Molecular Motions Involved in Na-K-Cl Cotransporter-mediated Ion Transport and Transporter Activation Revealed by Internal Cross-linking between Transmembrane Domains 10 and 11/12*

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Background: Na-K-Cl cotransporters (NKCCs) are responsible for volume and chloride homeostasis and chloride transport and are targets of loop diuretic drugs.

Results: Cross-links between transmembrane domains (TMs) 10 and 11/12 of NKCC1 are identified as inhibitory and stimulatory.

Conclusion: Activation of NKCC1 involves movement of TM12 relative to TM10.

Significance: This identifies movement of TM12 as a key step in the molecular mechanism of NKCC activation.

We examined the relationship between transmembrane domain (TM) 10 and TM11/12 in NKCC1, testing homology models based on the structure of AdiC in the same transporter superfamily. We hypothesized that introduced cysteine pairs would be close enough for disulfide formation and would alter transport function: indeed, evidence for cross-link formation would be close enough for disulfide formation and would alter transport function. Consistent with this, (a) cross-linking of P676C/I730C was inferred to transport while not affecting the dephosphorylation rate, thus uncoupling the phosphorylation and activation steps. Consistent with this, (a) cross-linking of P676C/I730C was dependent on activation state, and (b) mutants lacking the phosphorylation domain could still be activated by cross-linking. These results suggest a model of NKCC activation that involves movement of TM12 relative to TM10, which is likely tied to movement of the large C terminus, a process somehow triggered by phosphorylation of the regulatory domain in the N terminus.

NKCC1 regulates intracellular Cl− concentration in many cell types and is an essential part of the Cl− secretory pathway in salt secreting epithelia, whereas NKCC2 and Na-Cl cotransporter are responsible for a major fraction of Na+ and Cl− reabsorption in the renal tubule; NKCCs are inhibited by the loop diuretic drugs furosemide and bumetanide. A second branch of the cation-chloride cotransporter family includes K-Cl cointransporters that move K+ and Cl− out of the cell, thus balancing NKCC with regard to Cl− concentration and cell volume. Because these transporters are broadly involved in human electrolyte homeostasis, their pathophysiology is 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. Homology in the TM domain demonstrates that the small family of cation-chloride cotransporters is part of the APC (amino acid, polyamine, organocation) superfamily of transporters, for which a number of high resolution structures have recently been obtained. The key feature of the TM domain is an inverted repeat of TMs 1–5 and 6–10 that comprise a translocation domain surrounding a central ligand-binding cavity (3–6), a motif that is the structural core in five superfamilies of transporters (7). Our recent scanning analysis of highly conserved TM3 provides very strong support for the proposed alignment of TM3 as a pore-lining helix in structural homology between NKCC and amino acid transporters (8); however, there is lower confidence in the proposed alignments of other regions of the proteins. We have begun to further test the homology models utilizing a cysteine cross-linking approach, beginning here with an examination of the predicted relationship of TMs 10 and TM11/12.

On-off regulation of NKCCs occurs through phosphorylation of a set of threonine residues in the N terminus, in a region predicted to have little secondary structure. The C terminus also undergoes movement in this process, the two C termini in an NKCC dimer moving apart from one another when phosphorylation occurs in the N terminus (9). How these events are translated to the TM domain to switch the transport mechanism “on” is completely unknown. One hypothesis that receives

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3 The abbreviations used are: NKCC, Na-K-Cl cotransporter; APC, amino acid, polyamine, organocation transporter; CuPhe, cupric phenanthroline (1:3); TCEP, tris(2-carboxyethyl)phosphine; TM, transmembrane helix; MTSET, 2-(trimethyl-ammonium)ethyl methanethiosulfonate.
very strong support in the current study is that the signaling occurs by means of the tether linking TM12 to the C-terminal domain, which in turn affects the disposition of TM12 and the conformational movements in the rest of the transmembrane domain.

In the present study, we examine the relationship of TM10 and TM11/12 in human NKCC1 using a cysteine cross-linking approach (10). Homology models produced from outward open and occluded states of the APC transporter AdiC (4–6, 11) indicate a rotational movement of TM10 upon transition to the occluded state, and we predicted that cross-linking TM10 to nearby TM11 or TM12 would inhibit the activity of the transporter. In an initial set of eight potential cross-links, we found that three pairs did indeed show functional consequences on copper phenanthroline (CuPhe)-mediated and iodine-mediated disulfide formation, strongly confirming a model of conformational movement of TM10 important in transport. When we broadened the test region, we found that of 26 additionally tested pairs only one revealed cross-link behavior at low CuPhe concentrations, demonstrating specificity in identification of the TM10–12 residues and strongly supporting the homology model in the TM10–12 region. Inhibition by the Cu²⁺ ion itself was also observed in constructs derived from A675C, consistent with formation of a novel Cu²⁺-binding site in conjunction with Met382 of TM3. Surprisingly, in addition to inhibition by some of the cross-links, several of the identified cross-links were functionally stimulatory when studied after transporter deactivation; additional experiments demonstrated that the reason for increased function is that cross-linking “locks” the transporter in the activated state independent of phosphorylation. Conversely, several inhibitory cross-links were formed only in the inactive state, providing further support for the proposed TM12 movement. This directly implicates the TM10–12 interface in transporter activation and suggests that movement of TM12 may be a key event in transporter regulation.

