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A Dominant Negative Mutant of the KCC1 K-Cl Cotransporter

BOTH N- AND C-TERMINAL CYTOPLASMIC DOMAINS ARE REQUIRED FOR K-Cl COTRANSPORT ACTIVITY*

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K-Cl cotransport regulates cell volume and chloride equilibrium potential. Inhibition of erythroid K-Cl cotransport has emerged as an important adjunct strategy for the treatment of sickle cell anemia. However, structure-function relationships among the polypeptide products of the four K-Cl cotransporter (KCC) genes are little understood. We have investigated the importance of the N- and C-terminal cytoplasmic domains of mouse KCC1 to its K-Cl cotransport function expressed in Xenopus oocytes. Truncation of as few as eight C-terminal amino acids (aa) abolished function despite continued polypeptide accumulation and surface expression. These C-terminal loss-of-function mutants lacked a dominant negative phenotype. Truncation of the N-terminal 46 aa diminished function. Removal of 89 or 117 aa (ΔN117) abolished function despite continued polypeptide accumulation and surface expression and exhibited dominant negative phenotypes that required the presence of the C-terminal cytoplasmic domain. The dominant negative loss-of-function mutant ΔN117 was co-immunoprecipitated with wild type KCC1 polypeptide, and its co-expression did not reduce wild type KCC1 at the oocyte surface. ΔN117 also exhibited dominant negative inhibition of human KCC1 and KCC3 and, with lower potency, mouse KCC4 and rat KCC2.

Secondary active transport of chloride across cell plasma membranes is achieved by ion symport and antiport mechanisms. The major chloride symporters are members of the phylogenetically ancient CCC family. The transmembrane region of CCC polypeptides, based on data from KCC1, probably spans the lipid bilayer 12 times. Transmembrane helices 2, 4, and 7 of NKCC1 have been implicated in ion binding by site-directed mutagenesis studies. The two initial reports on structure-function relationships of K-Cl cotransporters concern aspects of their C-terminal cytoplasmic tails. Tyr1087 of rat KCC2, close to KCC2’s C terminus, and the analogous residue in rabbit KCC1 are each required for hypotonic activation of ion transport activity in Xenopus oocytes. However, neither residue is required for delivery to the oocyte surface or for inhibition of hypotonically activated transport activity by serine-threonine phosphatase inhibitors. In addition, Laufer et al. have shown that removal from Myc-tagged rabbit KCC1 of most of the C-terminal cytoplasmic domain abolished activation by N-ethylmaleimide (NEM) in HEK-293 cells while apparently decreasing but not abolishing surface expression.

Inhibition of the potassium efflux pathways mediating cell shrinkage has proven an increasingly important approach to the therapy of sickle cell disease. The major potassium efflux pathways of the sickle erythrocyte are K-Cl cotransport and the IK1 KCa channel (20). Although high potency inhibitors of the IK1 erythroid KCa channel are available, clinically tolerated (21), and in continued development, high potency inhibitors of K-Cl cotransport have not been identified. KCC inhibitors could serve as an adjunct treatment of sickle cell disease, as shown to date with clinical trials of oral magnesium supple-
mentation (22). Such specific KCC inhibitors would also provide a useful experimental tool to test the role of K-Cl cotransport in cell function.

We have initiated structure-function studies with the 1085-aa mouse KCC1 K-Cl cotransporter. We show here that both the C-terminal cytoplasmic domain and the membrane-proximate portion of the N-terminal cytoplasmic domain are absolutely required for transport function in Xenopus oocytes. In addition, we show that removal of the entire N-terminal cytoplasmic domain from KCC1 confers a dominant negative phenotype that requires the presence of the C-terminal cytoplasmic domain. The dominant negative mutant polypeptide associates physically with the wild type KCC1 polypeptide and exhibits its dominant negative phenotype when co-expressed with other KCC gene products. This dominant negative mutant should provide useful information on the mechanism of regulation of KCC K-Cl cotransporters.

**EXPERIMENTAL PROCEDURES**

**Polymerease Chain Reaction**—500 ng of plasmid pXmKCC1 encoding wild type mKCC1 cDNA (7) was subjected to hot start PCR in a total reaction volume of 50 μl, using the Expand High Fidelity PCR System (Roche Molecular Biochemicals) in the supplier's recommended buffer. PCR mixes lacking only primers were preheated at 82 °C for 1 min, after which appropriate primers were injected into the mixture by touch on mineral oil. The complete reaction mixtures were denatured for 5 min at 95 °C and then cycled through these conditions: denaturation for 45 s at 94 °C, annealing for 2 min at 60 °C, and elongation for 2 min at 72 °C. Final extension of 10 min at 72 °C was terminated by rapid cooling to 4 °C after 8–10 cycles. PCR products were analyzed in 1% agarose gels, purified from gel with the QIAquick Gel Extraction Kit (Qiagen), and cloned into the T-vector pCR2 (Invitrogen). DNA sequence integrity of the cloned PCR amplification products was verified with an ABI 373 DNA sequencer. DNA sequence analysis was carried out with the GCG suite of programs (University of Wisconsin Genetics Computing Group).

