

A Conserved Hydrophobic Tetrad near the C Terminus of the Secretory Na⁺-K⁺-2Cl⁻ Cotransporter (NKCC1) Is Required for Its Correct Intracellular Processing*

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Little is known about the intracellular folding and trafficking of integral membrane proteins. Here we identify a hydrophobic amino acid tetrad (ILLV) close to the C terminus of the secretory Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1) that is important for the proper intracellular processing of this protein. This tetrad appears in a C-terminal sequence pattern that is conserved across species in a number of members of the NKCC1 gene family (*slc12*) of electroneutral salt transporters. We studied the effects of various mutations of these amino acids on NKCC1 transiently transfected into HEK-293 cells. Our results show that mutation of two of these residues to alanine leads to a >50% reduction in expression and complex glycosylation levels and that multiple mutations to alanine have cumulative effects. By contrast, scrambling of these amino acids, or mutation of other nearby conserved C-terminal residues, has little effect on these parameters. Mutation of ILLV to AAAA reduces complex glycosylation of NKCC1 by ~90% and results in a protein that does not form stable dimers and is retained in the endoplasmic reticulum in a highly aggregated state. Our results are consistent with the hypothesis that mutation of the hydrophobic tetrad ILLV to AAAA leads to the *ab initio* misfolding and concomitant aggregation of this NKCC1 mutant, resulting in its retention in the endoplasmic reticulum.

Despite their abundance and obvious biological importance, relatively little is known about the processes involved in the folding and trafficking of polytopic membrane proteins. Integral membrane proteins destined for the cell surface or other cellular compartments are first inserted into the membrane of the endoplasmic reticulum (ER)³ via the translocon complex (1). In the ER, these nascent proteins acquire their mature tertiary and quaternary structures before being transferred to the

Golgi complex and ultimately to their final destinations (2, 3). These processes are mediated by ER chaperone activity and by targeting signals coded into the amino acid sequence of the protein itself. Quality control mechanisms in the ER typically ensure that proteins that do not fold or oligomerize correctly are retained in the ER and eventually degraded (2, 3). A growing number of diseases have been associated with the mistargeting, misfolding, and/or intracellular accumulation and aggregation of membrane proteins (4–10). As a result, there has been increasing interest in understanding and modifying these aberrant phenomena.

The electroneutral cation-chloride-coupled cotransporter gene family (*slc12*) contains nine members in vertebrates. These include two Na⁺-K⁺-2Cl⁻ cotransporters (NKCC1 and NKCC2), a Na⁺-Cl⁻ cotransporter (NCC), four K⁺-Cl⁻ cotransporters (KCC1, KCC2, KCC3, and KCC4), and two other homologues of unknown function. Members of the Slc12 family are known to play important roles in numerous physiological processes, including exocrine fluid secretion, renal salt and water absorption, hearing, olfaction, spermatogenesis, pain perception, visual processing, and other neuronal functions (11–14). Several studies have found evidence that residues near the end of the intracellular C terminus of Slc12 family members are required for their proper cellular processing. Thus, for example, in their investigations of mutations of NCC associated with Gitelman syndrome, Sabath *et al.* (8) found that several C-terminal truncation and point mutants exhibited impaired processing to the plasma membrane. In addition, in experiments from our own laboratory, we have found that truncating the C terminus of NKCC1 typically results in recombinant proteins that form intracellular aggregates (15). Studies of other integral membrane proteins have identified amino acids in their intracellular C termini that are associated with folding and/or trafficking (7, 16–26). In a number of cases, hydrophobic amino acids, particularly dileucine motifs, have been shown to be involved in these processes (16, 17, 19, 22, 23, 25).

A multiple alignment of Slc12 amino acid sequences reveals a C-terminal sequence pattern that is conserved in all vertebrate NKCCs, NCCs, and KCCs, as well as in a variety of Slc12 homologues from other nonvertebrate species, specifically xxxhxxxxhRGxxxxVhThYx, where h and x represent hydrophobic and variable amino acids, respectively. In many cases, the hydrophobic tetrad hhhh includes a central dileucine motif. Here we study the effects of mutations of the above sequence pattern using the secretory Na⁺-K⁺-2Cl⁻ cotrans-

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³ The abbreviations used are: ER, endoplasmic reticulum; NKCC, Na⁺-K⁺-2Cl⁻ cotransporter; NCC, Na⁺-Cl⁻ cotransporter; KCC, K⁺-Cl⁻ cotransporter; PBS, phosphate-buffered saline; DTSSP, 3,3'-dithiobis(sulfosuccinimidyl) propionate; pBK⁺, pBK-CMVlac⁺; WT, wild type; HA, hemagglutinin.

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porter NKCC1 as a model Slc12 protein. Our results indicate that modifications of the sequence downstream of the hydrophobic tetrad (ILLV in NKCC1) have no significant effect on the trafficking of NKCC1 from the ER to the Golgi. However, mutations of the amino acids of the hydrophobic tetrad itself to alanine result in the retention of NKCC1 in the ER and its aggregation, apparently as a result of the *ab initio* misfolding of the protein. Interestingly, it is conservation of residue hydrophobicity rather than residue identity that appears to underlie the role of this amino acid motif.

EXPERIMENTAL PROCEDURES

Materials—Phosphate-buffered saline (PBS) was obtained from Invitrogen. Leupeptin, pepstatin, and aprotinin were from Roche Applied Science. Bovine serum albumin, ouabain, and Triton X-100 were from Sigma. 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride was from ICN (Aurora, OH). *N*-Glycosidase F and restriction enzymes were from New England BioLabs (Ipswich, MA). EZ-Link Sulfo-NHS-LCLC-Biotin, 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP), and immobilized Streptavidin beads were from Pierce. Brefeldin A was obtained from Biomol (Plymouth Meeting, PA). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

The antibody α -wCT(r) was raised in rabbits against the recombinant C terminus of rat NKCC1 (27). The antibody α -wNT(r) was raised in rabbits against amino acids 3–202 of the (recombinant) N terminus of rat NKCC1 (28).