**EXPERIMENTAL PROCEDURES**

Constructs, Transfections, and Cells—Human NKCC1, yellow fluorescent protein-tagged on the N terminus, was expressed from a synthetic cDNA with unique silent restriction sites at ~150-bp intervals throughout the coding region (12) (our identifier NT17, Addgene 49085); mutations to cysteine were introduced by subcloning of synthetic ~150-bp cDNAs with appropriate base changes (Bio Basic Inc., Toronto, Canada; and GeneScript, Piscataway, NJ), and construction of double-cysteine mutants was by subcloning. Constructs containing F728C, V729C, W733C, and A735C (see Fig. 1D, asterisks, and Tables 1 and 2), also carried a C723S/C724V mutation. W732C was not studied because it has been found to occlude a Cu²⁺-binding site in conjunction with Met382 of TM3. Surprisingly, in addition to inhibition by some of the cross-links, several of the identified cross-links were functionally stimulatory when studied after transporter deactivation; additional experiments demonstrated that the reason for increased function is that cross-linking “locks” the transporter in the activated state independent of phosphorylation. Conversely, several inhibitory cross-links were formed only in the inactive state, providing further support for the proposed TM12 movement. This directly implicates the TM10–12 interface in transporter activation and suggests that movement of TM12 may be a key event in transporter regulation.

the C terminus was also replaced with that of NBCe. Sequences and plasmids of key constructs are available through Addgene.

HEK-293 cells were transfected with individual cDNAs 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 gentamicin (1 mg/ml) in a 37 °C humidified incubator.

**Western Blotting**—Cells in a 12-well plate 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 to nitrocellulose membrane, membranes were probed with the T4 antibody for total NKCC1 (13), R5 phospho-specific NKCC antibody (14), and secondary antibodies (goat anti-mouse IRDye® 800CW or goat anti-rabbit IRDye®680CW (LI-COR Biosciences, Lincoln, NE). Images were acquired using the Odyssey infrared imaging system (LI-COR).

**Immunofluorescence and Confocal Microscopy**—Transfected HEK cells were grown on polylysine-coated coverslips, fixed with methanol for 5 min, washed with PBS, and incubated in 0.1% BSA in PBS for 30 min at room temperature followed by incubation in anti-FLAG polyclonal antibody (Sigma-Aldrich; 1:500) overnight at 4 °C and followed by anti-rabbit Alexa-488 (Invitrogen) secondary for 1 h (room temperature). Cells were subsequently incubated in TO-PRO-3 iodide (Invitrogen) for 15 min and then washed and mounted with Vectashield (Vector Laboratories). Images were obtained using a laser scanning confocal microscope (Zeiss LSM 710; Carl Zeiss).

**86Rb⁺ Influx Assays**—NKCC function was assessed by measuring 86Rb⁺ influx into HEK cells in a robotic 96-well plate assay as described previously (9, 12, 15, 16). To optimize cell surface expression, cells were grown to confluence in 96-well polylysine-coated plates and moved to a 25 °C incubator 24 h prior to the experiment (17). Unless noted otherwise, solutions contained total 140 mM monovalent cation (Na⁺, K⁺, Rb⁺, and N-methyl glucamine) and 140 mM Cl⁻ or gluconate in addition to 1 mM Ca²⁺, 1 mM Mg²⁺, 1 mM SO₄²⁻, 1 mM PO₄³⁻, and 5 mM N-methyl glucamine HEPES at pH 7.4. We used 0 Cl⁻ medium to denote a solution in which all Cl⁻ has been replaced with gluconate and 0 Cl⁻ (SO₄²⁻) when SO₄²⁻ is the replacement anion (100 mM Na₂SO₄, 2.5 mM K₂SO₄).

To activate NKCC1, cells were exposed to a 0 Cl⁻ medium for 60 min (15). 86Rb⁺ influx was carried out for 1–6 min in flux medium (2 μCi/ml 86Rb⁺ with 5 mM Rb⁺, 135 mM Na⁺, and 0.1 mM ouabain) and terminated by washing plates with an isotonic MgCl₂ solution, and the wells were sucked dry. 86Rb⁺ was measured by phosphorimaging analysis of the 96-well plates. In most experiments, the results are normalized to a control sample in a neighboring row of the 96-well plate. The results are presented as ± S.E. of three to five samples within an experiment or among experiments as noted; all experiments were conducted at room temperature.

