Molecular Mechanisms of Cl⁻ Transport by the Renal Na⁺-K⁺-Cl⁻ Cotransporter

IDENTIFICATION OF AN INTRACELLULAR LOCUS THAT MAY FORM PART OF A HIGH AFFINITY Cl⁻-BINDING SITE*

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The 2nd transmembrane domain (tm) of the secretory Na⁺-K⁺-Cl⁻ cotransporter (NKCC1) and of the kidney-specific isoform (NKCC2) has been shown to play an important role in cation transport. For NKCC2, by way of illustration, alternative splicing of exon 4, a 96-bp sequence from which tm2 is derived, leads to the formation of the NKCC2A and F variants that both exhibit unique affinities for cations. Of interest, the NKCC2A variants also exhibit substantial differences in Cl⁻ affinity as well as in the residue composition of the first intracellular connecting segment (cs1a), which immediately follows tm2 and which too is derived from exon 4. In this study, we have prepared chimeras of the shark NKCC2A and F (saA and saF) to determine whether cs1a could play a role in Cl⁻ transport; here, tm2 or cs1a in saF was replaced by the corresponding domain from saA (generating saA/F or saF/A, respectively). Functional analyses of these chimeras have shown that cs1a-specific residues account for most of the A-F difference in Cl⁻ affinity. For example, \( K_{\text{m,Cl}^-} \) was \(-8 \text{ m}\) for saF/A and saA, and \(-70 \text{ m}\) for saA/F and saF. Intriguingly, variant residues in cs1a also affected cation transport; here, \( K_{\text{m,Na}^+} \), \( K_{\text{m,Rb}^+} \), and \( K_{\text{m,Cl}^-} \) all \(-20 \text{ m}\). Regarding tm2, our studies have confirmed its importance in cation transport and have also identified novel properties for this domain. Taken together, our results demonstrate for the first time that an intracellular loop in NKCC contributes to the transport process perhaps by forming a flexible structure that positions itself between membrane spanning domains.

The bumetanide-sensitive Na⁺-K⁺-Cl⁻ cotransporter (NKCC1) belongs to the cation-Cl⁻ cotransporter (CCC) family, which includes 3 other groups: the K⁺-Cl⁻ cotransporters, the Na⁺-Cl⁻ cotransporter, and the orphan CCCs (1–10). With the possible exception of the latter group for which no ionic substrates have been identified (10), the main function of a CCC is to couple the movement of Cl⁻ to that of Na⁺ and/or K⁺ across cellular membranes (11–14).

Within the NKCC group, 2 isoforms have been described: NKCC1, a widely distributed carrier that is expressed basolaterally in polarized cell types (13–15) and NKCC2, a kidney-specific carrier that is expressed apically in the thick ascending limb (3, 16–18). Alternative splicing of NKCC2 yields different gene products that are differentially distributed along this nephron segment (3, 16). These products include the NKCC2B, A, and F variants.

Except in squid axon, the stoichiometry of ion transport by NKCCs has generally been found to be 1Na⁺-1K⁺-2Cl⁻. Various kinetic models for this system predict that occupancy of each site occurs sequentially in the order Na⁺-Cl⁻-K⁺-Cl⁻, and that ion binding at one site induces the formation of other ion-binding sites (19–23). In some of the studies from which these models are derived, the kinetic properties of the 2 Cl⁻-binding sites have been shown to differ, e.g., \( K_{\text{m,Cl}^-} \) for the 1st site appears >5-fold higher than that for the 2nd site (21, 23).

Detailed analyses of the NKCC2 variants have demonstrated that \( 86\text{Rb}^- \) influx by these carriers, especially the “A” form, could be partially sodium-independent (24, 25). Based on the kinetic models discussed above (19–22), we have hypothesized that this behavior was due to \( \text{Rb}^-/\text{Kr}^+ \) exchange resulting from aborted reactions during the transport cycle, that is, from incomplete unloading of ions on either the intra- or extracellular (o) sides. Incomplete reactions have also been shown to result in Na⁺/Na⁺ exchange under certain conditions.

