A C-terminal Domain in KCC2 Confers Constitutive K\textsuperscript{+}-Cl\textsuperscript{−} Cotransport*

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The neuron-specific K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter KCC2 plays a crucial role in determining intracellular chloride activity and thus the neuronal response to γ-aminobutyric acid and glycine. Of the four KCCs, KCC2 is unique in mediating constitutive K\textsuperscript{+}-Cl\textsuperscript{−} cotransport under isotonic conditions; the other three KCCs are exclusively swelling-activated, with no isotonic activity. We have utilized a series of chimeric cDNAs to localize the determinant of isotonic transport in KCC2. Two generations of chimeric KCC4-KCC2 cDNAs initially localized this characteristic to within a KCC2-specific expansion of the cytoplasmic C terminus, between residues 929 and 1043. This region of KCC2 is rich in prolines, serines, and charged residues and encompasses two predicted PEST sequences. Substitution of this region in KCC2 with the equivalent sequence of KCC4 resulted in a chimeric KCC2 that was devoid of isotonic activity, with intact swelling-activated transport. A third generation of chimeras demonstrated that a domain just distal to the PEST sequences confers isotonic transport on KCC4. Mutagenesis of this region revealed that residues 1021–1035 of KCC2 are sufficient for isotonic transport. Swelling-activated K\textsuperscript{+}-Cl\textsuperscript{−} cotransport is abrogated by calyculin A, whereas isotonic transport mediated by KCC chimeras and KCC2 is completely resistant to this serine-threonine phosphatase inhibitor. In summary, a 15-residue C-terminal domain in KCC2 is both necessary and sufficient for constitutive K\textsuperscript{+}-Cl\textsuperscript{−} cotransport under isotonic conditions. Furthermore, unlike swelling-activated transport, constitutive K\textsuperscript{+}-Cl\textsuperscript{−} cotransport mediated by KCC2 is completely independent of serine-threonine phosphatase activity, suggesting that these two modes of transport are activated by distinct mechanisms.

The electroneutral cotransport of K\textsuperscript{+} and Cl\textsuperscript{−} across the plasma membrane is mediated by four members of the SLC12 cation-chloride cotransporter gene family, namely KCC1 (1), KCC2 (2), KCC3 (3–5), and KCC4 (3) (SLC12A4–A7, respectively). The four predicted KCC proteins are 65–75% identical and share a common predicted topology, with a large glycosylated extracellular loop between transmembrane domains 5 and 6 (3). Heterologous expression reveals that all four KCCs\textsuperscript{a} mediate cotransport of K\textsuperscript{+} and Cl\textsuperscript{−} (1, 3, 5–11), albeit with clear differences in ion affinities, anion series, and sensitivity to anion transport inhibitors (6, 9–11).

All four of the KCCs are swelling-activated, suggesting an important role in volume regulatory decrease (12). Indeed, K\textsuperscript{+}-Cl\textsuperscript{−} cotransport was first defined as a swelling-activated and NEM-activated K\textsuperscript{+} efflux mechanism in red cells (13, 14), which evidently coexpress KCC1 (7, 15), KCC3 (15), and KCC4 (16). Targeted deletion of KCC3 abolishes the volume regulatory decrease response of hippocampal neurons and attenuates the volume regulatory decrease in renal proximal tubular cells (17), providing dramatic genetic evidence for the role of the KCCs in cellular volume regulation. The loss of swelling-activated K\textsuperscript{+}-Cl\textsuperscript{−} cotransport may underlie the neuronal degeneration and peripheral neuropathy seen in both mice (17) and humans (18) with loss-of-function mutations in KCC3. Despite this evident physiological importance, the molecular determinants of swelling activation have not been elucidated in the KCCs. It is, however, known that swelling activation of red cell K\textsuperscript{+}-Cl\textsuperscript{−} cotransport (19) and of all four KCCs (7–11) is completely abrogated by the inhibition of protein phosphatase-1 with calyculin A. More recent data have implicated the WNK4 and SPAK serine-threonine kinases in the volume sensitivity of both K\textsuperscript{+}-Cl\textsuperscript{−} cotransport (KCC2) and Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransport (NKCC1) (20).

KCC2 has several characteristics that differentiate it from the other three KCCs. First, heterologous expression in Xenopus oocytes and mammalian cells reveals that KCC2 is unique in mediating constitutive K\textsuperscript{+}-Cl\textsuperscript{−} cotransport under isotonic conditions (6, 8, 9, 20); the other three KCCs are exclusively swelling-activated, without significant activity under isotonic conditions. KCC2 is, however, swelling-activated under hypotonic conditions (8, 9, 20). Second, a structural feature unique to KCC2 is an expanded domain of ~100 amino acids, rich in prolines, serines, and charged residues, near the end of the cytoplasmic C terminus. This region encompasses two predicted PEST (Proline/Exposure) (glutamate)/Serine/Threonine) sequences (21) that are completely unique to KCC2 (see also “Results” and “Discussion”). Third, KCC2 has perhaps the most clearly defined physiological role of the four KCCs. KCC2 is thus a neuron-specific gene (22, 23), with a crucial role in determining intracellular chloride activity ([Cl\textsuperscript{−}]) and the neuronal response to GABA and glycine (24, 25).

Early in postnatal life there is robust neuronal expression of the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter NKCC1 (26), with only minimal expression of KCC2. Inward-directed transport by NKCC1 increases [Cl\textsuperscript{−}] above its equilibrium potential, such that the activation of ligand-gated Cl\textsuperscript{−} conductance by the neurotransmitters GABA and glycine stimulates neuronal depolarization and excitation (27, 28). Several studies have demonstrated a dramatic induction of KCC2 within the first postnatal week.
(23, 29, 30), resulting in a dominance of \( K^-\)–Cl\(^-\) efflux and a decrease in [Cl\(^-\)], to below its equilibrium potential; this results in a shift in the GABA and glycine effect to hyperpolarization and neuronal inhibition (30).

