Scanning Mutagenesis of Transmembrane Helix 3 (TM3) of Na-K-Cl Cotransporter (NKCC1)∗,†

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Background: Na-K-Cl cotransporters (NKCCs) are essential in chloride homeostasis and salt transport.

Results: Mutations in NKCC1 transmembrane domain 3 (TM3) alter transport activity, ion binding, and inhibitor affinities.

Conclusion: This demonstrates a role for TM3 in the NKCC1 transport pathway.

Significance: This is the beginning of a systematic analysis of the Na-K-Cl cotransporter function in the context of structural models.

The Na-K-Cl cotransporter (NKCC) plays central roles in cellular chloride homeostasis and in epithelial salt transport, but to date little is known about the mechanism by which the transporter moves ions across the membrane. We examined the functional role of transmembrane helix 3 (TM3) in NKCC1 using cysteine- and tryptophan-scanning mutagenesis and analyzed our results in the context of a structural homology model based on an alignment of NKCC1 with other amino acid polyamine organocation superfamily members, AdiC and ApcT. Mutations of residues along one face of TM3 (Tyr-383, Met-382, Ala-379, Asn-376, Ala-375, Phe-372, Gly-369, and Ile-368) had large effects on translocation rate, apparent ion affinities, and loop diuretic affinity, consistent with a proposed role of TM3 in the translocation pathway. The prediction that Met-382 is part of an extracellular gate that closes to form an occluded state is strongly supported by conformational sensitivity of this residue to 2-(trimethylammonium)ethyl methanethiosulfonate, and the bumetanide insensitivity of M382W is consistent with tryptophan blocking entry of bumetanide into the cavity. Substitution effects on residues at the intracellular end of TM3 suggest that this region is also involved in ion coordination and may be part of the translocation pathway in an inward-open conformation. Mutations of predicted pore residues had large effects on binding of bumetanide and furosemide, consistent with the hypothesis that loop diuretic drugs bind within the translocation cavity. The results presented here strongly support predictions of homology models of NKCC1 and demonstrate important roles for TM3 residues in ion translocation and loop diuretic inhibition.

The Na-K-Cl cotransporters (NKCCs)3 are electroneutral transporters that mediate the inward transport of Na+, K+ and Cl− across the cell membrane. NKCC1 regulates intracellular Cl− concentration in many cell types and is a critical part of the Cl− secretory pathway in salt secreting epithelia whereas NKCC2 and NCC (Na-Cl cotransporter) are responsible for a major fraction of Na+ and Cl− reabsorption in the renal tubule. Both NKCCs are inhibited by the loop diuretic drugs furosemide and bumetanide. KCCs, related cation-chloride cotransporters which move K+ and Cl− out of the cell, balance NKCC with regard to Cl− concentration and cell volume. These transporters are thus broadly involved in human electrolyte homeostasis, and mutations in the transporters are associated with many disease states including hypertension and epilepsy (1, 2).

Cation chloride cotransporters have a basic structure consisting of large cytoplasmic N and C termini and a 12-transmembrane helix (TM) central domain. The N terminus of NKCCs and the C terminus of KCCs contain threonine residues whose phosphorylation state determines the rate of transport (3, 4). For both NKCCs and KCCs, the transporter exists as a homodimer in the cell membrane (5–8), and recent evidence suggests that dimeric interactions between NKCC C termini are decreased upon activation of the transporter (9).

To date, studies dissecting the function of the transmembrane domain of cation-chloride cotransporters have been rather limited. Three- to 5-fold differences in apparent ion affinity between shark and human NKCC1 were mapped to residues in TMs 2, 4, and 7 (10), and TM2 of NKCC2 has received considerable focus because it is encoded by three differentially spliced exons that confer different ion affinities in different segments of the thick ascending limb in the mammalian kidney (11). A functionally important residue has been implicated near the extracellular end of TM6 (12), and residues in TMs 11 and 12 have been found to affect the binding of loop

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3 The abbreviations used are: NKCC, Na-K-Cl cotransporter; APC, amino acid, polyamine, organocation; ICL, intracellular loop; KCC, K-Cl cotransporter; MTS, methanethiosulfonate; MTSES, 2-sulfonatoethyl methanethiosulfonate; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate; PDB, Protein Data Bank; TM, transmembrane domain.
diuretic drugs (10, 13). Until the recent availability of high resolution structures in the APC (amino acid, polyamine, organo-cation) superfamily, there has not been a good structural context in which to evaluate such studies.

The small family of cation-chloride cotransporters is part of the APC superfamily of transporters. High resolution structures of amino acid transporters APC superfamily members AdiC and ApoT have demonstrated that the key feature of the TM domain is an inverted repeat of TM-5 and 6-10 that comprise a translocation domain surrounding a central ligand binding cavity (14-17). This 5-5 inverted repeat architecture was first described in the LeuT superfamily (18) and is now recognized as the structural core in five superfamilies of transporters (19). In AdiC, hydrophobic residues in TM-11 and 12 form a dimerization interface in a homodimer (15).