CuPhe-mediated oxidation of cysteines to form disulfide cross-links was carried out in a 6- or 10-min incubation at indicated concentrations of 1:3 Cu²⁺-phenanthroline (expressed as

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4 Homodimeric cross-linking of NKCC1 at residues F728C, W732C, W733C, and A735C provides evidence for an NKCC1 dimer interface formed by TM11/12 (Suma Somasekharan, Michelle Y. Monette, and Blff Forbush, manuscript in preparation).
micromolar Cu$^{2+}$), diluted from freshly prepared stock solutions. For iodine oxidation, a fresh 0.5M I$_2$ stock was prepared in ethanol, and dilutions were made within 3 min before exposure of cells. DTT and TCEP were prepared fresh in 0 Cl$^-$-H$_2$O medium, DTT directly and TCEP by briefly dissolving in water at 0.2M, dilution, and adjustment of pH.

We noted physical differences in both Cu$^{2+}$ and I$_2$ solutions, when Cl$^-$ was replaced by gluconate. In the case of Cu$^{2+}$, the gluconate solution of CuPhe solution was a deeper blue, and as shown below, CuPhe cross-linking curves were right-shifted in gluconate. We also found that I$_2$ precipitated sharply from solution when diluted from ethanol to a concentration greater than 0.5 mM into a gluconate medium.

**RESULTS**

The available crystal structures of the bacterial arginine transporter AdiC show us an APC superfamily transporter in open to out and occluded states. As inferred from these structures, the closing of an extracellular “gate” is accomplished by small rotations and translocations primarily of TMs 10 and 6 (5, 6). These movements are illustrated in Fig. 1 (B and C), using homology models based on NKCC1-AdiC primary sequence alignments with TM11/12 alignment informed by our recent homodimeric cross-linking studies. To examine the hypothesis that occlusion of extracellular ions by NKCC takes place by a similar mechanism and to check the accuracy of this region of our current homology model, we designed cross-linking experiments to test the predicted proximity of residues in TM10 and TM11/12. Focusing on the hypothesized rotation and translocation (2 Å, 30°) of TM10 relative to TM12, we predicted that by adding cysteines at appropriate positions in both helices (Table 1), disulfide cross-linking would inhibit transport by preventing or slowing this conformational change.
Inhibitory Cross-linking of TM10–11/12 Residues—Cysteine residues were engineered in hNKCC1 in eight different pairs (pairs marked by dark dots in Fig. 1D; underlined in Table 2), replacing residues at positions in TM10, 11, and 12; these constructs were appropriately delivered to the plasma membrane and exhibited $^{86}\text{Rb}^+$ influx readily detectable above the HEK cell background (Table 2). We examined sensitivity of the cysteine pairs to oxidizing agents under conditions where the transporter was inactive after incubation in regular medium or had been previously activated by preincubation in the absence of extracellular Cl$^-$; this lowers intracellular [Cl$^-$] and brings about transporter activation by phosphorylation of the N terminus (15, 19). As illustrated in Fig. 2A, two of the constructs with paired cysteine mutations, P676C/A734C and I677C/A734C, were inhibited by CuPhe at micromolar concentrations, whereas wild type NKCC1 and the single-cysteine mutants were unaffected by CuPhe exposure. This strongly suggests that residues 676 and 677 in TM10 are close to residue 734 in TM12. We tentatively concluded that inhibitory disulfide cross-links had been formed, but further experimentation revealed the situation to be more complex.

During the course of these experiments, we became concerned that Cu$^{2+}$ itself might exhibit inhibitory binding to modified NKCC1. As shown in Fig. 2B, this is indeed the case for the two constructs inhibited by CuPhe exposure, whereas wild type NKCC1 and the single-cysteine constructs were again not affected. Because the single-cysteine constructs are not inhibited by copper, these data argue that in each case the two cysteines participate in a novel Cu$^{2+}$-binding site and thus are presumably within ~4 Å of one another. An alternative possibility is that Cu$^{2+}$ binds and directly oxidizes the cysteine pair to a disulfide (20). In either case, the proximity of these cysteine residues provides strong support for the overall homology model of NKCC1, and the inhibitory nature of the interaction supports a transport role of TM10 movement relative to TM12.

We attempted to distinguish between Cu$^{2+}$ binding and CuPhe-mediated cross-linking by means of further incubation of the cells with EDTA, expecting to reverse Cu$^{2+}$ binding but to have no effect on stable disulfide cross-links. As illustrated in Fig. 2C, these experiments demonstrated partial recovery of function in EDTA after both Cu$^{2+}$ and CuPhe and in both constructs. This intermediate result demonstrates that reversal of Cu$^{2+}$ inhibition is slow and/or incomplete, so that it is difficult to use this intervention to distinguish inhibitory binding from cross-link formation.

We also used molecular iodine to bring about disulfide cross-link formation; as illustrated in Fig. 2D, we found that brief exposure to I$_2$ produced inhibition in the P676C/A734C and I677C/A734C constructs, but not in wild type at the same concentration (I$_2$ was inhibitory to wild type NKCC1 above a concentration of 0.5 mM). This further supports the finding that residues 676 and 734 are close to one another.