Molecular Biology—The mammalian expression vector pBK-CMVlac⁻ (pBK⁻) (29) was used for expression studies. Mutants of wild-type rat NKCC1 in pBK⁻ (15) were generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions, except that mutagenic primers were designed using the method of Zheng *et al.* (30). An N-terminally truncated version of NKCC1 in pBK⁻ (nttNKCC1) (29) whose coding sequence begins at Met²⁰⁹, was modified (using the QuikChange kit) by inserting the sequence AYPYDVPDYAL (incorporating the HA tag) after the start codon; this HA-tagged clone is referred to as HA-nttNKCC1. All sequence modifications were confirmed by direct sequencing.

Cell Culture and Transient Transfection—The human embryonic kidney cell line HEK-293 was cultured in MEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 μ M each of penicillin and streptomycin. Cells were grown in 6-cm culture dishes in a humidified incubator at 37 °C and 5% CO₂ and subcultured every 2–3 days. Subconfluent (70–80%) HEK-293 monolayers were transiently transfected (2 days) with the expression vectors indicated using FuGENE6 (Roche Applied Science) following the manufacturer's instructions.

Membrane Preparation, Cross-linking, and Deglycosylation—A crude membrane fraction was prepared from HEK-293 cells essentially as described previously (15, 29). The cells growing in a 6-cm culture dish were washed twice in ice-cold PBS (pH 7.4) and then suspended in 200 μ l of ice-cold TEEA buffer, consisting of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 3 mM EGTA, 300 μ M 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride,

10 μ M leupeptin, 10 μ M pepstatin A, and 2.5 μ g/ml aprotinin. The suspended cells were then homogenized by passing five times through a 25-gauge needle. This material was centrifuged at 1000 \times *g* for 10 min, and the supernatant was saved. The pellet was resuspended in 200 μ l of TEEA buffer and rehomogenized and centrifuged as before. The combined supernatants from these two homogenization steps were centrifuged at 100,000 \times *g* for 30 min, and the resulting crude membrane fraction was resuspended in TEEA.

Cross-linking studies were performed on solubilized crude membranes prepared from transiently transfected HEK-293 cells as described previously (15). Briefly, membranes were suspended in PBS (pH 7.4) at a protein concentration of 1 mg/ml (measured using the Bio-Rad protein assay kit with bovine IgG as the standard) and solubilized in 0.3% Triton X-100. The solubilized membranes were then incubated with the homobifunctional amino acid cross-linking reagent DTSSP (1 mM) for 30 min, after which the cross-linking reaction was stopped by the addition of 100 mM glycine.

Aliquots of the crude membrane fraction were deglycosylated by treatment with *N*-glycosidase F as previously described (31).

Biotinylation of Intact HEK-293 Cells and Removal of Biotinylated Proteins on Streptavidin Beads—HEK-293 cells growing in a 6-cm culture dish were detached from the plate by incubation in citrate buffer containing 18.6 mM sodium citrate and 134 mM KCl at 37 °C. The suspended cells were then centrifuged at 400 \times *g* for 1 min, washed in 10 ml of PBS (this and all subsequent steps utilized ice-cold PBS at pH 8.0), and suspended in 4–5 ml of PBS. One-ml aliquots were transferred to 1.5-ml Eppendorf tubes, centrifuged at 1000 \times *g* for 5 s, washed again with 1 ml of PBS, and resuspended in 0.9 ml of PBS. One hundred μ l of freshly prepared EZ-Link Sulfo-NHS-LCLC-Biotin (25 mM in PBS) was then added to each tube. Tubes were incubated on ice for 2 h with mixing by inversion every 10 min, and then the biotinylation reaction was stopped by the addition of 110 μ l of 1 M glycine in PBS. Five min later, the cells were washed three times with 1 ml of PBS and solubilized in 0.6 ml of PBS containing 0.3% Triton X-100. After a 30-min incubation on ice, particulate matter was removed by centrifuging at 2000 \times *g* for 10 min. A 300- μ l aliquot of the supernatant was combined with 100 μ l of immobilized Streptavidin beads (a 50:50 slurry in PBS) and incubated with continuous mixing for 2 h at 4 °C. The material remaining in the supernatant after removal of the beads (*i.e.* the unbound material) was subsequently compared with the material in the supernatant before the addition of the beads by Western blotting (see "Results"). In control experiments (not shown), we have confirmed by Western blotting with horseradish peroxidase-conjugated avidin (see below) that this treatment with immobilized Streptavidin beads is sufficient to remove virtually all biotinylated proteins from the solubilized cell supernatant.

Co-immunoprecipitation Assay—Co-immunoprecipitation of NKCC1 or the mutant AAAA (see "Results") with HA-nttNKCC1 was carried out essentially as previously described (15). Briefly, HEK-293 cells in 6-cm dishes were transiently transfected with full-length NKCC1 (or AAAA) and HA-nttNKCC1 at a plasmid ratio of 1:8 (3 μ g of total DNA). Two days later, the

cells were washed with PBS, solubilized in PBS containing 0.3% Triton X-100, and immunoprecipitated with the antibody α -wNT(r), which had been pre-conjugated to Protein G beads (Pierce). After washing, the beads were eluted in 25 μ l of PBS containing 0.3% Triton X-100 plus 0.1% SDS for 30 min on ice. The beads were then centrifuged, and the eluate was removed and mixed with 6 μ l of 5 \times SDS-PAGE sample buffer. The material remaining on the beads was also extracted by adding 30 μ l of SDS-PAGE sample buffer to the pelleted beads. Equal volumes of each of these samples were probed by Western blotting (see below).

