Dependence of KCC2 K-Cl cotransporter activity on a conserved carboxy terminus tyrosine residue

KEVIN STRANGE, THOMAS D. SINGER, REBECCA MORRISON, AND ERIC DELPIRE

Anesthesiology Research Division, Laboratories of Cellular and Molecular Physiology, Departments of Anesthesiology and Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232

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Strange, Kevin, Thomas D. Singer, Rebecca Morrison, and Eric Delpire. Dependence of KCC2 K-Cl cotransporter activity on a conserved carboxy terminus tyrosine residue. Am J Physiol Cell Physiol 279: C860–C867, 2000.—K-Cl cotransporters (KCC) play fundamental roles in ionopic and osmotic homeostasis. To date, four mammalian KCC genes have been identified. KCC2 is expressed exclusively in neurons. Injection of Xenopus oocytes with KCC2 cRNA induced a 20-fold increase in Cl− dependent, furosemide-sensitive K+ uptake. Oocyte swelling increased KCC2 activity 2–3-fold. A canonical tyrosine phosphorylation site is located in the carboxy termini of KCC2 (R1081–Y1087) and KCC4, but not in other KCC isoforms. Pharmacological studies, however, revealed no regulatory role for phosphorylation of KCC2 tyrosine residues. Replacement of Y1087 with aspartate or arginine dramatically reduced K+ uptake under isotonic and hypotonic conditions. Normal or near-normal cotransporter activity was observed when Y1087 was mutated to phenylalanine, alanine, or isoleucine. A tyrosine residue equivalent to Y1087 is conserved in all identified KCCs from nematodes to mammals. Mutation of the Y1087 congener in KCC1 to aspartate also dramatically inhibited cotransporter activity. Taken together, these results suggest that replacement of Y1087 and its congeners with charged residues disrupts the conformational state of the carboxy terminus. We postulate that the carboxy terminus plays an essential role in maintaining the functional conformation of KCC cotransporters and/or is involved in essential regulatory protein-protein interactions.

neurons; potassium transport; cation-coupled chloride cotransport; cell volume regulation; furosemide

The potassium-chloride cotransporter plays fundamental roles in intracellular and extracellular ionic and osmotic homeostasis (4, 13, 14, 16, 20, 24). Extensive studies carried out on red blood cells have defined the basic properties of this transport pathway (4, 14). The cotransporter mediates electroneutral, obligatorily coupled, transmembrane K+ and Cl− movements. Potassium chloride flux is driven by the sum of the chemical potential differences for K+ and Cl−. Under normal physiological conditions, the K-Cl cotransporter functions as a solute efflux pathway driven by the outwardly directed chemical gradient for K+. The cotransporter is activated by cell swelling and plays an important role in cell volume regulation.

Gillen et al. (8) were the first to report the cloning of the K-Cl cotransporter protein, KCC1. Sequence analysis demonstrated that KCC1 is a member of the cation-chloride cotransporter (CCC) superfamily that includes the Na-K-2Cl cotransporter (NKCC1 and NKCC2) and the thiazide-sensitive Na-Cl cotransporter (NCC). The functional characteristics of heterologously expressed KCC1 resemble those of the K-Cl cotransporter in red cells and include activation by cell swelling and N-ethylmaleimide (NEM). KCC1 is expressed in numerous tissues, suggesting that it may be a “housekeeping” isoform responsible for cell volume regulation (8).

Three other K-Cl cotransporter isoforms have been cloned recently (8, 9, 17, 21). KCC3 is found predominantly in the heart, skeletal muscle, brain, and kidney (9, 17, 23). KCC4 is expressed widely; the most abundant expression is observed in heart and kidney (17).1 Both KCC3 and KCC4 are activated by cell swelling in heterologous expression systems (17, 23).

KCC2 is found only in neurons (20, 21, 24). Expression of this transporter varies during neuronal development (16, 24). Developmental regulation of KCC2 and the Na-K-2Cl cotransporter NKCC1 (22) appears to function critically in regulation of neuronal Cl− concentration. The concentration of Cl− in neurons has significant impact on GABAergic neuronal signaling and information processing (3, 24), which in turn may play an important role in neuronal development and synapse formation (7, 15, 19).

In addition to intracellular Cl− regulation, Payne (20) has proposed that KCC2 may function to regulate extracellular K+ concentration in the brain. During neuronal activity, extracellular K+ levels rise and must be tightly controlled to maintain normal neuronal function (18). Payne (20) has suggested that under
certain conditions, KCC2 may function as a KCl influx pathway, thereby reducing extracellular K⁺ levels.

