-galactosidase (−UHT + X) plates.

Two procedures were conducted to establish whether the behavior of YOULHs seeded on relevant plates could be ascribed to the occurrence of CCC-CCC interactions. 1) YOULHs were test for expression of the hybrid protein and for autonomous reporter gene activation on −UHL + X plates. 2) YOULHs were transformed with a CCC/pB42AD and tested for expression of a hybrid protein after selection of colonies on −UT plates as well as for autonomous gene activation on −UTL + X plates.

**Expression of CCCs in X. laevis Oocytes or HEK-293 Cells**—Mature oocytes were injected with H2O (controls) or cDNA-derived cRNAs (~15 ng for the KCCs and ~1 ng for NKCC1 unless mentioned otherwise) and maintained for 3 days at 18 °C in Barth medium plus 125 μM furosemide (15, 25, 32, 33). Native HEK-293 cells were subjected first to a pulse-chase experiment (34) in 100-mm dishes to label newly formed proteins through sequential incubations in 1) Met-free DMEM low (30 min), 2) Met-free DMEM low plus 100 μCi/ml [35S]Met (120 min), and 3) phosphate-buffered saline wash solutions. Subsequently, they were incubated in 12 ml of DMEM low plus 2.5 mM Met, transected through standard protocols (13, 35, 36) with KCC4 or pCDNA3 (25 μg of cDNA plus 60 μl of Lipofectamine™ 2000 and 3 ml of DMEM low), and maintained in the same (but cDNA-free) medium for 48 h.

**Pretreatment of X. laevis Oocytes**—In some cases, injected oocytes were subjected to another, ~2-h protocol and used for functional studies (Fig. 8) or localization analyses (Fig. 5). As previously described (15, 25, 26, 32, 33), this protocol included sequential incubations (at ~22 °C) in various media (detailed in Table 1) as follows: 1) removal of furosemide through rinses in Barth medium; 2) rinses in a low Cl− hypertonic medium (LH+); 3) 1-h incubations in medium LH+ (to stimulate NKCC1 but keep KCC inactive) with or without 1 mM Sulfo-NHS; 4) rinses in a normal-Cl− isotonic medium (R); 5) 45-min incubations in medium R with or without 1–2 μCl/ml 86Rb+; and 6) several rinses in a wash medium.

**Functional Studies**—Pretreated oocytes incubated in 86Rb+-containing solutions were transferred to 96-well plates prefilled with 2% SDS and scintillation fluid. After a brief stabilization period, 86Rb+ was detected with the TopCountNXT counter (Packard). For each condition tested, counts were averaged and converted into flux rates as explained in the legend to Fig. 8.

**Immunolocalization Studies**—Pretreated X. laevis oocytes expressing one or two different CCCs heterologously were examined by immunofluorescence as previously described (15, 26, 36). Anti-c-Myc or HA was used as primary antibody and Alexa Fluor-conjugated IgGs were used as secondary antibodies. Note that H2O-injected oocytes were used as negative controls.

**Protein Analyses**—Proteins were extracted from yeast, oocytes, or HEK-293 cells in lysis buffers supplemented with protease inhibitors (Table 1). In some cases, cell lysates (from nonpretreated oocytes) were subjected (at 4 °C in the same lysis buffer) to 1-h incubations with anti-c-Myc or anti-HA (1:100) followed by another 30-min incubation with 2% protein-Sepharose A (Amersham Biosciences) and centrifugation-wash cycles. In one series of assays, cell lysates (from oocytes exposed to Sulfo-NHS) were incubated at 4 °C with 4% agarose-coupled streptavidin before the centrifugation-wash cycles.