SDS-PAGE and Western Blotting—SDS-polyacrylamide gel electrophoresis and Western blotting were carried out as previously described (15). The primary and secondary antibodies used to detect NKCC1 and its C-terminal mutants in Figs. 2–6 were α -wCT(r), used at a dilution of 1:5000, and horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce), used at a dilution of 1:10,000, respectively. Blots from co-immunoprecipitations of HA-nttNKCC1 with NKCC1 or AAAA were probed with the peroxidase-conjugated rat anti-HA monoclonal 3F10 (Roche Applied Science) at a dilution of 1:1000 to detect HA-nttNKCC1 (Fig. 8) or with α -wNT(r) at a dilution of 1:5000 followed by Trueblue horseradish peroxidase-conjugated anti-rabbit IgG (eBioscience) at a dilution of 1:5000 to detect NKCC1 or AAAA (not shown). Detection of horseradish peroxidase was carried out using the ECL kit from Amersham Biosciences according to the manufacturer's directions. Blots were analyzed by densitometry.

Detection of biotin-labeled proteins by Western blotting was carried out as described above except that horseradish peroxidase-conjugated Immunopure Avidin (Pierce), used at a dilution of 1:1000, replaced the primary antibody, and no secondary antibody was used.

Immunofluorescent Staining and Confocal Microscopy—Immunofluorescent staining was performed as follows. Transiently transfected HEK-293 cells, grown on poly-L-lysine-coated circular glass coverslips, were rinsed three times with PBS (pH 7.4) and fixed with 4% paraformaldehyde in PBS for 30 min. The cells were then rinsed three times with PBS, incubated with 100 mM glycine in PBS for 20 min, permeabilized with methanol at -20°C for 5 min, and incubated in a mixture of 5% donkey serum and 0.5% bovine serum albumin in PBS for 20 min. Next, the cells were incubated with the antibody α -wCT(r) (diluted 1:3000) for 1 h and then with an Alexa Fluor 594-conjugated anti-rabbit secondary antibody (diluted 1:200; Invitrogen) for 30 min. After three additional washes in PBS (3 min each), the cells were treated with the SelectFX Alexa Fluor 488 endoplasmic reticulum labeling kit (Invitrogen) according to the manufacturer's instructions. Finally, the cells were washed again three times with PBS before being mounted onto a slide using Vectashield (Vector, Burlingame, CA) containing 4',6-diamidino-2-phenylindole. Fluorescence images were taken using a confocal laser-scanning microscope, Leica TCS-SP2, attached to an upright Leica DM-RE7 microscope (Leica, Wetzlar, Germany).

^{86}Rb Influx Assays—The ^{86}Rb influx experiments were performed as described previously (32).

A

rat NKCC1	DLPP	ILLV	RGNHQSVLTFYS.
rat NKCC2	NLPP	VLVV	RGNHKNVLTFFYS.
rat KCC1	GLER	VLVV	RGGGREVITIIYS.
rat KCC2	QLDR	VMLV	RGGGREVITIIYS.
rat KCC3	GLER	VLVV	RGGGSEVITIIYS.
rat KCC4	GLNR	VLVV	RGGGREVITIIYS.
shark NKCC1	DLPP	ILLV	RGNHQSVLTFYS.
shark NKCC2	NLPP	VIMI	RGNQKNVLTFFYS.
rat NCC	LSPV	VILLI	RGNQENVLTFYCQ
flounder NCC	LRPP	VLVV	RGNQENVLTFYCQ
Crab	DMPP	FLLI	RGNQTSVLTFFYS.
Sea urchin	GLDR	VLMV	RGGGREVITIIYS.
Fly_a	DMPP	FLFV	RGNQTSVLTFFYS.
Fly_b	GLEK	VLMV	RGGGREVITIIYS.
C_el_a	GLDR	VLVV	RGTGAEVVITIIYS.
C_el_b	NLPP	VLVV	RGNQSVLTFYS.
pattern	xxxx	hhhh	RGxxxxVhThYx

B

NKCC1 Mutants

	Name	C-terminal sequence
	WT	...LPP . ILL . V . RGNH . QSVL . TFYS
Group I		
1	IALV	...LPP . IAL . V . RGNH . QSVL . TFYS
2	ILAV	...LPP . ILA . V . RGNH . QSVL . TFYS
3	IAAV	...LPP . IAA . V . RGNH . QSVL . TFYS
4	ALLA	...LPP . ALL . A . RGNH . QSVL . TFYS
5	ALAA	...LPP . ALA . A . RGNH . QSVL . TFYS
6	AAAA	...LPP . AAA . A . RGNH . QSVL . TFYS
7	LVIL	...LPP . LVI . L . RGNH . QSVL . TFYS
8	ILLLV	...LPP . ILLLV . RGNH . QSVL . TFYS
9	Tr	...LPP
Group II		
10	RG/GR	...LPP . ILL . V . GRNH . QSVL . TFYS
11	V/M	...LPP . ILL . V . RGNH . QSML . TFYS
12	T/A	...LPP . ILL . V . RGNH . QSVL . AFYS
13	Y/A	...LPP . ILL . V . RGNH . QSVL . TFAS
Group III		
14	A^I	...LPPAILL . V . RGNH . QSVL . TFYS
15	A^R	...LPP . ILL . VARGNH . QSVL . TFYS
16	A^Q	...LPP . ILL . V . RGNHAQSVL . TFYS
17	A^T	...LPP . ILL . V . RGNH . QSVLTFYS

FIGURE 1. Slc12 C-terminal sequences and associated NKCC1 mutants. A, alignment of the C-terminal amino acids of rat NKCC1 (amino acids 1184–1203) with those of other representative members of the Slc12 family. Conserved residues and the “hydrophobic tetrad” (hhhh) (ILLV in NKCC1) are shaded in gray. See “Results” for additional details. The accession numbers for the sequences illustrated are rat NKCC1 (NP_113986), rat NKCC2 (NP_062007), rat KCC1 (NP_062102), rat KCC2 (NP_599190), rat KCC3 (XP_001066756), rat KCC4 (XP_001071999), shark NKCC1 (P55013), shark NKCC2 (AAM74966), rat NCC (NP_062218), flounder NCC (AAL26926), crab (AAF05702), sea urchin (XP_783625), Fly_a (*Drosophila*; NP_648572), Fly_b (*Drosophila*; NP_726378), C_el_a (*C. elegans*; NP_500910), C_el_b (*C. elegans*; NP_001076724). B, NKCC1 mutants studied in the paper. Mutated amino acids are shaded in gray. See “Results” for details.

