Ion and Bumetanide Binding by the Na-K-Cl Cotransporter

IMPORTANCE OF TRANSMEMBRANE DOMAINS

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The Na-K-Cl cotransporter (NKCC) plays a key role in electrolyte secretion and absorption across polarized epithelia. The structure of the Na-K-Cl cotransporter transport protein is not known, but from analysis of the primary amino acid sequence and biochemical studies, it has been inferred that the protein has large cytoplasmic N and C termini and a hydrophobic central domain containing 12 transmembrane helices. Both the central domain and the C-terminal domain are highly conserved within the cation-chloride cotransporter family. This paper examines the role of these three domains in interacting with the transported ions and with the inhibitor bumetanide. We have used a chimera approach, exploiting the functional differences between the structurally similar shark and human secretory Na-K-Cl cotransporters (sNKCC1 and hNKCC1). These transporters are 74% identical to one another and have similar transport and regulatory behaviors; however, sNKCC1 differs markedly from hNKCC1 with regard to apparent affinities for the cotransported ions and for bumetanide. We prepared six sNKCC1-hNKCC1 chimeras in which N and C termini were interchanged between species. When transfected in HEK-293 cells, each chimera carried out bumetanide-sensitive 86Rb influx, demonstrating transporter synthesis and cell surface delivery. Monoclonal antibodies J3 and J7 were used to detect the chimeric proteins, and the epitopes for these antibodies were localized to residues 49–196 and 1050–1168, respectively, in the shark sequence. For each of two chimeras that were examined, the time course of activation in low Cl– medium was the same as for the parent proteins; activation was found to proceed through a change in V_{max} rather than K_{m}. For the six chimeras, the apparent affinities for Na^{+}, K^{+}, Cl–, and bumetanide segregated exactly according to whether the large hydrophobic central domain was derived from sNKCC1 or hNKCC1. Significantly, the well-conserved C terminus does not appear to contain residues involved in the shark-human affinity differences. These results demonstrate that residues involved with ion translocation and inhibitor binding are within the large central domain that contains the 12 predicted transmembrane helices.

The Na-K-Cl cotransporter (NKCC) participates in net salt movement across epithelia by coupling the electroneutral cellular uptake of Na^{+}, K^{+}, and Cl– (cf. Refs. 1 and 2). There are two known isoforms of NKCC (also called BSC; Ref. 3), a member of the cation-chloride cotransporter family (1, 4). The secretory isoform (NKCC1), which is widely expressed in vertebrate tissues, has been cloned from several sources including the shark rectal gland (2), the rat kidney (5), and the human colon (6). The absorptive NKCC isoform, which is kidney-specific, has been found as 3 splice variants in the rabbit and mouse (7, 8). The cation-chloride cotransporter family also includes the thiazide-sensitive Na-Cl cotransporter (Ref. 9), as well as the K-Cl cotransporter recently cloned from rabbit kidney and rat brain (4, 10).

Translocation by the Na-K-Cl cotransporter requires the simultaneous binding of Na^{+}, K^{+}, and Cl–, and the apparent affinity for each ion depends on the concentration of the other cotransported ions (1, 11). The binding of the specific inhibitor bumetanide is also dependent on the simultaneous presence of Na^{+}, K^{+}, and Cl– in the medium (1). Similar observations have been made for binding of furosemide to K-Cl cotransporter (12).

Hydropathy analysis of the NKCC sequence delineates three broad regions as follows: large N and C termini and a ~500-residue central domain that is predicted to contain 12 transmembrane helices. Sites of regulatory phosphorylation have been identified in the N and C termini, demonstrating that at least parts of these domains are intracellular (2, 13). In the N terminus, only a small region encompassing the phosphorylation site is well conserved. In contrast, the C terminus is very well conserved among species and isoforms, with homology extending from cyanobacterium to man (14, 15). Candidate sites for N-linked glycosylation are found in a large loop between the 7th and 8th predicted transmembrane helices, and mutagenesis experiments confirm the glycosylation of these sites, showing that the loop is extracellular (16). These features are summarized in the models shown in Fig. 1.