All members of the CCC family share a common structure, illustrated by the model of shark (sa) NKCC2A in Fig. 1. Hydropathy plot analyses predict 12 transmembrane domains (tms) with intracellular N- and C-termini (1, 26, 27). Additional topological insight has come from the identification of epitope and phosphorylation sites in NKCC1 (28, 29) as well as from the membrane insertion properties of fusion proteins containing NKCC1 domains (30).

In previous studies (12, 22, 27, 31), structure-function analyses of shark and human (hu) NKCC1 chimeras have led to the identification of 3 domains, namely, tm2, 4, and 7, that mediate kinetic differences between species. The importance of tm2 in ion transport by the NKCCs was also confirmed through functional characterizations of NKCC2 variants (24, 32, 33). Indeed, these variants exhibit marked differences in their af-

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1 The abbreviations used are: NKCC, Na⁺-K⁺-Cl⁻ cotransporter; CCC, cation-Cl⁻ cotransporter; cs, connecting segment; hu, human; PBs, vector pBluescript; Pn, position; sa, S. acanthias; saF, S. acanthias NKCC2F; saA, S. acanthias NKCC2A; saA/F, chimera of the saNKCC2A (tm2) and saNKCC2F (cs1a); saF, S. acanthias NKCC2F; saF/A, chimera of the saNKCC2F (tm2) and saNKCC2A (cs1a); tm, transmembrane domain.
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**Experimental Procedures**

Vectors and cDNA Construction—For our experiments, the following vectors were used: (1) p Bluescript SK (pBS); (2) Pol1, a Xenopus laevis oocyte expression vector derived from pGEM and comprised of the T7 promoter, the X. laevis β-globin untranslated regions, and a poly(A) tract (10, 25, 26, 34); and (3) ptTZ19U, a phagemid designed to produce single-stranded DNA from cDNA templates (Bio-Rad).

Wild type constructs analyzed in this study (saNKCC2A/Pol1 and saNKCC2F/Pol1) are the same as those analyzed in previous studies (25, 26). Briefly, saNKCC2A/Pol1 and saNKCC2F/Pol1 were generated from a library-derived saNKCC2/pBS construct, each consisting of (1) a full-length saNKCC2AF, which contains the A and F exons in tandem, (2) a partial-length saNKCC2A, or (3) a partial-length saNKCC2F. For saNKCC2A/Pol1, the ~712-bp SphI-PflmI fragment of saNKCC2AF/pBS was replaced with that of saNKCC2ApBS, and the resulting insert was transferred to Pol1 as an EcoRI fragment. For saNKCC2F/Pol1, the fragment of saNKCC2F was used as replacement. For simplicity, saNKCC2A/Pol1 and saNKCC2F/Pol1 are called saA and saF hereafter.

Chimeric structures between shark A and F variants were produced by transferring the 2428-bp SphI-Bst1107I fragment of saA or saF into ptTZ19U digested with SphI. The mutagenic synthetic oligonucleotide cc acc tcc aac aac ttc att gtt cca tat aag cgg cat gag gat aat tg, which corresponds to a sequence of saNKCC2F comprised between bps 854 and 911 of the open reading frame and which encodes the tm2-cs1a junction point region, was hybridized to saNKCC2A/ ptTZ19U and extended with T7 polymerase to generate uracil-containing single-stranded DNA. Another oligonucleotide, ctg aac aca ttc gtt ggt cga aat ggc tga ggt aga caa ccc tgt taa gag, which corresponds to a sequence of saNKCC2A comprised between bps 853 and 904 of the open reading frame and which also encodes the tm2-cs1a junction point region, was hybridized to saNKCC2F/ptTZ19U and extended as above. Mutants were screened with Accl (the restriction site was removed from saNKCC2F/ptTZ19U and it was introduced in saNKCC2A/ptTZ19U), and positive clones were verified by sequencing. After these steps, the 1433-bp DraIII-MunI fragment of each mutant was reinserted in saA or saF to generate saA/F or saF/A. The former contains the tm2-coding sequence of saNKCC2A and the cs1a-coding sequence of saNKCC2F and the latter, the tm2-coding sequence of saNKCC2F and the cs1a-coding sequence of saNKCC2A.

