Identification of a Functionally Important Conformation-sensitive Region of the Secretory Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter (NKCC1)\(^*\)

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The secretory Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC1) is a member of a small gene family of electroneutral salt transporters that play essential roles in salt and water homeostasis in many mammalian tissues. We have identified a highly conserved residue (Ala-483) in the sixth membrane-spanning segment of rat NKCC1 that when mutated to cysteine renders the transporter sensitive to inhibition by the sulfhydryl reagents 2-aminoethyl methanethiosulfonate (MTSEA) and 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET). The mutation of Ala-483 to cysteine (A483C) results in little or no change in the affinities of NKCC1 for substrate ions but produces a 6-fold increase in sensitivity to the inhibitor bumetanide, suggesting a specific modification of the bumetanide binding site. When residues surrounding Ala-483 were mutated to cysteine, only I484C was sensitive to inhibition by MTSEA and MTSET. Surprisingly I484C showed increased transport activity in the presence of low concentrations of mercury (1–10 \(\mu\mathrm{M}\)), whereas A483C showed inhibition. The inhibition of A483C by MTSEA was unaffected by the presence or absence of sodium and potassium but required the presence of extracellular chloride. Taken together, our results indicate that Ala-483 lies at or near an important functional site of NKCC1 and that the exposure of this site to the extracellular medium is dependent on the conformation of the transporter. Specifically, our results indicate that the cysteine introduced at residue 483 is only available for interaction with MTSEA when chloride is bound to NKCC1 at the extracellular surface.

Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporters (NKCCs)\(^\ddagger\) mediate the electroneutral transport of Na\(^+\), K\(^+\), and Cl\(^-\) across cell membranes with a stoichiometry of 1Na\(^+\):1K\(^+\):2Cl\(^-\) (1, 2). By providing a concentrative chloride entry step in chloride secreting and absorbing epithelia, these transporters play a central role in trans-epithelial salt movements across these tissues (1, 2). The NKCCs belong to a small gene family with homologues in vertebrates, crustaceans, insects, worms, plants, and some microorganisms. Nine members of this family have been identified in vertebrates, and of these, seven have been shown to be electroneutral cation-chloride cotransporters (3). These include two Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter isoforms (NKCC1 and NKCC2), a Na\(^+\)-Cl\(^-\) cotransporter (NCC), and four K\(^+\)-Cl\(^-\) cotransporter isoforms (KCC1, KCC2, KCC3, and KCC4). The function of the remaining two vertebrate sequences as well as all of the homologues identified in lower organisms remains to be definitively established.

NKCC1, the “secretory” isoform of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporters, is the most extensively studied member of the cation-chloride cotransport family at the molecular level. This transporter is relatively widely expressed in both epithelial and nonepithelial tissues and has been of considerable interest because of its roles in cell volume regulation as well as trans-epithelial chloride secretion (1, 2). The activity of NKCC1 typically is highly regulated by physiological stimuli that can result in its phosphorylation as well as other as yet uncharacterized modifications (1, 4). Hydropathy analyses indicate that all of the vertebrate cation-coupled cotransporters share a common membrane topology consisting of large hydrophilic N and C termini (15–35 and ~50 kDa, respectively) on either side of a central hydrophobic transmembrane domain (~50 kDa). Topology studies of NKCC1 (5) have established that this hydrophobic domain consists of 12 (presumably \(\alpha\)-helical) membrane-spanning segments (MSSs).