Ion translocation must be mediated by amino acid residues within the plane of the lipid bilayer. In the simplistic model presented in Fig. 1, such residues are depicted to lie within the 12 predicted transmembrane helices. However, several short hydrophobic regions in the large C-terminal domain (2) introduce the possibility that segments of the C terminus may be embedded in the bilayer as β strand (17) or as short peptide loops (18). Furthermore, the extensive sequence conservation within the C terminus suggests an important functional role for this domain. Therefore, it is particularly important to test the model of Fig. 1 by determining experimentally which residues are involved in ion translocation.

To date, there has been no structure-function analysis of any

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1 The abbreviations used are: NKCC, Na-K-Cl cotransporter; bp, base pair(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonic acid.
of the cation-chloride cotransporters beyond the identification of phosphorylated residues involved in transport activation. For other transporters, mutational analysis has generally supported the hypothesis that residues in predicted transmembrane helices mediate solute translocation, whereas hydrophilic domains may be important through allosteric interactions. For instance, Noël and Pouyssegur (20) showed that the Na⁺-H exchanger is able to carry out antiport activity without its N- or C-terminal hydrophilic domains (19), but that the N-terminal domain is necessary for allosteric activation by intracellular acidification (20). Similarly, mutants of the Na-Ca exchanger that lack the large cytosolic domain between helix 6 and 7 are no longer regulated by free Ca²⁺ but retain transport activity (21). However, in some cases, allosteric interactions may be manifested as changes in ion affinity, as illustrated by the finding that modification of the cytoplasmic domain of the Na pump (22) results in changes in affinity for translocated K⁺.

Here we report the use of chimeras to explore the roles of the N terminus, C terminus, and transmembrane domain in ion and bumetanide binding by NKCC1. The analysis of chimeras between similar forms of a membrane protein is a powerful strategy to determine the role of the large structural domains. The exchange of regions between evolutionarily related carriers results in many conservative amino acid substitutions and is a rapid method to define important functional regions. As an alternative to selection of individual point mutations or deletion analysis, chimeras offer the advantage of determining the role of intact domains and limiting disruption in protein folding.

In this study we have begun to localize regions of NKCC1 responsible for ion and bumetanide binding, taking advantage of the fact that although the shark and human transporters are 74% identical, the Kₑ, for Na⁺, K⁺, and Cl⁻ and the Kᵦ for bumetanide are 4–6-fold higher in sNKCC1 compared with hNKCC1 (6). Functional analysis of these chimeras demonstrates that it is the central segment containing the transmembrane helices that specifies the kinetic characteristics of the NKCC proteins and that the cytoplasmic N and C termini have little if any role in determining the species differences in ion and inhibitor binding. A preliminary report has been presented (23).

**EXPERIMENTAL PROCEDURES**

Wild-type cDNAs and Vectors—The sNKCC1 and the hNKCC1 cDNAs, originally in the vector Bluescript SK−, were transferred to the mammalian expression vector pJB20 as KpnI-XhoI fragments. Subcloning in pJB20 required the addition of a second KpnI restriction site in the 5′ linker of sNKCC1/Bluescript SK− and hNKCC1/Bluescript SK− and of a new XhoI site in the 3′ linker of the vector pJB20; mutations in the linkers were created by inserting pairs of complementary synthetic oligonucleotides containing the appropriate restriction sites. The oligonucleotides containing the KpnI and XhoI sites were CTGAGATCCG-GTACCCTCTAGCT and CCGCCTCTAGGATCGATCTCGAGA, respectively. pJB20 (24) is derived from pCB6 (25) and contains intronic sequences at the 3′ end of the linker to improve transcript stability; transcription of the insert is under the control of the cytomegalovirus promoter, and selection of transfectants is achieved through geneticin (G418) resistance.