5 M. Y. Monette, S. Somasekharan, and B. Forbush, unpublished results.

TABLE 1
Activity and CuPhe inhibition of TM10–TM11/12 single-cysteine constructs

| Construct | Activity in regular media | Activity in 0 Cl media | Activity in CuPhe | Activity in CuPhe + bumetanide |
|-----------|---------------------------|------------------------|------------------|-------------------------------|
| N872C     | 0.95 ± 0.06               | 0.95 ± 0.06            | 0.89 ± 0.06      | 0.85 ± 0.06                   |
| V867C     | 0.93 ± 0.01               | 0.92 ± 0.01            | 0.86 ± 0.01      | 0.83 ± 0.01                   |
| A767C     | 0.89 ± 0.02               | 0.89 ± 0.02            | 0.84 ± 0.02      | 0.82 ± 0.02                   |
| V867C     | 0.91 ± 0.02               | 0.90 ± 0.02            | 0.85 ± 0.02      | 0.83 ± 0.02                   |
| P867C     | 0.87 ± 0.02               | 0.87 ± 0.02            | 0.82 ± 0.02      | 0.80 ± 0.02                   |
| I767C     | 0.85 ± 0.02               | 0.85 ± 0.02            | 0.80 ± 0.02      | 0.77 ± 0.02                   |
| F767C     | 0.83 ± 0.02               | 0.83 ± 0.02            | 0.79 ± 0.02      | 0.76 ± 0.02                   |

TABLE 2
Activity and CuPhe inhibition of TM10–TM11/12 double-cysteine constructs

| Construct | Activity in regular media | Activity in 0 Cl media | Activity in CuPhe | Activity in CuPhe + bumetanide |
|-----------|---------------------------|------------------------|------------------|-------------------------------|
| N872C     | 0.95 ± 0.06               | 0.95 ± 0.06            | 0.89 ± 0.06      | 0.85 ± 0.06                   |
| V867C     | 0.93 ± 0.01               | 0.92 ± 0.01            | 0.86 ± 0.01      | 0.83 ± 0.01                   |
| A767C     | 0.89 ± 0.02               | 0.89 ± 0.02            | 0.84 ± 0.02      | 0.82 ± 0.02                   |
| V867C     | 0.91 ± 0.02               | 0.90 ± 0.02            | 0.85 ± 0.02      | 0.83 ± 0.02                   |
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| I767C     | 0.85 ± 0.02               | 0.85 ± 0.02            | 0.80 ± 0.02      | 0.77 ± 0.02                   |
| F767C     | 0.83 ± 0.02               | 0.83 ± 0.02            | 0.79 ± 0.02      | 0.76 ± 0.02                   |

Inhibitory Cross-linking of TM10–11/12 Residues—Cysteine residues were engineered in hNKCC1 in eight different pairs (pairs marked by dark dots in Fig. 1D; underlined in Table 2), replacing residues at positions in TM10, 11, and 12; these constructs were appropriately delivered to the plasma membrane and exhibited $^{86}\text{Rb}^+$ influx readily detectable above the HEK cell background (Table 2). We examined sensitivity of the cysteine pairs to oxidizing agents under conditions where the transporter was inactive after incubation in regular medium or had been previously activated by phosphorylation of the N terminus (15, 19). As illustrated in Fig. 2A, two of the constructs with paired cysteine mutations, P676C/A734C and I677C/A734C, were inhibited by CuPhe at micromolar concentrations, whereas wild type NKCC1 and the single-cysteine mutants were unaffected by CuPhe exposure. This strongly suggests that residues 676 and 677 in TM10 are close to residue 734 in TM12. We tentatively concluded that inhibitory disulfide cross-links had been formed, but further experimentation revealed the situation to be more complex.

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**FIGURE 3.** *Enhancement of NKCC1 activity by cross-linking with CuPhe.*

HEK cells were incubated for 1 h in 0 Cl medium to activate, then for 6 min with various concentrations of CuPhe in regular medium, followed by a further 40 min in regular medium, and finally in a 6-min $^{86}$Rb flux assay. HEK cells expressed wild type NKCC1 (●), P676C/I730C (■), P676C/A734C (□), and I677C/A734C (▲). The data represent means and S.E. from three experiments, expressed as a ratio of activity in CuPhe treated cells to activity in untreated cells in neighboring wells.

That a disulfide cross-link is formed between residue 676 or 677 in TM10 and residue 734 in TM12 and that formation of the cross-link reduces or prevents ion transport mediated by this construct. Sensitivity of P676C/A734C to micromolar concentrations of CuPhe and to I$_2$ demonstrates that the paired residues are found in close proximity, and the inhibitory effect of cross-linking demonstrates that their conformational movement is necessary to give optimal transport. These results are thus strongly supportive of the proposed homology model in which TM10 and TM12 are near one another, and the inhibitory effect is in agreement with the hypothesis that movement of TM10 is part of the “occlusion” step in transport.