**Construction of N-terminally Truncated (ΔN) KCC1 Mutants**—Each forward oligodeoxynucleotide primer encoded the native Kozak initiation sequence of mouse KCC1 (mKCC1) followed by the desired amino acids of the KCC1 N-terminal cytoplasmic tail. The forward primers used were initi1 ΔN46F (5'-CAAGAAGCCGGCGATGGCCCTTCTT-

**Construction of C-terminally Truncated (ΔC) KCC1 Mutants**—For construction of mKCC1 C-terminal truncation mutant ΔC660, 660 bp of reverse primer KCC1.ΔC660R (5'-TGATGTAACATTCGT-CCGAGGACAT-

**Transcription and Translation of Wild Type and Truncated mKCC1 cRNAs and Polypeptides**—Capped cRNAs were transcribed with T7 polymerase from XhoI-linearized template (MEGAscript, Ambion) and purified (RNasey kit, Qiagen). In vitro transcription of polypeptide labeled with [35S]-Label (ICN) was performed with the nuclease-treated rabbit reticulocyte lysate system (Promega) in the presence of canine pancreatic microsomal membranes (Promega) in a 25-μl reaction volume, per manufacturer's protocol. Alternatively, coupled transcription-translation (Tnt; Promega) was used. The reaction was terminated, and microsomes were solubilized by the addition of 80 μl of immunoprecipitation (IP) buffer: 150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, and 2 mg/ml bovine serum albumin. SDS-polyacrylamide gel electrophoresis fluorography was as described (7).

**cRNA Expression in Xenopus Oocytes**—Female Xenopus anesthetized with 0.17% Tricaine were subjected to partial ovariectomy. Excised, minced ovarian segments were incubated for 1 h with gentle shaking at room temperature in 2 mg/ml type A collagenase (Roche Molecular Biochemicals) in ND 96, pH 7.4, containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 5 mM Hepes, and 2.5 mM sodium pyruvate, supplemented with 5 mg/100 ml gentamicin. Washed, manually defolliculated oocytes of stage V–VI were maintained at 19 °C. On the same day or the next day, oocytes were microinjected (Drummond manual microinjector) with 50 nl of water or solution containing 12.5 ng or the indicated quantity of cRNA. Oocytes were maintained in ND-96 plus gentamicin at 19 °C for 2–10 days, with daily change of medium (24), until used for ion transport assays.

**56Rb**-**Influx Assay**—mKCC1 function was assessed by measurement of 56Rb uptake into oocytes 4–10 days post-cRNA injection. Groups of 5–15 oocytes were preincubated in influx medium lacking isotope. The 60-min influx period was initiated by transfer of oocytes into 150 μl of...
medium containing 2.5–5 mCi of $^{86}$RbCl. $^{86}$Rb influx was terminated by five 50-ml washes at 4 °C in chloride-free medium lacking isotope. $^{86}$Rb content of individual oocytes was determined in a y counter (Cobra AutoGamma, Packard). Aliquots of influx medium were counted for determination of specific activity.

All media contained 5 μM butanedione and 200 μM ouabain to inhibit endogenous NKCC and Na,K-ATPase activities, respectively. Isotonic media were ND-96 or NMDG-96 (in which N-methyl-D-glucamine chloride substituted for sodium); results with either medium were indistinguishable. Chloride-free media contained gluconate salts. Hypotonic medium was NMDG-72. NEM was used at 1 mM.

Immunoblot Analysis of mKCC1 Polypeptide in Xenopus Oocytes—Oocytes were injected with 1 μl of cRNA or water. 2–4 days later, groups of 10 oocytes were manually homogenized at 4 °C in oocyte lysis buffer containing 500 mM NaCl (10 μl/oocyte) in a microcentrifuge tube with a fitted Teflon pestle ( Kontes) and then subjected to 30 min of vigorous shaking and 10 min of centrifugation in a microcentrifuge at 4 °C. Clarified supernatants were brought to 250 mM NaCl and then precleared with 5% normal rabbit serum. Precleared supernatants were incubated overnight at 4 °C with affinity-purified rabbit polyclonal antibodies to KCC1 N-terminal aa 1–14 and to mKCC1 C-terminal aa 1074–1085 and visualized by enhanced chemiluminescence (7).