Initial indications that GABA itself promotes the developmental induction of KCC2 (31) have not been supported by subsequent studies (32, 33). However, the overexpression of KCC2 in immature neurons is clearly sufficient to reduce [Cl\(^-\)], (34, 35), with a loss of the GABA-induced Ca\(^{2+}\) response (35). Most surprisingly, several correlates of GABAergic activity are induced by this ectopic overexpression of KCC2 (34), suggesting that KCC2 has direct effects on the development of GABAergic synapses. Finally, mice with a targeted deletion of KCC2 have impaired regulation of neuronal [Cl\(^-\)], (36), with hyperexcitability and early death from generalized seizures (37). Impaired expression and/or activity of KCC2 has also been implicated in several forms of neuronal injury (24, 38–40) and in the genesis of neuropathic pain (41).

The physiological role of KCC2-mediated swelling-activated \( K^-\)–Cl\(^-\) cotransport is not particularly clear, although one possibility is that it serves to limit neuronal/dendritic swelling in response to glutamatergic cotransport is not particularly clear, although one possibility is that it serves to limit neuronal/dendritic swelling in response to glutamatergic excitation (42). However, the isotonic activity of KCC2 is clearly suited to the constitutive maintenance of [Cl\(^-\)], a defined physiological role for this transporter (36). To begin a study of the molecular determinants of constitutive and swelling-activated KCC2 activity, we have utilized a directed series of chimeric and mutant cDNAs, using human KCC2 (9) and mouse KCC4 (3) as the starting point.

This effort has identified a KCC2-specific C-terminal domain that is both necessary and sufficient for constitutive \( K^-\)–Cl\(^-\) cotransport under isotonic conditions. To our knowledge this is the first identification of a discrete cytoplasmic domain that modulates the volume sensitivity of a defined class of ion transporter. Furthermore, this domain may be a critical cytoplasmic target for the post-transcriptional regulation of this important transporter.

**EXPERIMENTAL PROCEDURES**

**Construction of Chimeric and Mutant cDNAs**—For the purpose of constructing chimeric cDNAs between mouse KCC4 and human KCC2, we generated a number of silent restriction sites in the relevant cDNA constructs, all in the *Xenopus* expression vector pGEMHE (3, 9). The QuikChange mutagenesis system (Stratagene) was utilized for all of the mutations described herein; mutations were all confirmed by sequencing and subcloned back into the relevant expression constructs. To generate a silent EcoRI site within transmembrane domain 12 (TM12) of mouse KCC4 (codons 664–666), we utilized the sense primer 5′-GAGTGGGGGATGGAATTCGAGGAGGACGGCGATGGGGGAG-3′ and the antisense primer 5′-GTCGCTGGGGCCTGAATTCGGAGGTGCTGTTGGATGCAAGGTTTTCCCAGATCCAGGTCATGCACGTATGTTCCTGTCATGAATCAGTG-3′. We also generated two initial silent sites within the C terminus of KCC4. The primers 5′-AGAAGGAGGAGGACGGCGATGGGGGAG-3′ and 5′-GTCGCTGGGGCCTGAATTCGGAGGTGCTGTTGGATGCAAGGTTTTCCCAGATCCAGGTCATGCACGTATGTTCCTGTCATGAATCAGTG-3′ were thus utilized to generate a silent Mnul site in codons 951–952 of KCC4, whereas the primers 5′-AGGACCTCGAGATTTCCGTACCATGTGGTAACATCCAGTGTTAATAGG-3′ and 5′-CTCGGCACTGATTTCCGTACCATGTGGTAACATCCAGTGTTAATAGG-3′ were used to generate a silent SfuI site in codons 902–904. Finally, a silent BglII site was generated in codons 914–916 of human KCC2 using the primers 5′-GACGGGGTCCCAAGGATGCTGGGAACTGTAATCAGGACG-3′ and 5′-GACGGGGTCCCAAGGATGCTGGGAACTGTAATCAGGACG-3′.

Table 1 summarizes the various KCC4–KCC2 chimeras generated for this study. The junction points for these chimeras were generated within conserved segments, so as to maximize the likelihood that the chimeric cDNAs would be functional. These chimeras were all generated by PCR, using primers that incorporated restriction sites suitable for subcloning into the KCC4 or KCC2 backbone cDNA. Amplification were generally subcloned into pcR2.1 by TA cloning (Invitrogen) followed by sequence confirmation of the entire insert to verify the integrity of the DNA generated by PCR; fragments were then subcloned into the expression vectors using the relevant restriction enzymes, followed by sequence confirmation of insertion. For example, in the “K244” chimera, residues 1–138 of KCC4 were replaced by the N-terminal cyttoplasmic domain of KCC2. PCR amplification of the KCC2 template for this purpose utilized the sense primer 5′-TTACCAGGGGCAACATGCTGTAACCAACCTGAGGAGGACGGCGATGGGGGAGCACAACCTGCAGATGTTCCTGTCATGAATCAGTG-3′ and the antisense primer 5′-TCTTTGATACAGGTTTTCCCAGATCCAGGTCATGCACGTATGTTCCTGTCATGAATCAGTG-3′, which incorporate XmaI and AscI sites, respectively. The resulting fragment was subcloned from pcR2.1 into the modified KCC4 construct, using XmaI (5′-multiplicating site) and AscI (TM1).