**An Activating Cross-link**—Remarkably, as illustrated in Fig. 3, cross-linking the P676C/I730C construct with CuPhe was found to *increase* the transport activity, unlike the inhibitory effects reported above. This effect was most obvious when following CuPhe incubation, and prior to the flux assay, we included a further incubation in regular medium to deactivate normal transporters (see Fig. 4A); thus the data as presented in Fig. 3 show an increase in activity over basal activity levels. These results again demonstrate proximity of TM10 and TM11/12 residues, but in this case the cross-linked product must be functional. We conclude that the Cys$^{676}$–Cys$^{730}$ cross-link does not prevent the transporter from undergoing conformational movements necessary to bring about transport; as noted above, the predicted movement of TM10 is a primarily rotational movement (Fig. 1, B and C) that can apparently proceed in the presence of this cross-link.

P676C/A734C and I677C/A734C were shown above to be partially inhibited by CuPhe (Fig. 2A). Fig. 3 (thin lines) illustrates that upon return to basal conditions, these cross-linked...
NKCC Activation Mechanism Revealed by Cross-linking

constructs are similar to P676C/I730C in having greater activity than if they had not been treated with CuPhe. Thus these cross-links appear both to stabilize an activated state (Fig. 3) and to decrease the maximal transport rate (Fig. 2A); although this may appear paradoxical, it is readily understood if the activation mechanism and translocation machinery involve separate structural domains, with the activation mechanism acting as an on-off switch to regulate ion translocation.

How Does Cross-linking of Cys676–Cys730 Maintain Transport Activity at a Level above That of the Native Transporter?—One possibility is that the cross-link holds the transporter in an active state during the deactivation period. To test this, we examined the time course of the effect of CuPhe treatment on transport activity during a deactivation period. After incubation of HEK cells in low chloride medium to bring about trans-active state during the deactivation period. To test this, we experiment the data are best fit by a double exponential in monophasic decay of the wild type transporter (Fig. 4). A much larger difference in 0 Cl (gluconate) medium compared with regular medium, suggesting that the cross-link is more easily formed in an outward-facing conformation than in occluded or inward facing states (K_{0.5}(CuPhe) 1.4 ± 0.2 μM in 0 K^+ medium, 3.0 ± 0.7 μM in regular medium, n = 4). A much larger difference in 0 Cl (gluconate) medium was likely attributable to interaction of Cu^{2+} with gluconate, because it was not observed in 0 Cl (SO_4^{2-}) medium (K_{0.5}(CuPhe) 19.5 ± 3.8 μM in 0 Cl^- (gluconate) medium, 3.0 ± 0.5 μM in 0 Cl^- (SO_4^{2-}) medium).

Activation of Constructs Lacking a Phosphoregulatory Domain—If the P676C/I730 cross-link is sufficient by itself to maintain an active transporter, is it able to bring about activation in the complete absence of phosphorylation? To address this question, we engineered two different phospho-dead N termini into the P676C/I730C construct, and in one case we replaced the cytoplasmic C terminus with that from NBCe (solid symbols), but that this does not occur in the phosho-dead constructs without the P676C/I730C mutations (open symbols). Thus Cys676–Cys730 cross-linking is able to activate NKCC1 to ~20% of maximal WT flux (12), despite the absence of the phosphorylation sites in the N terminus or of the entire N-terminal phosphorylation domain. Interestingly, CuPhe treatment did not produce significant transport in a construct with the NKCC1 C terminus replaced by that from NBCe (solid triangles), suggesting that involvement of the C terminus may be necessary to permit the activating cross-link.

This is the first time that we or others have reported detectable transport activity of NKCC1 in the complete absence of regulatory phosphorylation. Thus we further characterized the behavior of the Cys676–Cys730 cross-linked N-terminal truncation mutant. As shown in Fig. 8, the cross-link-activated flux requires Na^+, Rb^+ (for K^+), and Cl^- for transport and is inhibited by bumetanide; the K_{0.5} value for each of these ligands is
FIGURE 4. Dephosphorylation and deactivation affected by CuPhe cross-linking of P676C/I730C. HEK cells transfected with wild type NKCC1 (NT17) or with P676C/I730C were incubated in 0 Cl\textsuperscript{-}/H\textsubscript{11002} medium for 1 h to activate the transporter and then treated with or without 1.5 mM CuPhe in 0 Cl\textsuperscript{-} medium for 10 min. After washing, cells were incubated in regular medium for various times during which the transporter became deactivated. In separate experiments, cells were functionally assayed in a 1-min \textsuperscript{86}Rb\textsuperscript{+} influx assay (A and D) or solubilized and analyzed by Western blotting with R5 antibody to detect phospho-NKCC1 and T4 antibody to detect total NKCC1 (B). D is as in A except that the 10-min treatment period included 0.3 mM CuPhe, 0 Cl\textsuperscript{-} control, 40 mM DTT, or 5 mM TCEP, as labeled. The data in A are triplicate flux assay determinations from one of four experiments with similar results; data in B are averages and S.E. from three experiments, one of which is illustrated in C, and data in D are averages and S.E. of four experiments.
NKCC Activation Mechanism Revealed by Cross-linking