Expression in the X. laevis Oocyte—The 4 cDNA/Pol1 constructs were linearized and in vitro transcribed with T7 RNA polymerase using the mMESSAGE mMACHINE T7 kit (Ambion). Defolliculated stage V-VI oocytes were injected with 25 nl H\textsubscript{2}O or with ~5 to 25 ng cRNA diluted in 25 nl H\textsubscript{2}O, and tested 3 to 4 days after injection. Before the kinetic studies, eggs were maintained at 18 °C in Barth’s medium supplemented with 125 μM furosemide.

**Kinetic Studies**—Ion transport rates were determined by 

\[ ^{86}\text{Rb}^- \text{ influx measurements at ~22 °C. As shown in Table I, various flux media were used for these studies. Each medium is derived from a basic solution that is isosmolar relative to the X. laevis extracellular fluid.} \]

The following multi-step protocol was used: 1) Removal of furosemide with several washes in a tracer-free basic solution. 2) Preincubation of oocytes for 1 h in a tracer-free hyperosmolar solution (obtained by supplementing the basic solution with 84 mosM sucrose) to activate cotransporter-mediated transport (25, 26); for some experiments, bumetanide was added at various concentrations (0 to 2 μM) during the last 15 min of this preincubation. 3) Reincubation of oocytes for 45 min in one of several isosmolar solutions containing 1 to 2 μM \[^{86}\text{Rb}^-\]. 4) After the washes, oocytes were transferred to Barth’s medium containing 10 μM ouabain, and 0 to 250 μM bumetanide. The various isosmolar solutions consisted of basic solutions in which Na\textsuperscript{+} was varied from 0 to 87 mM, [Rb\textsuperscript{+}] from 0.1 to 20 mM or [Cl\textsuperscript{−}] from 0 to 86 mM. In these solutions, Na\textsuperscript{+} or Rb\textsuperscript{+} was replaced with N-methyl glucamine and Cl\textsuperscript{−} with gluconate + SO\textsubscript{4}\textsuperscript{2−} (see Table I and figure legends). 4) Repeated washes in a tracer-free basic solution containing 250 μM bumetanide and 10 μM ouabain. 5) After the final washes, oocytes were transferred in 96-well plates (1 oocyte/well) and solubilized in 2% SDS. 

\[^{45}\text{Rb}^-\] was detected by liquid β-scintillation counting using the TopCountNXT microplate counter (Packard).

In each experiment, fluxes among 1 to 9 oocytes (usually from 4 to 6 oocytes) were averaged and normalized to other flux values or to

![Hydropathy plot models of saNKCC2.](Image)

Each form symbolizes amino acid residues. The model in the left panel includes the entire coding sequence of saNKCC2 (blue, saNKCC2A; darker blue, exon 4 of saNKCC2A). The 8 models in the right panel correspond to residues from the alternatively spliced exon 4 that encodes tm2 and cs1a (yellow, saNKCC2F; red, residues that are identical between the variants). These models were drawn using the program Plot by Biff Forbush.
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### RESULTS

**Functional Expression of NKCC2s**—As seen in Fig. 2, *X. laevis* oocytes injected with either wild type or mutant NKCC2s (saA, saF, saA/F, saF/A) exhibit bumetanide-sensitive \(\text{Rb}^+\) influxes that vary between 1.9 to 4.5 nmol/oocyte/h. For saA and saF, these rates are more than 8-fold above the usual background level (~0.4 nmol/oocyte/h), and they are also similar to those reported previously (25). For saF/A, on the other hand, cotransporter-specific activity is shown to be slightly lower (~4-fold above background). In these studies, flux measurements were performed after a 1-h incubation in hyperosmotic medium.

**Kinetics of Na\(^+\) Transport**—To determine whether variant residues in tm2 and/or cs1a account for differences in Na\(^+\) affinity between saA and saF, we measured the dependence of \(\text{Rb}^+\) influx on [Na\(^+\)] for the saA/F and saF/A chimeras, and compared results to those obtained in simultaneous experiments for wild type transporters. Data are illustrated in Fig. 3, A–D. The 2 chimeras display an affinity for Rb\(^+\) that is very similar to that displayed by the wild type high affinity NKCC2 carrier; as shown in panel E, for instance, \(K_m(\text{Rb}^+)\) is 2.2, 1.8, and 2.2 mM for saA/F, saF/A, and saA, respectively. These values are similar to those reported previously (24, 25). These results indicate that differences in the magnitude of Na\(^+\)-independent activity are probably conveyed to a large extent by variant residues in cs1a.