In the “K442” chimera, residues 665–1083 of KCC4, were replaced with the entire C terminus of KCC2, using the engineered EcoRI site in TM12 (see above) and an XbaI site in the 3′-multiplicating site. Parts of the central core of hydrophobic domains in KCC4 were also substituted with KCC2 in two “K424” chimeras. In the K424-A chimera, residues 133–664 of KCC4 (TM1–12, see Table 1) were replaced by KCC2 using the engineered AscI and EcoRI sites in TM1 and TM12. To generate a chimera in which TM1–2 of KCC4 were replaced by the (divergent) sequence of KCC2 (K424-B), we replaced codons 141–196 by KCC2, using the engineered HpaI and EcoRI sites in TM1 and just before TM3 (see above).

Sequence comparison of the mammalian KCCs indicates that KCC2 contains an expanded domain of ~100 amino acids, rich in prolines and charged amino acids (2, 9). In the two “K4UR2” chimeras, this “unique region” within the C terminus of KCC2 was substituted for the equivalent region of KCC4, with varying amounts of surrounding sequence. Thus in the K4UR2-A chimera, residues 952–1018 of KCC4 were replaced with residues 933–1051 of KCC2 by PCR-based subcloning, using the silent C-terminal Mnul site (see above) and an existing SfuI site in KCC4. In the K4UR2-B chimera, residues 903–1057 of KCC4 were replaced with residues 883–1090 of KCC2, using the silent C-terminal SfuI site (see above) and an existing Xbal site in KCC4. The corresponding “K2UR4” chimera was generated as well, replacing residues 915–1116 of KCC2 with residues 935–1083 of KCC4; this construct utilized the engineered BglII site (see above) and an Xbal site in the 3′-multiplicating site of KCC2.

Finally, a third generation of KCC4–KCC2 chimeras was generated to refine the localization of the sequence that conferred isotonic transport,
using the K4UR2-B chimera as the starting point. For this purpose, an existing BamHI site, corresponding to codons 437–438 of KCC4, was inactivated in K4UR2-B by mutagenesis. A new silent BamHI site (see Fig. 2A) was then generated in K4UR2-B, at codons 1019–1022 of the hybrid open reading frame, using the primers 5’-AAGGGAGGAGG-GATCCCGAGAAGGTCATCTCA-3’ and 5’-TGAGATGCACCT-TCTCCGGATCCGCTTCC-3’. An upstream Munl site was then added to this construct, using the primers 5’-AGAGGAGGAGG- GTGAAATTGATCCGATCAGATGTC-3’ and 5’-GAGACT- CTGATCGTGGATCAATTGCACCTCCTC-3’. Three “subdomain” chimeras were then generated, using PCR-based subcloning to revert sections of the KCC2 sequence in the K4UR2-B chimera to that of KCC4; see also Table 1 and Fig. 2B. In the K4SD-1 chimera, the region between the BamHI and Xhol sites was replaced by KCC4, thus retaining the proximal two-thirds of the unique region and both PEST domains. In the K4SD-2 chimera the region between the Sful and Munl sites was replaced by KCC4, removing the first PEST domain and retaining the distal two-thirds of the unique region. Finally, in the K4SD-3 chimera, the region between the Sful and BamHI sites was replaced by KCC4, retaining the distal one-third of the unique region of KCC2 (Fig. 2).

To prepare cRNA for injection, cDNA constructs were linearized at the 3’ end using Nhel, and cRNA was transcribed in vitro, using the T7 RNA polymerase mMESSAGE kit (Ambion). RNA integrity was confirmed on agarose gels, and concentration was determined by absorbance reading at 260 nm. cRNA was stored frozen in aliquots at −80 °C until used. Preparation of Xenopus laevis Oocytes—Adult female X. laevis frogs were purchased from NASCO (Fort Atkinson, WI) and maintained in a Biotech XR3 system (New Bedford, MA) under controlled light conditions at a water temperature of 16 °C. Oocytes were surgically extracted from animals anesthetized by 0.17% tricaine immersion; after several such procedures, anesthetized frogs were sacrificed by cardiac puncture. The use and care of the animals in these experiments were approved by the Institutional Animal Care and Use Committee at Harvard Medical School. After extraction, oocytes were incubated for 1 h with vigorous shaking in a Ca2++-free ND96 medium (mM: 96 NaCl, 2 KCl, 1 MgCl2, and 5 HEPES/Tris, pH 7.4, plus 2 mg/ml collagenase A). Oocytes were then washed four times in regular ND96, defolliculated by hand, and incubated overnight in ND96 at 16 °C. Mature oocytes were injected with 50 nl of water or with water containing 0.5 μg/μl of cRNA transcribed in vitro from the various KCC constructs. Oocytes were incubated for 4–5 days prior to transport assays, in ND96 at 16 °C supplemented with 2.5 mm sodium pyruvate and 5 mg/100 ml of gentamicin.