In the present study we analyze the function of cysteine- and tryptophan-substituted mutants of NKCC1, scanning most of the length of TM3. The results are evaluated in the context of structural models of NKCC based on homology to AdiC and ApoT which predict that TM-3 makes up one face of a translocation pathway that opens to the extracellular surface. We find excellent agreement between the predictions of the model and the results of the functional studies. The results are consistent with predicted pore-lining residues Tyr-383, Ala-379, Ala-375, Asn-376, Phe-372, Ile-368, and Gly-369 as functionally important in ion coordination and loop diuretic binding and with residue Met-382 as part of a gate that closes the translocation pathway in the occluded state. These experiments are the first step in a systematic effort to understanding the function of cation-coupled cotransporters in the context of high resolution structural models.

**EXPERIMENTAL PROCEDURES**

**Constructs, Transfections, and Cells**—Human NKCC1 DNA (20) constructs with single amino acid substitutions in TM-3 were generated using site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA). HEK-293 cells were transfected with individual constructs using Lipofectamine 2000 (Invitrogen) and selected with 1 mg/ml Genetin (Invitrogen) to generate mixed stable cell lines. Cell lines were maintained in DMEM, 10% FBS, penicillin (50 units/ml), streptomycin (50 units/ml), and Genetin (1 mg/ml) in a 37 °C humidified incubator.

**Western Blotting**—Protein expression was determined for each mutant using Western blotting. Stable cell lines were grown in 12-well plates. Cells were lysed in 1% Triton X-100 with protease inhibitor (Complete; Roche Applied Science) and then centrifuged at 14,000 rpm. Supernatant was measured for total protein concentration, and an equal amount of total protein for each cell line was loaded onto 7.5% Tris-glycine gels. After gel electrophoresis and transfer, membranes were probed with the T4 primary antibody (21) and an IRDye 800 CW goat anti-mouse IgG (LI-COR, Lincoln, NE). Images were acquired using the Odyssey infrared imaging system (LI-COR).

**Immunofluorescence Confocal Microscopy**—Cells were grown in polylysine-coated coverslips, fixed with methanol for 5 min, washed with PBS, and incubated in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature followed by incubations in an anti-HA monoclonal antibody (1:500 in 0.1% BSA in PBS; Covance Research products, overnight, 4 °C) and a secondary anti-mouse FITC-conjugated antibody. Images were obtained using a laser scanning confocal microscope (Zeiss LSM 510; Carl Zeiss).

**86Rb**-Influx Assays—NKCC function was assessed by measuring 86Rb+ influx into HEK cells in a 96-well plate as described previously (3, 10, 22), using an automated 96-well assay flux machine. To optimize cell surface expression (6), cells were grown to confluence in 96-well polylysine-coated plates and moved to a 25 °C incubator 24 h prior to the experiment. To activate NKCC1, cells were exposed to a low chloride hypotonic medium for 45 min (3, 22); in the experiments of Fig. 3, calyculin A was added to a set of samples for the last 15 min to assure maximal activation. 86Rb+ influx was carried out for 1 min in flux medium (135 mM NaCl, 5 mM 86RbCl (2 μCi/ml 86Rb+), 1 mM CaCl2, 1 mM MgCl2, 1 mM Na2HPO4, 2 mM Na2SO4, 0.1 mM ouabain, 5 mM HEPES, pH 7.4) with appropriate composition changes in ion or diuretic affinity measurements. Influx was terminated by washing plates with a high K+ buffer (135 mM K+ gluconate, 5 mM Na gluconate, 1 mM CaCl2/MgCl2, 1 mM Na2HPO4/Na2SO4, 15 mM Na-HEPES, pH 7.4), followed by a MgCl2 solution. Plates were allowed to dry, and 86Rb+ was measured by phosphorimaging analysis of the 96-well plates. Results are presented as mean ± S.E. of three to five experiments: significant difference from wild-type hNKCC1 at p < 0.05 from t test is indicated by asterisks in the figures. All experiments were conducted at room temperature.

In measurements of apparent ion affinity, ion concentrations were varied in the flux medium, with eight data points collected at eight concentrations. CI− was substituted with gluconate and Na+ and Rb+ with N-methyl glucamine. KNa values were determined from nonlinear least squares fits of individual experiments using the Michaelis-Menten equation for Na+ and Rb+ and the Hill equation (n = 1.6) (10) for CI−. We use “apparent affinity” to describe the inverse of KNa, following common usage and recognizing that this is not the true affinity of a transport site itself. The KNa of a transport protein is empirically determined and is a complicated function of all binding affinities and rate constants; true affinities cannot be obtained from transport measurements alone.

In measurements of bumetanide and furosemide affinity, cells were preincubated with low CI− hypotonic medium for 30 min followed by incubation in a medium with 20 mM CI− with five different concentrations of diuretic for 15 min (10). The same concentrations of diuretic were included in the flux medium for furosemide and in some experiments with bumetanide; in other experiments with bumetanide, the diuretic was omitted during the 1-min flux. Data were fit to a single binding site model of inhibition.