In a recent series of papers, Forsburg and colleagues (6–8) have shown that it is mainly the central hydrophobic domain of NKCC1 that determines its apparent affinities for sodium, potassium, and chloride as well as for the NKCC-specific inhibitor bumetanide (6–8). The cytosolic N and C termini have little effect on these properties and are thought to be mainly involved in the regulation of transport activity. In addition, these authors were able to identify a number of specific regions within the central hydrophobic domain that were involved in determining substrate affinities. They did this by taking advantage of the fact that although the shark and human NKCC1s are ~75% identical, they have quite different affinities for transported ions. By examining the properties of various shark-human chimeras, they were able to establish that sequence differences in MSSs 2, 4, and 7 could account for the differences in ion affinities between species. From appropriate point mutations, they were then able to narrow down the list of candidates to 13 residues (7). These residues are (in the sequence of rat NKCC1) Ser-311 and Val-312 (in MSS 2 associated with sodium affinity differences); Ala-316 and Met-317 (in MSS 2 associated with potassium affinity differences); Ala-405, Ile-406, Val-408, Val-410, and Leu-412 (in MSS 4 associated with potassium and chloride affinity differences); and Val-522, Ile-525, Ile-527, and Val-529 (in MSS 7 associated with affinity differences of all of the transported ions). The authors (7)
speculate that as few as four of these residues may be responsible for the affinity differences between shark and human NKCC1, two residues in MSS 2 and one each in MSS 4 and 7. Additional studies to further clarify the roles of these 13 candidate residues have yet to be performed.

The simplest interpretation of the above results is that these 13 residues, or some subset of them, are located at or near the ion binding sites of NKCC1. However, note that the approach taken in these experiments necessarily excluded the consideration of residues that are conserved between the shark and human NKCC1a. It is anticipated that many of these residues play important functional and structural roles in NKCC1 (hence their conservation) and that some of them also influence ion binding and affinities. Mutations of these latter amino acids would be expected to affect the properties of both shark and human NKCC1.

In contrast to the relatively simple findings described above for transported ions, Isenring et al. (7) found that the affinity differences for bumetanide between shark and human NKCC1 were associated with sequence differences in MSSs 2–7 as well as in MSS 11 and MSS 12. Moreover, a number of their shark-human chimerae had inhibition constants for bumetanide that were outside the range of both shark and human NKCC1, making these results difficult to interpret.

In this study, we continue the effort to identify and characterize important functional residues in NKCC1. To do this, we have made use of the substituted cysteine accessibility method (9), which involves replacing potentially interesting residues with cysteine and then assessing their accessibility and properties using cysteine-specific reagents. This method has been widely used to study the functional and structural properties of a number of membrane transporters and channels. Having only limited information that would allow us to select residues for mutation, in our initial studies, we chose amino acids that were predicted to be within the MSSs of NKCC1 but close to the extracellular surface and that could be conservatively mutated to cysteine. In a number of cases, these residues were chosen at or close to those identified above by Isenring et al. (7). Somewhat surprisingly, these latter cysteine mutants failed to be affected by the sulphydryl reagents tested, possibly indicating that these residues were located in relatively compact pockets accessible to substrate ions but otherwise protected from the extracellular solution. However, one of our mutants (A483C) reacted strongly with sulphydryl reagents applied from the extracellular surface and appeared to be exposed only during certain steps of the transport cycle.

**EXPERIMENTAL PROCEDURES**

**Materials**—The methanethiosulfonate (MTS) derivatives, MTSEA (2-aminoethyl methanethiosulfonate) and MTSET (2-(trimethylammonio)ethyl methanethiosulfonate), were from Toronto Research Chemicals. MTSEA and MTSET were prepared as 100-fold stock solutions in dimethyl sulfoxide (DMSO). Both of these reagents are positively charged with MTSEA being typically more widely reactive in all likelihood because of its somewhat smaller size (9). MTSEA has also been shown to be slightly membrane-permeant, whereas MTSET is membrane-impermeant (11). As illustrated in Fig. 1, neither 86Rb flux into mock-transfected HEK-293 cells (V) nor into those transiently transfected with wild-type rat NKCC1 (WT) is significantly affected by either of these reagents under our test conditions (3 min of incubation with 3 mM MTS reagent) (see Fig. 1 legend for details). As illustrated later in the paper (Fig. 5), these ouabain-insensitive 86Rb fluxes are completely inhibited by the NKCC-specific inhibitor bumetanide with Kd, 1–2 mU.