Data Presentation—All experiments were carried out three or more times with similar results. All quantitative results shown are means \pm S.E. from three or more independent experiments. p values \leq 0.05 (Student's t test) were taken to represent statically significant differences.

RESULTS AND DISCUSSION

Conserved C-terminal Sequence in Slc12 Family Members

In Fig. 1A, we show the C-terminal residues of various Slc12 gene family members, illustrating that a number of these proteins from diverse species have a similar sequence pattern at their C-terminal end, namely xxxhhhhRGxxxxVhThYx.

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This pattern is conserved in all vertebrate NKCCs, NCCs, and KCCs sequenced to date. Since all of these latter transporters are known to be plasma membrane proteins and since, as already discussed, the C termini of a number of integral membrane proteins have been shown to play important roles in their intracellular folding and/or trafficking, we wondered whether the conserved features of this pattern might play a similar role for these Slc12 proteins. To explore this possibility, we generated a number of C-terminal (rat) NKCC1 mutants. These mutants can be broken down into three general groups (Fig. 1B).

Group I, Mutations of the Hydrophobic Tetrad ILLV—Here we mutated 1–4 of the amino acids in the hydrophobic tetrad ILLV to alanine (mutants 1–6), scrambled their order in the NKCC1 sequence (ILLV to LVIL; mutant 7), added an additional hydrophobic amino acid (ILLV to ILLLV; mutant 8), or truncated the NKCC1 sequence at Pro¹¹⁸⁷ immediately before the hydrophobic tetrad (mutant 9).

Group II, Mutations of the Highly Conserved Amino Acids Downstream of the ILLV Hydrophobic Tetrad—Mutations were generated by switching amino acid positions (Arg¹¹⁹² and Gly¹¹⁹³; mutant 10) or by replacement with methionine (mutant 11) or alanine (mutants 12 and 13).

Group III, Insertion Mutants—Here we inserted an extra alanine at various positions in the NKCC1 C-terminal sequence (mutants 14–17). Our rationale here was to attempt to disrupt potentially important sequence patterns by changing the relative spacing between residues.

Expression Levels and Complex Glycosylation of NKCC1 Mutants

NKCC1 contains two glycosylation consensus sites in the extracellular loop between its seventh and eighth membrane-spanning segments. In cells, NKCC1 is first core-glycosylated at one or both of these sites as it is synthesized in the ER and then subsequently complex glycosylated after it reaches the Golgi apparatus. When wild-type NKCC1 is exogenously expressed in HEK-293 cells, both the core and complex glycosylated forms are typically observed, the latter being the more abundant (15). Fig. 2A is a Western blot of wild-type (WT) NKCC1 and the IAAV mutant (Fig. 1B), illustrating the presence of the core and complex (*com*) glycosylated proteins. In Fig. 2B, we show the effect of *N*-glycosidase F on wild-type NKCC1 and on the AAAA mutant. This experiment illustrates that both the core and complex glycosylated bands are shifted downward by *N*-glycosidase F treatment, the former by ~5 kDa and the latter by ~40 kDa, confirming that both of these bands represent glycosylated forms of the protein. Note also that the AAAA mutant is predominantly expressed in the core glycosylated form (as quantitated in Fig. 2D; see below).

In Fig. 2, C and D, we illustrate the effects of our various mutations of the NKCC1 C terminus on protein expression levels (measured relative to wild-type NKCC1) and complex glycosylation (measured relative to core glycosylation), respectively. Somewhat surprisingly, the glycosylation patterns of all of the mutants in Groups II and III are not significantly different from wild-type NKCC1, and only two of these mutants (Y/A and A^I) show a decrease in protein expression levels (~40%