To determine inhibition by methanethiosulfonate (MTS) sulphydryl-modifying agents, cells were preincubated in low CI− hypotonic medium for 30 min followed by the addition of 3 mM MTSET or 5 mM MTSES (Toronto Research Chemicals, Toronto, ON, Canada) for 10 min prior to a 1-min flux (23). In the experiment presented in Fig. 7, B and C (below), we measured the conformation dependence of NKCC modification as a function of MTSET concentration. Cells were preincubated in
hypotonic low chloride medium to activate the transporter followed by incubation in medium with 20 mM Cl– with or without 30 μM bumetanide and subsequent addition of MTSET after 5 min. After a 10-min incubation with MTSET, cells were washed three times and incubated in 3 mM Cl– medium for 30 min, followed by a 1-min flux assay. Assuming that the rate of inhibition is proportional to the concentration of the irreversible inhibitor MTSET (M), the results were fit to the equation \[ V = V_{\text{max}} e^{-\left(\frac{M}{M_{t10}}\right)} \], where \( V \) is the remaining flux rate, and \( t10 \) is the fixed 10-min time.

Homology Models of NKCC1 Transmembrane Domain—A multiple sequence alignment of truncated NKCC1 (supplemental Fig. 1) was generated using ClustalW and modified using results from I-Tasser (24); there is a high degree of confidence in the TM3 region in part because of the highly conserved ICL1 (intracellular loop 1) preceding TM3. Using this alignment and the program Modeler (25) homology models (supplemental open and occluded homology models) were built to the outward-facing structure of AdiC (PDB 3QB6) (17) and to occluded structures of ApcT (PDB 3GIA) (14) and AdiC (PDB 3LIL) (16) using the following parameters: library_schedule = autosched.slow, max_var_iterations = 300, md_level = refine.slow, repeat_optimization = 2, max_molpdf = 1e6. The best models were selected according to GA341 and normalized DOPE scores.

RESULTS

Homology Model of NKCC1—The results presented here will be evaluated in the context of a homology model of NKCC1 (Fig. 1) which is based on available structures of amino acid transporters AdiC and ApcT in the same APC superfamily. We chose to begin a detailed examination of NKCC1 structure-function by testing the validity of the homology model in the region of TM3. TM3 is an attractive starting point because over much of its length it is seen to line the presumed translocation pathway in the outward-facing APC transporters, potentially providing insight into the ion translocation process. The ICL1 loop that immediately precedes TM3 is the most highly conserved region in this family of membrane proteins, and TM3 itself also contains a number of very highly conserved residues. Thus, our expectations are high that the alignment is correct in this region and that a model of TM3 built on amino acid transporters will accurately predict the behavior of NKCC mutants.

In the outward-facing structures of ApcT and AdiC and in the NKCC1 homology model derived from them (Fig. 1A), a clear translocation pathway is visible from the extracellular surface to ICL1 (yellow in Fig. 1) with a narrowing at Phe-372 (NKCC1; Tyr-93 in AdiC) approximately halfway through the membrane. In the occluded structure (Fig. 1B), the extracellular portions of TMs 6 and 10 have rotated and bent against TMs 3, 8, and 1, contacting TM3 at Met-382 and Tyr-383 (in NKCC1), thus forming an extracellular “gate” that completely closes off the pathway from the extracellular medium. In AdiC, the binding site for transported arginine is found inside this occluded cavity, between the gate residues and Tyr-93 (Phe-372 in NKCC1) (16), and in ApcT, the cavity is seen to be filled with water molecules inward to ICL1 (14). The conformational changes that allow intracellular exit of translocated ligands is unknown but has been the matter of some speculation (14, 17). One possible mechanism for NKCC1 will be discussed below with reference to Fig. 1, C and D.

Expression and Membrane Localization—We performed scanning mutagenesis of residues 368–385 in TM3 of human...
NKCC1, substituting cysteine and tryptophan for the native residues. Cysteine is particularly useful because its unique reactivity allows specific chemical modification and because it is a small residue that is often well tolerated in substitution experiments. Tryptophan, on the other hand, is a bulky aromatic residue that is a severe test of steric constraints; it has also proven very useful in a scanning approach (26). Wild-type and mutant hNKCC1s were stably expressed in HEK cells, and full-length NKCC1 protein was detected for all of the constructs in the form of an immature band at 140 kDa and for most constructs in a mature glycosylated form at \(190\) kDa (Fig. 2).

All of the cysteine mutants and a majority of the tryptophan mutants show an expression pattern similar to wild-type NKCC1, with most of the protein in the mature form and localized at the plasma membrane by immunofluorescence (supplemental Fig. 2). Clear exceptions were three tryptophan mutants, V380W, A381W, and V384W, which had little if any of the mature glycosylated band (190 kDa, Fig. 2) and were seen to be mostly retained in the endoplasmic reticulum by immunofluorescence (supplemental Fig. 2B). These three residues are predicted to be in an interhelical region between TMs 4/9 and TM3 in the homology model (Fig. 1), and mutation of these residues may disrupt correct protein folding. Some constructs, most notably V380C, express considerably more protein than the wild-type NKCC1; immunofluorescence localization reveals that much of this is due to retention in an intracellular compartment (supplemental Fig. 2A).

**Functional Analysis of Cysteine and Tryptophan Mutants**

To determine the role of TM3 residues in the transport function of hNKCC1, we carried out \(^{86}\)Rb influx assays after incubation with calyculin A to inhibit protein phosphatase 1 and bring about maximal phosphorylation and transport activation (3). As seen in Fig. 3A, whereas most of the cysteine substitution mutants exhibited \(^{86}\)Rb fluxes near that of the wild-type hNKCC1, maximal transport activity was reduced 50% or more in I368C, G369C, A379C, M382C, and Y383C mutants. Substitution by tryptophan had much larger effects; as seen in Fig. 3B, only four residues retained...
Scanning Mutagenesis of NKCC1 TM3

50% or greater of the wild-type activity. In the model these residues are seen in a cluster (Fig. 1F, white) with Leu-370, Ala-373, and Phe-374 facing the lipid environment where tryptophan is well tolerated; Ile-371 is between TM3s 13 and 12 but apparently does not disrupt folding.