Chimera Nomenclature and Construction—In our nomenclature, single letters (s, h) are used to designate segments from the shark and the human NKCC1 (Fig. 1), and a number (x.x) indicates the location of the chimeric junctions relative to the 12 putative transmembrane domains (0. indicates the N terminus, 1. to 12. indicate transmembrane regions, and 13. indicates the C terminus; each domain is roughly divided in 10 subdomains from 0 to 9). For example, sh0.9 represents a chimera with a shark N terminus and the remainder comprised of human sequence; the junction lies at the end of the N terminus near the transmembrane domain. The two junction points utilized in this study are at 0.9 (at His²⁴⁷/Val²⁴⁸ in sNKCC1 and Tyr²⁷⁵/Thr²⁷⁶ in hNKCC1) and 13.1 (at His⁷⁸⁵/Val⁷⁸⁶ in sNKCC1 and His⁸⁶⁵/Val⁸⁶⁶ in hNKCC1).

sNKCC1/hNKCC1 chimeras were generated directly in pJB20 by fragment exchange at common restriction sites. For this purpose we used a unique KpnI site in the 5′ linker of both constructs and a unique PmlI site in a conserved region of the C terminus, 25 amino acids after the last transmembrane domain (bp 2703 of sNKCC1 and 2511 of hNKCC1). In the N terminus, a convenient BstI restriction site is unique in hNKCC1/pJB20 (bp 986), at a position encoding the 15th amino acid before the first transmembrane domain. A corresponding BstI site was created silently at bp 1175 in sNKCC1 and for hs13.1 and sh13.1, the BstI-PmlI linker of both constructs and a unique KpnI-PmlI linker of the vector pJB20; mutations in KpnI and PmlI fragments of sNKCC1/hNKCC1 obtained by fragment exchange to generate a full-length construct sNKCC1/pJB20.

Chimeras sh0.9 and hs0.9 were generated by ligating the appropriate KpnI-BstI 1107I fragments from sNKCC1/pJB20 and hNKCC1/pJB20, and for hs13.1 and sh13.1, the KpnI-PmlI fragments of sNKCC1/pJB20 and hNKCC1/pJB20 were used. sh0.9/13.1 was created by ligating the KpnI-PmlI fragments of sh0.9 and sNKCC1/pJB20; hs0.9/13.1 was created by ligating the KpnI-PmlI fragments of hs0.9 and hNKCC1/pJB20.

The deletion mutant sNKCC1-1350–2161/pJB20 was generated by cutting sNKCC1/pJB20 at the unique restriction sites BstI and Bpu1102I that flank a region of sNKCC1 corresponding to residues 50–216 of the N terminus. After complete digestion, the 5′ recess ends were filled with the Klenow fragment in restriction digest buffer supplemented with 33 μM dNTPs. The larger fragment containing the vector, the UTRs, residues 1–48, and residues 217 to the stop codon was gel-purified and blunt-end ligated using T4 DNA ligase.

![Fig. 1. Models for sNKCC1, hNKCC1, and the six chimeras based on proposed structure (6). Circles symbolize amino acid residues (black = sNKCC1 and gray = hNKCC1).](https://www.nature.com)
The truncation mutant sNKCC1\textsubscript{J3,1004-1191} was generated by cutting sNKCC1/Bluescript SK\textsuperscript{+} at the unique restriction site \\textit{attII}. The 3' recess ends were removed using the Klenow fragment in restriction digest buffer supplemented with 33 \mu M dNTPs. The unique fragment was gel-purified and blunt-end ligated. The resulting construct contains 19 missense residues from 1004 to 1012 (AGGPSMTETV) before the new stop codon; a PstI-HindIII fragment of this construct was moved into corresponding sites in sNKCC1/pJB20 for expression.

All constructs were analyzed by automated sequencing or restriction analysis.

**Functional Expression**—The function of the proteins encoded by the wild-type cDNAs and the six chimeras were examined by isolating stable transfected HEK-293 cell lines. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 \mu g/ml) in a humidified incubator (5% CO\textsubscript{2} at 37 °C).