Immunoprecipitation of mKCC1 Polypeptides from Xenopus Oocytes—Oocytes were injected with 10 μl of [35S]methionine (1 mCi/ml) and with cRNA or water. 2–4 days later, groups of 10 oocytes were manually homogenized at 4 °C in oocyte lysis buffer containing 500 mM NaCl (10 μl/oocyte) in a microcentrifuge tube with a fitted Teflon pestle ( Kontes) and then subjected to 30 min of vigorous shaking and 10 min of centrifugation in a microcentrifuge at 4 °C. Clarified supernatants were brought to 250 mM NaCl and then precleared with 5% normal rabbit serum. Precleared supernatants were incubated overnight at 4 °C with affinity-purified anti-KCC1 antibodies (in the presence of 0.5% Tween 20) or anti-KCC1 C-terminal antibodies, and immune complexes were precipitated with protein A-agarose. Pellets were washed at 4 °C six times in 1 ml of lysis buffer containing 500 mM NaCl and six more times in 1 ml of buffer without NaCl and then analyzed by SDS-polyacrylamide gel electrophoresis fluorography (25).

The same immunoprecipitation protocols were followed using 1% Triton X-100 extracts of wild type KCC1 and Δc1077, preserved in oocyte lysis buffer containing 500 mM NaCl and washed three times in lysis buffer lacking NaCl and then mixed with SDS-loading buffer, electrophoresed on a 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and subjected to immunoblot analysis with affinity-purified antibodies to KCC1 N-terminal aa 1–14 and to mKCC1 C-terminal aa 1074–1085 and visualized by enhanced chemiluminescence (7).

Surface Biotinylation of KCC1 Polypeptides—All procedures except polyacrylamide gel electrophoresis were conducted at 4 °C. Oocytes were subjected to three 2-min washes in phosphate-buffered saline (PBS), pH 9.0, and then incubated for 30 min in the same buffer supplemented with 0.5 mM sulforaphane (4-S-biotinylmercaptosilane, Pierce) and 0.1 μg/ml of avidin-biotin complex (Vector). This solution was replaced with fresh solution, and incubation was continued for a second 30-min period. The biotinylated oocytes were washed and then quenched for 20 min in PBS containing 10 mM glycine. After three washes in ice-cold PBS, groups of five oocytes were homogenized in lysis buffer containing 500 mM NaCl, 20 mM Tris, pH 7.4, 1% Triton X-100, 0.1% SDS, and Complete® protease inhibitor. After a 30-min incubation, the lysates were cleared by centrifugation. The cleared lysates were incubated with lysis buffer-prewashed streptavidin-agarose beads (10 μl) for 1 h with end-over-end rotation. The beads were washed three times in ice-cold lysis buffer containing 500 mM NaCl and washed three times in lysis buffer lacking NaCl and then mixed with SDS-loading buffer, electrophoresed on a 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and subjected to immunoblot analysis.

Confocal Immunofluorescence Microscopy—Five days after cRNA or water injection, 6–10 oocytes in each group, along with uninjected controls, were fixed in 1 ml of PBS containing 3% paraformaldehyde for 4 h at room temperature. Fixed oocytes were extensively rinsed with PBS, exposed to 1% SDFs for 1–5 min, and then blocked for 1 h in PBS with 1% bovine serum albumin and 0.005% saponin. Overnight incubation with primary antibody was followed by several washes in PBS. After overnight incubation with secondary antibody and further washes in PBS, oocytes were dehydrated in methanol for 1 h and incubated overnight in BA-BB solution (23). 4–10 such oocytes expressing a single form or coexpressing two forms of KCC1 were aligned along a plexiglass groove and imaged with the Bio-Rad MRC-1024 laser-scanning confocal microscope. Images were acquired from at least two independent sets of cRNA injections for each KCC1 construct. Representative images of median intensity were compiled with Adobe Photoshop 5.0.

RESULTS

C-terminal Truncation of mKCC1 Leads to Loss of Function—mKCC1 expressed in Xenopus oocytes was stimulated an average of 5.9-fold by hypotonic swelling (Fig. 2A, p < 0.001; the mean stimulation of just the heterologous KCC1-mediated influx was 8.6-fold). Even in isotonic conditions, $^{86}$Rb influx was higher in oocytes expressing mKCC1 than in water-injected oocytes (p < 0.001). Our previous studies of mKCC1 in Xenopus oocytes (7) and other studies of rabbit (5, 19) and human KCC1 in 293 cells (6) and of native Xenopus oocyte K-Cl cotransporter (14) have shown that this activated $^{86}$Rb influx requires bath chloride and is inhibited by the serine-threonine phosphatase inhibitors okadaic acid and calyculin and by the diuretic diindenylalkanoic acid. Removal of only the eight C-terminal residues from the C-terminal cytoplasmic tail of mKCC1, as in Δc1077, preserved the low level of activity in isotonic medium (p = 0.008) but abolished stimulation of $^{86}$Rb uptake elicited by hypotonicity. More extensive truncation of the C-terminal cytoplasmic domain after residue 940, 805, 698, or 660 abolished activity (Fig. 2A).