At least a 50% decrease in activity resulted from tryptophan substitution at positions that generally conform to a pattern of α-helical periodicity, and most are predicted to lie on the pore-lining face of TM3 in the homology model. Ala-375, Asn-376, and Ala-379 are in the region corresponding to the AdiC ligand binding site, a region bounded on the top by Met-382 and Tyr-383, which appear to form part of the extracellular occlusion gate. Mutation of each of these residues affects transport, either in the case of cysteine or tryptophan substitution. The decrease in activity observed upon substitution at residues Ile-368 and Gly-369 which are found toward the intracellular end of TM3 is somewhat surprising. The homologous residues are at the intracellular end of the water-filled cavity seen in ApcT (14), and they are in contact with ICL1 residues. Although this region has not been implicated in substrate binding in other 5-5 transporters, TM2 residues which are at this level in the structure have been shown to determine the apparent ion affinity differences among NKCC2 splice variants (27). Thus, it seems likely that part of the ion binding pathway involves Ile-368 and Gly-369.

**Apparent Affinity for Transported Ions**—NKCC1 requires four ions to be bound for transport to take place, and available kinetic data support an ordered binding model in which ions bind in the order Na⁺, Cl⁻, K⁺(Rb⁺), Cl⁻ from the outside of the membrane (28). To look for interactions between TM3 residues and translocated ions, we examined the apparent affinity of each of the ions in supporting coupled transport, measured as 86Rb influx in each construct which exhibited sufficient flux to be readily distinguishable above the HEK cell background. As illustrated in Fig. 4, the largest changes in these parameters were found for proposed pore-lining residues Phe-372, Asn-376, and Met-382 as well as for Val-378, which is predicted to lie between TM3 and TM10.

**Further Examination of Residues in Translocation Pathway**—We carried out additional analysis of key pore-lining residues, with results summarized in Fig. 5; results for Met-382 will be discussed further below. Tyr-383 appears in the model to be part of the extracellular occlusion interface and to project into the translocation cavity. Both Y383C and Y383W are inactive and as shown in Fig. 5A. Even the conservative Y383F mutant does not support transport, demonstrating a requirement for the Tyr-383 hydroxyl.

Struck by the sensitivity of Ala-379 to cysteine substitution and its location in the region analogous to the ligand binding region of AdiC, we mutated this residue to glycine, serine, and leucine. A379L was inactive as A379C and A379W, suggesting that a hydrophobic side chain is not tolerated in this position. However, both A379S and A379G retained most of the native activity (Fig. 5A) but with a reduction in apparent ion affinities, particularly for Na⁺ and Cl⁻ (Fig. 5B).

We also examined highly conserved Phe-372, a prominent residue in the aqueous pathway (Fig. 1, A and D). Although this has been described as the “floor” of the cavity in AdiC (15), a water-filled cavity clearly extends inward beyond this point to

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**FIGURE 4.** Kₘ values for Na⁺, K⁺, and Cl⁻. Kₘ for Cl⁻, Rb⁺, Na⁺ in cysteine- and tryptophan-substitution mutants are expressed relative to the wild-type hNKCC1 values: Kₘ(Na⁺) = 18.1 ± 1.6, Kₘ(Rb⁺) = 1.6 ± 0.16, Kₘ(Cl⁻) = 47.9 ± 1.6. A, cysteine substitution mutants. B, tryptophan substitution mutants. Mutants indicated in light gray are nonfunctional and were not analyzed. The shaded bar indicates α-helical periodicity as in Fig. 3. Data shown are a mean ± S.E. (error bars; n = 3–5 experiments) with significant difference from wild-type hNKCC1 indicated (⁎) when p < 0.05.
tion of the transporter and the presence of all three transported ions (29). Binding and inhibition are inhibited by high concentrations of Cl\(^{-}/H_2O\), consistent with the hypothesis that bumetanide binds at the second Cl\(^{-}/H_2O\) binding site, but also consistent with other models of competition. Bumetanide binding and dissociation are relatively slow (dissociation about 0.1 min\(^{-1}\)) (29), and bumetanide becomes "occluded" with Na, K, and Cl (30). There are very few available data regarding the kinetics of furosemide inhibition, other than that it has lower affinity and much faster dissociation rate compared with bumetanide (fully reversible within 1 min).\(^4\)

We examined inhibition of the cysteine- and tryptophan-substituted NKCCs by furosemide and bumetanide, with the results presented in Fig. 6. Experiments were conducted with the rapidly reversible inhibitor furosemide present only in the flux solution or with bumetanide present in a 20-min preincubation (with 20 mM Cl\(^{-}/H_2O\) to optimize binding). Mutants with greatly reduced bumetanide affinity have a higher bumetanide dissociation rate allowing bumetanide to dissociate before the flux assay; thus, these mutants were also characterized with bumetanide maintained in the flux solution (Fig. 6B, hatched bars).