### Table 1

| [Im] | Na\(^+\) | Rb\(^+\) or K\(^+\) | Cl\(^-\) | Ca | Mg | PO\(_4\) | SO\(_4\) | HEP | NMG | GLU | SUC | OSM |
|------|---------|-----------------|--------|----|----|--------|--------|-----|-----|-----|-----|-----|
| Basic isoosmolar | 87 | 5 | 86 | 2 | 2 | 1 | 1 | 10 | 0 | 0 | 200 |
| Hyperosmolar (SUC) | 87 | 5 | 86 | 2 | 2 | 1 | 1 | 10 | 0 | 0 | 284 |
| Barth’s medium | 90.4 | 1 | 90 | 0.7 | 0.8 | 0.8 | 10 | 0 | 0 | 0 | 200 |
| Rb\(^+\) dependence | 87 | 0.1–20 | 86 | 2 | 2 | 1 | 1 | 10 | 0–20 | 15 | 0 | 230 |
| Na\(^+\) dependence | 0–87 | 5 | 86 | 2 | 2 | 1 | 1 | 10 | 0–87 | 0 | 0 | 200 |
| Cl\(^-\) dependence | 87 | 5 | 0–86 | 2 | 2 | 1 | 1–10 | 10 | 0 | 0–78 | 0 | 200 |
| Wash | 19 | 73 | 8 | 2 | 2 | 1 | 1 | 10 | 0 | 78 | 0 | 200 |

**Kinetics of Rb\(^+\) Transport**—To determine whether tm2 and cs1a also convey differences in affinity for the other cotransported cation, the dependence of \(\text{Rb}^+\) influx on [Rb\(^+\)] was measured as above for saA/F and saF/A as well as for the wild type transporters (see Fig. 4, A–E). For these experiments, the data is again fit by a model of ion binding at a single site and \(K_m\) values derived from the activity versus [ion] relationships are depicted in Fig. 3E.

**Kinetics of Cl\(^-\) Transport**—Fig. 5 presents results for the Cl\(^-\) dependence of \(\text{Rb}^+\) influx and \(K_m\) values derived from the activity versus [ion] relationships. Similar to the observed differences in cation affinities, the wild type carriers exhibit marked differences in Cl\(^-\) affinity. As shown in panels A and B, by way of illustration, \(K_m(\text{Cl}^-)\) is 6.9 mM for saA and 69.1 mM for saF/A.
Km anions also differs substantially between the chimeras. Indeed, that constitute part of a high affinity Cl⁻ transport but that tm2, on the other hand, does not mediate appreciable differences in Cl⁻ affinity. Results presented in Fig. 5 also point to the possibility that cs1a contains residues that are partly involved in Cl⁻ transport to some extent, e.g., it could constitute part of a high affinity Cl⁻-binding site.

In a recent publication (25), we have observed a non sigmoidal relationship between the activity of certain variants and [Cl⁻]. For saA, e.g. this relationship was better fit with a model of Cl⁻ binding at a single site, whereas for saF, it was better fit with a model of Cl⁻ binding at 2 sites. In Fig. 5 (panels A and B), Hill coefficients for the wild type carriers are also seen to be different, consistent with previously reported behaviors. Quite interestingly, in addition, Fig. 5 shows that the 2 chimeras are not reciprocal in regard to this behavior. In fact, Hill coefficients for both saA/F and saF/A are exactly the same, that is, they are closer to 1. These results suggest that tm2 is also involved in Cl⁻ transport to some extent, e.g. it could constitute part of a low affinity Cl⁻-binding site.