Measurement of K+–Cl− Cotransport—K+–Cl− cotransport was assessed by measuring Cl−-dependent 86Rb+ uptake (PerkinElmer Life Sciences) in X. laevis oocytes, as described previously (3, 9–11). Rubidium uptake was assessed 4–5 days after injection under both isotonic and hypotonic conditions, as noted (see Figs. 1 and 2A and B). A 30-min incubation period in a Na+– and Cl−-free medium (mM: 50 N-methyl-D-glucamine gluconate, 10 K+ gluconate, 4.6 Ca2+ gluconate, 1 Mg2+ gluconate, 5 HEPES/Tris, pH 7.4) containing ouabain (1 mM) was followed by a 60-min uptake period in a Na+–free medium (mM: 50 N-methyl-D-glucamine-Cl, 10 KCl, 1.8 CaCl2, 1 MgCl2, 5 HEPES/Tris, pH 7.4) supplemented with ouabain and 2.5 μCi of 86Rb+ per ml (PerkinElmer Life Sciences). Isotonic conditions were generated by supplementing the same solutions with 3.5 g/100 ml sucrose to reach isotonic conditions for oocytes (~210 mosmol/kg). Ouabain was added to inhibit 86Rb+ uptake via Na+–K+–ATPase, and removal of extracellular Na+ prevented 86Rb+ uptake via the endogenous Na+–K+–2Cl− cotransporter. All uptakes were performed at 32 °C. At the end of the uptake period, oocytes were washed three times in ice-cold uptake solution without isotope to remove extracellular fluid tracer. Oocytes were dissolved in 10% SDS, and tracer activity was determined for each oocyte by β-scintillation counting. The uptake experiments all included at least 15 oocytes in each experimental group; statistical significance was defined as two-tailed p < 0.05, and results were reported as means ± S.E. All of the transport experiments shown were performed at least two times, with the appropriate controls (water-, KCC2-, and/or KCC4-injected groups) as shown.

Western Blotting and Surface Biotinylation—Western blotting was performed using an affinity-purified rabbit polyclonal antibody specific for residues 22–40 of mouse KCC4 (43). Total cellular protein for Western blot analysis was prepared from groups of 15–20 X. laevis oocytes injected with cRNA for the various constructs. After 4–5 days, oocytes in ND96 were transferred to Eppendorf tubes on ice and were lysed in lysis buffer (mM: 10 Tris-HCl, 150 NaCl, 1 EDTA, 1% Triton) supplemented with protease inhibitors. After clearing the lysate of yolk and cellular debris by centrifugation at 15,000 × g for 15 min, the supernatant was stored at −80 °C. Western blotting was performed using the KCC4 antibody at a titer of 1:1000, as described (44).

Surface biotinylation was also assessed, as a measure of protein expression at the cell membrane. For this purpose, groups of 20 oocytes were injected with 25 ng of KCC cRNA and kept at 16 °C for 4–5 days in ND96 medium. Oocytes were then washed with ND96 and incubated for 1 h in 1 ml of ND96 containing 1 mM EZ-link sulfo-NHS biotin (Pierce). After this incubation period, oocytes were washed three times with 3 ml of ND96 and lysed (20 μl/oocyte) in a lysis buffer containing protease inhibitors. Lysis was achieved by incubating the oocytes for 10 min on ice in the lysis buffer, followed by trituration with a pipette, an additional 15 min of incubation on ice, and a final centrifugation step of 15,000 × g for 15 min. The supernatant was saved and diluted with lysis buffer to a final volume of 1 ml. Streptavidin (50 μl) was then added and incubated with the samples for 2 h at 4 °C. The samples were then spun and washed three times with lysis buffer. After the final wash, the pellet of streptavidin beads was resuspended in 65 μl of sample buffer containing 4% β-mercaptoethanol, heated at 50 °C for 20 min, and subjected to 7.5% SDS-PAGE and Western blot analysis with the KCC4-specific antibody (43).

RESULTS

Isotonic Activity Is Conferred by the C-terminal KCC2 Unique Region—The ability of a given segment of KCC2 to confer isotonic transport in KCC4–KCC2 chimeras was studied using Cl−-dependent 86Rb+ uptake in Xenopus oocytes, measured under both isotonic and hypotonic conditions. Characterization of the first generation of chimeras reveals that the C-terminal cytoplasmic domain of KCC2, residues 645–1116, contains a sequence that confers isotonic transport (K442 chimera, Fig. 1A). In contrast, neither the K244 chimera nor the two K424 chimeras mediate isotonic transport; however, they do mediate significant swelling-activated K+–Cl− cotransport under hypotonic conditions (Fig. 1, A and B). Therefore, neither the N terminus nor the central hydrophobic core of KCC2 confers isotonic transport, whereas the C terminus is clearly required. An obvious candidate for the domain involved is the KCC2-specific expansion of the cytoplasmic C terminus; between residues 929 and 1043, K4UR2-A and K4UR2-B, chimeras in which this region is substituted for the equivalent section of KCC4 (see Table 1), mediate considerable isotonic transport. In contrast, the loss of this domain in KCC2
in the K2UR4 chimera results in a loss of isotonic K\(^+\)-Cl\(^-\) transport with preserved swelling activation (Fig. 1, C and D).

As shown in Fig. 2A, the KCC2-unique C-terminal domain encompasses a large expansion that is rich in prolines, glutamate, serine, lysine, and arginine, features reminiscent of a PEST domain (see “Discussion”). The web-based program PESTfind (bioweb.pasteur.fr/seqanal/interfaces/pestfind.html) predicts two PEST sequences in this region, between residues 952–966 and 975–1000 (PESTfind scores of +8.35

TABLE 1

| Chimera | Comment | KCC4 residues\(^a\) | KCC2 residues\(^a\) |
|---------|---------|---------------------|---------------------|
| K244    | N terminus of KCC2 | 139–1083 | 1–117 |
| K442    | C terminus of KCC2 | 1–664 | 645–1116 |
| K424-A  | TM1–TM12 of KCC2 | 1–132; 665–1083 | 112–644 |
| K424-B  | TM1–TM2 of KCC2 | 1–140; 197–1083 | 120–175 |
| K2UR4   | "Unique region" of KCC4 | 935–1083 | 1–914 |
| K4UR2-A | Unique region of KCC4 | 1–951; 1019–1083 | 933–1051 |
| K4UR2-B | Unique region of KCC4 | 1–902; 1058–1083 | 883–1090 |
| K4SD-1  | Proximal 2/3rds of UR domain | 1–951; 1058–1083 | 973–1090 |
| K4SD-2  | Distal 2/3rds of UR domain | 1–973; 1058–1083 | 1000–1090 |
| K4SD-3  | Distal 1/3rd of UR domain | 1–973; 1058–1083 | 1000–1090 |

\(^a\) The content of the KCC2 or KCC4 sequence is defined by the specific codons incorporated in the chimeric cDNAs.