As seen in Fig. 6, substantial changes in diuretic affinity were observed in 9 of the 15 residues tested. Generally, the magnitude of the decrease in bumetanide affinity was considerably greater than the change in apparent ion affinities seen in Fig. 4, arguing against kinetic coupling of ion and diuretic binding as an explanation. Rather, the results lend strong support to the longstanding hypothesis that loop diuretics bind in the translocation pocket of NKCC. For the most part, the pattern of changes was similar for bumetanide and furosemide, supporting the idea that both diuretics bind at the same site. There are, however, clear exceptions, as would be expected by the considerable differences in structure of the inhibitors; for instance, the significant effect on furosemide and bumetanide affinities was in opposite directions with V378W. In addition, there were

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\(^4\) B. Forbush, unpublished data.
strong effects on bumetanide affinity at M382W and F372C with little change in furosemide affinity and a greater effect on furosemide affinity with I368C.

Remarkably, mutation of Met-382 to tryptophan produced a 200-fold decrease in the $K_i$ for bumetanide, practically producing a bumetanide-insensitive NKCC1 with half of the maximal flux of the wild type (Fig. 3) and with similar apparent ion affinities (Fig. 4). In the homology model, Met-382 forms part of an extracellular gate in the occluded state, close against TM10. The bulk of the tryptophan side chain is apparently critical for this effect because substitution with phenylalanine has very little effect on bumetanide affinity (Figs. 5 and 6) or on ion flux (Fig. 5). It seems likely that the bulky tryptophan residue blocks bumetanide entry into the pocket while still allowing ion entry. Val-378 is next to Met-382 in the helix, and the Val-378 side chain is between TM3 and TM10; this may explain the effect of tryptophan substitution at Val-378 in decreasing bumetanide affinity (Fig. 6).

Most of the other residues with large effects on diuretic affinity are in the cytoplasmic half of TM3 (Fig. 6), suggesting that the diuretic binding site may be near the intracellular end of the translocation cavity. Among these, Phe-372 is clearly a pore-lining residue, whereas Val-378 and Ile-371 are predicted to be buried between TM3 and TMs 10 and 12; large effects on both furosemide and bumetanide binding at the latter positions indicate that displacement of the TM3 backbone may play a role.

Modification of Cysteine Residues by MTS Reagents—We tested the effect of treatment of cysteine-substituted NKCC1s with membrane-impermeant cysteine-modifying reagents MTSET and MTSES. As illustrated in Fig. 7, only the most extracellular residues in the TM3 helix exhibited a functional change after reaction with these compounds. Inhibition with either compound was striking at residue Met-382, which in the homology model is at the opening of the translocation pocket at the site of the extracellular gate. Because this position tolerates substitution by tryptophan, phenylalanine, alanine, and cysteine, but not by glutamate or lysine (Fig. 5, above), it is likely that the introduction of a charge, either positive or negative, by modification with MTSET or MTSES, respectively, produces inhibition.

Inhibition by MTSET is also seen at Val-385; this residue is outside of the occlusion pocket and between TM3 and TM10, and it would seem unlikely that it is directly involved in ion movement. Quite possibly modification at this position affects the large relative movement of TM10 relative to TM3 that accompanies the occlusion event.

Surprisingly, neither MTSET nor MTSES produced substantial inhibition within the predicted translocation pore, at 5 mM or 10 mM concentrations, respectively (except for a small but significant inhibition at Ala-381 and Val-378). To test the possibility that modification takes place without effect on the flux rate, we looked for changes in the affinity for bumetanide fol-

**FIGURE 7. Inhibition by MTS reagents.** A, fractional inhibition of cysteine scanning mutants by 3 mM MTSET or 5 mM MTSES as described under “Experimental Procedures.” Nonfunctional mutants (light gray) were not analyzed. Data are corrected for base-line inhibition in wild-type hNKCC1 (−5.8 ± 2.1% with MTSET, −9.5 ± 5.2% with MTSES) and are presented as mean ± S.E. (error bars; n = 3) with significant difference from wild-type hNKCC1 indicated (*) when $p < 0.05$. The shaded bar indicates $\alpha$-helical periodicity as in Fig. 3. B, concentration dependence of MTSET inhibition of M382C and V385C in a 10-min exposure in regular medium (●) or in the bumetanide-occluded condition (○). This experiment is one of three, error bars show the duplicate variation within the experiment. Data are fit with $V = V_{\text{max}} e^{-k[M_{\text{TS}}]}$, as described under “Experimental Procedures.” C, rate constant ($k$) for modification of M382C and V385C by MTSET in regular medium (open bars) or with bumetanide (shaded bars). The results are means ± S.E. from three experiments including the one in B.
lowing exposure of predicted pore-lining residues (N376C, A375C, F372C) to MTS reagents and found that there were was no significant effect at these positions (data not shown). Our conclusion from these negative results is that MTSET and MTSES are unable to enter the translocation pathway at sufficient concentration to bring about effective modification.

As seen in Fig. 1, B and C, Met-382 is predicted to be exposed to the medium in the outward-open state and hidden against TM10 as part of the extracellular gate in the occluded state; its reactivity should therefore depend on the transporter state. Thus, we compared MTSET reactivity of M382C in regular medium or in the presence of bumetanide to bring about a bumetanide-occluded state, with flux analysis carried out after washing for 40 min to remove bumetanide. As illustrated in Fig. 7, B and C, MTSET reactivity was decreased 40-fold with the transporter in the occluded state, strongly supporting the occlusion model. An alternative explanation would be that bumetanide blocks MTSET reactivity by steric interaction, although this is not likely if bumetanide binds deep in the cavity as proposed above.