Kinetics of Bumetanide Inhibition—Earlier studies have shown that bumetanide inhibits the Na⁺-K⁺-Cl⁻ cotransporter by interacting with the protein at a site that is presumably extracellular (35, 36). More recent studies have also revealed that residues near tm2 or within tm2 are partly involved in inhibitor binding (31, 33). To determine whether cs1a plays a role in this regard as well, we measured bumetanide inhibition of 86Rb⁺ influx in oocytes expressing the wild type or mutant saNKCC2s prepared in this study.

Looking first at the results for the wild type saNKCC2s (Fig. 6, panels A, B, and E), we observe a small albeit significant 3-fold difference in bumetanide affinity between the variants. Here, $K_{i(bumetanide)}$ is 0.26 µM for saA and 0.83 µM for saF. This
difference is relatively similar to that reported by another group (33) and confirms the presumed importance of the tm2-cs1a region in bumetanide binding.

Looking next at the chimeras, it is seen that $K_m$, bumetanide, are also different. As illustrated in Fig. 6, the affinity constant is 0.39 μM for saA/F (panel C), thus closer to that for saA (panel A), whereas it is 1.46 μM for saF/A (panel D), thus closer to that for saF (panel B). Taken together, the findings reported in Fig. 6 indicate that variant residues specific to tm2 are responsible for the differences in bumetanide affinity observed between the wild type NKCC2 carriers, whereas variant residues specific to cs1a play little if any role.

**DISCUSSION**

In this study, we have examined the role of tm2 and cs1a in ion transport and bumetanide binding by NKCC2 splice variants. Functional analyses of chimeric saNKCC2s in which tm2 or cs1a were interchanged between the A and the F variants have demonstrated that differences in cation and anion affinities are mediated by variant residues present in both domains, whereas the difference in bumetanide affinity is mediated by variant residues present in tm2 only. Intriguingly, the way in which these domains affect cation transport differs from that in which they affect anion transport (see below).

Because the affinity for one ion during the transport of 1Na⁺-1K⁺-2Cl⁻ is, as mentioned earlier, affected by the affinity for other ions (11, 12, 19–23), it may be difficult in this work to interpret functional changes resulting from residue substitutions. However, previous analyses (12, 19, 22) have demonstrated that the interdependence of kinetic constants could be predicted within the scheme of ordered substrate-carrier interactions characteristic of the NKCCs (11, 12, 19–23). For the secretory isoform, in particular, it was shown that high [Na⁺] or [Rb⁺], changes in either Na⁺ or Rb⁺ affinity have small effects on $K_m$Cl⁻ whereas at high [Cl⁻], a change in Cl⁻ affinity has a larger effect on $K_m$Na⁺ and $K_m$Rb⁺ (22). Considering that the mode of operation of NKCC2 is analogous to that of NKCC1, the pattern of interdependence among kinetic parameters is probably similar for both isoforms.

Looking at changes in Cl⁻ affinity in the context of the codependence profiles described in this work, it is noteworthy that substitutions in either of tm2 or cs1a led to reciprocal changes in $K_m$. In these studies, for example, $K_m$Cl⁻ for saA/F

**Fig. 5.** Dependence of $^{86}$Rb⁺ influx on [Cl⁻] for saA, saF, saA/F, or saF/A. Within each experiment, averaged fluxes among 1–9 oocytes were normalized to the value measured at the highest [ion] as for Na⁺ and Rb⁺. The data in each panel are shown as an average of these normalized fluxes among of 2 to 5 experiments. In panel A–D, [Cl⁻] was varied from 0 to 86 mM, whereas [Na⁺] and [Rb⁺] were maintained at 87 and 5 mM, and data points were fit by the Michaelis-Menten equation (panels A, C, and D) or the Hill equation with n = 2 (panel B). In panel E, $K_m$Cl⁻ shown were obtained by a fit of the averaged data. *, significantly different statistically ($p < 0.05$) compared with high affinity carriers (black bars) based on $K_m$ values obtained by averaging $K_m$ from individual experiments.