In all studies, pellet-bound or solubilized proteins from whole cell extract were incubated for 2 min at 100 °C in protein sample buffer, migrated on polyacrylamide SDS-Tricine gels, and revealed by Coomassie Blue staining, direct autoradiography, or chemiluminescence (Amersham Biosciences). For the latter detection procedure, proteins were first transferred to Immobilon-P nylon blots (Millipore), where they were incubated sequentially with a primary and a secondary antibody. When appropriate, whole-cell extract proteins were quantified with the DC protein assay (Bio-Rad), and protein bands separated by gel electrophoresis were quantified numerically.
induction, isolated by sonication in pBS, and immobilized
on glutathione-coupled Sepharose beads. Radioactive
proteins (used as prey) were synthesized from CCC/pCDNA3 in reticu-
locyte lysates. The final reaction solution was as follows (in 50 µl):
1 µg of cDNA, 40 µl of reticulocyte lysate mixed with amino
acids, and 0.4 mCi/ml [35S]Met or 20 µl cold Met. The assays
per se were carried out as in Ref. 14 by incubating −4 µg of
bead-immobilized proteins with 10 µl of labeled prey for −6 h
at 4 °C in a binding buffer (200 mM NaCl, 1 mM EDTA, 2.0%
Triton X-100, and 20 mM Tris-HCl) followed by several washes
in the same buffer supplemented with 800 mM NaCl.

Reverse Transcription-PCR Analyses—To determine which
KCCs are expressed in HEK-293 cells, gene-specific primers
(see Table 1S in the supplementary material) mixed with
cDNA templates (prepared from HEK-293 cells or present in
a Clontech human kidney library) were diluted in a PCR
buffer (50 µl total) and subjected to 35 rounds of thermal
cycling (annealing temperatures 45–55 °C). After amplifica-
tion, one-fifth of the PCR products were migrated on ethidium bromide-stained agarose gels.

Sequence Analyses and Statistics—DNA characterizations
were performed by restriction analyses and automated
sequencing using vector- or CCC-specific primers. For DNA or
protein sequence analyses, we used a combination of programs,
including DNASTar (Lasergene) and online bioinformatic work
tools. When pertinent, all data (band densities, flux rates, etc.)
were averaged among replicate experiments and expressed as
mean values ± S.E. In some instances, differences between
various groups of variables were also analyzed by Student’s two-tailed t
tests, rejecting the null hypothesis for p > 0.05.

RESULTS

Yeast Two-hybrid Studies—Forty-eight protein segments
were tested for their ability to support interactions in yeast.
Various residue stretches belonging to the putative cytosolic Ct
of various CCCs (NKCC1, KCC1, KCC2, KCC3, and KCC4)
were included in 38 of these protein segments, 19 of which were
fused to LexA and 19 to HA. Those used to illustrate the behav-
ior of yeast are depicted through horizontal bars in Fig. 1S and
are termed according the accompanying figure legend). The
remaining protein segments (not illustrated in this work)
included the putative cytosolic Nts of KCC1, KCC2, KCC3A,
KCC3B, and KCC4.

As a prerequisite to the testing of bait-prey interactions in
this system, Western analyses (conducted as described in the
legend to Fig. 1S) confirmed that yeast transformed with any of
the 48 CCC-encoding constructs and selected on appropriate
plates were able to express hybrid proteins even in the absence
of reporter gene activation (results not shown). As another pre-
requisite to the testing of interactions in this system, various
analyses also confirmed that none of the LexA- or HA-coupled
Ct-derived protein segments formed in yeast were able to auto-
induce the reporter genes (results also not shown). Unfortu-
nately, this was not the case for the CCC-derived Nt protein
segments, which were all shown to activate the reporter genes
autonomously and thus could not be used in further analyses.

The behavior of YOULHTs (yeast transformed with the con-
struct p8op-lacZ and different combinations of protein seg-
ments as described under “Experimental Procedures”) is shown
through representative examples in Fig. 2S (provided as supple-
mental material along with Fig. 1S to illustrate the protein seg-
ments used). In each horizontal line, a unique transformant
coeexpressing a pGilda- and a pB42AD-derived protein segment
(columns i and ii, respectively) was seeded on −UHTL plates
and −UHT + X plates (columns iii and iv, respectively), where
the occurrence of an interaction is suggested by the ability of
yeast to develop into colonies or to generate strong β-galacto-
sidase activity.

When the coexpressed protein segments were from KCC1 or
KCC3, we observed that the reporter genes Leu⁴⁺/lacZ failed to
become activated whether the expressed residue stretch included
a truncated Ct, a full-length Ct, or an extended Ct and
whether they were from the same isofrom or from both of these
isosforms. Relevant examples are provided in Fig. 2S (1) along
with Fig. 1S to illustrate the protein segments used. The other
combinations tested (over 50) were carried out with additional
protein segments that varied in length but included at least one
putative oligomerization domain based on alignments with
NKCC1. Among other possibilities, these findings suggest that
KCC1 and KCC3 are devoid of self-interacting properties, that
their assemblies into quaternary structures are not mediated by
the Ct, unlike NKCC1 (14), or that factors required for the
association of certain CCC domains are lacking in yeast.