**Flux Media**—The following media were used in the flux experiments.

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*S. M. Saitoh and R. J. Turner, unpublished data.*
As already indicated, the amino acids we have chosen for mutation to cysteine (Fig. 1) are thought to be located within the MSSs of NKCC1 close to the extracellular surface (5). In most cases, we chose residues at or close to those identified by Isenring et al. (7) as being potentially associated with substrate binding sites. Ser-311, Val-312, and Ala-316 are in the second MSS of NKCC1 and are thought to be at or near the sites of interaction of the cotransporter with sodium and potassium, Ala-405 is located in the fourth MSS of NKCC1 at or near a region associated with the interactions of potassium and chloride, and Ala-528 and Ser-530 are located in the seventh MSS close to a region associated with the interactions of all three substrate ions (see Introduction). Finally, Ala-483 is located in the sixth MSS of NKCC1 at or near a region associated with the interactions of potassium and chloride, and Ala-528 and Ser-530 are located in the seventh MSS close to a region associated with the interactions of all three substrate ions (see Introduction). Finally, Ala-483 is located in the sixth MSS of NKCC1, a region of the protein that is highly conserved, especially between the NKCCs and NCCs (Fig. 2). As illustrated in Fig. 1, all of these cysteine mutants are functional when transiently transfected into HEK293 cells, but only A483C is inhibited by MTSEA and MTSET under our experimental conditions.

The dependence of the inhibition of A483C on MTSEA and MTSET concentrations is illustrated in Fig. 3, A and B. The analyses of these data yield \( K_{0.5} \) values for the effects of MTSEA and MTSET of 0.36 \( \pm \) 0.07 and 1.48 \( \pm \) 0.37 mM, respectively (see Fig. 3 legend). From Fig. 3A, it can be seen that the inhibitory effect of MTSEA is essentially complete after 3 min of incubation with 3 mM reagent. This was confirmed in time course studies where we found that the \( t_{1/2} \) for the effect of 3 mM MTSEA was <15s (data not shown, times up to 10 min were studied). After 3 min of incubation with 3 mM MTSEA, we found that the average residual \(^{86}\)Rb flux into A483C-transfected cells is 4.7 \( \pm \) 0.3(n = 17) nmol/mg protein/min. By comparison, the average \(^{86}\)Rb flux attributed to HEK-NKCC in mock-transfected cells (3.4 \( \pm \) 0.2 nmol/mg protein/min (n = 25)) is significantly lower. Thus, the reaction with MTSEA (and in all likelihood also with MTSET) (cf. Fig. 3B) does not appear to result in complete inhibition of A483C. These results also indicate that transient transfection of NKCC1 into HEK-293 cells does not suppress endogenous HEK-NKCC activity as has been suggested to occur in HEK-293 cells stably transfected with NKCC1 constructs (6, 13).

The above experiments demonstrate that the mutation of Ala-483 to cysteine renders NKCC1 sensitive to sulphydryl
Fig. 4. Sodium, rubidium, and chloride dependence of fluxes via wild-type NKCC1, HEK-NKCC, and A483C. $^{86}$Rb fluxes were measured in HEK-293 cells transiently transfected with wild-type NKCC1 (circles), empty pBK-CMVvec vector (squares), and A483C (triangles) in the presence of the concentrations of bumetanide indicated. In these experiments, bumetanide was present with $^{86}$Rb in the uptake medium as well as during the 10-min incubation with chloride-free medium plus 0.1 mM ouabain (see Experimental Procedures) for other experimental details. Wild-type NKCC1 and A483C fluxes have been corrected for $^{86}$Rb uptake via HEK-NKCC, and all of the fluxes have been normalized to that observed in the absence of bumetanide. The data were fit to a model that assumes a single inhibitory site for bumetanide. The solid line is a fit to the wild-type NKCC1 results ($K_{0.5} = 2.4 \pm 0.7 \mu M$), and the dashed line is a fit to the A483C data ($K_{0.5} = 0.37 \pm 0.02 \mu M$). A fit to the HEK-NKCC data (not shown) yielded $K_{0.5} = 0.43 \pm 0.09 \mu M$.

