THE RESIDUES DETERMINING DIFFERENCES IN ION AFFINITIES AMONG THE ALTERNATIVE SPLICE VARIANTS F, A, AND B OF THE MAMMALIAN RENAL NA-K-CL COTRANSPORTER (NKCC2)

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Running Title: Residues Determining Ion Affinities in NKCC2 Splice Variants
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Three alternatively spliced variants of the renal Na-K-Cl cotransporter (NKCC2) are found in distinct regions of the thick ascending limb of the mammalian kidney; these variants mediate Na+K+2Cl transport with different ion affinities. Here we examine the specific residues involved in the variant-specific affinity differences, utilizing a mutagenic approach to change the NKCC2B variant into the A or F variant, with functional expression in Xenopus oocytes. The splice region contains the second transmembrane domain (TM2) and the putative intracellular loop (ICL1) connecting TM2 and TM3. It is found that the B variant is functionally changed to the F variant by replacement of 6 residues, half of the effect being brought about by three TM2 residues and half by three ICL1 residues. The involvement of the ICL1 residues strongly suggests that this region of ICL1 may actually be part of a membrane-embedded domain. Changing six residues is also sufficient to bring about the smaller functional change from the B to the A variant; three residues in TM2 appear to be primarily responsible, two of which correspond to residues involved in the B-to-F changes. A B-variant mutation reported in a mild case of Bartter’s disease was found to render the cotransporter inactive. These results identify the combination of amino acid variations responsible for the differences among the three splice variants of NKCC2 and they support a model in which a reentrant loop following TM2 contributes to the chloride binding and translocation domains.

The Na-K-Cl cotransporter (NKCC) carries out the coupled movement of 1 Na, 1 K, and 2 Cl ions across the cell membrane, taking advantage of the sodium electrochemical gradient to accumulate Cl. Two isoforms are encoded by separate genes: NKCC1 (Slc12a2), a nearly ubiquitous isoform with an important role in chloride secretory epithelia, and NKCC2 (Slc12a1), a kidney specific isoform that is the target of the present investigation. NKCC2 provides the salt reabsorption mechanism necessary for renal salt and water conservation, and is the clinical target for loop diuretics -- mutations in NKCC2 are responsible for Bartter’s disease (1).

NKCCs are thought to be conventional 12-transmembrane-helix transport proteins, with large cytoplasmic N- and C-termini. The transmembrane region is responsible for ion binding and translocation (2), whereas the N-terminal cytosolic domain contains the regulatory phosphoacceptors (3) as well as a binding site for the regulatory kinase (4,5) and in NKCC1 a binding site for the regulatory phosphatase (6). Three TMs have been shown to be involved in ion translocation, as determined with a chimera and single-residue mutation approach (7,8); one of these is TM2, which is encoded by the NKCC2 splice region.

NKCC2 is a highly specialized protein whose expression is restricted to the apical membrane of thick ascending limb (TAL) cells in the kidney. The NKCC2 gene contains three forms of the fourth exon, which through alternative
splicing give rise to the three variants of NKCC2, termed F, A, and B. The variants have distinct and almost separate distribution along the TAL, with F present primarily in the inner aspect of the outer medulla, A primarily in the outer part of outer medulla and in the cortex, and B is localized primarily in macula densa (9,10).

The three splice variants of NKCC2 differ in their affinities for the three transported ions, in a way that effectively matches the transport $K_m$s to the concentration of ions in the renal tubule (11-13). Thus, as concentrated NaCl enters the outer medullary portion of the TAL it is handled by low affinity NKCC2F, and as the fluid becomes diluted in more distal segments of the TAL, intermediate-affinity NKCC2A is appropriately matched for effective transport. As to NKCCB, it is still a puzzle why this macula densa-specific variant, which plays a role in the Cl-sensing step of tubuloglomerular feedback, has evolved to have the highest apparent affinities of the three variants (9,10).

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The NKCC2 alternatively spliced exons encode the predicted second transmembrane domain (TM2) and the first part of the following intracellular connecting loop (ICL1). Beginning towards the end of predicted TM2 is the stretch of amino acid sequence that is best-conserved among all members of the cation-chloride superfamily of cotransporters, which includes the NKCCs, the thiazide-sensitive Na-Cl cotransporter (NCC) and the K-Cl cotransporters (KCCs), as well as two transporters whose role is yet unknown. Among 50 amino acid residues, including the signature GGYAYYLIS sequence, more than 40 are identical or highly conserved in most family members. By itself, this degree of conservation suggests a central role in the membrane transport function of the protein, and we have noted that the periodic spacing of these conserved residues suggests a functionally important alpha-helical structure of the first part of ICL1 (14). This hypothesis has been strongly supported by the preliminary evaluation of the work reported here (15), as well as by related studies in shark NKCC2 (16,17).

Here we present a detailed study of the residues determining ion affinity differences among the three splice variants of mammalian NKCC2. Utilizing a multiple site-directed mutagenesis approach, with functional evaluation in Xenopus oocytes, we have pinpointed residues that together appear to be fully responsible for the functional differences among the three variants. No single residue change was found to confer the majority of the difference – rather, it appears that two sets of three residues make up the structural domains that are most involved with ion affinity changes. Importantly, one of these sets of residues is in a region previously sketched to be in ICL1 – with a clear involvement in ion translocation we propose that this region is alpha-helical and membrane embedded.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis. Full-length cDNA for rabbit NKCC2B in an oocyte expression vector (11) was subjected to site-directed mutagenesis using PhiTurbo DNA polymerase (QuickChange, Stratagene). One substitution was performed at a time, and multiple substitutions were created using previous mutants. Individual substitutions were confirmed by sequencing a 600bp region encompassing the splice region, and end-point mutants containing quadruple, quintuple or sextuple substitutions were fully sequenced.