![Fig. 2](http://www.jbc.org/) Dominant Negative Mutant of KCC1 K-Cl Cotransporter

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**Fig. 2.** mKCC1 C-terminal truncations lead to loss-of-function. A, $^{86}$Rb influx into oocytes expressing WT mKCC1 or the indicated Δc mutants, measured in isotonic (–) or hypotonic (+) medium. Values are means ± S.E. for n experiments, each evaluating 5–25 individual oocytes. For all Δc mutants, hypotonic and isotonic values are indistinguishable. B, $^{86}$Rb influx into oocytes expressing WT mKCC1 or the indicated Δc polypeptides, measured in isotonic medium in the absence (–) or presence of 1 mM NEM (+). Values are means ± S.E. for n experiments, each evaluating 5–20 individual oocytes. For all Δc mutants, NEM and control values are indistinguishable.
Treatment of oocytes with 1 mM NEM stimulated \(^{86}\text{Rb}^+\) influx 2.1-fold in oocytes expressing wild type mKCC1 (p = 0.011) This stimulation was abolished by removal of the C-terminal eight amino acids and by all of the more extensive C-terminal truncations tested (Fig. 2B).

These mutant mKCC1 \(\Delta_c\) polypeptides accumulated in \(Xenopus\) oocytes to levels lower than for WT KCC1; \(\Delta_c\)940 polypeptide failed to accumulate to any detectable level (Fig. 3, A and B). Confocal immunofluorescence microscopy with anti-mKCC1 anti-peptide antibody directed against either cytoplasmic N-terminal aa 1–14 (\(\alpha\)NT) or cytoplasmic C-terminal aa 1074–1085 residues of mKCC1 (\(\alpha\)CT) detected wild type mKCC1 at or near the cell surface (Fig. 3B). \(\alpha\)CT antibody staining was greatly reduced in oocytes expressing mKCC1 \(\Delta_c\)1077 and was lost in oocytes expressing other \(\Delta_c\) mutants. However, \(\alpha\)NT staining suggested that \(\Delta_c\)1077, \(\Delta_c\)805, and \(\Delta_c\)660 retained considerable expression at or near the oocyte surface despite their loss of transport activity (Fig. 3B). The absence of staining in oocytes injected with mKCC1 \(\Delta_c\)940 (Fig. 3B) correlated with the absence of polypeptide on immunoblot (Fig. 3A): mKCC1 \(\Delta_c\)698 did not accumulate detectably at the oocyte surface despite its modest accumulation within the oocyte.

Following construction and functional analysis of these engineered mKCC1 \(\Delta_c\) mutants, we found in the database a variant hKCC1 transcript, expressed sequence tag AI 799106. The presence of the variant transcript was confirmed in human RNA from 293T cells (by DNA sequence) and in placenta and T84 cells (by RT-PCR with two primer pairs). In this transcript, selective deletion of exon 18 (hKCC1 \(\Delta\)Ex18) encodes a polypeptide in which Q747 (the terminal codon of exon 17) is followed by 33 novel, exon 19-encoded, frameshifted amino acid residues before termination at position 780 (GenBank\textsuperscript{TM} number AY026038). As is true for the engineered mKCC1 \(\Delta_c\) polypeptides, this physiological hKCC1 \(\Delta_c\) variant exhibited no detectable transport function in \(Xenopus\) oocytes (not shown). However, no \(\Delta\)Ex18 form of mKCC1 mRNA was detected by RT-PCR in mouse brain, heart, kidney, ES cells, and MEL cells.

N-terminal Truncation of mKCC1 Leads to Loss of Function—The absence of the N-terminal 46 residues of mKCC1 (\(\Delta_c\)46 mKCC1) led to \(^{86}\text{Rb}^+\) influx indistinguishable from that of WT mKCC1 but greater than in water-injected controls (Fig. 4, \(p < 0.001\)). \(\Delta_c\)46 mKCC1 exhibited diminished hypotonic activation of \(^{86}\text{Rb}^+\) influx, 47% of wild type levels representing 3.3-fold stimulation of mKCC1-mediated influx (\(p = 0.004\)) compared with hypotonically treated wild type mKCC1-expressing or water-injected oocytes; \(p < 0.05\) for simple pairwise comparison with isotonic conditions, but \(>0.05\) when corrected for 71 such comparisons). More extensive N-terminal deletion of 89 (\(\Delta_c\)89) or 117 residues (\(\Delta_c\)117) completely abolished activation of \(^{86}\text{Rb}^+\) influx by hypotonicity (Fig. 4A). The 1.6-fold stimulation by NEM of \(^{86}\text{Rb}^+\) influx into oocytes expressing mKCC1 \(\Delta_c\)46 was not statistically significant. As true for hypotonic stimulation, NEM stimulation and basal activity were also abolished by more extensive N-terminal truncation of mKCC1 (Fig. 4B).