Nearly confluent cells were split 1:5 in a 10-cm dish the day before transfection. At 50–70% confluence, HEK-293 were transfected with 40 \mu g of cDNA by calcium phosphate precipitation (140 \mu M NaCl, 25 mM HEPES, 0.75 mM Na\textsubscript{2}HPO\textsubscript{4}, 125 mM CaCl\textsubscript{2}, pH 7.1). Forty-eight hours after transfection, cells were selected for G418 resistance (950 \mu g/ml) over 3-week period. Individual well-separated colonies (generally 12–16) were then isolated, amplified, and screened for \textsuperscript{86}Rb influx assay.

**Immunofluorescence Microscopy**—HEK-293 cells were plated on poly-L-lysine pre-coated coverslips and grown to 50–60% confluence. They were then fixed in 3% paraformaldehyde, permeabilized in 0.3% Triton X-100, and incubated 2 h at room temperature with J7 or J3 diluted to 5 \mu g/ml in blocking buffer. The coverslips were reincubated 1 h with goat anti-mouse IgG-conjugated with rhodamine at a 10 \mu g/ml dilution and mounted on glass slides with aquamount (Learner, Inc.). Immunofluorescence was performed on a Zeiss Axioskop microscope using Tmax-400 black and white film (Kodak).

**Binding and Flux Studies**—HEK-293 cells were sub-cultured onto 96-well plates pre-coated with poly-L-lysine and grown to confluence (6 to 8 days) at 37 °C, 5% CO\textsubscript{2}. Ion transport rates were determined by \textsuperscript{86}Rb uptake measurements, and bumetanide binding was assessed by inhibition of \textsuperscript{86}Rb uptake activity. All experiments were done at room temperature (−22 °C), and the solutions were at pH 7.4. Before each flux assay, the cells were incubated in hypotonic low Cl\textsubscript{\textsuperscript{−}} (163 mosm, 2 mM) medium for 1 h (67.5 mM Nagluconate, 2.5 mM Kgluconate, 0.5 mM CaCl\textsubscript{2}/MgCl\textsubscript{2}, 0.5 mM Na\textsubscript{2}HPO\textsubscript{4}/Na\textsubscript{2}SO\textsubscript{4}, and 7.5 mM NaHEPES) to activate the cotransporter. The fluxes were performed for 1 min in a flux medium containing 135 mM NaCl, 5 mM RbCl (2 \mu M \textsuperscript{86}RbCl), 1 mM CaCl\textsubscript{2}/MgCl\textsubscript{2}, 1 mM Na\textsubscript{2}HPO\textsubscript{4}/Na\textsubscript{2}SO\textsubscript{4}, and 0.1 mM ouabain; when necessary, Na\textsuperscript{+} or Rb\textsuperscript{+} was replaced with equimolar amounts of N-methylglucamine and Cl\textsuperscript{−} with glucocotate. To determine bumetanide-insensitive influx, 250 \mu M bumetanide was added directly to the flux medium; residual bumetanide-insensitive flux was largely Na\textsuperscript{+} and Cl\textsuperscript{−} independent (data not shown) indicating that it was not due to incomplete inhibition of NKCC activity by bumetanide. \textsuperscript{86}Rb uptake was terminated by addition of, and three rinses in, ice-cold stop solution (135 mM Kgluconate, 5 mM Nagluconate, 1 mM CaCl\textsubscript{2}/MgCl\textsubscript{2}, 1 mM Na\textsubscript{2}HPO\textsubscript{4}/Na\textsubscript{2}SO\textsubscript{4}, 15 mM NaHEPES, 250 \mu M bumetanide, and 0.1 mM ouabain). Cells were solubilized in 2% SDS and assayed for protein content and for \textsuperscript{86}Rb by counting Cerenkov radiation. Each concentration curve was carried out in a single row of the 96-well plate, and in a typical experiment there were 4 replicate rows. Data were handled on a per-row basis, and counts were normalized to the value at the highest concentration. Data are expressed as means ± S.E.M. among all rows in 3–7 experiments, as described in the figure legends, and non-linear least-squares curve fitting was carried out using the Simplex algorithm.