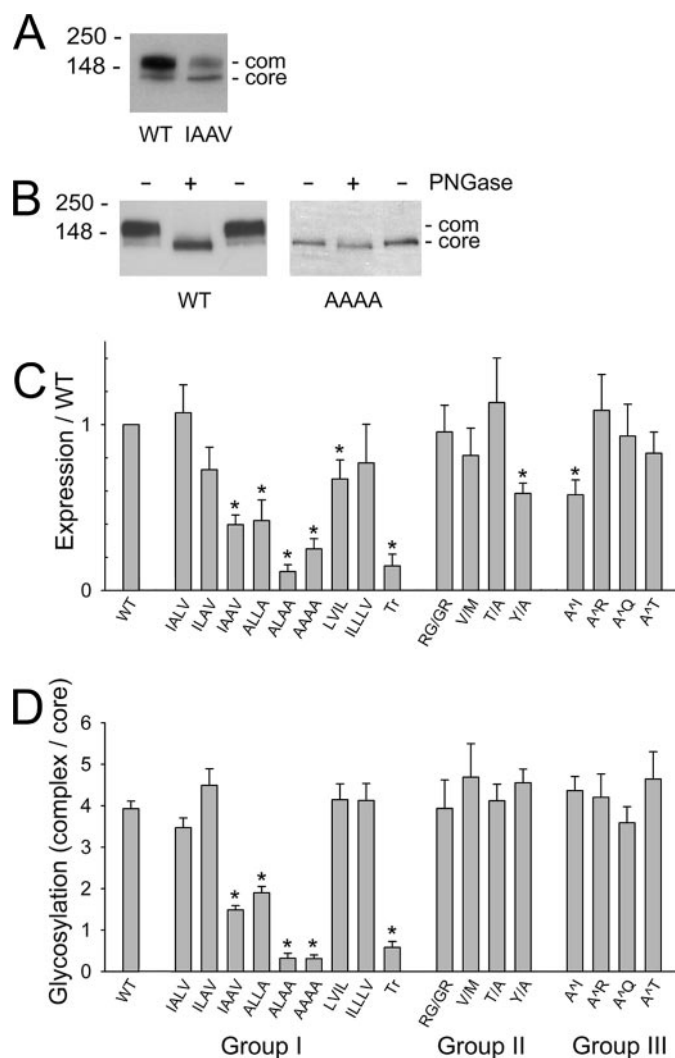


FIGURE 2. Protein expression and complex glycosylation levels of wild-type NKCC1 and C-terminal mutants. A, Western blot of crude membrane fractions prepared from HEK-293 cells transiently transfected with WT NKCC1 or the IAAV mutant (see "Experimental Procedures" for details). The positions of the core and complex (*com*) glycosylated proteins are indicated. B, Western blot of crude membrane fractions from HEK-293 cells transiently transfected with WT NKCC1 or the AAAA mutant. Membranes were treated with (+) or without (–) *N*-glycosidase F (*PNGase*) as indicated (see "Experimental Procedures" for details). C, protein levels of the various C-terminal NKCC1 mutants transiently expressed in HEK-293 cells, measured relative to WT NKCC1 expression levels. In additional studies (not shown), we have established that most of the reactivity of the antibody α -wCT(r) used in these experiments is directed against the sequence between amino acids 913–998 and that the mutations studied here (after amino acid 1187) have no detectable effect its reaction with NKCC1. D, ratio of complex to core glycosylated protein levels of WT NKCC1 and the C-terminal NKCC1 mutants. Results in C and D are means \pm S.E. from four or more independent determinations.

relative to wild type). Thus, all of these mutated proteins appear to be relatively well tolerated by the HEK-293 cells and properly processed and trafficked from the ER to the Golgi. Interestingly, although the complex glycosylation pattern of our V/M mutant does not differ from wild-type NKCC1, Sabath *et al.* (8) found that the corresponding NCC mutant, V995M, which has been associated with Gitelman's disease, exhibited decreased complex glycosylation relative to wild-type NCC.

In contrast to the mutants in Groups II and III, a number of the mutations of the hydrophobic tetrad ILLV (Group I) show

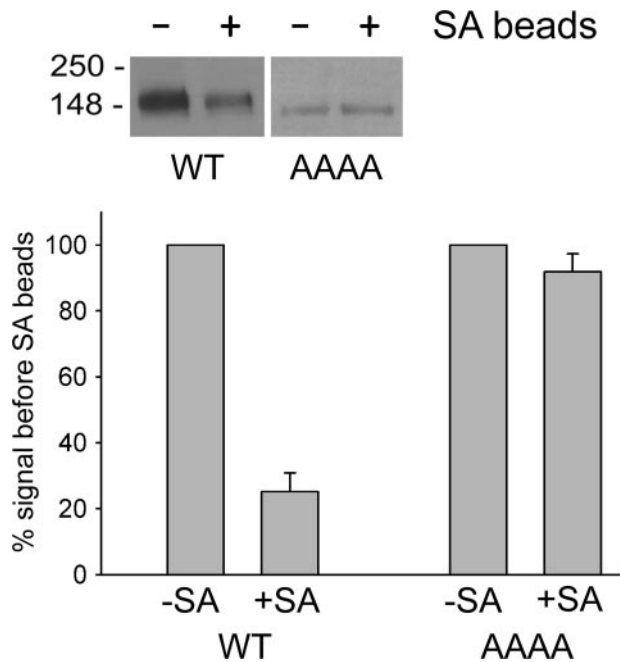


FIGURE 3. Cell surface expression of wild-type NKCC1 and the AAAA mutant. HEK-293 cells were transiently transfected with WT NKCC1 or the AAAA mutant and then incubated with EZ-Link Sulfo-NHS-LCLC-Biotin. The cells were then solubilized, and biotinylated proteins were precipitated on streptavidin (SA) beads (see "Experimental Procedures" for details). Aliquots of the cell lysate before (–) and after (+) streptavidin precipitation were probed by Western blotting (*inset*), and the amount of WT NKCC1 or AAAA mutant was quantitated in each case. The *bar graph* shows the amount of each protein found in the lysate before (–SA) and after (+SA) precipitation on the beads. Results have been normalized to the signal observed before the addition of the streptavidin beads. Means \pm S.E. from three independent experiments are shown.

both significantly reduced protein and complex glycosylation levels compared with wild-type NKCC1. In particular, mutations of two of these hydrophobic amino acids to alanine (mutants IAAV and ALLA) reduced both expression and complex glycosylation levels by $>50\%$. Mutations of three or four of these amino acids to alanine (mutants ALAA and AAAA) resulted in even larger reductions in expression and complex glycosylation. Similar effects were seen by completely truncating the C terminus of the protein upstream of the hydrophobic tetrad (mutant Tr). On the other hand, mutation of one hydrophobic amino acid to alanine (mutants IALV and ILAV), scrambling of the hydrophobic tetrad (mutant LVIL), or insertion of an additional hydrophobic amino acid (mutant ILLV) had little or no significant effect on expression or complex glycosylation levels.

Localization and Structural State of the AAAA Mutant

The above data suggest that mutation of two or more of the amino acids in the hydrophobic tetrad ILLV to alanine results in defective processing of NKCC1 from the ER to the Golgi and consequently in reduced complex glycosylation of the protein. To investigate this effect in more detail, we carried out several experiments designed to characterize the localization and structural state of the AAAA mutant. In the experiment shown in Fig. 3, we biotinylated the extracellular surface of intact HEK-293 cells transfected with either wild-type NKCC1 or the AAAA mutant and then solubilized the cells and precipitated