For simplicity, multiple substitutions are denoted with a shorthand nomenclature. In B-to-F mutants, target residues selected from NKCC2B (A238, T240, G243, T249, A253 and Y257, see Fig. 1, below) are listed before a ‘/’, followed by the corresponding product residues from the NKCC2F sequence, as in “ATG•/SVT•”. B-to-A mutants are handled similarly – here the original residues are G236, A238, V239, T240, G243, and Y257. For further clarity we mark the junction between TM2 and ICL1 with a ‘•’, as in “A•TAY/S•MCV”.

*Functional expression in Xenopus laevis oocytes.* cRNA was synthesized from linearized cDNA templates using T7 RNA polymerase (mMessage-MMachine kit, Ambion) and injected in stage IV-VI defolliculated Xenopus oocytes (~70-90 ng RNA/oocyte). Oocytes were maintained for three days at 17°C in ND96 media supplemented with penicillin-streptomycin prior to flux experiments.
We measured the function of NKCC2 using a $^{86}$Rb influx assay as reported previously (11). Briefly, in the standard influx experiment, oocytes are incubated for 40 minutes in an influx medium containing: 96 mM NaCl, 2 mM RbCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM Na$_2$HPO$_4$, 2 mM Na$_2$SO$_4$, 0.1 mM ouabain, 20 µCi/ml $^{86}$RbCl, 5 mM Hepes (pH 7.4 at room temperature). Ion substitution experiments were carried out by replacement of Cl with gluconate, and by replacement of Na or Rb with N-methylglucamine. Influx was terminated by rinsing with ice-cold 96 mM K gluconate, 2 mM Na gluconate, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM Na$_2$HPO$_4$, 2 mM Na$_2$SO$_4$, 5 mM Hepes, 0.25 mM bumetanide and 0.1 mM ouabain. Solution changes were executed by transferring oocytes from one well to another in a 48-well plate, and $^{86}$Rb in individual oocytes was determined at the end of the experiment by Cerenkov radiation in a scintillation counter. We have previously shown that >90% of the $^{86}$Rb uptake in NKCC2-injected oocytes is accounted for by the activity of exogenously expressed cotransporter, and that all of the NKCC2-mediated flux is bumetanide sensitive. (11).

For most of the experiments in this paper, we used a modification of the above procedure that allowed us to make three sequential influx determinations with a single set of oocytes. Following each of three 10 min influx periods in different influx solutions (20 µCi/mL $^{86}$Rb) oocytes (6 oocytes per well) were rinsed in ND96, then transferred to a scintillation counter for a 2 minute counting period; after counting they were returned to the flux plate for washes and the next influx incubation period. Influx rates were calculated from the difference in counts in the oocytes before and after the influx period, correcting (less than 5%) for counts that left the oocytes during the counting procedure by counting the washes. This method offers a substantial advantage in that it gives internally paired values for fluxes under three different conditions in the same set of oocytes; it has some statistical limitation in the fact that differential influx is harder to measure as the total number of counts in the oocytes increases.

Usually we utilized the triple-flux procedure to determine $^{86}$Rb influx at 10 mM, 40 mM, and 100 mM extracellular [Cl] in the same set of oocytes. Incubations were carried out in the 10-40-100 order, because this gives the largest influx when the oocytes have accumulated the most counts, thus optimizing signal-to-noise. Control experiments showed that derived $K_m$ were indistinguishable when the order of incubations was changed, and the $K_m$s from this method were also the same whether this method or the standard method was employed (see Fig. 2, below, and compare NKCC2A,B,F to previous reports).

$K_m$ values were obtained by fitting individual experiments with the Michaelis-Menten (Na curves) or Hill equations using non-linear least squares analysis and the simplex algorithm. To determine $K_{m(Cl)}$, the Hill coefficient was fixed at 1.6 (8). It should be noted that $K_{m(Cl)}$ becomes difficult to determine when it is near or above the highest available Cl concentration, as is the case in NKCC2F and similar constructs. In that regard, the fit routines did not converge for data from two mutants (see Fig. 3, legend), and we have obtained estimated $K_m$ values by fitting with $V_{max}$ fixed at 6 nmol/oocytes/hr, which is in the vicinity of similar constructs. We use “apparent affinity”, and less precisely “affinity”, to describe the inverse of $K_m$, following common usage and recognizing that this is not the true affinity of a transport site itself. The $K_m$ of a transport protein is empirically determined and is a complicated function of all binding affinities and rate constants; true affinities can not be obtained from transport measurements alone. Results are presented as ± SEM of 3-20 experiments (see table 1, below); evaluations of statistical significance are carried out with Student’s t test.

For some mutants we carried out Western blot analysis to compare the levels of total NKCC2 protein. Oocytes (4-6 per group) were solubilized in 100 µl of homogenization buffer (1% Triton X-100) and 6 uL of that homogenate were run on SDS gels, blotted and evaluated with the T4 antibody (18).