**RESULTS**

**Deletion Mutants**—In principal, the involvement of individual domains can be assessed by analyzing the behavior of deletion mutants. For example, both the Na-H exchanger (NHIE-2) and band 3 (AE-1) are able to carry out antipporter activity in the absence of their N-terminal domains (19, 28).

**Mapping J3 and J7 Epitopes**—The chimeras described here provide a powerful means to test the hypotheses regarding the epitope localization of our anti-sNKCC1 antibodies. Largely through analysis of proteolytic fragmentation patterns, Lytle et al. (27) concluded that the J7 epitope is located in the C-terminal 24 kDa of the sNKCC1 protein and that the J3 epitope is located within a central 20–25 kDa region that is now known to include the last three transmembrane helices. It was not possible, however, to accommodate all of the fragmentation data within the model. Fig. 2 illustrates the results of Western blot analysis of the eight NKCC constructs expressed in HEK-293 cells, using J3 and J7 antibodies. These antibodies are specific for the shark protein, and neither the native HEK-cell transporter nor hNKCC1 was recognized.

**J7 was found to recognize only the chimeras that include the C terminus of sNKCC1 (Fig. 2, bottom)**, and it did not recognize the truncation mutant sNKCC1\textsubscript{J3,1004-1191} (data not shown). This demonstrates that the J7 epitope is within the region

2 B. Forbush, personal communication.

3 R. Behnke, P. Isenring, and B. Forbush, unpublished observations.
containing residues 1004–1191 of the C-terminal shark sequence. Since J7 does not recognize hNKCC1, the epitope must include residues that are different between sNKCC1 and hNKCC1; they include residues 1050–1168. This localization is consistent with our earlier hypothesis that the J7 epitope is in the 20-kDa C-terminal segment of the protein.

In contrast, J3 labeled only chimeras that include the N terminus of sNKCC1 (Fig. 2, top), and the positive signal with sh0.9 demonstrates that the N terminus of the shark protein is indeed sufficient for antibody recognition. Thus our earlier assignment of the J3 epitope to a central domain based on proteolytic fragmentation patterns was incorrect. The localization to the N-terminal domain is further narrowed to residues 49–196 by the failure of J3 to bind to the deletion mutant sNKCC1del:50–216 (data not shown; J7 detected this truncated protein).

Cell Surface Expression of NKCC1 Chimeras—Immunofluorescence studies using the J3 antibody (27) localized sNKCC1, sh0.9, sh13.1, and shs0.9/13.1 to the cell surface, as illustrated for shs0.9/13.1 in Fig. 3. Some intracellular staining is also observed, a common finding with overexpression of membrane proteins. J3 recognizes the N terminus of sNKCC1, and constructs in which the N terminus was from human (hNKCC1, hs0.9, ha13.1, and hshs0.9/13.1) were negative with J3, as were HEK-293 cells (see hNKCC1 in Fig. 3). The intensity of the rhodamine signal was homogeneous for all the cells on a coverslip, suggesting that the technique used to select individual colonies allowed true clonal expansion.

Functional Expression of NKCC1 Chimeras—When stably expressed in HEK-293 cell lines, each of the chimeras in Fig. 1 exhibited robust levels of bumetanide-sensitive 86Rb influx, illustrated in Fig. 4. Although comparisons of absolute flux rates among individual clonal lines must be made with caution, it may be noted that NKCC1 constructs that contain the human transmembrane domain were found to mediate 86Rb influx at about the same rate as hNKCC1 (1st 4 pairs of bars), 7–9-fold above the endogenous cotransport activity of mock-transfected HEK-293 cells. Similarly, constructs containing the shark transmembrane domain exhibited transport rates similar to sNKCC1, 4–6-fold above the endogenous rate. It is likely that the difference in 86Rb influx rates between sNKCC1 and hNKCC1 is due in part to the fact that the ion binding sites of the shark cotransporter are not saturated with ions in our regular flux medium (see Ref. 2 and below).