All \(\Delta_c\) mutant polypeptides accumulated to levels lower than wild type level (Fig. 5A). However, \(\Delta_c\) mutant polypeptides were present at or near the oocyte surface at levels similar to that of wild type mKCC1, as detected by confocal immunofluorescence microscopy with \(\alpha\)CT antibody (Fig. 5B). \(\alpha\)NT antibody failed to detect any N-terminally truncated mKCC1 polypeptide (Fig. 5B).
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The Loss-of-Function Mutant \(\Delta_{805}\) Is Not a Dominant Negative Mutant—We tested the hypothesis that among the mKCC1 loss-of-function mutants generated by progressive deletion of C-terminal and N-terminal cytoplasmic tails might be one or more that exhibit a dominant negative phenotype. Wild type and \(\Delta_{805}\) mKCC1 were coexpressed in Xenopus oocytes and tested for transport function and for surface expression. Fig. 6A shows that even when expressed in a 3-fold molar excess of cRNA, mKCC1 \(\Delta_{805}\) did not inhibit hypotonicity-activated \(^{86}\)Rb\(^+\) influx mediated by coexpressed wild type mKCC1. There was no statistical correlation between mole fraction of \(\Delta_{c605}\) mKCC1 and \(^{86}\)Rb\(^+\) influx (\(p = 0.10\)). Moreover, both mutant and wild type polypeptides were expressed at or near the cell surface, and mutant coexpression did not reduce the abundance of wild type mKCC1 at the oocyte periphery (Fig. 6B). Co-injection of oocytes with wild type mKCC1 and with any one of the mKCC1 mutants \(\Delta_{c1077}, \Delta_{c698}, \) or \(\Delta_{c660}\) at 1:1 cRNA ratios similarly failed to inhibit wild type mKCC1-mediated \(^{86}\)Rb\(^+\) uptake (not shown). We also tested the hypothesis that coexpression of \(\Delta_{89}\) and \(\Delta_{c117}\) mKCC1 mutants might complement rescue function. However, coexpression of mKCC1 \(\Delta_{c117}\) with mKCC1 \(\Delta_{805}\) did not rescue \(^{86}\)Rb\(^+\) uptake stimulated by hypotonicity (not shown).

The Loss-of-Function Mutant \(\Delta_{c117}\) Is a Potent Dominant Negative Mutant—Co-expression of the itself inactive mKCC1 mutant \(\Delta_{c117}\) with an equal cRNA amount of wild type mKCC1 suppressed \(^{86}\)Rb\(^+\) uptake by 80–90% (Figs. 7A and 8; \(p < 0.001\)). Co-expression of the inactive mKCC1 mutant \(\Delta_{c89}\) with an equal cRNA amount of wild type mKCC1 produced a 50% decrease in activity (not shown). Expression of wild type mKCC1 polypeptide at or near the oocyte surface was not diminished by coexpression of mKCC1 \(\Delta_{c117}\), itself present at or near the surface at WT levels (Fig. 7B). Fig. 7C shows that both WT KCC1 and \(\Delta_{c117}\) KCC1 were biotinylated at the oocyte surface in comparable quantities (lanes 3 and 7), although biotin-accessible surface mKCC1 polypeptide represented a very small proportion of total oocyte mKCC1 (compare lane 1 with lane 3 and lane 6 with lane 7). Moreover, abundance of neither total (lane 1) nor surface-biotinylated WT KCC1 (lane 3) was reduced by coexpression of \(\Delta_{c117}\) KCC1 (lanes 4 and 5). Thus, dominant negative suppression of WT KCC1 function by \(\Delta_{c117}\) KCC1 is not achieved by diminution of WT KCC1 expression or surface accumulation.

mKCC1 \(\Delta_{c117}\) significantly suppressed function of coexpressed wild type mKCC1 in a dose-dependent manner, even at mole fractions of \(\leq 0.1\). When expressed at mole fractions of \(\geq 0.5\), mKCC1 \(\Delta_{c117}\) completely suppressed function of wild type mKCC1 (Fig. 8; \(p < 0.001\)). Interestingly, the itself inactive compound truncation mutant, mKCC1 \(\Delta_{c117}/\Delta_{805}\), did not inhibit wild type mKCC1 function when coexpressed at a 1:1 cRNA ratio (not shown). Specificity of the dominant negative phenotype was also demonstrated by lack of suppression

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2 In the immunoblot presented in Fig. 7C, apparent \(M_\text{r}\) values differ for \(\Delta_{c117}\) mKCC1 in whole oocyte lysate (lanes 4 and 6) and in streptavidin precipitates from surface-biotinylated oocytes (lanes 5 and 7). This difference reflects the presence in whole oocyte lysate of abundant yolk platelet lipoproteins with \(M_\text{r}\) just above that of \(\Delta_{c117}\), which consequently is displaced lower in the gel. These lipoproteins are essentially absent from the surface-biotinylated protein preparations in the streptavidin precipitates.