**RESULTS AND DISCUSSION**

The three NKCC2 splice variants are most readily distinguished by their apparent chloride affinity: the $K_{m(Cl)}$ ranges from 9 mM in high-affinity NKCC2B to 113 mM in low-affinity
NKCC2F, and NKCC2A is intermediate, with \( K_{\text{m(Cl)}} = 45 \text{ mM} \) (values as per (11)). To elucidate the molecular determinants of this affinity difference, we used the rabbit NKCC2B sequence as a template and made residue substitutions according to the sequence in rabbit NKCC2F (B-to-F mutants) and rabbit NKCC2A (B-to-A mutants). The exon in the NKCC2 gene affected by alternative splicing encodes 32 amino acids (Fig. 1, residues 230 to 261 in rabbit NKCC2); our analysis was focused on the variations which pose a significant structural change and which are conserved across species. As highlighted in Fig. 1, A238, T240, G243, T249, A253 and Y257 were modified in B-to-F mutants, and G236 and V239 were additionally modified in B-to-A mutants.

Residues determining the differences in chloride affinity between splice variants B and F. Fig. 2 presents typical data from characterization of the B-to-F mutants. Western blot analysis of NKCC2 expression (Fig. 2b) confirms that similar levels of protein expression are attained in each of these constructs. Typical flux data, shown in Fig. 2a, illustrate that in addition to changes in \( K_{\text{m(Cl)}} \), discussed above, the magnitude of the fluxes is also altered by some of these replacements (see also Table 1). Most dramatic is the case of T249M, which shows a 5-fold reduction in cotransport activity. This is most likely due to an effect on \( V_{\text{max}} \) of the cotransporter, since the total expression is similar for all of the constructs (Fig. 2a), although it could also be a result of decreased cell-surface delivery of this construct. The transport \( V_{\text{max}} \) in T249M is substantially rescued by simultaneous mutation of Y257 and further by mutation of A253, demonstrating a cooperative interaction of these three residues; whereas mutation of the three TM2 residues did little to increase the flux rate (Table 1).

\( K_{\text{m(Cl)}} \) values were obtained from the three-point flux assays for 28 of the 63 possible combinations of the B-to-F site changes, and the results are presented in Fig. 3. It is seen that by themselves the single substitutions each trended towards lower affinity (higher \( K_{\text{m}} \)), although only T240V (\( p=0.02 \)), and G243T (\( p=0.03 \)) and T249M (\( p=0.03 \)) reached significance. Individually, it is clear that none of the single substitutions came close to accounting for the full B-to-F effect.

Some combinations of two or more substitutions showed additive changes, but there was a wide range of synergism. For residues in TM2, none of the dual combinations produced a significant affinity decrease over individual residue substitutions. However, when all three substitutions were made (ATG•/SVT•) the chloride affinity was lowered 5-fold, to half that of NKCC2F. This result is consistent with the three residues being related in a structurally and functionally important way.

Substitution of the three ICL1 residues (•TAY/•MCV) produced a 4.5-fold change in chloride affinity compared to NKCC2B, similar to exchanging the three TM2 residues. In this case the effect was matched or exceeded by two of the dual exchanges, •TA/•MC and •TY/•MV. From comparison of the point mutants and the dual substitutions in these experiments, T249 appears to be the most important single residue in determining Cl affinity.

Together, mutation of all 6 target B-to-F residues (ATG•TAY/ SVT•MCV) brought about the full change from B to F chloride affinity (bottom bar, Fig. 3). Comparing this result with the triple-mutants in TM2 and ICL1, this is clearly consistent with a combined effect of TM2 and ICL1 sets of residues. This finding supports the identification of these 6 residues as those involved in the Cl affinity difference between NKCC2B and NKCC2F.

Overall, the outcomes of 4- or 5-change mutations in the B-to-F series were in general agreement with expectations from the simpler combinations. Residues in ICL1 are seen to have a larger effect than those in TM2, and among the ICL1 residues the order of importance is T249 \( > > \) A253 \( > > \) Y257 as it is in the double mutations. Within TM2, the individual effects of G243 in combination with ICL1 changes are small but significant (see G•TAY/S•MCV vs •TAY/•MCV, \( p=0.04 \)) whereas only T240 has a clearly substantial effect on the B-to-F transition by itself. Overall, residues in TM2 thus rank T240 \( > > \) G243 \( > > \) A238 in B-to-F importance.

Chloride-dependence curves for \(^{86}\text{Rb} \) influx are illustrated in Fig. 4a for B-to-F constructs ATG• /SVT•, •TAY/•MCV and ATG•TAY/ SVT• MCV. It can be seen that the 6-
Residue B-to-F mutant behaves as a wild type NKCC2F, while constructs bearing triple-substitution of one of the two amino acid regions, TM2 or ICL1, have intermediate chloride affinities. These results are in excellent agreement with results obtained from the 3-point flux assays in Fig. 3.

**Na dependence of ion transport in B-to-F mutants.**

We examined the Na dependence of $^{86}$Rb influx in the constructs ATG/SVT and TAY/MCV as well as in NKCC2B and NKCC2F and the 6-residue B-to-F mutant (ATG•TAY/SVT•MCV). The 6-residue mutant had a $K_m$(Na) affinity similar to NKCC2F (Fig. 4b), as was the case for $K_m$(Cl) (Fig. 3,4a). Thus the 6 identified residues account for the kinetic differences between variants B and F in translocation of Na as well as Cl.