We also observed a smaller state-dependence of reactivity at V385C, with greater reactivity seen in the occluded conformation (Fig. 7, B and C). The homology model does not provide an explanation for this difference, because Val-385 appears to be less exposed upon occlusion. However, this is near the outer end of the TM helices, and it is quite possible that nonmodeled extracellular loop regions may play a role.

DISCUSSION

The present work applies cysteine and tryptophan substitution scanning to examine the properties of TM3 of NKCC1. Our results show that substitution of TM3 residues affects the maximal rate of ion transport and apparent ion affinities in a periodic pattern that is consistent with the most important residues being found on one face of an α-helix (Figs. 3 and 4). By evaluating our results in the context of a homology model based on APC superfamily amino acid transporters AdiC (14–16) and ApcT (14) (Fig. 1), we propose that these TM3 residues face the translocation pathway and provide strong support for both the role of TM3 in transport activity and for the applicability of our models.

Consideration of Specific Pore-lining Residues—Examining our results in greater detail beginning at the extracellular end of the translocation cavity, our data provide strong support for the importance of Met-382 and Tyr-383 as part of a contact surface in an extracellular gate that is fully closed in the occluded conformation. In the homology model, Tyr-383 is seen to point into the pocket in both the open and occluded state (Fig. 1, B, E, and G). Because the corresponding Y383F mutant is inactive as well as Y383C and Y383W (Fig. 5), it seems likely that the Tyr-383 hydroxyl takes part in ion coordination. This position corresponds to the Met-104 in AdiC, which interacts with the aliphatic portion of the bound arginine, and to the highly conserved Tyr-108 in LeuT that is involved with coordinating the carbonyl group of a bound leucine and in stabilizing a close interaction with TM1 (18).

Met-382 is predicted to be at the external opening of the cavity in the outward-open conformation and in close contact with TM1 and TM10 in the occluded conformation (Fig. 1B). The conformational change in exposure of Met-382 is strongly supported by the large decrease in MTS reactivity seen when the transporter is in the bumetanide-occluded conformation (Fig. 7, B and C). Although Met-382 tolerates substitution with various hydrophobic residues, introduction of a charged group either by glutamate or lysine substitution or by modification with MTSET or MTSES results in loss of function. This may be due to hindrance of ion entry by charge repulsion, or it may reflect a requirement for a hydrophobic residue in the TM3-TM10 interface to favor closure of the extracellular gate. Finally, the mutant M382W is nearly bumetanide-insensitive, further evidence of a role of Met-382 at a critical site in the extracellular occlusion gate; presumably, the bulk of the substituted tryptophan in this position blocks bumetanide entry into the ion translocation pathway.

Within the translocation pathway, residue Ala-379 clearly has strict requirements to permit ion translocation because mutation to cysteine, leucine, or tryptophan is met with loss of function, whereas glycine or serine is tolerated with a decrease in apparent ion affinities (Fig. 5). The neighboring Val-380 is homologous with Asn-101 in AdiC, a residue involved with arginine binding. Val-380 is not exposed to the pore in the NKCC1 homology model, apparently due to differences caused by the absence of a bulky residue corresponding to AdiC Trp-293 in TM8, and cysteine substitution at Val-380 in NKCC1 has relatively minor effect. In the next turn of the helix, Ala-375 and Asn-376 are both predicted to be exposed to the pore, the homologs in AdiC (Ala-96, Cys-97) being involved in coordination of the guanidinium group of arginine (16). Substitution with tryptophan at either position leads to loss of function, whereas cysteine substitution results in 2-fold changes in apparent ion affinities in the form of an increase at Ala-375 and a decrease at Asn-376 (Figs. 3 and 4).

Phe-372 is homologous to Tyr-93 in AdiC, at the intracellular end of the ligand binding region of the pore in AdiC and ApcT. On the one hand, substantial changes in apparent ion affinities on substitution (Figs. 4 and 5) attest to the importance of Phe-372 in the translocation pocket; on the other hand, it is somewhat surprising that side chains in a broad range of sizes are tolerated in substitution for this highly conserved aromatic residue (Fig. 5). It is intriguing to note that tryptophan substitution of this residue had a large effect on apparent Na+ affinity and none on K affinity, opposite to a K-only effect at Val-378 higher in the pocket. This result would be consistent with Na+ binding at the innermost site, consistent with the Na+-Cl- -K+-Cl binding order obtained from kinetic measurements (28). This conclusion should be tempered, however, by the observation that cysteine mutants showed no such pattern (Fig. 4A). It will clearly require additional studies of pore lining TMs 1, 6, 8, and 10 to further define ion binding parameters.

Many of the 5–5 inverted repeat transporters are Na-coupled cotransporters, and it has been noted that the position of the bound Na+ ion, coordinated by residues in TMs 1 and 8, is remarkably conserved from one superfamily to another (e.g. Ref. 14). It would thus be tempting to guess that the Na+ binding site is at a similar position in NKCC1. The current NKCC1 homology model does not provide obvious support for this...
hypothesis because two of the homologous TM8 residues in NKCC1 (Ala-610 and Ser-613) are somewhat above and rotated out of the apparent occlusion pocket. The amino acid transporters within the APC superfamily are not Na-coupled, and it seems a likely possibility that within the APC lineage the NKCCs have evolved their own unique Na⁺ binding site.