3 Since the epitope for neither \(\alpha\)NT nor \(\alpha\)CT antibodies was present in the mKCC1 compound truncation mutant \(\Delta_{c117}/\Delta_{805}\), the absence of dominant negative phenotype exhibited by this mutant could reflect either a requirement for the C-terminal cytoplasmic tail or too little accumulation of this mutant polypeptide.
confocal immunofluorescence images show that the inactive $\Delta N_{117}$ mKCC1 or isotonic media (open bars) or closed bars: lanes 1, 4, 6, and 8). When expressed in Xenopus oocytes, the inactive $\Delta N_{117}$ mKCC1 and of $\Delta N_{117}$ mKCC1 in oocytes expressing these polypeptides individually or together. Immunoblots with $\alpha$NT (upper blot) and $\alpha$CT antibodies (lower blot) compare KCC1 content in 0.1 oocyte equivalent of total lysate (C, lanes 1, 2, 4, 6, and 8) with that in strepavidin precipitates from lysate of five oocytes previously treated with (−) or with (+) sulfo succinimidyl-6-(biotinamido)hexanoate. One of four similar experiments is shown.

of WT KCC1-mediated $^{86}$Rb$^+$ influx by unrelated polytopic membrane transport proteins.$^4$

$mKCC1$ $\Delta N_{117}$ Physically Associates with Wild Type $mKCC1$—The dominant negative functional phenotype of $\Delta N_{117}$ mKCC1, as well as its co-expression with wild type

$^4$ A second criterion for specificity was examined by co-expression of either of two loss-of-function mutants of an unrelated polytopic membrane protein, the Cl /HCO$_3$- exchanger eAE1. Neither mutant AE1 polypeptide itself increased $^{86}$Rb$^+$ influx into oocytes. The inactive human eAE1 mutant R760P (Band 3 Prague I), a cause of autosomal dominant hereditary spherocytosis, does not appear at the surface of the red blood cell or of the Xenopus oocyte and does not display a dominant negative anion exchanger phenotype (22). Similarly, AE1 Prague does not suppress mKCC1-mediated $^{86}$Rb$^+$ influx activity even when co-expressed at a 5-fold cRNA excess. In contrast, the mouse eAE1 mutant E699Q, inactive in the absence of sulfate, is expressed at the oocyte surface (23). As is true for hAE1 Prague, mouse eAE1 E699Q did not suppress mKCC1-mediated $^{86}$Rb$^+$ influx activity.
mKCC1 in immunoprecipitates from lysates of metabolically labeled oocytes (not shown).

ΔN117 mKCC1 Is a Potent Dominant Negative Suppressor of Co-expressed KCC3 and Less Potently Suppresses Co-expressed KCC4 and KCC2—The KCC gene family has two branches, one represented by the closely related KCC1 and KCC3 (~77% identical) and the other comprising KCC2 and KCC4 (each about 65% identical to KCC1) (4, 26). Since single cell types can express more than one KCC gene product, the utility of a dominant negative KCC1 construct to inhibit K-Cl cotransporter activity in intact cells will depend on its ability to inhibit K-Cl cotransport activity mediated by the polypeptide products of other KCC genes.

Fig. 10A shows that mKCC1 ΔN117 at very low injected cRNA levels suppressed hypotonically stimulated activity of co-expressed wild type hKCC3 as potently as that of wild type mKCC1. mKCC1 ΔN89 also potently suppressed hypotonically stimulated activity of coexpressed hKCC3 (not shown). Fig. 10B shows that hypotonically stimulated activity of co-expressed wild type mKCC4 also was suppressed by mKCC1 ΔN117, although less potently than were KCC1 and hKCC3. KCC4 activity was inhibited 67% at a 1:1 cRNA ratio and 84% at a wild type/mutant cRNA ratio of 1:2. The higher functional activity of mKCC4 in Xenopus oocytes has been noted previously (26). Fig. 10C shows that hypotonically stimulated activity of rKCC2 was also inhibited by co-expressed mKCC1 ΔN117 but less potently still than against KCC4. 1:1 and 1:3 wild type/mutant ratios of injected cRNA ratios led to 31 and 67% inhibition, respectively, of KCC2 transport activity in isotonic medium. Higher relative amounts of ΔN117 led also to rKCC2 inhibition in isotonic medium. Substantial basal activity of KCC2 in isotonic medium has been noted previously (18, 27).

DISCUSSION

We have initiated structure-function analysis of the N-terminal and C-terminal cytoplasmic tails of the KCC1 K-Cl cotransporter. We have shown that removal of small terminal segments of either cytoplasmic tail sufficed to abrogate KCC1-mediated 86Rb+ influx into Xenopus oocytes stimulated either by hypotonicity or by 1 mM NEM. All C-terminal truncations exhibited loss-of-function phenotypes regardless of accumulation at or near the oocyte surface, but none displayed dominant negative properties.