The Na-K-Cl cotransporter is thought to translocate four ions across the membrane in a single-file mechanism in the order Na,Cl,K,Cl (19). It is thus intriguing to propose that the TM2 site might bind one ion pair, and ICL1 the other ion pair. If so, we would expect to see Na affinity changed by modifications at one site, and K affinity changed by modifications at the other site, subject to the caveat that changes in true affinity for one ion are bound to be at least partially reflected in changes measured $K_m$s for all ions (see (7)). Examining TM2 and ICL1 mutants (intermediate curves in Fig. 4b), it is seen that these two sets of residues are about equally involved in determining kinetic differences $K_m$(Na). This appears to rule out the hypothesis that these two sites are involved in binding ion pairs, one for Cl-Na, and the other for Cl-K. In fact the situation was unlikely to have been that simple, since we previously found that two sets of residues in the beginning of TM2, upstream of the B-to-AF residues (corresponding to GV232 and GL237 in the NKCC2B sequence, Fig. 1), mediate affinity differences for Na and K between shark and human NKCC1s (2). Considered together, the NKCC1 and NKCC2 findings argue either (a) that at least some of the observed $K_m$ differences are due to long range effects, or (b) that TM2/ICL1 is not one extended helix, but has at least one hairpin enabling more than one set of amino acid residues to interact with the same transported ion or ion pair.

**Residues responsible for the difference in chloride affinity between splice variants A and B.**

Variants A and B of the rabbit NKCC2 cotransporter differ significantly only in chloride affinity (11). For B-to-A changes, the only ICL1 difference is the conservative change Y257F, the other candidates all being in TM2: G236L, A238S, V239T, T240M and G243S (Fig. 1). As seen in Fig. 5, in mutants with these individual substitutions, only the construct T240M exhibits a significant decrease in chloride affinity compared to wild-type NKCC2B ($p=0.001$).

A construct bearing all 6 substitutions (GAVTG•Y/LSTMS•F) was indistinguishable from NKCC2A (Fig. 5, bottom), confirming the importance of the candidate residues. We tested a limited set of intermediate B-to-A combinations, as illustrated in Fig. 5. The results demonstrate that V239, T240, and G243 (in that rank order) are involved in the B-to-A difference, and together they appear to be sufficient to produce an A-like construct. While there may be effects of A238, G236, and Y257, they are not statistically significant in these experiments. Interestingly, when compared to the B-to-F TM2 substitution ATG•/SVT•, the B-to-A substitution AVTG•/STMS• exhibits less change from parent NKCC2B ($p=0.05$), which stresses the critical role of synergistic interaction among different residues in determining ion affinities.

**A Bartter mutation in NKCC2 variant B.** Inherited mutations in the NKCC2 gene (SCL12A1) are responsible for the human disease Bartter syndrome type I, which is characterized by salt-wasting, metabolic alkalosis, hypokalemia, and frequently hypotension with normal renin and aldosterone. A genetic analysis of families affected by Bartter syndrome has recently identified a patient with a mild Bartter phenotype bearing a single mutation in exon 4 of NKCC2 that results in a G243D (G224D human) substitution only in the B variant (20). We analyzed the functional consequence of this mutation by expression of a G243D rabbit NKCC2 construct in oocytes. Although Western blot analysis demonstrated that G243D was apparently not different from wild-type NKCC2B in its expression (not shown), we found that there was no measurable $^{86}$Rb influx carried by the mutated cotransporter (Table 1, water vs. G243D, $p=0.5$).
In molecular terms this is not surprising, since the mutation introduces fixed negative charge in a region that appears to be involved in coordination of the transported Cl ions, and G243 is one of the residues shown in the present study to be involved in determining high chloride affinity in NKCC2B. The fact that the patient bearing G243D was reported to have only a mild type I Bartter phenotype, is consistent with recent knockout experiments demonstrating overlap of function of NKCC2A and NKCC2B (10).

A threonine-rich transmembrane domain. To assist in consideration of structure-function relationships, the TM2-3 region is presented in Fig. 6 as one long alpha helix to enable visualization of combinations of residues as potential helix neighbors, but without bias intended as to the extent of alpha helical content in this region. Previously proposed membrane crossings are indicated by grey bars. Residues are color-coded to illustrate (A) hydrophobicity, (B) conservation among CCC’s, (C) residues implicated as important in determining ion affinity in NKCC2 in this study and previously in NKCC1, and (D) serine, threonine, and glycine residues.

We have previously noted that TM2 of NKCC is unusually rich in threonine and serine residues (14); these tend to form a stripe on a face of an alpha helix (Fig. 6D). Here we report that the two most important residues in the B-to-AF changes are T240 and T249; loss of either one results in significant loss in apparent Cl affinity. Thrreonine and serine residues could fulfill one of two roles in this transport protein, and paradoxically the two potential roles are essentially exclusive of one another. On the one hand, threonine and serine residues have been demonstrated to stabilize helix-helix interactions, when present as a motif on one face of a transmembrane alpha helix (21). It is possible that interactions between TM2 and TM8, also rich in threonine and serine, could be stabilized by these residues. If that is the case, affinity shifts observed with mutation would be indirectly due to general structural changes.

A second potential role for these residues is that the serine-threonine-rich faces of TM2 and TM8 may be involved in coordination of the transported ions, uncharged polar residues presenting a minimal thermodynamic barrier to passage of both cations and anions. Recent high resolution structures have illustrated participation of serine and threonine side chain hydroxyls in the binding of Cl by halorhodopsin (22) and by a CLC H-Cl exchanger (23), and of Na and leucine in a Na/Cl-dependent neurotransmitter transporter (24); in each case, the translocation site relies on hydrogen bonding interactions and partial charges from the protein rather than on direct interaction with formally charged residues. Our data do not compel a choice between the above alternatives. On the one hand T240 and T249 play the largest role in B-to-F transitions, and for each residue, mutation results in an apparently lowered affinity of the Cl binding site, consistent with a potential role for threonine in direct coordination of the Cl ion. On the other hand, the changes in these residues exhibit pronounced effects only when accompanied by changes elsewhere in TM2 or ICL1, possibly indicative of more generalized structural changes.