FIGURE 1. Functional characterization of KCC4-KCC2 chimeras. A, isotonic conditions. The C terminus of KCC2 contains the determinant of isotonic, constitutive transport. Chloride-dependent 86Rb\(^+\) transport was measured in Xenopus oocytes expressing KCC2, KCC4, or KCC4-KCC2 chimeras with the N terminus (K244), C terminus (K442), or varying segments of the transmembrane core (K424-A/B) of KCC2 replacing the equivalent segment of KCC4, B, hypotonic conditions. All of the chimeras in A function as swelling-activated K\(^+\)-Cl\(^-\) cotransporters. C, isotonic conditions. The unique region in the C terminus of KCC2 (residues 929–1043) contains the determinant of isotonic, constitutive transport. Chloride-dependent 86Rb\(^+\) transport was measured in Xenopus oocytes expressing KCC2, KCC4, or KCC4-KCC2 chimeras in which the unique region of KCC2 was inserted into KCC4 (K4UR2-A/B) or removed and substituted with the equivalent region of KCC4 (K2UR4). D, hypotonic conditions. All of the chimeras in C function as swelling-activated K\(^+\)-Cl\(^-\) cotransporters. * refers to p < 0.0001 compared with KCC4-expressing cells.
C-terminal Domain in KCC2 Determines Isotonic Transport

To begin to identify residues required for isotonic transport, we initially reverted individual amino acids in K4SD-3 to the residue present at that position in KCC4. The first mutants (Fig. 4) targeted serines and prolines scattered along the KCC2 insertion, specifically S1013L, S1022D, P1023T, and S1025P; the assumption was that these residues might be important for intracellular signaling events that control isotonic transport, functioning, for example, in proline-directed serine phosphorylation. We also generated alanine mutants of a sequence that could confer isotonic transport. We initially generated the D995S/P998S mutant of KCC4, mutating the residues corresponding to Ser-1022 and Ser-1025 of KCC2. Sequential mutation ultimately reverted residues 994–998 of KCC4-KCC2 (KDTGP to HKGPSPVS, residues 1018–1025 of KCC2). We thus proceeded to generate alanine mutants, aspartate mutants of serine residues, and KCC4 revertants in this more restricted region. Whereas the G1020N and P1021K mutants preserved isotonic transport (data not shown), the S1022A, P1023A, and S1025A mutations all reduced isotonic transport without affecting swelling-activated transport (Fig. 5). Mutants of serine 1026 did not affect isotonic transport; however, deletion of the glutamate at codon 1027 of KCC2, which is absent in KCC4 (Fig. 2A), inhibited isotonic but not hypotonic transport. Finally, C-terminal extension of this analysis revealed that the I1029F, F1032L, and M1035L mutations significantly reduced isotonic transport in K4SD-3 without affecting swelling-activated transport (Fig. 6).

This mutational study suggested that residues 1022–1037 contained the “isotonic domain” (ISO domain) required for constitutive, isotonic transport. We then set out to define the minimum domain within this sequence that could confer isotonic transport on KCC4. We initially generated the D995S/P998S mutant of KCC4, mutating the residues corresponding to Ser-1022 and Ser-1025 of KCC2. Sequential mutation ultimately reverted residues 994–998 of KCC4-KCC2 (KDTGP to HKGPSPVS, residues 1018–1025 of KCC2) followed by the addition of the glutamate at codon 1027 of KCC2 (at codon 1000 of KCC4) to generate the “sextuplet” mutant in Fig. 7. None of these KCC4-to-KCC2 mutants exhibited isotonic transport (Fig. 7 and data not shown) nor did more N-terminal mutants (e.g. H991N/R992K/N993G, data not shown). Although an extension of the sextuplet mutant (K994P/D995S/T996P/G997V/P998S/E1000plus) to add H991N/R992K/N993G did not result in isotonic transport, the C-terminal extension to add the F1001F, P1032L, and M1035L mutations significantly reduced isotonic transport in K4SD-3 without affecting swelling-activated transport (Fig. 6).

FIGURE 2. C-terminal domains in KCC2 and selected KCC4-KCC2 chimeras. A, alignment of KCC2 and KCC4, showing the position of silent restriction sites in KCC4 and in the K4UR2-B chimera that were utilized for C-terminal chimeras (see Table 1 and the text for details). The two predicted PEST domains within the unique region of KCC2 (residues 929–1043) are underlined, as is the minimal ISO domain that confers constitutive isotonic transport on KCC4 (see Fig. 6). B, schematic illustration of the C-terminal KCC2-specific domains retained in the subdomain chimeras, K4SD-1/2/3. These chimeric cDNAs were generated by replacing KCC2 sequence in the K4UR2-B chimera with KCC4 (see Table 1 and the text for details). Only the extreme C terminus of these chimeras is shown, corresponding to the alignment shown in A; the N terminus, TM1–12 domains, and proximal C terminus are identical to KCC4. Not drawn to exact scale.
Immunoblotting and Cell Surface Biotinylation—It is evident in Figs. 4–6 that several mutations in K4SD-3 have significant effects on both constitutive and swelling-activated K⁺-Cl⁻ cotransport, whereas other mutations primarily affect constitutive transport. To address which mutations affect processing and/or stability of the chimeric K4SD-3 protein, we performed Western blot analysis of injected oocytes, using an N-terminal KCC4-specific antibody (11, 43). This revealed a marked and reproducible reduction in the expression of the S1013L and P1023T mutants, indicating that these mutations affect processing and/or stability of the protein (Fig. 8). Although other mutants had intermediate expression, some are clearly expressed at equivalent levels to that of “wild-type” K4SD-3 and KCC4, indicating a selective effect of these mutations on constitutive, isotonic transport.