Fig. 5. Dose response of the effect of bumetanide on wild-type NKCC1, HEK-NKCC, and A483C. $^{86}$Rb fluxes were measured in HEK-293 cells transiently transfected with wild-type NKCC1 (circles), empty pBK-CMVvec vector (squares), and A483C (triangles) in the presence of the concentrations of bumetanide indicated. In these experiments, bumetanide was present with $^{86}$Rb in the uptake medium as well as during the 10-min incubation with chloride-free medium plus 0.1 mM ouabain (see Experimental Procedures) for other experimental details. Wild-type NKCC1 and A483C fluxes have been corrected for $^{86}$Rb uptake via HEK-NKCC, and all of the fluxes have been normalized to that observed in the absence of bumetanide. The data were fit to a model that assumes a single inhibitory site for bumetanide. The solid line is a fit to the wild-type NKCC1 results ($K_{0.5} = 2.4 \pm 0.7 \mu M$), and the dashed line is a fit to the A483C data ($K_{0.5} = 0.37 \pm 0.02 \mu M$). A fit to the HEK-NKCC data (not shown) yielded $K_{0.5} = 0.43 \pm 0.09 \mu M$.

Fig. 6. Dose response of the effect of inorganic mercury on wild-type NKCC1, HEK-NKCC, and A483C. $^{86}$Rb fluxes were measured in HEK-293 cells transiently transfected with wild-type NKCC1 (circles), empty pBK-CMVvec vector (squares), and A483C (triangles) in the presence of the concentrations of mercury (as HgCl$_2$) as indicated. In these experiments, mercury was present with $^{86}$Rb in the uptake medium as well as during the 10-min incubation with chloride-free medium plus 0.1 mM ouabain (see Experimental Procedures) for other experimental details. Wild-type NKCC1 and A483C fluxes have been corrected for $^{86}$Rb uptake via HEK-NKCC, and all of the fluxes have been normalized to that observed in the absence of mercury. The wild-type NKCC1 and HEK-NKCC data were fit to a model that assumes a single inhibitory site for mercury. The solid line is a fit to the wild-type NKCC1 results ($K_{0.5} = 27.8 \pm 8.9 \mu M$). A fit to the HEK-NKCC data (not shown) yielded $K_{0.5} = 24.4 \pm 2.9 \mu M$.

Characterization of A483C—To better understand the significance of the A483C mutation, we next compared the behavior of A483C to that of wild-type NKCC1 and HEK-NKCC. Fig. 4 illustrates the sodium, rubidium, and chloride dependence of $^{86}$Rb fluxes via these transporters. In the upper panels, we have plotted (normalized) total fluxes versus substrate concentration, whereas in the lower panels, wild-type NKCC1 and A483C fluxes have been corrected for flux via HEK-NKCC. Overall, the transport characteristics of A483C are very similar to the wild-type transporter except possibly for a marginally lower affinity for chloride (see Fig. 4 legend). In contrast, as illustrated in Fig. 5, A483C as well as HEK-NKCC shows a 6-fold higher sensitivity to bumetanide than wild-type NKCC1. The fact that the mutation of Ala-483 to cysteine has little or no effect on the affinities of all three substrate ions suggests that this modification results in only minor perturbations in NKCC1 conformation and function. The larger effect on bumetanide affinity appears to be specific to bumetanide, possibly...
NKCC1 Conformation-specific Residue

Effects of Inorganic Mercury—Inorganic mercury is also known to react strongly with free sulfhydryl groups. Fig. 6 shows the effects of mercury on wild-type NKCC1, HEK-NKCC, and A483C. It is clear that wild-type NKCC1 and HEK-NKCC have a similar sensitivity to mercury with $K_{0.5}$ values of 25 and 30 $\mu M$ (see Fig. 6 legend). However, A483C shows a far greater sensitivity. Approximately, 70% of the $^{86}$Rb flux via A483C is inhibited by mercury with $K_{0.5} < 1$ $\mu M$, whereas the remainder appears to have a similar sensitivity to that of wild-type NKCC1 (we have not attempted to fit these data to a two-component model because of the scatter on the points). This behavior is reminiscent of the effects of MTSEA and MTSET documented in Fig. 3 in that the inhibition by mercury at its high affinity site does not result in complete blockade of A483C.