When, on the other hand, the protein segments coexpressed
in YOULHTs were from KCC2 or KCC4, we found that they were
able to induce Leu⁴⁺/lacZ as long as both corresponded to
full-length Cts. Fig. 2S (2) shows representative examples,
including YOULHTs rendered β-galactosidase-positive and resis-
tant on −Leu plates by the expression of KCC2-(640–1116)
with KCC2-(640–1116), KCC2-(640–1116) with KCC4-(661–
1083), or KCC4-(661–1083) with KCC4-(661–1083). Even if
these findings do suggest that certain KCCs can form homool-
genic units in cells, as suggested in part by the yeast data, coimmunoprecipitation studies
show that none of the YOULHTs tested behave as if bait-prey
interactions have occurred, in contrast to a positive control that
consisted of YOULHTs expressing the proximal and distal Ct of
NKCC1 (14). These results suggest on more that the localization
or binding requirements of oligomerization domains vary
among the carriers or that KCC1 and KCC3 do not behave as
self-interacting structures.

Homooligomeric Assemblies between KCCs—To confirm that
certain KCCs can form homooligomeric units in cells, as sug-
gested in part by the yeast data, coimmunoprecipitation studies
were carried out with oocyte lysates expressing each isofrom as
a c-Myc- and/or HA-tagged protein. Results are shown in Fig. 1,
A–D, but only for KCC1 and KCC3A, since they were similar
for all of the isofroms tested (including KCC3B). In each panel,
lanes 2 served as controls to establish that the signals observed
in lanes 1 were not due to the antibody used for immunopre-
Coimmunoprecipitation studies using *X. laevis* oocytes coexpressing differentially tagged but otherwise identical KCC isoforms. Oocytes were microinjected with cRNAs derived from epitope-tagged cDNAs and incubated 3 days in Barth medium before cell lysis. *A–D*, proteins were from total cell lysates (lanes 2) or corresponded to protein-Sepharose A-bound antigens (lanes 1, 3, and 4). Isolforms expressed were c-Myc-tagged and/or HA-tagged KCC1 (A and B) and c-Myc-tagged and/or HA-tagged KCC3A (C and D). Studies carried out with oocytes expressing c-Myc-tagged KCC2 and HA-tagged KCC2 or expressing c-Myc-tagged KCC4 and HA-tagged KCC4 revealed identical results (not shown). For each of the panels shown, lysates were obtained by solubilizing oocytes in a protease inhibitor-supplemented solution (Table 1), and immunoprecipitated antigenes were obtained by incubating these lysates with a primary antibody (mouse anti-c-Myc or anti-HA) and protein-Sepharose A. The quantity of solubilized protein was ~100 μg/lane, and the quantity of immunoprecipitated proteins was from ~1 mg of total lysates. Detection was carried out by chemiluminescence assays (exposure times of ~1 min) after incubating the blots sequentially with mouse anti-c-Myc (1:400) or mouse anti-HA (1:1000) and a horseradish peroxidase-coupled anti-mouse IgG (1:5000). Each of the blot images included in this figure was chosen to illustrate the results of 3–4 experiments. Note that in the examples provided, differences between the intensities of certain antigens were specific, i.e., a direct c-Myc-HA interaction by conducting similar experiments (compare lanes 2 versus lanes 1 in B or C) suggest that coimmunoprecipitation efficiency was unexpectedly high. At the same time, less pronounced differences in other experiments (~5-fold) imply that certain factors (quantity of samples loaded per lane, protein degradation, etc.) might have accounted for some of the findings. Importantly, however, the results of longer exposure times always failed to reveal signals for the negative controls. Note also that in most lanes, positive signals appear as broad doublet-like bands, as would be expected for a CCC family member (1, 12, 17, 20, 29, 36). Note also that in D, the signal of lane 1 appears as a narrower band with altered electrophoretic mobility compared with lane 2, suggesting that a specific form of post-translationally modified carrier might have been selected for through immunoprecipitation. On the other hand, it should be noted that longer exposure times revealed a broad doublet-like band in this lane as well, indicating that the carrier immunoprecipitated had not been completely confined to intracellular domains. M, c-Myc; H, HA; IP, antibody used for immunoprecipitation.