Highly conserved glycines in the TM2-TM3 region. As illustrated in Fig. 6D, our region of interest also includes a number of highly-conserved glycine residues, including two of the most conserved residues of the CCC family, the GG of the GGAYYLIS signature sequence. Glycine residues have been show to play two roles in membrane protein structure, as structural backbone elements and as critical hinge residues. A) The motif GXXXG forms a strong transmembrane helix packing motif, the small glycine residues allowing close packing of neighboring helices (25); the extended version of this motif, the “glycine zipper” is a strong predictor of membrane packing (26). In NKCCs, three highly-conserved glycine residues at the end of predicted ICL1 form a potential glycine zipper, suggesting that this region may actually be embedded in the membrane. B) Because of its small size, glycine confers flexibility in the peptide chain, and a gly-gly motif is often seen as a hinge point in protein structure (cf. (27,28)). We propose that the gly-gly of the GGAYYLIS sequence, conserved throughout the CCC family, forms a hinge that is important in the conformational transition involved in ion transport. The functional importance of these residues is underscored by the finding that
mutation of the second of these glycines to glutamate has been identified as the genetic defect in an antenatal Bartter patient (20).

**Structure in the TM2-TM3 region.** All CCC’s share a remarkably highly conserved region that includes ICL1 and the end of predicted TM2 – about 40 of 50 amino acids are identical or very similar throughout the family (Fig. 6B). Without question this region must play an essential role in the structure and function of all members of the transporter family and is presumably directly involved in the transport process – over a decade ago we noted the probable significance of the region and noted a pattern of conserved residues that suggested helical structure to the first part of ICL1 (14). This proposal that the initial part of ICL1 is an extension of the TM alpha helix is strongly supported by the pattern of residues (T,A,Y in NKCC2B) found to mediate much of the NKCC2 variant difference. In contrast however, similar results with shark NKCC2 have been interpreted in the context of a flexible segment (16). Interestingly, the entire region TM2-ICL1-TM3 appears to be coordinately inserted in the membrane during protein synthesis (29), further suggesting unusual characteristics of this part of the protein structure.

In order to develop testable hypotheses, it may be useful to propose a new working model for the TM2-ICL1 region. In Fig. 6E we use the old working model to highlight and summarize points which are likely to require further examination and in Fig. 6F we present our new model which addresses these issues. (a) A conserved GXXXG motif suggests an earlier initiation of TM1. (b) The NKCC2B T,A,Y residues may be on one face of an alpha helix, involved in transport, thus extending the membrane helix with the first part of the old ICL1. (c) The region of residues which appear to be involved in transport is very extensive, suggesting an internal hairpin structure that brings these groups in proximity with one another. (d) The highly-conserved gly-gly motif of the GGAYYLIS sequence is quite possibly a hinge involved in conformational change. (e) Very high conservation in putative ICL1 suggests that this region is actually embedded in the membrane and part of the translocation machinery, possibly in a reentrant “pore loop” like structure. (f) A conserved glycine zipper motif at the end of putative ICL1 may form the beginning of a transmembrane domain. An attractive possibility is that glycine zippers in TM1 and TM3 hold these two helices together (not shown in 2-D in Fig. 6F) to stabilize the transport region; alternatively one or the other of these could be involved in homooligomerization as in ion-channel pore structures (26).

The pore loop motif has been demonstrated to be an essential structural and functional element in both ion (30) and water channels (31), comprising the selectivity filter for ions and part of the conduction pathway for water. Reentrant pore loops have also been implicated in the function of a number of transporters, including the glutamate transporter (32,33). For both the water channel and the glutamate transporter, early predictions based on experimental evidence (32,34) were later borne out by the 3-D structures (31,33). Since in each of the latter structures there are two pseudo-symmetrical pore loops that enter from opposite sides of the membrane and meet in the interior, it is tempting to search for a similar paired structure in CCCs: the loop following TM8 seems to be the only reasonable candidate, but with the difficulty that current evidence places this region on the same side of the membrane as ICL1.

Subsequent to initial review of this paper, we became aware of the proposal of a reentrant loop structure in xCT, a component of the x-cysteine-glutamate exchange transporter (35). Heteromeric amino acid transporters are very distant relatives of cation-chloride cotransporters, with about 18% identity through the aligned membrane domains, but without the large C-terminal domain that is ubiquitous in CCCs. Significantly, the GGAYYLIS motif sequence of ICL1 in CCCs is partially matched by GGHY in xCT. By means of scanning cysteine accessibility studies, Gasol and coworkers arrived at a 12-TM membrane topology similar to that supported for the CCCs(29), and with GxY at the apex of a reentrant loop corresponding directly to the placement of GxY in Fig. 6f (35). This concordance of independent models for distantly homologous transporters adds considerable confidence to our proposal of a reentrant pore loop structure involved in cation-chloride cotransport.
Clearly, the region encompassed by the NKCC2 splice residues and neighboring ICL1 are at the core of the functionality of cation-chloride cotransporters. The current study and those of others (16,17) have highlighted residues that determine ion affinities in NKCC2 and are presumed to be part of the translocation pocket, complementing related findings in NKCC1 (2).

The importance of the region is underscored by the finding of Bartter disease mutations (20). Further insight into structure-function relationships in the transport process awaits additional experiments on TM2-ICL1 residues, and actual determination of 3-D structure.