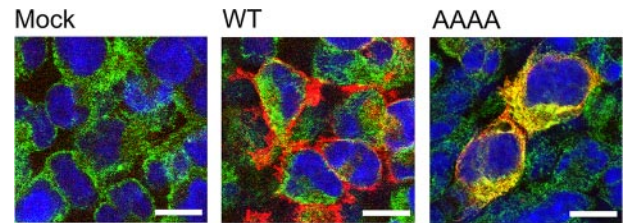


FIGURE 4. Immunofluorescent localization of wild-type NKCC1 and the AAAA mutant in HEK-293 cells. HEK-293 cells were transiently transfected with empty vector (pBK⁻; *Mock*), WT NKCC1, or the AAAA mutant and then fixed, permeabilized, and stained for NKCC1 and AAAA (*red*), the ER marker protein-disulfide isomerase (*green*), and nuclei (*blue*); see "Experimental Procedures" for details. The *white bars* represent 10 μ m. Note that in these experiments, the NKCC1 antibody shows no detectable signal for the endogenous (human) NKCC1 in the HEK-293 cells. This is because the antibody used is relatively selective for the rat protein (15).

biotinylated proteins with streptavidin (SA) beads. The *bars* in the figure illustrate the amount of wild-type NKCC1 and AAAA mutant in the solubilized cell lysate before (–) and after (+) the removal of biotinylated proteins on the beads. Whereas $\sim 75\%$ of the wild-type NKCC1 is bound by the beads and was therefore exposed and biotinylated on the cell surface, no significant binding of AAAA to the beads was observed. Thus, the AAAA mutant apparently fails to reach the plasma membrane.

In Fig. 4, we compare the cellular localization of wild-type NKCC1 and the AAAA mutant immunohistochemically. From *left to right*, the *three panels* in Fig. 4 show HEK-293 cells transiently transfected with empty vector (pBK⁻; *Mock*), wild-type NKCC1, or the AAAA mutant, respectively. The cells have been fixed, permeabilized, and stained for NKCC1 or AAAA (*red*), the ER marker protein-disulfide isomerase (*green*), and nuclei (*blue*). In cells transfected with wild-type NKCC1 (*middle panel*), NKCC1 staining is mainly localized to the periphery of the cells in a region that is distinct from the ER marker. Thus, this result is consistent with that of Fig. 3, which showed that most of the wild-type protein is in the plasma membrane. On the other hand, virtually all of the AAAA mutant staining (*right panel*) overlaps with the ER marker. Taken together, the results of Figs. 2–4 provide strong evidence that the AAAA mutant is mainly localized to the ER and does not reach the Golgi apparatus or the plasma membrane.

Previous studies from our laboratory have demonstrated that NKCC1 exists as a homodimer in the cell membrane (15, 29). These dimers are stable after mild membrane solubilization and can be cross-linked using the amino cross-linking reagent DTSSP. This effect is illustrated in the *left-hand panel* of Fig. 5. Here crude membranes from HEK-293 transfected with wild-type NKCC1 were solubilized in 0.3% Triton X-100 and exposed to DTSSP. Following DTSSP treatment, we see that almost all of the wild-type NKCC1 immunoreactivity migrates at a molecular weight that is twice its monomeric size (15, 29). The *two remaining panels* of Fig. 5 show the results of a similar experiment carried out with crude membranes prepared from cells transfected with the AAAA mutant; the *middle panel* is a short exposure, and the *right-hand panel* is a long exposure of the same Western blot. These results show that almost all of the AAAA immunoreactivity is found near the top of the gel, indicating that most of the AAAA protein is in a highly aggregated state.

A Conserved Hydrophobic Tetrad in NKCC1

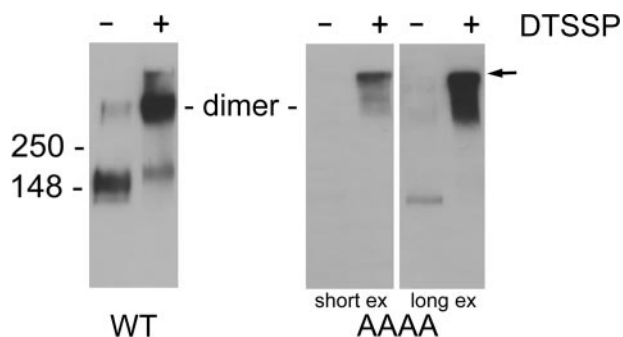


FIGURE 5. Cross-linking of wild-type NKCC1 and the AAAA mutant by DTSSP. Crude membrane fractions from HEK-293 cells transfected with wild-type NKCC1 (WT) or the AAAA mutant were solubilized in 0.3% Triton X-100, treated with cross-linker DTSSP, and analyzed by Western blotting (see "Experimental Procedures"). The middle and right-hand panels show short and long exposures, respectively, of the same blot. The arrow indicates the top of the gel.

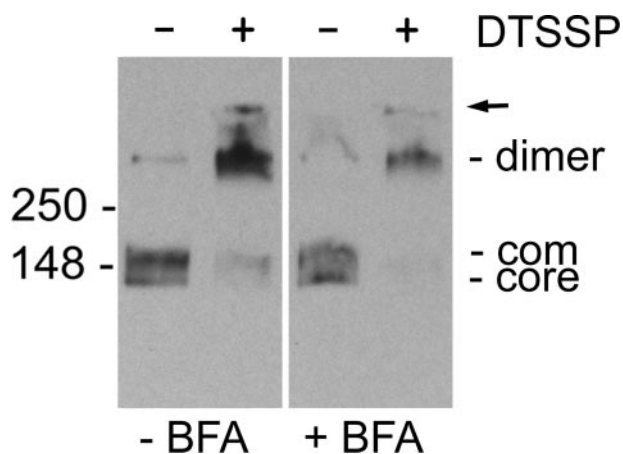


FIGURE 6. Effect of brefeldin A treatment on NKCC1 dimerization. HEK-293 cells transiently transfected with NKCC1 were treated with (+BFA) or without (-BFA) brefeldin A (5 μ g/ml) for 2 h in culture medium at 37 °C. Crude membranes were then harvested from the cells and treated with (+) or without (-) DTSSP as described under "Experimental Procedures." The positions of the core and complex (com) glycosylated proteins are indicated. The arrow indicates the top of the gel.