Effects of Substrates and Inhibitors on the Inactivation of A483C by MTSEA—Because Ala-483 is located in a highly conserved (Fig. 2) and apparently functionally important region of the NKCCs, we wondered whether its accessibility to sulfhydryl reagents might be influenced by substrates or inhibitors of NKCC1. In Fig. 7, we illustrate that when A483C-transfected HEK-293 cells are exposed to MTSEA in sodium-free or potassium-free medium or in the presence of 100 $\mu M$ furosemide, its effect is not significantly different from that observed under control (sodium-, potassium-, and chloride-replete) conditions. However, when exposure to MTSEA is carried out in the absence of chloride, its inhibitory effect is markedly blunted. This result suggests that the reaction of MTSEA with A483C is dependent on transporter conformation and, more specifically, that the conformations of the transporter present in the absence of extracellular chloride are insensitive to MTSEA. A more detailed discussion of the significance of this result is given later in the paper.

Cysteine-scanning Mutagenesis of MSS 6—In Fig. 8, we show the effects of 3 $mM$ MTSEA and MTSET on NKCC1 mutants in which nine residues surrounding Ala-483 have been individually changed to cysteine. $^{86}$Rb uptake into HEK-293 cells transiently transfected with two of these mutants, P487C and A488C, is not significantly different from that seen with mock-transfected cells (cf. Fig. 1), indicating that these mutations do not result in a functional transporter at the plasma membrane. Fluxes via the remaining seven cysteine mutants are 50–100% of wild-type levels, but only I484C in addition to A483C is inhibited by MTSEA and MTSET. In Fig. 9, we examine the sensitivity of these mutants to inorganic mercury. Most of the mutants behave quite similarly to wild-type NKCC1 (Fig. 9, solid line in all panels); however, I484C (middle panel, squares) shows a dramatic stimulation of $^{86}$Rb uptake over the mercury concentration range of 1–10 $\mu M$ followed by inhibition at higher mercury concentrations. A similar but blunted effect appears to also occur for S480C (upper panel, triangles) and F486C (lower panel, triangles).

DISCUSSION

We have used substituted cysteine mutagenesis in combination with sulfhydryl specific reagents to examine the properties of selected residues in NKCC1. Bumetanide-sensitive $^{86}$Rb fluxes via these mutants were measured in transiently transfected HEK-293 cells. HEK-293 cells have been extensively employed by previous investigators to study a number of facets of NKCC1 function (6–8, 12–15). These past studies were carried out on stably transfected clonal cell lines. The present experiments demonstrate that comparable $^{86}$Rb fluxes can be obtained from transient transfectants, allowing for a significant reduction in time and labor in high throughput-type experiments such as the screening of mutants.

In their experiments, Isenring et al. (6–8, 12) have extensively characterized human NKCC1 in this expression system. They found $K_{m}$ values of 15, 1.95, and 31 $mM$ for the substrates sodium, rubidium, and chloride, respectively, and $K_{0.5}$ values of 56 $\mu M$ for mercury and 0.28 $\mu M$ for the NKCC-specific inhibitor bumetanide. In the present experiments with wild-type rat NKCC1, we found $K_{m}$ values of 61, 1.85, and 48 $mM$ for sodium, rubidium, and chloride, respectively, and $K_{0.5}$ of 29 $\mu M$ for mercury and 2.4 $\mu M$ for bumetanide (Figs. 4–6). In their $^{86}$Rb flux measurements, Isenring et al. (6–8) used 5 $mM$ unlabeled rubidium, whereas we have used 1 $mM$. Because higher rubidium concentrations are expected to increase the affinity of NKCC1 for sodium, chloride, and bumetanide, this may ac-
count for at least some of the differences between our results and theirs. In their studies, Isenring et al. (12) also found that HEK-NKCC, the endogenous Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter of the HEK-293 cells (a human cell line), had rather different properties from their human NKCC1 clone. More specifically, they found \(K_m\) values of 35, 15, and 42 mM for sodium, rubidium, and chloride, respectively, and \(K_{0.5}\) values of 133 µM for mercury and 0.08 mM for bumetanide. In our hands, the behavior of HEK-NKCC is more similar to that of the rat NKCC1. We found \(K_m\) values of 41, 1.47, and 3.6 mM for sodium, rubidium, and chloride, respectively, and \(K_{0.5}\) values of 24 µM for mercury and 0.4 µM for bumetanide (Figs. 4–6). This difference may be related to the source of HEK-293 cells used in our experiments, but we have not explored this matter further.