REFERENCES

1. Simon, D. B., Karet, F. E., Hamdan, J. M., DiPietro, A., Sanjad, S. A., and Lifton, R. P. (1996) Nat Genet 13(2), 183-188.
2. Isenring, P., Jacoby, S. C., and Forbush, B., 3rd. (1998) Proc Natl Acad Sci U S A 95(12), 7179-7184.
3. Darman, R. B., and Forbush, B. (2002) J Biol Chem 277(40), 37542-37550.
4. Dowd, B. F., and Forbush, B. (2003) J Biol Chem 278(30), 27347-27353.
5. Piechotta, K., Lu, J., and Delpire, E. (2002) Journal of Biological Chemistry 277(52), 50812-50819.
6. Darman, R. B., Flemmer, A., and Forbush, B. (2001) J Biol Chem 276(37), 34359-34362.
7. Isenring, P., Jacoby, S. C., Chang, J., and Forbush, B. (1998) J Gen Physiol 112(5), 549-558.
8. Isenring, P., and Forbush, B., 3rd. (1997) J Biol Chem 272(39), 24556-24562.
9. Igarashi, P., Vanden Heuvel, G. B., Payne, J. A., and Forbush, B., 3rd. (1995) Am J Physiol 269(3 Pt 2), F405-418.
10. Castrop, H., Lorenz, J. N., Hansen, P. B., Friis, U., Mizel, D., Oppermann, M., Jensen, B. L., Briggs, J., Skott, O., and Schnermann, J. (2005) American Journal of Physiology - Renal Physiology 289(6), F1185-1192.
11. Gimenez, I., Isenring, P., and Forbush, B. (2002) J Biol Chem 277(11), 8767-8770.
12. Plata, C., Meade, P., Vazquez, N., Hebert, S. C., and Gamba, G. (2002) J Biol Chem 277(13), 11004-11012.
13. Gagnon, E., Forbush, B., Caron, L., and Isenring, P. (2003) Am J Physiol Cell Physiol 284(2), C365-370.
14. Payne, J. A., and Forbush, B., 3rd. (1994) Proc Natl Acad Sci U S A 91(10), 4544-4548.
15. Gimenez, I., and Forbush, B. (2002) FASEB Journal 16, 26a.
16. Gagnon, E., Bergeron, M. J., Brunet, G. M., Daigle, N. D., Simard, C. F., and Isenring, P. (2004) J Biol Chem 279(7), 5648-5654.
17. Gagnon, E., Bergeron, M. J., Daigle, N. D., Lefoll, M. H., and Isenring, P. (2005) Journal of Biological Chemistry 280(37), 32555-32563.
18. Lytle, C., Xu, J. C., Biemesderfer, D., and Forbush, B., 3rd. (1995) Am J Physiol 269(6 Pt 1), C1496-1505.
19. Lytle, C., McManus, T. J., and Haas, M. (1998) Am J Physiol 274(2 Pt 1), C299-309.
20. Vargas-Poussou, R., Feldmann, D., Vollmer, M., Konrad, M., Kelly, L., van den Heuvel, L. P., Tebourbi, L., Brandis, M., Karolyi, L., Hebert, S. C., Lemmink, H. H., Deschenes, G., Hildebrandt, F., Seyberth, H. W., Guay-Woodford, L. M., Knoers, N. V., and Antignac, C. (1998) Am J Hum Genet 62(6), 1332-1340.

21. Dawson, J. P., Weinger, J. S., and Engelman, D. M. (2002) Journal of Molecular Biology 316(3), 799-805

22. Kolbe, M., Besir, H., Essen, L. O., and Oesterhelt, D. (2000) Science 288(5470), 1390-1396

23. Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Nature 415(6869), 287-294

24. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Nature 437(7056), 215-223

25. Russ, W. P., and Engelman, D. M. (2000) Journal of Molecular Biology 296(3), 911-919

26. Kim, S., Jeon, T. J., Oberai, A., Yang, D., Schmidt, J. J., and Bowie, J. U. (2005) Proceedings of the National Academy of Sciences of the United States of America 102(40), 14278-14283

27. Vila, R., Ponte, I., Jimenez, M. A., Rico, M., and Suau, P. (2002) Protein Science 11(2), 214-220

28. Grant, G. A., Xu, X. L., and Hu, Z. (2000) Biochemistry 39(24), 7316-7319

29. Gerelsaikhan, T., Parvin, M. N., and Turner, R. J. (2006) Biochemistry 45(39), 12060-12067

30. Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280(5360), 69-77

31. Sui, H., Han, B. G., Lee, J. K., Walian, P., and Jap, B. K. (2001) Nature 414(6866), 872-878

32. Grunewald, M., Bendahan, A., and Kanner, B. I. (1998) Neuron 21(3), 623-632

33. Yernool, D., Boudker, O., Jin, Y., and Gouaux, E. (2004) Nature 431(7010), 811-818

34. Jung, J. S., Preston, G. M., Smith, B. L., Guggino, W. B., and Agre, P. (1994) J Biol Chem 269(20), 14648-14654

35. Gasol, E., Jimenez-Vidal, M., Chillaron, J., Zorzano, A., and Palacin, M. (2004) J Biol Chem 279(30), 31228-31236

FOOTNOTES

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The abbreviations used are: NKCC, Na-K-Cl cotransporter; A, NKCC2 variant A; B, NKCC2 variant B; F, NKCC2 variant F; CCC, cation-chloride cotransporter, TM, transmembrane domain; ICL, intracellular connecting loop; rb, rabbit; sa, shark (spiny dogfish); fu, fugu; hu, human; m, mouse
LEGENDS TO FIGURES

Fig. 1. NKCC2 alternative splice region alignment. Comparison of the 32 amino acid sequence encoded by the alternatively spliced exon in NKCC2 and the same sequence in other members of the Na-dependent cation-chloride cotransporters. The top bar indicates the previously proposed TM2 and ICL1 (14). The residues investigated in the present study are bold typed and inside shaded boxes. NKCC: Na-K-Cl cotransporter; NCC: Na-Cl cotransporter; rb: rabbit; sa: shark (spiny dogfish); fu: fugu; hu: human; m: mouse.