Effect of Disruption of ER to Golgi Trafficking on Wild-type NKCC1

The aggregation of the AAAA protein illustrated in Fig. 5 could be a cause or an effect of its retention in the ER. For example, if the hydrophobic tetrad were a trafficking signal, its mutation might lead to retention of NKCC1 in the ER, followed by its aggregation. To explore the possibility that retention of NKCC1-like proteins in the ER might lead to their aggregation, we treated HEK-293 cells transfected with wild-type NKCC1 with brefeldin A, a compound that blocks protein trafficking from the ER to the Golgi (33). In Fig. 6, we compare the effects of DTSSP on wild-type NKCC1 in membranes from cells treated with (+BFA) or without (-BFA) brefeldin A. In cells treated with brefeldin A, we see that complex glycosylation of NKCC1 is reduced, consistent with the effect of this drug on disrupting exit from the ER. However, cross-linking of NKCC1 from brefeldin A-treated cells demonstrates that this protein is mainly found in dimers, as seen in untreated cells. In particular, there is no evidence for the appearance of high molecular weight aggregates like those seen for AAAA after exposure to

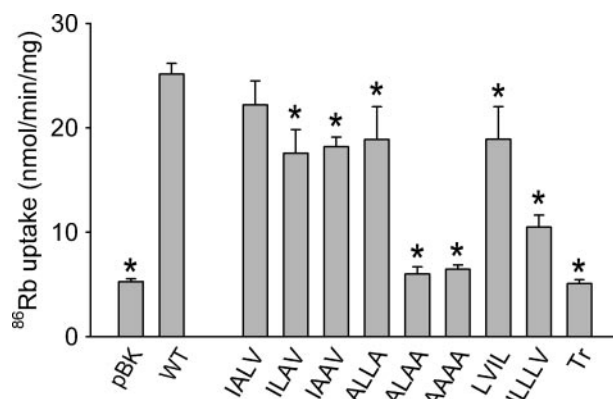


FIGURE 7. Bumetanide-sensitive ⁸⁶Rb fluxes via NKCC1 mutants. ⁸⁶Rb fluxes were measured as previously described (32) in HEK-293 cells transiently transfected with the NKCC1 mutants indicated. Flux measured in the presence of 250 μ M bumetanide was subtracted from that measured in its absence to obtain the bumetanide-sensitive (*i.e.* NKCC-specific (32)) component of flux illustrated. Values are means \pm S.E. from four or more independent determinations.

DTSSP (Fig. 5), consistent with the hypothesis that retention in the ER does not itself lead to NKCC1 aggregation.

Function of NKCC1 Mutants

Fig. 7 shows the bumetanide-sensitive (*i.e.* NKCC-specific (32)) component of ⁸⁶Rb influx in HEK-293 cells transiently transfected with the NKCC1 mutants in Group I of Fig. 1B (*viz.* mutations of the hydrophobic tetrad ILLV). Transfection with NKCC1 itself yields a ~5-fold stimulation of ⁸⁶Rb uptake over the basal level observed with the empty vector pBK⁻; this basal bumetanide-sensitive flux is known to be due to the endogenous (human) NKCC1 found in the HEK-293 cells (32). Flux via the mutant AAAA, which shows minimal trafficking to the cell surface (Fig. 3), is similar to basal levels, as are fluxes via the mutants ALAA and Tr. All of the remaining mutants show at least some level of functional activity. Generally speaking, mutations that lead to decreased protein expression also lead to decreased flux of ⁸⁶Rb. However, closer inspection of the data reveals a somewhat more complex pattern. For example, the mutants ILAV, LVIL, and ILLLV have significantly higher levels of protein expression and complex glycosylation than IAAV ($p < 0.04$ and $p < 0.0001$, respectively, in all cases), but fluxes via IAAV are comparable with those via ILAV and LVIL and significantly higher than those via ILLLV. Thus, these data suggest that the hydrophobic tetrad ILLV may also be involved in the functional properties of NKCC1. Further examination of this possibility is beyond the scope of the present paper.

Co-immunoprecipitation of NKCC1 and AAAA with HA-nttNKCC1

Since AAAA-transfected HEK-293 cells exhibit bumetanide-sensitive ⁸⁶Rb fluxes comparable with mock-transfected cells (Fig. 7), the endogenous HEK-293 NKCC1 in these cells is presumably fully functional and properly processed and trafficked to the plasma membrane; in particular, the endogenous NKCC1 is expected to be present in dimers (15, 29). However, very little if any of the AAAA mutant reaches the plasma membrane (Figs. 3 and 4) or appears to be in a dimerized state (Fig. 5). Since recombinant rat WT NKCC1 is expected to dimerize

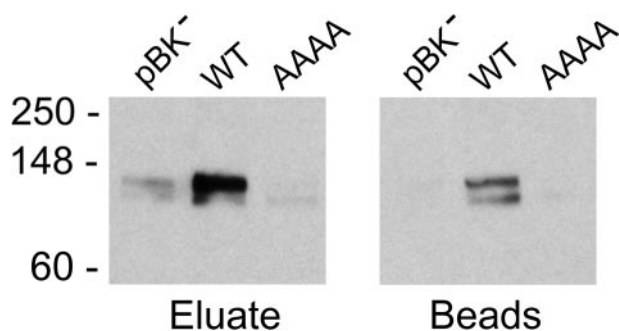


FIGURE 8. Co-immunoprecipitation assay: HA-nttNKCC1 is co-immunoprecipitated with NKCC1 but not with AAAA. HEK-293 cells were transiently transfected with HA-nttNKCC1 and either pBK⁻, WT NKCC1, or the AAAA mutant, as indicated. Two days later, the cells were solubilized and immunoprecipitated with Protein G beads to which the antibody α -wNT(r), raised against the N terminus of rat NKCC1, had been conjugated. The material eluted from the immunoprecipitate by 0.1% SDS (*Eluate*) as well the material remaining on the beads (*Beads*) was then probed by Western blotting with an anti-HA antibody to detect HA-nttNKCC1 (all procedures are described under "Experimental Procedures"). In additional assays in which we probed these blots with the antibody α -wNT(r) (not shown; see "Experimental Procedures"), we have established that the amount of AAAA immunoprecipitated in these experiments is 0.52 ± 0.02 ($n = 2$) times that of WT NKCC1 (although, as shown in Fig. 2A, AAAA is expressed at ~ 0.2 times the level of WT NKCC1, these experiments (15) are carried out with cell extract sufficient to oversaturate the Protein G-conjugated antibody when immunoprecipitating from WT NKCC1-expressing cells).