Time Course of Activation of NKCC by Reduced [Cl−]—We have previously reported that NKCC1 is activated when intracellular [Cl−] is lowered and that this form of regulation appears to occur in HEK-293 cells as well as in the shark rectal gland (2, 29). For the shark cotransporter, we have identified threonine residues in N and C termini (Thr189 and Thr1114) whose phosphorylation appears to be involved in cotransporter activation (2, 13). Fig. 5 illustrates time courses of NKCC activation in experiments in which the cells were preincubated in a low Cl− hypotonic medium to reduce [Cl−]. The data show that the time course of activation is similar for sNKCC1 and for hNKCC1, as well as for the two chimeras in which both termini are exchanged (t1/2 ~10 min). This finding demonstrates that the chimeras are functionally intact with regard to regulation...
as well as transport, which suggests that shark termini can effectively interact with the human transmembrane domain and vice versa. It is also interesting that N and C termini can be interchanged separately as well as together, indicating that coordinate interaction of the two termini, if present, is retained in the sNKCC1/hNKCC1 chimeras.

The data in Fig. 5 also illustrate that the activation of NKCC1 takes place through a change in \( V_{\text{max}} \) and not through a change in \( K_m \) for Cl\(^-\). This result provides evidence that activation does not occur by a modest modulation of the ion affinities of the transporter but that it occurs by up-regulation from a functionally silent population of transporters (the result does not rule out a very large change in \( K_m \)). This all-or-none activation could occur by translocation from a subcellular compartment or by up-regulation at the cell surface. Our recent experiments (30) are in disagreement with the former hypothesis and in favor of modulation by direct phosphorylation (29).

**Kinetics of Ion Translocation and Bumetanide Inhibition**—To determine which domains confer the sNKCC1/hNKCC1 differences, we measured the dependence of \( ^{86}\text{Rb} \) influx on Na\(^+\), Rb\(^+\), Cl\(^-\), and bumetanide concentration for sNKCC1, hNKCC1, and the six chimeras. Data are illustrated for \( ^{86}\text{Rb} \) influx as a function of [Na\(^+\)] in Fig. 6, fit by a model of Na\(^+\) binding at a single site; the \( K_m \) values are summarized in Fig. 8A.

The \( K_m \) for Na\(^+\) is 15.2 ± 1.5 mM for hNKCC1 and 113 ± 11 mM for sNKCC1, values that are similar to those reported previously (2, 6). Clearly, the data for the chimeras fall into two categories as follows: constructs that include the transmembrane domain of hNKCC1 (upper panels in Fig. 6) exhibit the high apparent affinity for Na\(^+\) characteristic of hNKCC1, and those with the transmembrane domain of sNKCC1 exhibit the low apparent affinity for Na\(^+\) seen in sNKCC1.

Fig. 7 and Fig. 8C present results for the Cl\(^-\) dependence of \( ^{86}\text{Rb} \) influx. The data illustrate a sigmoidal relationship consistent with two binding and translocation sites for Cl\(^-\). Using a simple model of two sites, the \( K_m \) values of 31 ± 1 and 102 ± 0.1 (not shown); this is consistent with the (inexact) fit of the Hill equation to models in which two sites of similar affinity must be occupied for transport to occur (n ~ 1.6; B. Forbush III, unpublished data.).

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**FIG. 6.** \( ^{86}\text{Rb} \) influx as a function of Na\(^+\) concentration for sNKCC1, hNKCC1, and the six chimeras. Cells were incubated in low Cl\(^-\) and hypotonic medium for 1 h and assayed for \( ^{86}\text{Rb} \) uptake in 0, 4.8, 9.6, 19.3, 38.5, 77, 115.5, or 154 mM Na\(^+\). The basic flux medium was as described in Fig. 4. The Na\(^+\) concentration was modified by replacing NaCl, Na\(_2\)HPO\(_4\)/Na\(_2\)SO\(_4\), and NaHEPES with N-methylglucamine Cl, N-methylglucamine PO\(_4\)/SO\(_4\), and N-methylglucamine HEPES, respectively. In each experiment, individual uptakes were normalized to the counts measured at the highest Na\(^+\) concentration (154 mM). These normalized counts were averaged (n = 14–25), and the first seven points were fitted using the Michaelis-Menten equation for binding at a single Na\(^+\) site.