Removal of the N-terminal 89 or 117 amino acids from mKCC1 also produced loss-of-function mutants that exhibit wild type levels of expression at or near the oocyte surface. The ΔN117 mKCC1 mutant proved to be a potent dominant negative inhibitor of wild type ion transport function while not decreasing wild type polypeptide abundance at the oocyte surface. The ΔN117 mutant and wild type polypeptides were associated in immunoprecipitates, whether prepared from oocytes...
in which they were co-expressed or from in vitro co-translation reactions in the presence of pancreatic microsomes. In contrast, the nondominant negative loss-of-function mutant ΔC46 mKCC1 polypeptide did not associate with wild type polypeptide in either setting. Both dominant negative suppression of wild type transport function by ΔC117 and the ability of ΔC117 to associate with wild type polypeptide required portions of the C-terminal tail beyond residue 805. ΔC117 mKCC1 also exhibited potent dominant negative inhibition of hKCC3-mediated $^{86}$Rb$^+$ uptake and, less potently, of $^{86}$Rb$^+$ uptake mediated by mKCC4 and rKCC2.

**Functional Requirement for Both C-terminal and N-terminal Cytoplasmic Domains**—The requirement of the C-terminal residues for stimulation of mKCC1 by either hypotonicity or by NEM extends earlier findings on the importance of rabbit KCC1 Tyr$^{1056}$ and the analogous rat KCC2 Tyr$^{1087}$ to hypotonic stimulation (18). Our results also corroborate and extend those recently reported for NEM stimulation of rabbit KCC1 in HEK 293 cells (19). We have found that truncation of as few as eight C-terminal amino acids from mKCC1 abolished function without decrease in surface expression. Although all C-terminal truncation mutant polypeptides except ΔC940 accumulated in oocytes, distinct ΔC mutants were expressed at variable levels at the cell surface.

Following construction and functional analysis of our engineered mKCC1 ΔC mutants, we noted in the data base the variant hKCC1 expression sequence tag Al 799106. In this transcript, selective deletion of exon 18 encodes a polypeptide in which Gln$^{747}$ (the terminal codon of exon 17) is followed by 33 novel, exon 19-encoded, frameshifted amino acid residues before termination at position 780. The transcript is present in human RNA from 293T cells, T84 cells, and placenta. In agreement with all tested mKCC1 ΔC polypeptide mutants, this physiological hKCC1 ΔC variant exhibited no detectable transport function in *Xenopus* oocytes (not shown). Thus, our findings with engineered mKCC1 C-terminal truncation variants are relevant to at least one physiological hKCC1 transcript.

In contrast to the requirement for the entire C-terminal tail, removal of 46 N-terminal residues from mKCC1 preserved partial hypotonic stimulation. Retention of ΔC46 mKCC1 stimulation by NEM did not reach statistical significance. However, removal of 89 or 117 N-terminal residues abolished stimulation by both stimuli. These are the first data demonstrating a required role for the N-terminal cytoplasmic domain in KCC function. The N-terminal cytoplasmic domain, however, appears to be unnecessary for delivery to and accumulation of mKCC1 at the oocyte surface.

The N-terminal cytoplasmic tail of dogfish NKCC1 harbors a functional consensus binding site for protein phosphatase I, which dephosphorylates NKCC1 aa 184 to inactive ion transport (28). This consensus sequence is absent from both cytoplasmic termini of mKCC1. However, many candidate phosphorylation sites are present within the N-terminal cytoplasmic region defined by deletion as critical for stimulation by hypotonicity or by NEM.

**Oligomeric Structure of mKCC1**—Two observations support the hypothesis that recombinant mKCC1 exists as a homomultimer in *Xenopus* oocytes. First, the ΔC117 mutant of mKCC1 suppresses ion transport function of co-expressed wild type mKCC1. Second, the mutant and wild type polypeptides co-expressed in vitro and in oocytes can be co-immunoprecipitated by αNT antibody that recognizes only wild type mKCC1. A homodimeric state has been proposed for rat parotid gland NKCC1 based on chemical cross-linking experiments (29). Chemical cross-linking and gel filtration also demonstrate covalent multimerization of mKCC1 as well as of other KCC gene products. αNT immunoprecipitates contain an additional band of ~170 kDa, representing a distinct mKCC1-associated polypeptide absent from water-injected oocytes and not detected in αCT immunoprecipitates (Fig. 9).

**Dominant Negative Functions of CCC Family Members**—ΔC117 mKCC1 is the first reported dominant negative mutation among KCC K-Cl cotransporters and the first engineered dominant negative construct among CCCs. However, two examples of naturally occurring dominant negative CCCs have been reported. The first is the inactive C4 variants of mouse NKCC2, the shorter of two NKCC2 C-terminal polypeptide variants (30, 31). Co-expression of inactive C4 polypeptide (A4) with the longer and functionally active but cAMP-insensitive C9 variant of NKCC2 (F9) inhibited its cation transport activity in a manner that was partially reversed by cAMP-isobutyl-methylxanthine (31). Since C4 and C9 isoforms of NKCC2 are coexpressed in mouse thick ascending limb of Henle (28), this interaction was proposed to underlie the ability of vasopressin to activate NKCC2 activity in thick ascending limb of the mouse kidney (31).