FIGURE 5. Enhancement of P676C/I730C activity by cross-linking of active or inactive transporters. HEK cells were incubated for 1 h in 0 Cl- medium to activate or in regular medium to maintain an inactive state and then for 6 min with various concentrations of CuPhe in regular medium. All cells were then incubated for 40 min in 0 Cl- to activate transporters followed by 40 min in regular medium to deactivate and finally in a 6-min 86Rb+ flux assay. The data represent the means and S.E. of fractional increase in four experiments after normalizing the individual experiments (relVmax 2.5, 2.4, 2.0, and 2.0) to the average. Light lines show the individual results for inhibition in the inactive state, suggesting that in one of four experiments NKCC1 was not completely inactivated.

FIGURE 6. Expression of phospho-dead NKCC1 constructs in HEK cells. Western blot with anti-FLAG antibody. Cell lines are wt NKCC1 (NT17) (lane a), N terminus serines and threonines mutated (NT51) (lane b), truncated N terminus (NT53) (lane c), P676C/I730C with N terminus serines and threonines mutated (NT95) (lane d), P676C/I730C with truncated N terminus (NT96) (lane e), and P676C/I730C with C terminus replaced by NBCe C terminus (NT94) (lane f).

FIGURE 7. 86Rb+ influx in phospho-dead constructs. Transfected cells were incubated for 20 min in regular medium and then for 10 min with various concentrations of CuPhe, as indicated, followed by a 6-min 86Rb+ influx assay. Transfected constructs (our identifier, sequences in Addgene) were: solid symbols, P676C/I730C; open symbols, controls; ⊘, ⊙ and □, N terminus serines and threonines mutated (NT51); ⊘, ⊙, and □, truncated N terminus (NT95, NT53, NT94); and ⊘, ⊙, and □, C terminus replaced by NBCe C terminus (NT94).

approximately the same as previously reported values for NKCC1 (see figure legends). This suggests that the machinery involved with coordination and translocation of ions is the same in this cross-link-activated construct as in native NKCC1.

Specificity of Cross-link Reactions—As shown above and in Table 2 and Fig. 1D, for five of the eight residue pairs initially proposed to be within close proximity, treatment with CuPhe produced functional effects consistent with high efficiency cysteine cross-linking or Cu2+ inhibition consistent with participation of the novel cysteine pairs in a binding site. To further test the specificity of these interactions, we engineered cysteine pairs to test for potential cross-linking of TM10 with residues 730 and 734 of TM11/12 and also for TM11/12 with residue 676 in TM10; 25 of the 31 new pairs were sufficiently expressed at the membrane for evaluation (Tables 1 and 2). Except for A675C (see below), the single cysteine mutations did by not by themselves introduce substantial CuPhe sensitivity (Table 1; P676C and S679C were also affected, but <20%).

As indicated schematically in Fig. 1D, most of the novel cysteine pairs exhibited little or no effect of exposure to CuPhe even at high concentrations, indicating that they are not close enough to one another to form functionally detectable cross-links. In some cases (light pink in Fig. 1D), CuPhe-mediated cross-linking resulted in inhibition at concentrations greater than 100 µM, indicative of less favorable geometry for cross-link formation or less frequent excursions to a conformation permitting cross-linking. However, these cross-links were very sensitive to transporter conformation and thus informative as to the geometry of the different conformational states. One of these is I674C/I730C, a cross-link that was formed preferentially in the bumetanide-inhibited state of the transporter, thus suggesting that tight binding of the loop diuretic involves movement of TM10 relative to TM12.

Cross-linking of the Inactive State—Importantly N680C/W733C, N680C/W734C, and I677C/A735C were inhibited by CuPhe only when the transporter was in an inactive state (Table 2, column b), providing evidence as to TM10–TM12 residues in proximity when the transporter is inactive. As illustrated in red in Fig. 1 (B and C; also see Fig. 11), Asn680 is one helix further into the membrane compared with Pro676 of the activation-requiring cross-links, suggesting that the deactivation process involves inward moment of TM12 relative to TM10 by a distance on the order of 5 Å.