![Diagram](https://via.placeholder.com/150)
Structural Organization of the CCCs

![Diagram](image_url)

**FIGURE 3.** Coimmunoprecipitation studies using *X. laevis* oocytes coexpressing different CCC isofoms. Oocytes were microinjected with cRNAs derived from epitope-tagged cDNAs and were incubated for 3 days in Barth medium before cell lysis. Proteins were from total cell lysates (lanes 2) or protein-Sepharose A-bound antigens (lanes 1, 3, and 4). Isolates expressed were c-Myc-tagged NKCC1 with or without HA-tagged KCC1 (A), or they were HA-tagged KCC1 with or without c-Myc-tagged NKCC1 (B). The number of experiments from which illustrative blot images are shown, the results of longer exposure times, and the overall conditions employed were also as described in the legend to Fig. 1. Although differences between the intensities of certain bands on a given blot tended to vary among experiments (as in Figs. 1 and 3), the positive and negative controls revealed that the signals observed, as well as the antibodies used, were all specific here as well. M, c-Myc; H, HA; IP, antibody used for immunoprecipitation.

To determine whether Na$^{+}$-independent CCCs interact with each other to form heterooligomers, coimmunoprecipitation studies were conducted once more as above except that each oocyte was microinjected with different combinations of CCC isofoms (one c-myc-tagged and the other HA-tagged). Results, which are shown in Fig. 3, A and B, reveal specific bands in all lanes regardless of which antibody was used as AbI or for detection. Various controls, such as those used to test for KCC homooligomerization, also confirmed that the bands observed and antibodies used were specific.

**FIGURE 4.** Coimmunoprecipitation studies using *X. laevis* oocytes coexpressing different CCC isofoms. Oocytes were microinjected with cRNAs derived from epitope-tagged cDNAs and were incubated for 3 days in Barth medium before cell lysis. Proteins were from total cell lysates (lanes 2) or protein-Sepharose A-bound antigens (lanes 1, 3, and 4). Isolates expressed were c-Myc-tagged NKCC1 with or without HA-tagged KCC1 (A), or they were HA-tagged KCC1 with or without c-Myc-tagged NKCC1 (B). The number of experiments from which illustrative blot images are shown, the results of longer exposure times, and the overall conditions employed were also as described in the legend to Fig. 1. Although differences between the intensities of certain bands on a given blot tended to vary among experiments (as in Figs. 1 and 3), the positive and negative controls revealed that the signals observed, as well as the antibodies used, were all specific here as well. M, c-Myc; H, HA; IP, antibody used for immunoprecipitation.

HA-KCC1 alone, c-myc-NKCC1 and HA-KCC1, or c-myc- NKCC1 and HA-KCC4. Results, which were identical for both CCCs, are presented in Fig. 4 for KCC1 only. Quite interestingly, it is seen through this example that KCC1 and NKCC1 can be coimmunoprecipitated with either anti-c-Myc- or anti-HA (lanes 4), that the observed bands are carrier-specific (lanes 1 versus 2), and that the Ab,s used were specific as well (lanes 1 versus 3).

**Localization of CCCs in Oocytes—Immunofluorescence studies and Western analyses of cell surface–biotinylated CCCs were performed to determine whether the cellular localization of a CCC in amphibian cells was affected substantially by the coexpression of another CCC and, thus, to exclude the possibility that the interactions identified only took place in subcellular structures.** Fig. 5 is used to illustrate the data, but only for KCC1 and NKCC1 in A (similar results were obtained for other combinations, including KCC4 and NKCC1) and only for KCC4 and NKCC1 in B.