**Fig. 6.** Inhibition of $^{86}$Rb⁺ influx by bumetanide for saA, saF, saA/F, or saF/A. Within each experiment, averaged fluxes among 1–8 oocytes were normalized to the value measured at lowest [bumetanide]. The data in each panel are shown as an average of these normalized fluxes among of 3 to 5 experiments. In these studies, [bumetanide] was varied from 0 to 2 μM whereas [Na⁺], [Rb⁺], and [Cl⁻] were maintained at 87, 5, and 86 mM; here bumetanide was also added during the last 15 min of the preincubation period at the same concentration as during incubation with $^{86}$Rb⁺. In panels A–D, the data points were all fit by the Michaelis-Menten equation, and in panel E, $K_m$Cl⁻ shown were obtained by a fit of the averaged data. *, significantly different statistically ($p < 0.05$) compared with high affinity carriers (black bars) based on $K_m$ values obtained by averaging $K_m$ from individual experiments. Bu, bumetanide.
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Fig. 7. Alignments of the tm2-cs1a regions of 9 NKCCs. On the left side, each number in a black circle symbolizes an amino acid. Here, number 1 is attributed to the first residue of the tm2 α-helix according to the proposed model of NKCC1 and 2 (1, 3, 25, 26). The positions of residues relative to the hydrophobic face of the helix (represented by the gray area) are based on a helical wheel model of tm2 drawn with 100° angles. Gray letters, residues that are predicted to be in contact with the lipid bilayer; black letters, residues that are predicted to be on the hydrophilic face of the wheel or in the cytosol; boxed letters, residues that are different between saA and saF; and arrows, residues that might constitute part of a high affinity Cl\(^-\) binding site.

was identical to that for saF, whereas \(K_{m,\text{Cl}}\) for saF/A was identical to that for saA. These results indicate that most of the difference in Cl\(^-\) affinity between the A and F forms are conveyed by variant residues in cs1a and, therefore, that this domain is probably very important in determining the true affinity for Cl\(^-\).

Although saF and saAF were shown to exhibit similar \(K_{m,\text{Cl}}\); atr saF/A was identical to that for saA. These results indicate that most of the difference in Cl\(^-\) affinity between the A and F forms are conveyed by variant residues in cs1a and, therefore, that this domain is probably very important in determining the true affinity for Cl\(^-\).

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It is important to recognize that the Hill coefficient is a lower limit for the number of specific ions that are included in the transport process (25). Accordingly, it is possible that the transport cycle for saA, saA/F, and saF/A does not involve 2 Cl\(^-\) ions and 2 binding sites of fairly dissimilar affinities but that it involves only one Cl\(^-\) ion and 1 binding site. Additional experiments examining the electrogenticity of transport will be necessary to determine whether the stoichiometry for certain NKCC2 is actually 1Na\(^+\)-1K\(^+\)-1Cl\(^-\).

In regard to the kinetics of cation transport, the pattern of changes due to residue substitutions was unlike that observed for Cl\(^-\). Indeed, exchanging either of tm2 or cs1a between the carriers led to \(K_m\)s that were identical to those displayed by saA. These results suggest that differences in \(K_{m,\text{Na}}\) and \(K_{m,\text{Rb}}\), are mediated by variant residues present not only in tm2 but present in cs1a as well. Based on more on the expected changes in \(K_m\)s stemming from the ion-dependence of ion binding by NKCC1, and based on the importance of cs1a in determining Cl\(^-\) affinity, it is possible that residue substitutions in cs1a led to changes in the affinity for cations largely through changes in Cl\(^-\) affinity.

In the current work, a large fraction of \(^{86}\text{Rb}\) influx through saA and saAF/A was seen to be \(\text{Na}_{\text{in}}\)-independent. This behavior for the A variant has already been reported in the past (24, 26) and is not due to contamination of the flux medium by Na\(^+\) (based on various measurements and on the experimental design used for the assays). Hence, we consider 2 explanations for this \(\text{Na}_{\text{in}}\)-independent component. Could \(\text{Rb}_{\text{in}}/\text{K}_{\text{out}}\) occupy the Na\(^+\) site under some conditions, e.g., when [\(\text{Rb}_{\text{in}}/\text{K}_{\text{out}}\)] is very high? At present, there is no direct evidence for or against this hypothesis. However, a sigmoidal dependence of \(^{86}\text{Rb}\) influx on [\(\text{Rb}_{\text{in}}/\text{K}_{\text{out}}\)] has not been described for this system to date (2). Could some of the NKCC2s exhibit substantial \(\text{Rb}_{\text{in}}/\text{K}_{\text{out}}\) exchangers? Here, interestingly, differences in the \(\text{Na}_{\text{in}}\)-independent component were conveyed by variant residues in cs1a, a domain that also encodes strongly polarized behaviors in regard to Cl\(^-\) transport kinetic. Hence, it is possible that the properties of the Cl\(^-\)–binding sites, perhaps of the one that is occupied last in the series Na\(^+\)–Cl\(^-\)–K\(^+\)–Cl\(^-\), determine to a large extent the degree at which NKCC2s behave as K\(^+\)/K\(^+\) exchangers.