A number of the residues we have mutated to cysteine in this study (S311, Val-312, Ala-316, Ala-405, Ala-528, and Ser-530) have been previously suggested to be at or near the binding sites for sodium, potassium, or chloride on NKCC1 (7). Interestingly, none of these mutations rendered NKCC1 sensitive to MTSEA or MTSET under our experimental conditions (Fig. 1). In additional experiments (data not shown), we have confirmed that the sensitivity of these mutants to inorganic mercury is likewise not significantly altered from that of wild-type NKCC1. These results indicate either that the cysteines introduced at these sites are inaccessible to the sulfhydryl reagents we have tested or that the modifications resulting from these reagents have no effect on transport. Although additional experiments are required to distinguish between these possibilities, given the rather convincing evidence that modifications in at least some of these residues can dramatically affect substrate affinities (7), we would suggest that the former explanation is more likely to be the correct one. If this is the case, it would appear that the NKCC1 ion binding sites may be located in regions that are relatively inaccessible except to substrates, for example, in pockets buried below the protein surface.

In contrast to the above mutations, we found that the NKCC1 mutant A483C was inhibited by MTSEA and MTSET (Figs. 1 and 3) and showed a dramatically higher sensitivity to inorganic mercury than wild-type NKCC1 (Fig. 6). Because MTSET is membrane-impermeant, its reaction with A483C must be from the extracellular solution. After 3 min of incubation with 300 µM reagent, we found that it has achieved 50% of its maximal effect, indicating a \(t_{1/2}\) of ~3 min. However, reaction rates of MTSEA and MTSET with accessible protein cysteines can exceed 10^4 M\(^{-1}\) s\(^{-1}\) (11, 16, 17) in which case the \(t_{1/2}\) for reaction with 300 µM reagent would be 0.23 s. The much longer \(t_{1/2}\) we observe with A483C suggests that the reactive cysteine is not directly accessible from the extracellular solution. Consistent with this finding,
hydropathy analyses and topology studies (5) indicate that Ala-483 is located within MSS 6 of NKCC1 close to but not at the extracellular surface. This region of the protein is highly conserved among NKCCs and their homologues (Fig. 2), suggesting an important role in transporter structure or function.

The relatively conservative mutation of Ala-483 to cysteine results in little or no change in substrate affinities (Fig. 4), but it does produce a 6-fold increase in the affinity of NKCC1 for bumetanide (Fig. 5). The fact that this mutation produces a bumetanide-specific effect suggests that it may result in a relatively small change that only affects the bumetanide binding site. In their studies, Isenring et al. (7) found that the residues associated with the differences in bumetanide affinity between human and shark NKCC1 were in MSS 2–7 as well as MSS 11 and MSS 12. The present results indicate that MSS 6 is also associated with bumetanide binding.

When residues surrounding Ala-483 were mutated to cysteine, only I484C showed a dramatic change in sensitivity to MTS reagents and inorganic mercury (Figs. 8 and 9). Surprisingly, I484C showed increased transport activity in the presence of low concentrations (1–10 μM) of mercury (Fig. 9), whereas A483C showed inhibition (Fig. 6). The reason for this dramatic difference in transport behavior remains to be determined, but it is again consistent with the hypothesis that MSS 6 plays an important role in the function of NKCC1. When the residues surrounding Ala-483 are plotted on a helical wheel (Fig. 10), it is clear that Ala-483 and Ile-484 lie on a relatively polar face of a (putative) helix whose opposite side is strongly hydrophobic. Interestingly, Ser-480 whose cysteine mutant also shows some stimulation at low concentrations of mercury (Fig. 9) and Pro-487 whose cysteine mutant shows no function (Fig. 8) also lie on the same face (Ser-480, Ala-483, Ile-484, and Pro-487 are identified by asterisks in Fig. 10). Because little is really known regarding the structure of NKCC1, it is difficult to speculate about the involvement of MSS 6 in helix packing; however, the highly hydrophobic face of MSS 6 on the left side in Fig. 10 almost certainly faces the membrane lipid, indicating that MSS 6 lies on the periphery of the intact NKCC1 molecule.