The immunofluorescence studies, which are shown in Fig. 5A, reveal that the carriers tested can reach the cell surface or a compartment just beneath it, whether they are expressed alone (Fig. 5A, 3 and 6) or in combination (Fig. 5A, 7 and 8), and that the signals observed are of similar intensity among these subpanels. Regarding the Western analyses, which are shown in Fig. 5B with the data of three separate experiments, they reveal that the quantity of cell surface c-Myc-NKCC1 (corresponding to the upper bands of the middle subpanel) only decreases modestly in the presence of KCC4 (compare lanes 2 versus lanes 3 or lanes 4). In conjunction with the coimmunoprecipitation studies, which show high levels of oligomeric formation in oocytes (see Figs. 1, 3, and 4), the localization studies suggest that the cell surface of oocytes do express CCC-enclosing oligomers.
Analyses of CCC-CCC Interactions Using Other Approaches—
Because the yeast studies could have led to false positive or negative results, as they can do at times (14, 37, 38), and because the interactions described thus far were identified primarily in the yeast or oocyte environment, we exploited other experimental systems to confirm our main findings and determine whether other interactions could have been missed. Results for these additional studies are described below and shown through representative experiments in Figs. 6 and 7.

One of the assays exploited was to pulse-label native CCCs in HEK-293 cells (KCC1, KCC4, and NKCC1 but not KCC2 and KCC3, according to Fig. 6A), to incubate the cells in a chase medium, to transfect them with c-myc-KCC4, and to carry out coimmunoprecipitation studies with anti-c-Myc and T₄, a NKCC-specific antibody (39). Based on Fig. 6, we observe that c-Myc-immunoprecipitated c-Myc-KCC4 can be revealed with anti-c-Myc (Fig. 6B), confirming heterologous protein synthesis and that it can associate with endogenous, 125–175-kDa radioactive proteins (Fig. 6C) but that it cannot be coimmunoprecipitated with detectable quantities of NKCC1 (Fig. 6D). At the same time, however, Fig. 6 shows that T₄-immunoprecipitated NKCC1 can be detected with anti-c-Myc (Fig. 6E), suggesting that the radioactive proteins in Fig. 6C probably included endogenous NKCC1 in addition to endogenous KCC4. It should be mentioned that T₄ has often worked poorly in our hands when used as a detecting antibody in coimmunoprecipitation studies (36).

The other assay exploited was to determine whether radioactive NKCC1-(759–1212) or KCC4-(1–1083) could interact with GST-KCC4-(921–1057) more than it could with GST. The results of these studies, which were identical among three experiments and are presented in Fig. 7 (through Coomassie-stained and autoradiographed protein gels that were loaded with washed protein-coupled Sepharose mixtures), showed that the beads were highly enriched in GST-KCC4-(921–1057) or GST (Fig. 7A) but retained prey only when enriched in GST-KCC4-(921–1057) (Fig. 7B). Taken together, these results confirm the KCC4-KCC4 and KCC4-NKCC1 interactions and suggest that the yeast studies did lead to false negatives.

Functional Studies in Oocytes—To determine whether some of the oligomers identified could behave as functionally relevant structures, we performed pilot experiments in which CCC-dependent ⁸⁶Rb⁺ transport rates were measured in oocytes expressing NKCC1, KCC4, or both of these transporters under conditions that were intended to stimulate transport rates for NKCC1 but not for KCC4. The results of these studies, which are summarized in Fig. 8, show that both NKCC1 and KCC4 behave as predicted under such conditions but, interestingly, that transport rates by oocytes expressing both NKCC1 and KCC4 increase 2-fold relative to those by ⁸⁶Rb⁺-transfected NKCC1 but maintain KCC4 inactivated and lysed in a protease inhibitor-supplemented solution at the end of the procedure (Table 1). The quantity of streptavidin-immunoprecipitated proteins was from −1 mg of total lysates. Detection was carried out by chemiluminescence assays (exposure times of −1 min) after incubating the blots sequentially with mouse anti-c-Myc (1:50) and a horseradish peroxidase-coupled anti-mouse IgG (1:5,000). Each of the micrographs or blot images shown was chosen to illustrate the results of 3–4 experiments. M, c-Myc; H, HA; IP, antibody used for immunoprecipitation; *, RNA of HA-tagged KCC4 diluted 1:5 (−3 ng total) relative to the quantity used in all other experiments.