**FIG. 7.** \( ^{86}\text{Rb} \) influx as a function of Cl\(^-\) concentration for sNKCC1, hNKCC1, and the six chimeras. Cells were incubated in low Cl\(^-\) hypotonic medium for 1 h and assayed for \( ^{86}\text{Rb} \) uptake in 0, 4.5, 9, 18, 36, 72, 108, or 144 mM Cl\(^-\). As in Fig. 6, the normalized counts were averaged (n = 12–28) but fitted with a Hill coefficient of 2 using the Michaelis-Menten equation.
of hNKCC1 had $K_m$ values 31–37 mM, whereas for can identify only residues that confer differences in kinetic important functional role.

sequence conservation through the C terminus suggests an that might have been expected to interact with the membrane C-terminal domain has several short hydrophobic segments significant effect on the characteristics of ligand binding. The binding between sNKCC1 and hNKCC1 are exclusively deter-

ior of the six chimeras, the differences in ion and bumetanide translocation and bumetanide binding. As shown by the behav-

DISCUSSION

The results reported here illustrate the importance of the transmembrane domain of the Na-K-Cl cotransporter in ion translocation and bumetanide binding. As shown by the behavior of the six chimeras, the differences in ion and bumetanide binding between sNKCC1 and hNKCC1 are exclusively determined by the large central domain that includes the predicted 12 helices. It is especially noteworthy that interchanging the large C terminus between sNKCC1 and hNKCC1 has no significant effect on the characteristics of ligand binding. The C-terminal domain has several short hydrophobic segments that might have been expected to interact with the membrane and to participate in ion translocation, and the high level of sequence conservation through the C terminus suggests an important functional role.

An obvious limitation of the chimera approach is that one can identify only residues that confer differences in kinetic behavior; residues that are critically involved in transporter function will be missed if they are the same in shark and human forms of the protein. In the present study we examine three large domains of the cotransporter. It seems unlikely that affinity-modifying residues and those crucial for the binding and translocation of ions would be found in different domains of the protein. Therefore, it is reasonable to extrapolate our results to the prediction that both ion binding and inhibitor binding involve principally residues in the transmembrane domain.

We have reported here measurements of Michaelis constants for transport and inhibition ($K_m$ and $K_i$) or “apparent affinities.” These experimentally measured parameters are related to the true binding site affinities in a complex manner that involves all of the rate constants for the transport cycle. It is possible, for instance, that when we determine a difference in $K_m$ comparing two transporters, it is actually a rate constant for conformational change that is different and not the ion binding parameters. This is of particular concern here: the apparent affinity for each of the three transported ions is lower by 4–6-fold in shark compared with human, and this is just the behavior that would be seen if the change in apparent affinity were caused by a change in a rate-limiting step. Preliminary data indicate that this is not the case; in examining mutations within the cotransporter transmembrane domain, we have found some that differentially alter the affinity of the three ions (31). This behavior is inconsistent with the idea that the differences are due to changes in a conformational transition and thus argues strongly that the differences in $K_m$ reflect structural differences in the ion binding sites.

The results reported here are the first mutational analysis of structure-function relationships in the cation-chloride cotransporter family. The chimeras used in these studies represent conservative mutations, and, as demonstrated, exhibit all the hallmarks of functional NKCC proteins as follows: glycosylation, cell surface delivery, inhibition by bumetanide, kinetic behavior consistent with 1Na+:1K+:2Cl⁻ stoichiometry, and activation by preincubation in low Cl⁻. Our results have clearly shown that the central region of the protein, which includes the 12 presumed transmembrane helices, contains all of the residues conferring shark/human ion and bumetanide affinity differences in NKCC1. In future studies it will be informative to extend this chimera approach to allow the investigation of individual transmembrane helices.

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