A second, naturally occurring, dominant negative form of CCC was found through functional characterization of a novel CCC cDNA identified in the expressed sequence tag data base and sharing 27% amino acid sequence identity with NKCC1 (32). Named CIP (for CCC-interacting protein, this transcript expressed in heart, placenta, brain, muscle, and kidney traffics to the cell surface, but is itself inactive as a transporter of rubidium or sodium. CIP selectively inhibited co-expressed NKCC1 but lacked dominant negative activity when co-expressed with the equally (remotely) homologous NKCC2 or with KCC1. Epitope-tagged heterologous CIP in lyses of transfected 293 cells could (in one condition) be co-immunoprecipitated with endogenous NKCC1 polypeptide.

**Stoichiometric Considerations Arising from the Dominant Negative Phenotype of mKCC1 ΔC117**—The C4 variant of NKCC2 inhibited C9 NKCC2 activity only minimally in oocytes injected with equimolar quantities of cRNA but inhibited nearly completely when the C4/C9 ratio was 2:1 (31). A 1:1 ratio of injected CIP and NKCC1 cRNAs led to >80% inhibition of NKCC1 function (32). Inhibition by ΔC117 mKCC1 of co-expressed wild type mKCC1 and hKCC3 activities in oocytes (Figs. 7A, 8, and 10) was of comparable or greater potency. Oligomeric stoichiometry of interaction between mutant and wild type membrane transport protein subunits in *Xenopus* oocytes is generally calculated based on relative mole fractions of injected cRNAs. Such calculations assume that the mass ratio of injected cRNAs reflects equivalent ratios of the encoded polypeptides at the cell surface or other interaction sites and further assume equivalent interaction affinities for homo- and heterooligomers. These assumptions are believed to be met for a growing number of both engineered (33) and genetically encoded dominant negative variants of homotetrameric K$^+$ channels (34, 35) but remain inadequately tested for the interaction of ΔC117 KCC1 with WT KCC1 or for other dominant negative CCC interactions in heterologous expression systems. Modeling the mKCC1 coexpression data in Fig. 8 with the assumption of a binomial distribution of hetero- and homo-oligomers (33) does not discriminate between dimeric and tetrameric states (linear fit of the ln/ln plot of this data yields a slope 3.2 ± 0.9, not shown). Chemical cross-linking and gel filtration data$^5$ indicate that all wild type KCC polypeptide gene products are at least homodimeric, consistent with the cross-linking of NKCC1 dimers (29). In addition, each mKCC1 truncation mutant examined in

$^5$ S. Casula, A. S. Zolotarev, and S. L. Alper, unpublished observations.
the current work can be covalently cross-linked to the homo-dimeric state. Thus, the presence of neither cytoplasmic domain of mKCC1 is required for homo-oligomerization.

**Hetero-oligomeric Interactions among Polypeptide Products of Different KCC Genes**—The ability of ΔN117 mKCC1 potently to inhibit ion transport function of hKCC3 and less potently to inhibit mKCC4 and rKCC2 strongly suggests that the products of the different KCC genes can associate to form hetero-oligomeric polypeptides with considerable combinatorial complexity. Since the ion affinities, regulatory properties, and sensitivity to inhibitors varies among the different KCC gene products (4, 12, 26, 27), some of those macroscopic properties may also differ in cells expressing putative hetero-oligomers. This may be especially so for regulatory properties that might arise via altered affinity of homo-oligomeric (or hetero-oligomeric) interaction.

**Utility of the ΔN117 mKCC1 Dominant Negative Mutant**—Specific and potent pharmacological inhibitors of KCC K-Cl cotransporters are currently unavailable. Correlative experiments examining changes in KCC isoform expression during whole animal physiological manipulations have been initiated (36). More direct tests of the physiological functions of KCC polypeptides and the consequences of loss of K-Cl cotransporters are currently unavailable. Correlative experiments examining changes in KCC isoform expression during whole animal physiological manipulations have been initiated (36). More direct tests of the physiological functions of KCC polypeptides and the consequences of loss of K-Cl cotransport activity will require knockout or knock-down experiments. These will involve either generation of homoygous knockout animals or transgenic expression of dominant negative or antisense constructs.

KCC K-Cl cotransporters are widely expressed among tissue and cell types. Some cell types probably express more than one KCC gene product. Thus, the possibility of functional compensation of a knockout of one gene product by unaltered or up-regulated expression of cognate genes is a serious one. In this setting, overexpression of ΔN117 or similar mutants offers a currently unique nonpharmacological tool for functional inhibition of all or most KCC gene products.

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