with the endogenous (human) protein as well as with itself (15), a simple interpretation of these results is that the AAAA mutant is unable to form dimers and thereby is without effect on the endogenous HEK-293 NKCC1. To test this hypothesis, we employed an NKCC1 co-immunoprecipitation assay previously developed in our laboratory (15). Briefly stated (see "Experimental Procedures"), we transiently co-expressed WT NKCC1 or the AAAA mutant along with HA-nttNKCC1, an HA-tagged, N-terminally truncated version of NKCC1. We then carried out an immunoprecipitation from solubilized cells with the antibody α -wNT(r) conjugated to Protein G beads. We have previously established that the cytosolic C terminus of NKCC1 is required for its dimerization and that truncation of its N terminus has no effect on dimer formation (15). Since the sequence against which the antibody α -wNT(r) was raised is missing from HA-nttNKCC1 (see "Experimental Procedures"), we expect this truncated protein to only appear in the α -wNT(r) immunoprecipitate as a component of NKCC1/HA-nttNKCC1 or AAAA/HA-nttNKCC1 dimer pairs.

The results of such an experiment are shown in Fig. 8. Here we have eluted the immunoprecipitated material (on Protein G beads) with 0.1% SDS, which, we have previously shown, disrupts most WT NKCC1 dimer pairs (15, 29), and performed Western blots using an anti-HA antibody on this eluate as well as on the material remaining on the eluted beads to detect HA-nttNKCC1. A strong HA-nttNKCC1 signal is seen in the eluate from cells cotransfected with WT NKCC1 and HA-nttNKCC1, indicating the dimerization of these proteins. A much weaker HA-nttNKCC1 signal is seen in the eluate from cells cotransfected with empty vector (pBK⁻) and HA-nttNKCC1, presumably reflecting dimerization of HA-nttNKCC1 with the endogenous NKCC1 of the HEK-293 cells, as previously suggested (15). However, no evidence for an increased signal relative to that seen with pBK⁻ is evident in either the eluate or the beads

from cells cotransfected with AAAA and HA-nttNKCC1. We quantitated these results by expressing the total amount of immunoprecipitated HA-nttNKCC1 (eluate plus beads) relative to that measured in cells transfected with pBK⁻. These analyses show that the amounts of immunoprecipitated HA-nttNKCC1 from cells expressing WT NKCC1 and AAAA were 4.8 ± 1.2 times and 1.2 ± 0.4 times, respectively, that found with pBK⁻. Although the amount of AAAA immunoprecipitated in these assays is $\sim 50\%$ of that of WT NKCC1 (see the legend to Fig. 8), these experiments nevertheless provide no evidence for an HA-nttNKCC1 signal representing dimerization or coimmunoprecipitation of HA-nttNKCC1 with AAAA. Thus, these results indicate that, in contrast to WT NKCC1, there is no detectable stable interaction between AAAA and HA-nttNKCC1, consistent with the above conjecture that AAAA is unable to form dimers.

Conclusions

Here we have identified a hydrophobic amino acid tetrad ILLV close to the C terminus of NKCC1 that is important for the proper intracellular processing of this protein and its appearance in the plasma membrane. This hydrophobic tetrad is a feature of a number of vertebrate and nonvertebrate Slc12 family members (Fig. 1A). Although other C-terminal amino acids are conserved in these same Slc12 proteins (Fig. 1A), our results indicate that these residues do not play a large role in the intracellular processing of NKCC1 (Fig. 2). Our experiments show that mutations of two of the amino acids in the hydrophobic tetrad to alanine (our mutants ALLA and IAAV) lead to a $>50\%$ reduction in complex glycosylation and expression levels of the mutant proteins relative to wild-type NKCC1, whereas additional mutations to alanine (our mutants ALAA and AAAA) lead to even more dramatic effects (Fig. 2). In experiments where we explored the fate of the AAAA mutant, we found that it failed to reach the cell surface (Fig. 3) and colocalized with an ER marker (Fig. 4). These results provide strong evidence that this mutant is trapped in the ER. When we investigated the structural state of AAAA, we found that this protein was predominantly in a highly aggregated state (Fig. 5).

Several observations indicate that mutation of the hydrophobic tetrad ILLV to AAAA leads to the misfolding and concomitant aggregation of this NKCC1 mutant and that this is the reason for its retention in the ER. First, it is well established that misfolded and/or aggregated proteins are typically retained in the ER (2, 3), consistent with the localization of AAAA in this compartment. Second, our experiments with brefeldin A demonstrate that preventing wild-type NKCC1 from exiting from the ER does not lead to its aggregation (Fig. 6); thus, the aggregation of AAAA does not appear to be a result of its retention in the ER but rather a cause. Finally, we find no evidence that AAAA forms stable dimers (Fig. 8). Taken together, these results indicate that the AAAA mutant misfolds soon after its synthesis, making it incapable of stable dimer formation and resulting in its aggregation and retention in the ER.

Our results do not address the nature of the physical role of the hydrophobic tetrad identified here in the folding and processing of the Slc12 proteins. We anticipate that structural studies beyond the scope of the present paper will be required to

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give a definitive understanding of its significance. Interestingly, the data presented here as well as comparison with other Slc12 sequences (Fig. 1A) suggest that it is conservation of residue hydrophobicity rather than residue identity that underlies the role of this amino acid motif.

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