Cu2+ Binding to A675C—Many of the Cu2+-inhibited cysteine pairs include A675C in TM10 (Fig. 1D, pink outline), and indeed the A675C mutant by itself was found to be strongly inhibited by CuPhe and Cu2+ (Table 1 and Fig. 9). It is interesting that A675C/V729C is stimulated by CuPhe cross-linking but inhibited by Cu2+ (Fig. 9), suggesting that cross-linking of Cys729C with Cys75C competes with Cu2+ binding, and a similar competition may also explain the relatively smaller CuPhe effect on A675C/A734C. These data show that Cys675 forms part of a Cu2+-binding site in conjunction with backbone atoms or side chains from the same or other TMs, potentially Glu670 of TM10 or Met382 of TM3. In addition to predicted proximity of Met382 illustrated in Fig. 10, we found that A675C is like M382C (8) in being strongly inhibited by extracellular cysteine reagents 2-[(trimethylammonium)-ethyl methane-thiosulfonate and 2-[(trimethyl-ammonium)]ethylnmethanethiosulfonate (MTSET). The involvement of Met382,
would be especially intriguing because based on structural models as well as on conformation-sensitivity of MTSET block and reduced bumetanide affinity of M382W, Met382 appears to be part of the extracellular gate determining access to the translocation pathway (8).

DISCUSSION

We have found that NKCC1 with cysteines engineered in TMs 10 and 11/12 can form functionally significant cross-links when treated with low concentrations of CuPhe or with I2. These results demonstrate proximity of TM10 and TM12 residues, in general agreement with homology models of NKCC1 based on APC amino acid transporters and are in specific agreement with an alignment of TM11/12 based on homodimeric cross-linking studies.4 The Cys676–Cys734 and Cys677–Cys734 cross-links are inhibitory to NKCC1-mediated 

$^{86}$Rb$^+$ transport (Fig. 2), suggesting that movement of TM10 relative to TM12 is a necessary step in transport as is predicted by the homologous transition in the APC transporter AdiC. Surprisingly, the P676C/I730C cross-link was found to stimulate transport by locking the transporter in an activated state, a finding that sug-

gests movement of TM12 as a key step in the mechanism of regulatory activation.

The results of cross-linking experiments described here provide strong support for the proximity of TMs 10 and TMs 12, visualized in homology models based on APC superfamily amino acid transporters. The APC transporters have a 5-5 helix inverted repeat structure shared with the LeuT in the NSS

FIGURE 8. $^{86}$Rb$^+$ influx mediated by cross-linked N-truncated P676C/I730C. Transfected HEK cells were incubated for 20 min in regular medium and then 10 min in 1.5 mM CuPhe to cross-link Cys676–Cys730. $^{86}$Rb$^+$ influx was measured over an 8-min flux period in the presence of various concentrations of Na$^+$ (top left panel), Rb$^+$ (top right panel), Cl$^-$ (bottom left panel), and bumetanide (bottom right panel), as plotted; lines represent least square fit solutions for a single Na$^+$ or Rb$^+$ sites or for two Cl$^-$ sites. As determined in this and three other experiments, $K_m$(Na$^+$) = 21.4 ± 2.9 mM, $K_m$(Rb$^+$) = 1.24 ± 0.24 mM, $K_m$(Cl$^-$) = 58.8 ± 3.5 mM, nearly the same as those previously reported for human NKCC1 (18, 1.6, and 48 mM, respectively) (8). $K_i$(bumetanide) = 1.29 ± 0.49 μM, is not directly comparable to previous values because the bumetanide incubation conditions are different in the current experiment.

FIGURE 9. Effect of Cu$^{2+}$ and CuPhe on A675C and A675C/V729C. Experimental protocol is the same as in Fig. 2 (A and B). The results are the averages and S.E. from four or six experiments (NT17, A675C n = 6; A675C/V729C n = 4).

FIGURE 10. Model of NKCC1, highlighting proximity of Met382 and A675C. The model is the same as that in Fig. 1. A, view from extracellular solution. B, side view from within the membrane.
superfamily and with several other transporter superfamilies (21), in which the translocation pathway is roughly along the axis of inverted symmetry in the first 10 helices (22). The APCs have two additional TM s, 11 and 12, which are near helices 3, 6, and 10 and in the case of AdiC are found to form a homodimerization interface. The experiments in this paper were originally conceived to test the relative proximity of TMs 10 and 12 in NKCC1 to test the homology of NKCC1 with the APC transporters, and indeed the results provide substantial support for this homology.

The details of the TM11/12 region are poorly determined by homology models alone, because there is a very low level of sequence conservation in this region. In addition there is a significant difference between AdiC and ApCT amino acid transporters in that AdiC has an additional turn at the top of TM 11.

We have analyzed this region in NKCC1 by studying homodimeric cross-linking of single cysteine residues introduced into the two TMs* and arrived at the alignment in the homology model of Fig. 1 (B and C). With TM12 fixed by this alignment, there are quite specific predictions (Fig. 1D, dots) regarding potential cross-links between TMs 10 and 12. These predictions are very well borne out by the results reported here (Figs. 1D, 2, and 3).