Fig. 2. Analysis of NKCC2B point mutants. Oocytes were injected with wild type NKCC2B or with point mutants. A) $^{86}$Rb influx rates at three concentrations of extracellular chloride (mean±SEM, n>3). B, Western blotting analysis of NKCC expression levels 3 days after injection of cRNA into Xenopus oocytes. Blots were probed with T4 antibody.

Fig. 3. $K_{m(Cl)}$ for B-to-F mutants. $^{86}$Rb influxes mediated by NKCC2 variants and mutants were measured in Xenopus oocytes at three different Na concentrations and data were fit to the Hill equation (Hill coefficient=1.6), as described in methods. For ATG•TY/SVT•MV and ATG•TA/SVT•MC it was necessary to assume $V_{max}$=6 nmol/oo/hr in order to obtain a stable fit. Data are from 3-20 experiments for each construct (see Table 1A for n’s).

Fig. 4. $^{86}$Rb influx into Xenopus oocytes as a function of Cl and Na concentration. A, Cl dependence. B, Na dependence. Oocytes were injected with cRNA for NKCC2B (●), NKCC2F (■), ATG/SVT (▲), TAY/MCV (□), and ATG•TAY/SVT•MCV (○) (mean±SEM, n=4, except n=1 for Cl dependence of the ATG•TAY/SVT•MCV mutant). Curves are from least squares curve fits with Hill coefficient=1.6.

Fig. 5. $K_{m(Cl)}$ for B-to-A mutants. As in Fig. 3, data were fit to the Hill equation (Hill coefficient=1.6); n’s are given in Table 1B.

Fig. 6. Structural considerations in cation-chloride cotransporters. A-D, Alpha-helical presentations of the primary sequence of NKCC2B. Gray bars back the regions previously assigned to TM2 (top) and TM3 (bottom). A, Residues are colored by a K-D hydrophobicity scale (red=hydrophobic). B, Residues are colored by degree of homology among CCC’s (red=identity). C, Highlighted are residues implicated as important in determining ion affinity in NKCC2 in this study and previously in NKCC1 (orange). D, Serine and threonine (red), and glycine (green) are colored, with highly conserved residues in brighter colors. E, Previous model of the TM1-TM3 region of NKCC2 (primary sequence of NKCC2A is shown), highlighting regions and residues discussed in the text (a-f refer to points in the text). F, New working model of TM1-TM3, revised from E according to discussion in the text.


### Table 1A: Kinetic analysis of B-to-F mutants.

|                | $K_m$ ± flux ± n |
|----------------|------------------|
| water          | 0.08 ± 0.05 ± 4  |
| G243D          | 0.05 ± 0.01 ± 5  |
| NKCC2B         | 11.2 ± 0.8 ± 5.0 ± 0.5 ± 20 |
| NKCC2 A        | 38.7 ± 3.2 ± 4.6 ± 0.6 ± 18 |
| NKCC2F         | 122.8 ± 18.8 ± 1.4 ± 0.2 ± 13 |
| B-to-F mutants |                  |
| A238S          | 14.0 ± 2.8 ± 3.8 ± 1.1 ± 8  |
| T240V          | 16.0 ± 2.3 ± 2.4 ± 0.4 ± 9   |
| G243T          | 21.8 ± 7.7 ± 2.1 ± 0.5 ± 7   |
| T249M          | 21.8 ± 6.5 ± 0.6 ± 0.1 ± 10  |
| A253C          | 13.8 ± 1.1 ± 5.0 ± 0.2 ± 3   |
| Y257V          | 15.2 ± 4.1 ± 4.4 ± 1.2 ± 5   |
| AT*/SV*        | 21.0 ± 2.5 ± 2.5 ± 0.8 ± 3   |
| AG*/ST*        | 15.1 ± 2.6 ± 2.6 ± 0.5 ± 3   |
| TG*/VT*        | 20.5 ± 3.4 ± 2.1 ± 0.4 ± 6   |
| *TA*/MC        | 104.1 ± 40.1 ± 0.3 ± 0.0 ± 4 |
| *TY*/MV        | 47.0 ± 10.2 ± 1.8 ± 0.5 ± 5  |
| *AY*/CV        | 17.9 ± 6.4 ± 7.8 ± 1.4 ± 4   |
| A*T/S*M        | 24.6 ± 4.3 ± 0.8 ± 0.1 ± 7   |
| ATG*/SVT*      | 53.7 ± 7.0 ± 4.9 ± 0.8 ± 6   |
| *TAY*/MCV      | 47.6 ± 8.8 ± 3.9 ± 1.2 ± 6   |
| TG*T/VT*M      | 149.2 ± 86.2 ± 0.4 ± 0.1 ± 3 |
| TG*Y/VT*V      | 36.8 ± 8.7 ± 5.4 ± 0.5 ± 5   |
| ATG*T/SVT*M    | 100.5 ± 21.1 ± 0.8 ± 0.2 ± 6 |
| ATG*A/SVT*C    | 61.4 ± 9.3 ± 4.6 ± 1.3 ± 6   |
| ATG*Y/SVT*V    | 38.0 ± 5.1 ± 10.1 ± 1.6 ± 6  |
| A*TAY/S*MCV    | 63.2 ± 3.5 ± 8.0 ± 3.0 ± 3   |
| T*TAY/V*MCV    | 117.6 ± 6.6 ± 4.3 ± 1.3 ± 3  |
| G*TAY/S*MCV    | 81.4 ± 4.4 ± 5.0 ± 1.2 ± 3   |
| ATG*TA/ SVT*MC | 110.0 ± 15.9 ± 3.1 ± 0.4 ± 4 |
| ATG*TY/ SVT*MV | 86.7 ± 7.0 ± 3.7 ± 0.3 ± 4   |
| ATG*AY/ SVT*CV | 45.2 ± 4.5 ± 4.9 ± 0.3 ± 3   |
| TG*TY/ VT*MV   | 150.6 ± 27.9 ± 2.2 ± 0.3 ± 3 |
| ATG*TAY/ SVT*MCV | 119.0 ± 14.2 ± 4.5 ± 1.5 ± 3 |
Table 1B: Kinetic analysis of B-to-A mutants.