Loop Diuretic Inhibition—It has long been proposed that loop diuretic drugs bind within the transport pocket based on the observation that bumetanide binding is competitive with one of two chloride ions (29). In the present experiments large decreases in inhibitor affinities were seen when TM3 residues were mutated to cysteine or tryptophan (Fig. 6). Other than the nearly complete block of bumetanide binding at the extracellular entry point (Met-382) the most dramatic decreases in inhibitor affinity were caused by mutations at the inner end of the pore, at Phe-372 and Ile-371. Thus, the current results are consistent with the hypothesis that loop diuretics bind within the pore and suggest that the binding site may be near the intracellular end of the pocket.

In the course of these efforts, we have identified a single point mutation, M382W, which produces a virtually bumetanide-insensitive NKCC but retains furosemide affinity only 4-fold less than the wild-type transporter. It is anticipated that this construct will be useful in future studies to help functionally distinguish a mutated transporter from endogenous NKCC or to provide a distinct partner in studies of potential dimer interactions.

Intracellular Gating—One of the surprises in the current results is the importance of residues toward the intracellular end of TM3, evidenced by loss of function with mutation of Gly-369 and in I368W (Figs. 3 and 5) and changes in apparent ion and loop diuretic affinities with mutation at Ile-371, Leu-370, and Ile-368 (Figs. 4 and 6). This region is seen as a water-filled cavity in ApcT (14) bounded by TMs 2, 3, 6, 8, and 10 (Fig. 1D), including the TM2 residues shown to be involved in apparent ion affinity differences among NKCC2 splice variants (11). Thus, it seems likely that in addition to clearly evidenced ion coordination in the extracellular half of the pathway, ions are coordinated within this more cytoplasmic part of the pore, either in the outward-open conformation or in an inward-open transport conformation, for which there is no APC structure available.

We propose that the inward gate consists primarily of ICL1, which is seen in outward-open and occluded models in Fig. 1 to completely block the pore in contact with TMs 2, 3, 6, 8, and 10. Because these transporters were originally cloned it has been noted that ICL1 is the most highly conserved region in the cation-chloride family of transport proteins, suggesting a specialized role for these 20 residues. We suggest that the ICL1 structure may be specifically tuned to provide an intracellular gate, which moves out of the pore during the transport cycle and opens a pathway for intracellular access (Fig. 1D). This proposed mechanism of intracellular gating is quite different from other proposed mechanisms which are based on inward-outward symmetry within the 5-5 repeat of TM helices and which involve a “rocking bundle” of helices and very large scale conformational movements (31). Strong support for these models has been obtained from inward-open crystal structures of Mhp1 (32) and vSGLT (33) and partial agreement from a LeuT inward-open structure (34); in the APC superfamily a similar model has been suggested for AdiC (17). One piece of evidence that different transporters may utilize different mechanisms is in the turnover rate of the transport cycle; we note that the temperature-adjusted turnover rate for NKCC1 (~1000/s at 25 °C, based on 3500/s at 37 °C (35) and 3.5-fold difference in flux between 37 °C and 25 °C (36) is >20-fold faster than the turnover rates of LeuT (0.1/s) (36), SERT (10/s) (37), SGLT1 (13/s) (38), and Gly1 (50/s) (39). This rapid turnover rate of cation-chloride cotransporters may very well require much smaller conformational changes in the transport cycle.

The present results provide strong confirmation of a role of TM3 of NKCC1 in the binding and translocation of ions as well as in the binding of loop diuretic inhibitors, in excellent agreement with predictions from homology models built to other APC superfamily members. The data support the orientation of TM3 with regard to the translocation pocket as well as a direct role for TM3 in an extracellular occlusion gate. Intriguingly, the results also suggest a critical role for residues near the intracellular end of TM3, perhaps as part of the translocation pathway in an inside-facing conformation. Future studies will test and refine this and similar homology models, continuing to examine the translocation pore and defining distances and movements by cysteine scanning and cross-linking experiments. The results should be generally applicable to other members of the cation-chloride cotransporter family which have much identity to NKCC in the transmembrane domains but also have clear differences in pore-lining residues; these differences must determine the different specificities of transport.