To assess whether the ISO domain of KCC2 affects membrane expression of the various KCCs, we performed cell surface biotinylation followed by Western blotting of biotinylated protein with the KCC4-specific antibody (11, 43). Surface biotinylation of wild-type KCC2 did not differ from that of K4SD-3 (Fig. 9A) or other chimeric KCCs; these experiments were repeated three times, with equivalent results. The cell surface biotinylation of K4SD-3 point mutants reproducibly tracked with their relative expression in oocytes, as measured by Western blotting of whole cell extracts (Fig. 9B). Of note, the K4SD-3 protein differs significantly from the KCC4 protein, with the substitution of residues 974–1057 of KCC4 by residues 1000–1090 of KCC2; divergence outside of the “ISO” domain may affect membrane expression of this chimeric KCC. Therefore, we also compared surface biotinylation of these KCCs to that of the sextuple KCC4 mutant (see above) and to the KCC4-ISO mutant that exhibits isotonic transport (see above and Fig. 7). None of these proteins differed in relative cell surface biotinylation (Fig. 9A) or in relative expression in oocytes (Fig. 9B). Thus it appears that the ISO domain of KCC2 does not affect the expression of the KCC protein at the plasma membrane of Xenopus oocytes.

Effect of Kinase and Phosphatase Inhibition—Swelling activation of human KCC2 and other KCCs is blocked by the serine-threonine phosphatase inhibitor calyculin A (7–11). KCC2, K442, and K4SD-3 are completely resistant to calyculin A under isotonic conditions (Fig. 10A),
whereas hypotonic activation of K4SD-3 is inhibited ~90% by this inhibitor (Fig. 10B). We have reported previously that human KCC2 (9), mouse KCC4 (10), and human KCC3 (11) are sensitive to calyculin A under hypotonic conditions. The ISO domain in KCC2 that confers isotonic transport contains a number of prolines and serines, suggesting the possibility that proline-directed serine kinases might modulate isotonic transport. However, K⁺-Cl⁻ cotransport in cells expressing K4SD-3 and K4UR2-B that were treated with staurosporine (nonspecific kinase inhibitor), olomoucine (inhibitor of cyclin-dependent kinases) (45), or SB203580 (p38 MAPK inhibitor) did not differ from vehicle-treated control cells (Fig. 10C). Therefore, basal isotonic transport in *Xenopus* oocytes is not dependent on serine-threonine kinases that are sensitive to these three inhibitors.

**DISCUSSION**

The two major subtypes of cation-chloride cotransport, Na⁺-K⁺-2Cl⁻ and K⁺-Cl⁻ cotransport, have opposing physiological roles that are subject to reciprocal regulation. In neurons, inward-directed Na⁺-K⁺-2Cl⁻ cotransport (NKCC1) and outward-directed K⁺-Cl⁻ cotransport (KCC2) play dominant roles in the regulation of [Cl⁻]ᵢ, with opposing effects on neuronal excitability (30, 36, 37, 46, 47). NKCC1 is activated by cell shrinkage and by decreases in [Cl⁻]ᵢ (48, 49) but inhibited by the serine-threonine phosphatase-1 (PP1) (50, 51), the kinase inhibitor staurosporine (51, 52), and the thiol-alkylating agent N-ethylmaleimide (NEM) (53). In contrast, K⁺-Cl⁻ cotransport is activated by cell swelling (13) and by increases in [Cl⁻]ᵢ (48) and is activated by PP1, staurosporine, and NEM (54). Progress in this area has accelerated recently, with revelations that the serine-threonine kinases WNK4 and SPAK function in the reciprocal regulation of Na⁺-K⁺-2Cl⁻ and K⁺-Cl⁻ cotransport (20, 55–57). SPAK may furthermore play a key role in the volume sensitivity of other transport systems (58). However, the specific protein domains and/or phospho-acceptor site(s) that are targeted by these novel kinases have yet to be defined.

Functional comparison of the KCCs has indicated that KCC2 has minimal (6) or reduced (9) activation by cell swelling, with significant constitutive activity under isotonic conditions. We have exploited these characteristics to identify a cytoplasmic domain in KCC2 that can con-
fer isotonic transport on KCC4, its closest paralog. For reasons that are not clear, we find that KCC2 and KCC4 mediate reproducibly equivalent swelling-activated K\(^{+}\)-Cl\(^{-}\) cotransport in *Xenopus* oocytes, in contrast to our prior data indicating considerably higher swelling-activated transport in KCC4-injected cells (9). In consequence, we did not identify residues that confer absolute or quantitative differences in swelling activation. Alternative approaches will be necessary to characterize the molecular determinants of swelling activation in KCC2 and the other KCCs; presumably the relevant domains will encompass phospho-acceptor sites that are differentially targeted by serine-threonine kinases and phosphatases.