5 As explained in a previous study by our group (14), translation of NKCC1-(759–1212) in rabbit reticulocyte lysates produces two major bands. One band migrates at −50 kDa and probably corresponds to residue stretch 794–1212 initiated at a non-AUG (CUG, AGC, and GUG) triplet (as can be frequently encountered in this system) (13, 41). The other band migrates at −46 kDa and probably corresponds to residue stretch 794–1212 initiated at the first AUG.
Figure 6. CCC-CCC interactions in HEK-293 cells. A, reverse transcription-PCR analyses. Oligonucleotides used are listed in supplemental material (Table 1S), and templates used (per reactions) were from 0.2 μg of total RNA (HEK-293 cells) or corresponded to −2 μg of cDNA library (human kidney). The image shown is from an ethidium bromide-stained agarose gel that was used to separate the various reverse transcription-PCR products. Bands are expected to migrate at 408 bp for KCC1, 707 bp for KCC3A, 844 bp for KCC3B, and 589 bp for KCC4. For KCC4, therefore, the band observed in the KID lane is unexplainably higher than expected. B–E, communoprecipitation studies using HEK-293 cell lysates. For these experiments, cells were first pulse-labeled with [35S]Met, after which they were incubated in a chase medium and transiently transfected with c-myc-KCC4 or pCDNA3 as control. For each of the panels shown, lysates obtained by solubilizing cells in a protease inhibitor-supplemented solution (Table 1) were incubated with mouse anti-c-Myc (1:100) or mouse T4 (1:100), an NKCC-specific antibody that does not recognize KCC4, and with protein-Sepharose A afterward. Bound antigens were revealed in gel through autoradiography (C) or after their transfer onto nylon blots through ECL detection (B, D, E), using mouse anti-c-Myc (1:250) or mouse T4 (1:500) as primary antibody and a horseradish peroxidase-coupled anti-mouse IgG as secondary antibody (1:5000). Each of the images shown was chosen to illustrate the results of two experiments. M, c-Myc; IP, antibody used for immunoprecipitation; KID, kidney; 7d (in C), 7-day exposure time before developing the autoradiogram.

Figure 7. GST pull-down assays. A, Coomassie Blue stain of an SDS-polyacrylamide Tricine gel illustrating the quantity of GST-coupled Sepharose beads loaded relative to that of GST-KCC4-(921–1057) coupled Sepharose beads (n-fold difference between band intensities is −1.5). B, autoradiogram of another Coomassie-stained gel illustrating the quantity of radiolabeled prey” (full-length KCC4 or protein segment KCC4-(1579–1212)) retained by the GST-coupled Sepharose beads relative to that retained by the GST-KCC4-(921–1057)-coupled Sepharose beads (n-fold differences between band intensities are 50 for each of the prey tested). Both the gel image and autoradiogram shown were chosen to illustrate the results of three experiments.

Discussion

In this work, communoprecipitation studies have shown that several of the CCCs (NKCC1 and KCC1, KCC2, KCC3, and KCC4) have the potential to form homooligomeric units when heterologously expressed in X. laevis oocytes and that they have the potential to form heterooligomeric units as well by interacting with each other in this cell type; in particular, we demonstrated that certain of these heterooligomers could be composed of the secretory NKCC1 linked physically to a KCC. We have also found that the some of the associations identified in amphibian cells could occur in HEK-293 cells as suggested by an experiment in which endogenous NKCC1 or KCC4 was shown to communoprecipitate with heterologously expressed KCC4.

Various studies aimed at determining whether CCC-enclosing oligomers occur in native tissues or untransfected cell lines were not conducted to complement this work, since antibodies are not available for most of the isoforms. Still, it is unlikely that such studies would have allowed resolution of this issue with certainty based on the following rationale: 1) tissue lysates from most sources contain several cell types that cannot be sorted out easily; 2) colocalization of two proteins in a cellular domain through imaging analyses is not a proof of interaction; and 3) antibodies often lack the sensitivity to detect a protein that is produced at low levels, especially if purified by immunoprecipitation beforehand (as found in this study, for instance). The latter rational also implies that immunolocalization studies in which variant tissue distributions were observed for certain CCCs (9, 16–21) should be interpreted cautiously.

Yeast two-hybrid studies and GST pull-down assays carried out to determine which domains underlie the formation of CCC-enclosing oligomers in oocytes and HEK-293 cells sug-
gest that the cytosolic Ct probably plays an important role. As will be explained below, however, these studies did not lead to the identification of interacting domains for all of the CCCs (i.e. the Ct domains of KCC1 and KCC3 were found to be devoid of self-interacting properties based on the yeast analyses). Left alone, these results would imply that the Ct domains of KCC1 and KCC3 can support the formation of NKCC1-NKCC1, KCC2-KCC2, KCC4-KCC4, or KCC2-KCC4 oligomers but not that of other CCC-CCC combinations.