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REFERENCES

1. Delpire, E., and Mount, D. B. (2002) Human and murine phenotypes associated with defects in cation-chloride cotransport. Annu. Rev. Physiol. 64, 803–843
2. Aronica, E., Boer, K., Redeker, S., Spliet, W. G., van Rijen, P. C., Troost, D., and Gorter, J. A. (2007) Differential expression patterns of chloride transporters, Na⁺-K⁺-2Cl⁻ cotransporter and K⁺-Cl⁻ cotransporter, in epilepsy-associated malformations of cortical development. Neuroscience 145, 185–196
3. Darman, R. B., and Forbush, B. (2002) A regulatory locus of phosphorylation in the N terminus of the Na-K-CI cotransporter, NKCC1. J. Biol. Chem. 277, 37542–37550
4. Rinehart, J., Maksimova, Y. D., Tanis, J. E., Stone, K. L., Hodson, C. A., Zhang, J., Risinger, M., Pan, W., Wu, D., Colangelo, C. M., Forbush, B., Joiner, C. H., Gulcicek, E. E., Gallagher, P. G., and Lifton, R. P. (2009) Sites of regulated phosphorylation that control K-CI cotransporter activity. Cell 138, 525–536
5. Moore-Hoon, M. L., and Turner, R. J. (2000) The structural unit of the secretory Na⁺-K⁺-Cl⁻ cotransporter (NKCC1) is a homodimer. Biochemistry 39, 3718–3724
6. Pedersen, M., Carmosino, M., and Forbush, B. (2008) Intramolecular and intermolecular fluorescence energy transfer in fluorescent protein-tagged Na-K-CI cotransporter (NKCC1): sensitivity to regulatory conformational change and cell volume. J. Biol. Chem. 283, 2663–2674

5 C. Lytle, unpublished data.
Scanning Mutagenesis of NKCC1 TM3

7. Simard, C. F., Brunet, G. M., Daigle, N. D., Montminy, V., Caron, L., and Isenring, P. (2004) Self-interacting domains in the C-terminus of a cation-Cl\(^-\) co-transporter described for the first time. J. Biol. Chem. 279, 40769–40777

8. Blassle, P., Guillaum, J., Schindler, J., Schweizer, M., Delpère, E., Khroug, L., Friauf, E., and Nothwang, H. G. (2006) Oligomerization of KCC2 correlates with development of inhibitory neurotransmission. J. Neurosci. 26, 10407–10419

9. Monette, M. Y., and Forbush, B. (2012) Regulatory activation is accompanied by movement in the C-terminal of the Na-K-Cl cotransporter (NKCC1). J. Biol. Chem. 287, 2210–2220

10. Isenring, P., Jacoby, S. C., Chang, J., and Forbush, B. (1998) Mutagenic mapping of the Na-K-Cl co-transporter for domains involved in ion transport and bumetanide binding. J. Gen. Physiol. 112, 549–558

11. Giménez, I., Isenring, P., and Forbush, B. (2002) Spatially distributed alternative splice variants of the renal Na-K-Cl cotransporter exhibit dramatically different affinities for the transported ions. J. Biol. Chem. 277, 8767–8770

12. Dehaye, J. P., Nagy, A., Premkumar, A., and Turner, R. J. (2003) Identification of a functionally important conformation-sensitive region of the secretory Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter (NKCC1). J. Biol. Chem. 278, 11811–11817

13. Castañeda-Bueno, M., Vázquez, N., Bustos-Jaimes, L., Hernández, D., Rodríguez-Lobato, E., Pacheco-Alvarez, D., Carino-Cortés, R., Moreno, E., Bobadilla, N. A., and Gamba, G. (2010) A single residue in transmembrane domain 11 defines the different affinity for thiazides between the mammalian and flounder NaCl transporters. Am. J. Physiol. Renal Physiol. 299, F1111–F1119

14. Shaffer, P. L., Goehring, A., Shankaranarayanan, A., and Gouaux, E. (2009) Structure and mechanism of a Na\(^+\)-independent amino acid co-transporter. Science 325, 1010–1014

15. Fang, Y., Jayaram, H., Shane, T., Kolmakaova-Partensky, L., Wu, F., Williams, C., Xiong, Y., and Miller, C. (2009) Structure of a prokaryotic virtual proton pump at 3.2 Å resolution. Nature 460, 1040–1043

16. Gao, X., Zhou, L., Iao, X., Lu, F., Yan, C., Zeng, X., Wang, J., and Shi, Y. (2010) Mechanism of substrate recognition and transport by an amino acid co-transporter. Nature 463, 828–832

17. Kowalczyk, L., Ratera, M., Paladino, A., Bartocciioni, P., Errasti-Munugarren, E., Valencia, E., Portella, G., Bial, S., Zorzano, A., Fitó, I., Orozco, M., Carpena, X., Vázquez-Ibar, J. L., and Palacín, M. (2011) Molecular basis of substrate-induced permeation by an amino acid co-transporter. Proc. Natl. Acad. Sci. U.S.A. 108, 3935–3940

18. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Crystal structure of a bacterial homologue of Na\(^+\)/Cl\(^-\)− dependent neural transporters. Nature 437, 215–223

19. Khafizov, K., Staritschbichler, R., Stamm, M., and Forrest, L. R. (2010) A study of the evolution of inverted-topology repeats from LeuT-fold transporters using AlignMe. Biochemistry 49, 10702–10713

20. Carmonoso, M., Giménez, I., Caplan, M., and Forbush, B. (2008) Exon loss accounts for differential sorting of Na-K-Cl co-transporters in polarized epithelial cells. Mol. Biol. Cell 19, 4341–4351

21. Lytle, C., Xu, J. C., Biemeisderfer, D., and Forbush, B., 3rd (1995) Distribution and diversity of Na-K-Cl co-transport proteins: a study with monoclonal antibodies. Am. J. Physiol. 269, C1496–1505

22. Monette, M. Y., Rinehart, J., Lifton, R. P., and Forbush, B. (2011) Rare mutations in the human Na-K-Cl co-transporter (NKCC2) associated with lower blood pressure exhibit impaired processing and transport function.