The first set of chimeric cDNAs (Fig. 1) revealed that the large C-terminal cytoplasmic domain of KCC2 contains the domain responsible for isotonic activity. This contrasts with the evolving importance of the N terminus of NKCC1, which contains interaction motifs for both PP1alpha and SPAK (55), in addition to a cluster of threonines that are expected to form a random coil in the KCC2 protein, followed by a stretch of \(\alpha\)-helices at the end of the C terminus. This sequence does not constitute a known signaling motif or protein-protein interaction domain (63), nor does it exhibit isotonic transport or the KCC4-ISO mutant with isotonic activity (replacement of residues 994–1007 of KCC4 with 1021–1035 of KCC2). Twenty oocyte equivalents per lane were utilized for Western blotting of membrane protein (KCC4 antibody) recovered after surface biotinylation. B, Western blotting of whole cell lysates from the same cells utilized in A, 0.1 oocyte equivalent per lane. Surface biotinylation of KCC4-expressing cells does not differ from that of oocytes expressing K4SD-3 or KCC4 mutants with or without isotonic transport. Although whole cell expression was reduced, mutants of K4SD-3 without isotonic transport could be detected by surface biotinylation.

KCCs. A large C-terminal expansion in the KCC2 protein was also an early candidate for the domain that confers constitutive transport (2, 9). This expanded KCC2-unique region (“UR2” in the chimeric cDNAs, Table 1) is rich in prolines, serines, and charged residues; although not initially appreciated (2, 9), this region encompasses two predicted PEST sequences (21) (see also “Results”).

The K4UR2 chimeras incorporating residues 933–1051 and 883–1090 of KCC2 (K4UR2-A and K4UR2-B, respectively, see Table 1) mediate robust isotonic transport. In contrast, the replacement of this region in the K2UR4 chimera abrogates isotonic transport, with intact swelling-activated transport (Fig. 1). Notably, the two KCC2-specific PEST domains do not support isotonic transport (K4SD-1 chimera, see Table 1 and Fig. 3), nor does the substitution of both PEST domains in the K4SD-3 chimera lead to the loss of isotonic transport. Rather, extensive mutagenesis of the K4SD-3 chimera indicated that residues 1021–1035 might contain the critical domain required for isotonic transport; this was confirmed by sequential mutagenesis of KCC4 (Fig. 7).

The minimal sequence that confers isotonic transport on KCC4 is predicted to form a random coil in the KCC2 protein, followed by a stretch of \(\alpha\)-helices at the end of the C terminus. This sequence does not constitute a known signaling motif or protein-protein interaction domain (63), nor does it exhibit homology to proteins other than KCC2. Notably, the sequence is 100% conserved in the mammalian KCC2 orthologs but is not found in the three *Caenorhabditis elegans* KCCs or in the C-terminal isoforms of the *Drosophila* KCC protein (GenBank\textsuperscript{TM} accession numbers AAM68277 and AAF47099). Should any of these invertebrate KCCs function under isotonic conditions, we assume that a different mechanism is involved. With the exception of P1023T, S1025A, and M1035L, mutations within this domain did not affect K4SD-3 protein expression in *Xenopus* oocytes (Fig. 8), suggesting that

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**FIGURE 7.** Definition of the minimal domain that confers isotonic transport. Muta- tion of the K4SD-3 chimera (Figs. 4–6) suggested that residues 1022–1037 of KCC2 contained the iso domain required for constitutive, isotonic transport. To define the minimal domain, we sequentially mutated this region of KCC4 to KCC2, beginning with the D995S/P998S mutant, in which we mutated KCC4 to the residues corresponding to Ser-1022 and Ser-1025 of KCC2. Selected mutants in this series are shown; isotonic transport did not emerge until residues 994–1007 of KCC4 were all mutated completely to the sequence PSPVSSEGIKDFFSM, corresponding to residues 1021–1035 of KCC2. To generate this mutant, the triple F1001I/L1004F/L1007M was generated in the sextuple mutant (SM) (K994P/D995S/T996P/G997V/P998S/E1000plus). * refers to \(p < 0.000000001\) compared with KCC4-expressing cells.

**FIGURE 8.** Western blot analysis. Mutants were generated in the K4SD-3 chimera, in which residues 974–1057 of KCC4 are replaced by residues 1000–1090 of KCC2; point mutants are labeled based on the equivalent residue in the KCC2 protein. Western blotting of total oocyte lysates utilized an antibody specific for the N terminus of KCC4 (11, 43), using the equivalent of one oocyte per lane.

**FIGURE 9.** Surface biotinylation; the isotonic domain does not increase expression at the plasma membrane. A, surface biotinylation. In the left panel, surface biotinylation of *Xenopus* oocytes expressing KCC4 was compared with that of oocytes expressing K4SD-3 or several mutants of K4SD-3. In the right panel, surface biotinylation of oocytes expressing KCC4 was compared with that of oocytes expressing K4SD-3, the sextuple KCC4 mutant (KCC4-SM: K994P/D995S/T996P/G997V/P998S/E1000plus) that does not exhibit isotonic transport or the KCC4-ISO mutant with isotonic activity (replacement of residues 994–1007 of KCC4 with 1021–1035 of KCC2). Twenty oocyte equivalents per lane were utilized for Western blotting of membrane protein (KCC4 antibody) recovered after surface biotinylation. B, Western blotting of whole cell lysates from the same cells utilized in A, 0.1 oocyte equivalent per lane. Surface biotinylation of KCC4-expressing cells does not differ from that of oocytes expressing K4SD-3 or KCC4 mutants with or without isotonic transport. Although whole cell expression was reduced, mutants of K4SD-3 without isotonic transport could be detected by surface biotinylation.
C-terminal Domain in KCC2 Determines Isotonic Transport

They do not affect isotonic transport via major changes in secondary structure or nonspecific effects on transport activity.