Despite the yeast data, other observations obtained from previous investigations and from this work as well suggest that several, if not all, of the CCCs do have the potential of behaving as self-interacting partners. These observations are as follows. 1) All of the CCCs studied thus far (NKCC1, NKCC2, NCC, and KCC2) appear to be organized as oligomers (12, 28–31). 2) We have identified residue sequences that behave as self-interacting domains for many CCCs, including NKCC1, NKCC2, KCC2, and KCC4 (14, 15). 3) The CCCs are all highly conserved through evolution, implying that important functional domains should be found at the same locations among the isoforms. 4) In pull-down assays, used as an alternative strategy to identify oligomerization domains in the CCCs, NKCC1-(759–1212) was found to interact with KCC4-(921–1057), although it was unable to do so in yeast. 5) Our own coimmunoprecipitation studies yielded clear cut results.

One reason that might have accounted for the inability of certain Ct-derived proteins segments to interact in yeast is that the bait or prey tested were missing key residues. In this study, such a hypothesis was verified for KCC1 and KCC3 by using bait or prey that extended from transmembrane domain 12 to the end of the Ct, but interactions still failed to occur. Attempts were also made to determine whether the missing residues could belong to the proximal extremity of these carriers, but this possibility could not be tested given that the Nts of all CCCs were found to activate the yeast reporter genes. In this regard, however, a study by Moore-Hoon and Turner (12) showed that a mutant NKCC1 lacking all of its Nt was still able to assemble into homooligomers in a cell line. Accordingly, and based on the observations that have already been detailed above, it would be surprising if the Nt played an important role in carrier assembly.

An alternative reason that could explain the yeast data for KCC1 and KCC3 is that improper folding of certain protein segments in this expression system precluded associations that normally occur in vivo when carriers are intact. This conformational constraint, often incriminated as a cause of false negative results in yeast two-hybrid studies (14, 37, 38), would imply that the Ct of various CCCs might have to adopt isoform-specific structural configurations for oligomerization to occur and that the stringency of this requirement is variable among the carriers. It would perhaps also explain why the proximal and distal Ct of various CCCs failed to interact with each other in this system. Additional studies aimed at identifying oligomerization domains for all of the CCCs and at confirming that KCC1-CCCx or KCC3-CCCx oligomers do occur in various cell types will be necessary to confirm these hypotheses.

As stated earlier, various studies have shown that some cells do express more than one CCC subtype; human erythrocytes, for example, were shown to produce at least five CCC isoforms/variants (10, 11), and HEK-293 cells (through this work) were shown to produce at least three different CCCs. In view of these observations, one might predict that CCC coexpression occurs in other cell types as well, a possibility that is supported further by at least three different observations: 1) Some CCCs (NKCC1 and KCC1) are widely distributed (1, 2, 4, 16, 39); 2) gene redundancy resulting from the coexpression of functionally homologous proteins is a common feature among several cell types; and 3) inactivation of any given CCC in mammals is generally non-lethal even for those carriers that exhibit wide distributions (40, 42–44).

An alternative reason that could explain the yeast data for KCC1 and KCC3 is that improper folding of certain protein segments in this expression system precluded associations that normally occur in vivo when carriers are intact. This conformational constraint, often incriminated as a cause of false negative results in yeast two-hybrid studies (14, 37, 38), would imply that the Ct of various CCCs might have to adopt isoform-specific structural configurations for oligomerization to occur and that the stringency of this requirement is variable among the carriers. It would perhaps also explain why the proximal and distal Ct of various CCCs failed to interact with each other in this system. Additional studies aimed at identifying oligomerization domains for all of the CCCs and at confirming that KCC1-CCCx or KCC3-CCCx oligomers do occur in various cell types will be necessary to confirm these hypotheses.
assemble with one another in various cell types to form heterooligomeric complexes (45).

For the CCCs, the potentially important implication of forming such complexes in native cells stems from the differential characteristics that these carriers exhibit in regard to substrate specificities and kinetic behaviors. In contrast to NKCC1, for instance, the KCCs behave as Na\(^+-\)independent carriers that are modestly sensitive to bumetanide (4, 16, 25, 26, 32, 33), and compared with KCC1, KCC2 exhibits higher affinity for Cl\(^-\) (16, 22–25). Heterooligomers that are composed of NKCC1 and a KCC or of different KCCs could thus display characteristics that differ substantially from those of NKCC1 or KCC homooligomers, leading to diversity in the physiological and pharmacologic properties of cation-Cl\(^-\) cotransport among various tissues.