Two available crystal structures of AdiC show the transporter in open to out and occluded conformations. Only modest movement of TMs appears to be involved in the transition between these forms, primarily rotation and bending of TMs 6 and 10 (Fig. 1, C and D). Our finding here that cross-linking of TM10 and 12 inhibits ion transport is consistent with a movement of TM10 in NKCC1 during occlusion of extracellular ions, similar to the step that occludes arginine in AdiC. It can be argued that the data do not uniquely pinpoint the occlusion-related conformational change in NKCC1 as the inhibited step because other conformational transitions may also involve TM10 movement. However, it may be noted that models of an inward open transporter based on the GadC crystal structure (23) do not display further movement of TM10 relative to TM12, and an alternative suggestion for inward opening of NKCC1 involving movement of intracellular loop 1 out of the pore would also not involve TM10 (8). Thus it is reasonable to propose that the movement of TM10 relative to TM12 that is inhibited by CuPhe cross-linking is the movement involved in formation of the occluded state from the outward open state.

Remarkably, we found that that CuPhe cross-linking of P676C/I730C substantially increased the level of ion transport by NKCC1, particularly when examined under conditions where the transporter would normally have become deactivated following exposure of the active transporter to CuPhe. In fact even constructs that were partially inhibited by cross-linking (Cys676–Cys734 and Cys677–Cys734) were found to be more active than wild type transporters under deactivated conditions (Fig. 3). Examining deactivation of the transporter upon removal of the stimulus for regulatory phosphorylation, we found that after activation and cross-linking, the cross-linked transporter remained locked in an activated state that is no longer dependent on the phosphorylation state of residues in the N-terminal regulatory domain (Fig. 4). This cross-link that locks the transporter in the activated state was also effective in bringing transport activity to NKCC1 mutants that are totally lacking in the N-terminal phosphoregulatory residues (Fig. 7), conclusively demonstrating that the TM12-TM10 interaction is a sufficient intermediate step in the overall activation process.

Consistent with the fact that the Cys676–Cys730 cross-link prevents transition to an inactive state, formation of the cross-link was found to be completely dependent on the transporter being active at the time of CuPhe exposure (Fig. 5). The fact that the P676C/I730C cross-link does not inhibit the conformational movements of the active transporter (i.e., it is not inhibitory) is consistent with the observation that its formation was largely independent of the ionic composition of extracellular media, cross-linking being only slightly favored in 0 K medium compared with regular medium. In this regard, it is possible that the inhibitory effect of the P676C/A734C and I677C/A734C cross-links arises from slowing of conformational transitions, rather than from completely blocking conformational movement.

These experiments also provided a clue as to the amount of movement involved in activation. Formation of inhibitory cross-links in two constructs containing N680C (Figs. 1, B and C, red, and 11) was found to occur only in the inactive state of the transporter. This identifies a different TM10–TM12 configuration in the inactive state, one that involves a TM10–TM12 cross-link further into the membrane. The fact that I677C/A735C exhibits similar behavior (Tables 1 and 2) suggests that the TM12 movement may be somewhat less than the 5 Å of a full helix turn.

It has long been known that phosphorylation of the N terminus of NKCC1 is absolutely required for transporter activity (15, 24), but it is still unclear how the phosphorylation events in the N terminus are translated to allow conformational movements within the TM translocation domain. One clue was obtained in a study of movement of the large C-terminal cytoplasmic domain; we found that upon phosphorylation and activation of the transporter, the two C termini in an NKCC1 dimer become further from one another, raising the possibility that the C termini could exert regulatory tension on the transmembrane domain (9).
The present finding that TM12-TM10 proximity on the extracellular side of the membrane is a crucial aspect of the NKCC1 activation machinery adds one more piece to the regulatory puzzle. Thus, together with previous observations, the result suggests a model (Fig. 11) in which phosphorylation of the N-terminal regulatory domain results in movement of the C terminus by a yet unknown mechanism; the C terminus in turn exerts tension on TM12 and affects an important relationship between TM12 and TM10, in turn allowing or blocking conformational changes in TMs 1–10. The homology between NKCCs, Na-Cl cotransporters, and K-Cl cotransporters argues that the same basic mechanism is responsible for regulation of all of the cation-chloride cotransporters, but with different degrees and directions of regulation depending on phosphoregulatory regions in the N and C termini (25).

Clearly absent from the model in Fig. 11 is the feature by which phosphorylation of the N terminus triggers the rest of the activation mechanism. An activating interaction of the phosphoregulatory domain is favored over an inhibitory interaction of the dephosphorylated domain by the fact that all N-terminal truncation mutants have been found to be inactive (Fig. 7). Presumably the phosphorylated N terminus interacts either with a region in the C terminus or with intracellular loops of the transmembrane domain to bring about transition seen in Fig. 11. Further experiments will be necessary to uncover this interaction as well as to further test the current model and refine the relationships of C terminus, the TM12-C terminus linker helix as well as to further test the current model and refine the membrane domain to bring about transition seen in Fig. 11.

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