The mechanism(s) whereby this short cytoplasmic sequence activates $K^+\cdotCl^-$ cotransport is not as yet clear. However, surface biotinylation experiments indicate that the presence or absence of this domain does not affect the expression of KCCs at the plasma membrane (Fig. 9), such that it is unlikely to exert a major effect on trafficking of the transporter protein. Of the four serines in the minimal sequence only Ser-1022, Ser-1025, and Ser-1034 are predicted to be substrates for protein kinases (64); a limited survey of kinase inhibitors suggests that phosphorylation by serine-threonine kinases is not required for isotonic activity.

We had reported previously that calyculin A did not affect isotonic transport mediated by KCC2 (9), whereas others had found that rat KCC2 was sensitive to calyculin A under isotonic conditions (8). It was conceivable that this discrepancy occurred as a function of experimental variation in oocyte expression, given the relatively modest isotonic transport mediated by KCC2 (typically 5–10-fold higher than water-injected controls). However, the KCC4-KCC2 chimeras with isotonic transport are considerably more active than KCC2 (see below), as much as 40-fold higher than water-injected controls (K4SD-2 chimera, Fig. 3A); all of these chimeras were completely insensitive to calyculin A under isotonic conditions (Fig. 10). The swelling activation of all four KCCs (7–11) and of K4SD-3 (Fig. 10) in Xenopus oocytes is completely dependent on serine-threonine phosphatase activity, such that swelling-activated and constitutive transport are evidently regulated by different mechanisms. It is perhaps not surprising that KCC2 has evolved a distinct mechanism of isotonic transport, given the reciprocal regulation of Na$^+\cdotK^+\cdot2Cl^-$ and $K^+\cdotCl^-$ cotransport by cell volume (48, 54). The presence of this isotonic domain allows for separate regulation of constitutive and swelling-activated $K^+\cdotCl^-$ cotransport.

The interaction between cytoplasmic domains that control swelling-activated and constitutive transport is clearly an important issue for future study; however, the determinants of swelling-activated transport are as yet unknown. Regardless, there are already indications that KCC2 is regulated under isotonic conditions by pathways that are implicated in swelling activation (20). Thus the coexpression of SPAK and WNK4 kinases reduces swelling activation of KCC2 in Xenopus oocytes; the coexpression of kinase-dead SPAK, with a presumptive dominant-negative effect, results in a significant activation of constitutive isotonic transport, suggesting that some of the phospho-acceptor sites targeted by SPAK are phosphorylated by this kinase under isotonic conditions (20).

We have utilized Xenopus oocytes as the expression system in which to define a regulatory domain in KCC2, a neuronal specific transporter. Despite their acknowledged limitation as a model system for neurons, Xenopus oocytes have several advantages for this purpose; these include a low background activity of the endogenous $K^+\cdotCl^-$ cotransporter (65) and robust activity of expressed KCCs (7–11, 62). Of particular importance, the swelling-activated response in Xenopus oocytes replicates the in vivo behavior of the KCCs (13, 17, 54). Several other volume-sensitive transporters and ion channels have also been studied in detail in Xenopus oocytes, with the appropriate physiological response (20, 66–68). In addition, although measurement of $K^+\cdotCl^-$ cotransport in brain has generally been indirect (34–36, 69–71), it is evident that KCC2 mediates constitutive Cl$^-$ transport in neurons, as it does in Xenopus oocytes. Notably, however, the isotonic activity of KCC2 does not require expression in neuronal cells, suggesting that the isotonic domain serves to either change the intrinsic characteristics of the transporter or to alter its interaction with cellular factors that are common to both neurons and oocytes. That being said, it remains to be seen how this domain is affected by expression in mature neuronal cells. Indeed, it appears that post-translational modification is required for the full expression of KCC2 activity in mature neurons (72).

All of the KCC4-KCC2 chimeras demonstrate a reproducible gain-
of-function in isotonic transport; for example, the K442 chimera has ~4-fold higher activity than that of KCC2 under isotonic conditions (Fig. 1). We assume that the inclusion of the first 665 or the first 971 residues of KCC4 in these chimeras (see Table 1) plays a major role in this phenomenon. One possibility is that the central core of KCC4 has intrinsically higher transport activity; however, we note that swelling-activated transport mediated by these chimeras was equivalent or even lower than that of the full-length KCC2 or KCC4 proteins (Figs. 1 and 3). An alternative explanation is that other domains within KCC2 serve to tonically inhibit constitutive transport. The greater isotonic transport mediated by the K4SD-2 and K4SD-3 chimeras is particularly interesting in this regard, because the first predicted PEST domain of KCC2 (see Fig. 2) is deleted from both of these proteins. PEST domains (21) and related sequences (73) can serve to target proteins for calpain-dependent degradation, such that the loss of this sequence may reduce proteolytic degradation of the transporter. Prominent examples of transport proteins that are regulated by calpain-dependent proteolysis include N-methyl-d-aspartic acid receptor subunits (74), the GLT1 glycine transporter (75), and the ABCA1 lipid transporter (76).

In summary, by using KCC4-KCC2 chimeras we have identified a KCC2-specific C-terminal domain that is both necessary and sufficient for constitutive K⁺-Cl⁻ cotransport under isotonic conditions. To our knowledge, this is the first identification of a discrete cytoplasmic domain that modulates the volume sensitivity of a given class of ion transporter. The effect of this domain is independent of the reciprocal regulation that governs the volume sensitivity of the Na⁺-K⁺-2Cl⁻ and K⁺-Cl⁻ cotransport.

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