|                | $K_m$ (mM) | ±   | flux (nmol/oocyte/hr) | ±   | n  |
|----------------|------------|-----|-----------------------|-----|----|
| NKCC2B         | 11.25      | 0.82| 5                     | 0.5 | 20 |
| NKCC2 A        | 38.67      | 3.25| 4.57                  | 0.6 | 18 |
| NKCC2F         | 122.8      | 18.8| 1.45                  | 0.2 | 13 |
| B-to-A mutants |            |     |                       |     |    |
| G236L          | 6.0        | 1.2 | 3.4                   | 1.3 | 4  |
| A238S          | 12.3       | 2.6 | 3.7                   | 0.8 | 10 |
| V239T          | 9.2        | 2.7 | 2.1                   | 0.9 | 6  |
| T240M          | 18.7       | 2.5 | 3.7                   | 0.8 | 10 |
| G243S          | 11.1       | 3.1 | 2.4                   | 0.4 | 7  |
| Y257F          | 18.7       | 8.4 | 0.2                   | 11  |
| TG/MS          | 18.4       | 4.5 | 0.2                   | 12  |
| ATG•/SMS•      | 20.2       | 1.0 | 6.5                   | 1.2 | 7  |
| AVT•/STM•      | 27.5       | 1.3 | 9.3                   | 3.6 | 3  |
| AVG•/STS•      | 23.1       | 1.8 | 7.3                   | 2.6 | 3  |
| TGY•/MSF•      | 15.8       | 2.2 | 1.2                   | 0.2 | 4  |
| VTG•/TMS•      | 32.3       | 10.1| 6.5                   | 3.7 | 3  |
| AVTG•/STMS•    | 33.7       | 4.3 | 4.1                   | 2.1 | 5  |
| GAVTG•Y/LSTMS•F| 39.8       | 6.1 | 5.5                   | 1.1 | 9  |

Table 1, Kinetic analysis of NKCC2 splice variants mutants. Summary of $K_m$ (mM) and $^{86}$Rb influx level (nmol/oocyte/hr) for constructs described in this paper. $^{86}$Rb influxes mediated by NKCC2 variants and mutants were measured at three different Na concentrations (10, 40, 100 mM) and data were fit to the Hill equation (Hill coefficient=1.6), as described in methods. For ATG•TY/SVT•MV and ATG•TA/SVT•MC (Table 1A) it was necessary to assume $V_{max}$=6 nmol/oo/hr in order to obtain a stable fit. “Flux” presented in the third data column is the $^{86}$Rb influx value (nmol/oocyte/hr) obtained at 100 mM Cl.
|          | TM2                                                                 | ICL1                                                                 |
|----------|---------------------------------------------------------------------|---------------------------------------------------------------------|
| rbNKCC2B | LGIVIIGLAVTVTGITSALTSAIANTNHYVRGG                                 |                                                                     |
| rbNKCC2A | LGIVIILLSTMTSITGLSTSAIATNGFVRGG                                   |                                                                     |
| saNKCC2A | LGIVIIVLLATITSTITGLSTSAISTINGCVRGG                                 |                                                                     |
| rbNKCC2F | LGIVIVGLSVVVTTLTGISMSAICTNGVVRGG                                  |                                                                     |
| saNKCC2F | LGIVICLSTVVTVLTCLISMSAICTNGVVRGG                                  |                                                                     |
| fuNKCC2F | LGIVIILLSCVTITCLISMSAICTNGIVVRGG                                  |                                                                     |
| huNKCC1  | LSVLVIMMATVTITGLSTSAIATNGFVRGG                                    |                                                                     |
| saNKCC1  | LALLVIGTATVTTITGLSTSAITTNGFVRGG                                   |                                                                     |
| mNCC     | LTWLILLSVMVTSITGLSISAIINSTNGKVKSG                                  |                                                                     |
Figure 2, Giménez & Forbush
Figure 3, Giménez & Forbush
Figure 6, Giménez & Forbush
The residues determining differences in ion affinities among the alternative splice variants F, A, and B of the mammalian renal Na-K-Cl cotransporter (NKCC2)
Ignacio Giménez and Biff Forbush

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