Another implication to the formation of these complexes is that the residues forming each of the ion- or inhibitor-binding sites in an oligomer could be contributed by different carriers. As such, and presuming that all of the residues included in a binding site operate as a coordinated ensemble, oligomers generated through KCC-KCC or NKCC-KCC associations would be expected to interact with K\(^+\), Cl\(^-\), and furosemide regardless of the isoform present in the complex but not with Na\(^+\). An alternative scenario would be that all of the CCCs can interact with both Na\(^+\) and K\(^+\) but that they exhibit a preference for K\(^+\) when the subunits at work only include KCC isoforms. This scenario would explain why the KCCs behave as Na\(^+-\)independent systems when expressed heterologously (4–8, 16, 22–26) and why NKCC1 can mediate some levels of K\(^+\)-Cl\(^-\) cotransport in the absence of extracellular Na\(^+\) (13, 32, 33, 46).

In the current study, we have begun to address the interesting question of whether oligomeric assemblies between certain CCCs could lead to the formation of functionally unique structures by conducting ion transport studies in oocytes injected with NKCC1 with or without KCC4. To our surprise, we found that KCC4 coexpression led to a 2-fold increase in \(^{86}\)Rb\(^+\) flux rates even if the assays were designed to stimulate transport activity by NKCC1 but prevent that by KCC4. In conjunction with the biotinylation studies of Fig. 5B, the observed increase in \(^{86}\)Rb\(^+\) transport suggests that NKCC1 and KCC4 behaved in oocytes as cooperating subunits rather than independent carriers, leading to changes in \(^{86}\)Rb\(^+\) transport by simple summation. They are thus consistent with the idea that certain types of CCC-enclosing heterooligomers play a relevant role in vivo.

Several studies have shown that the KCCs not only differ in kinetic behaviors but also differ in their responses to changes in cell volume, intracellular [Cl\(^-\)], and cell surface kinase/phosphatase activities (16, 26). As mentioned previously, the behavior of NKCC1 in this regard differs from that of KCCs to a considerable extent (4, 16, 27). If, in such a context, heterooligomeric associations between various KCCs or between NKCC1 and KCCs did take place in certain cell types, the molecular mechanisms of CCC regulation would probably be more complex than foreseen initially. For example, would each subunit within an oligomer be regulated independently from one another? And if so, would the regulation of one unit affect that of other units or of the ensemble?

Another question raised by the in vivo occurrence of different types of CCC-CCC complexes is whether certain factors could play a role in determining the ratio of homo- to heterooligomer formation. Conceivably, such factors would vary from cell to cell based on the abundance of any given isoform and type of auxiliary subunits present. Differences in the affinities at which given CCCs combine with each other could also correspond to an important factor. Although very challenging in essence, future studies are warranted to determine how much native cells are able to exploit this type of potential structural diversity by controlling the formation rate or composition of various assemblies.

Several questions that have been raised thus far concerning the functional relevance of certain associations between various CCCs will remain unanswered until additional experiments are carried out to complement this study. In particular, the ion dependences, inhibitor sensitivities, and saturabilities of CCC activity in cell types that express different combinations of isoforms and are subjected to relevant environmental stimuli will have to be determined. The data obtained through these measurements will also have to be interpreted based on the quantity of homooligomers versus heterooligomers that can form in untransfected cells or expression systems and based on model-based kinetic predictions to determine whether coexpressed carriers promote ion movement through cooperative associations or by summation.

In conclusion, our finding that several CCCs can assemble with each other into both homooligomeric and heterooligomeric units represents a key finding in the investigative field of (Na\(^+\))-K\(^+\)-Cl\(^-\) cotransport. Indeed, this new information will have to be integrated to that obtained from other studies in which molecular mechanisms for CCC family members have been or are being explored. Our findings are also highly relevant from the perspective of drug development, given that the CCCs are probably underexploited targets for the treatment of diseases, such as sickle cell anemia, systemic hypertension, and epilepsy.

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