It has generally been assumed that bumetanide and Cl\(^-\) bind to the same site on NKCC (33, 35–37). However, recent kinetic analyses using saNKCC1-huNKCC1 chimeras have provided evidence against this hypothesis by showing that regions involved in inhibitor binding are different from those determining Cl\(^-\) affinity (12, 22, 31). In this work, we have also demonstrated a similar distinction between such regions; by way of illustration, the chimera with a higher bumetanide affinity (saF/A) had lower Cl\(^-\) affinity, and the chimera with a lower bumetanide affinity (saF/A), a higher Cl\(^-\) affinity. These results suggest further that the bumetanide-binding site is not the same as the Cl\(^-\)-binding site. They indicate, nonetheless, that tm2 does contribute to bumetanide binding in a, as yet unidentified manner.

The characterization of mutant NKCC2s allows us to extend structure-function studies of the tm2-cs1a region begun previously (12, 22, 31). As illustrated in Fig. 7, the chimeras induce 9 substitutions in tm2 (Pn 1–5, 7, 9, 12, and 13, relative to the beginning of this domain) and 5 substitutions in cs1a (Pn 15, 16, 18, 22, and 26). Based on the behavior of saNKCC1-huNKCC1 chimeras reported earlier, one might predict that tm2- or cs1a-specific residues differing between saNKCC1 and huNKCC1 as well as between high affinity and low affinity NKCCs would be those that are important in determining cation affinities, whereas tm2- or cs1a-specific residues differing only between high affinity and low affinity NKCC2s would be those that are important in determining anion affinity.

In regard to cations, the only residue fitting the evoked criteria is at Pn 22; for instance, it is T in saNKCC1 and an A in huNKCC1, and it is an A, S, or E in high affinity carriers and a C or T in low affinity NKCCs. Hence, the current analysis
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does not allow the identification of individual residues that might be important in determining affinity. In regard to anions on the other hand, several residues (9, 13, 16, 18, and 26) fit the criteria; here, residue at Pn 15 is excluded because it is conserved between high and low affinity carriers and residue at Pn 22 is also excluded because it differs between saNKCC1 and huNKCC1. Based on kinetic measurements reported in this study and on the site of the chimeric junction point (between residues 14 and 15), we propose that residue(s) 9 and/or 13 modulate Cl\(^-\) affinity at one site and that residue(s) 16 and/or 26 modulate Cl\(^-\) affinity at another site.

Groups of residues within the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter probably operate in concert to bring about ion binding. In such a case, given combinations of residues rather than individual residues could be a more important determinants of affinity. In the outer region of tm2, by way of illustration, residues at Pn 1, 4, 7, and 8 are predicted to be in close proximity to one another on the most hydrophilic face of a helical wheel model (Fig. 7) and these are combined differently among carriers. Hence, the chimera approach exploited here was probably informative in permitting to assess the role of intact residue groups. For the same reasons, pursuing with this approach in the future, e.g. by exchanging domains at different junction points, may be a more advantageous avenue than single point substitutions to infer the role of individual residues.

In conclusion, we have shown for the first time that an intracellular loop within the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter is involved in ion transport and more specifically, that it probably modulates the true affinity for Cl\(^-\). We propose that this domain forms part of a high affinity Cl\(^-\)-binding site within the Na\(^+\)-K\(^+\)-Cl\(^-\)-binding pocket. On a structural level, such a role for cs1 may indicate that NKCCs possess a pore region that is analogous to that of voltage-sensitive cation channels (38–40).

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