Because there are other native cysteines on NKCC1, it is possible in principle that mutating Ala-483 could result in a change in conformation that leads to the exposure of a previously hidden cysteine and that the effects we observe here are because of reactions at that site. In fact, there are only two candidates for this putative cysteine. Of the 12 native cysteines on wild-type rat NKCC1 (18), five are thought to be intracellular and therefore inaccessible to MTSET and five are located in a long glycosylated extracellular loop between MSS 7 and MSS 8 and therefore already accessible to extracellular reagents in wild-type NKCC1. The remaining two cysteines are located near the middle of MSS 11 (5). There are several reasons why we feel that it is very unlikely that our results could be the result of the exposure a native cysteine. First, the mutation of Ala-483 to cysteine is a relatively conservative one and is unlikely to result in a major conformational change in the protein. Thus, the reactive cysteine in A483C is much more likely to be Cys-483 than a previously non-reactive native cysteine. Second, the transport properties of A483C are almost identical to those of wild-type NKCC1, again arguing strongly against a major change in conformation. Third, the effects of mercury on A483C and I484C are dramatically different (Figs. 6 and 9). It is very unlikely that these two disparate effects could both be accounted for by the exposure of a previously inaccessible cysteine.

The mutant A483C has one other very interesting property. It is insensitive to inhibition by 3 mM MTSEA and MTSET in the absence of extracellular chloride (Fig. 7). This result indicates that the reactivity of the cysteine introduced at position 483 is dependent on transporter conformation. The significance of this observation can be best appreciated by considering a model for the function of NKCC1. Fig. 11 shows the NKCC1 transport cycle proposed by Lytle et al. (19). The binding of substrates is ordered with sodium binding first to the empty transporter on the extracellular side followed by a chloride, potassium, and then a second chloride. NKCC1 is thought to have "mirror symmetry," meaning that substrate dissociation on the intracellular side occurs in the same order that the substrates bind on the extracellular side, i.e. sodium dissociates first, followed by potassium, followed by chloride, potassium, and chloride (see Fig. 11). Coupling is “tight,” i.e. only the completely empty and fully loaded transporters are capable of the conformational change that reorients the substrate binding pocket between its outward-facing and inward-facing forms (the transitions between conformations I and X and between V and VI, respectively). During incubation in sodium-, potassium-, and chloride-replete medium, all of the conformations of NKCC1 will be populated so that the inhibition of A483C by MTS reagents (Fig. 3) under these conditions could be attributed to a reaction with any or all of them. In the absence of extracellular chloride, conformations III, IV, and V can still be populated from conformation VI; however, conformations III and V will only be present transiently because chloride will dissociate from them and cannot be replaced. Accordingly, because the reaction with 3 mM MTSEA is rapid (t_{1/2} < 15 s, see above) and chloride loss from HEK-293 cells in isotonic chloride-free medium is much slower (t_{1/2} ~ 40 min; see Ref. 12), we expect that all of the conformations of NKCC1 other than III and V would be available for reaction with MTSEA when these cells are switched to chloride-free medium. Because MTSEA is in fact without effect under these conditions (Fig. 7), we conclude that only conformations III and V could be sensitive to MTSEA. By similar arguments, conformations III and V would be expected to be available for reaction in extracellular sodium-free and potassium-free conditions, whereas conformations II and IV, respectively, would not. Consistent with our argument that only conformations III and/or V are MTSEA-sensitive, MTSEA is in fact inhibitory in sodium-free and potassium-free media (Fig. 7). Other models of NKCC1 function would obviously lead to appropriately modified conclusions, but in general, our results indicate that the cysteine introduced at site 483 is only available for interaction with MTSEA when NKCC1 is in an externally oriented conformation with chloride bound.

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