The signaling mechanisms that regulate K-Cl cotransport are incompletely understood. Serine/threonine protein phosphatase inhibitors inhibit K-Cl cotransporter activity (2, 11, 26), whereas inhibition of protein kinase activity with NEM (12) or staurosporine (1) activates the transport pathway. The tyrosine kinase inhibitor genistein inhibits the effects of both NEM and staurosporine (6). In red cell ghosts, volume-sensitive K-Cl cotransport is increased by vanadate, a tyrosine phosphatase inhibitor, and decreased by genistein (25). K-Cl cotransport activity is substantially elevated in red cells from knockout mice deficient in the Fgr and Hck Src family tyrosine kinases (5). These findings indicate that both serine/threonine and tyrosine phosphorylation play important roles in KCC regulation. However, the identities of the phosphorylation substrate proteins are unknown.

The postulated physiological roles of KCC2 in the central nervous system underscore the importance of defining how the transporter is regulated. A canonical tyrosine kinase motif is located in the carboxy terminus of KCC2. This motif is also present in KCC4 but not in any of the other identified K-Cl cotransporters isoforms, suggesting that KCC2 and KCC4 may be uniquely regulated by direct tyrosine phosphorylation. To test this possibility, we developed KCC2 expression assays using cRNA-injected Xenopus oocytes and transiently transfected Chinese hamster ovary (CHO) cells. We demonstrate here that tyrosine phosphorylation events do not appear to be important for regulating KCC2 activity in heterologous expression systems. However, mutation of the tyrosine residue in the kinase motif dramatically alters transporter function. We suggest that this tyrosine residue, which is conserved in all identified KCC isoforms, may participate in regulatory protein-protein interactions and/or be responsible for maintaining the active conformational state of the cotransporter.

MATERIAL AND METHODS

Expression of K-Cl cotransporters in Xenopus oocytes. cDNA clones for rat KCC2 (rtKCC2) and rabbit KCC1 (rbKCC1) were generous gifts from Drs. John Payne (University of California, Davis) and Bliss Forbush (Yale University), respectively. The open reading frames of both clones were subcloned into the oocyte expression vector pBF (kindly provided by Dr. Bernd Falker, University of Tubingen). Linearized template DNA was transcribed in vitro by Sp6 RNA polymerase using a mMessage mMachine kit (Ambion, Austin, TX). Capped cRNA was precipitated in ethanol and resuspended in RNase-free H₂O.

Stage V and VI oocytes from Xenopus laevis were manually defolliculated and maintained in modified L-15 medium (Life Technologies, Gaithersburg, MD) diluted to an osmolality of 195 mosmol/kgH₂O. One day after isolation, oocytes were microinjected with 50 nl of cRNA solutions. In preliminary studies, oocytes were injected with 1–100 ng of cRNA coding for rtKCC2. The highest levels of cotransporter activity were observed 3–4 days after injection. Optimal expression was observed with injection of 27.5 ng of cRNA. Injection of larger amounts of cRNA reduced transport activity. Given these results, all additional studies were carried out using oocytes injected with 27.5 ng cRNA. Functional studies were performed 3 days after injection.

Transient transfection of Chinese hamster ovary cells. For transient transfections, KCC2 was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). Stock cultures of Chinese hamster ovary (CHO-K₁) cells were grown at 37°C and 5% CO₂ in Ham’s F12 medium containing 10% fetal bovine serum (HyClone, Logan, UT). For transfection, cells were seeded at ~70% confluence into 6-well culture dishes. After 24 h, cells were rinsed briefly with OptiMEM (Life Technologies), and the culture medium in each well was replaced with 1 ml of OptiMEM containing 1 μg DNA and 15 μg Lipofectamine (Life Technologies). After incubation for 5.5 h in a 37°C incubator, the DNA/Lipofectamine medium was replaced with Ham’s F-12 medium. This medium was changed after 24 h, and cells were allowed to grow for another 24 h before fluxes were performed.

[^86Rb] uptake assays. To prevent[^86Rb] uptake via the endogenous Na-K-Cl cotransporter and Na⁺-K⁺-ATPase, all uptake measurements in oocytes were performed using a Na⁺-free flux medium containing 1 mM ouabain. The flux medium contained 48 mM choline chloride, 3.5 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, and 5 mM HEPES (pH 7.4, 195 mosmol/kgH₂O). Medium osmolality was adjusted by addition or removal of sucrose. Chloride removal studies were performed by replacing Cl⁻ with NO₃⁻.

Oocytes were washed three times with flux medium and then transferred to individual wells of a six-well culture plate. Oocytes were incubated at room temperature in 300 μl of flux medium containing 25 μCi/ml[^86Rb]. Uptake was terminated by washing oocytes five times in ice-cold flux medium.[^86Rb] content was determined by liquid scintillation counting after digestion of individual oocytes in 200 μl of 0.1 N NaOH. Preliminary studies demonstrated that[^86Rb] uptake was linear for at least 1 h. All flux measurements were therefore performed using a 1 h uptake period.

[^86Rb] uptake measurements on transiently transfected CHO cells were performed using a Na⁺-free flux medium containing 140 mM N-methyl-d-glucamine (NMDG)-Cl, 5 mM KCl, 1.2 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, and 0.5 mM ouabain (pH 7.4, 95 mosmol/kgH₂O). Chloride removal studies were carried out by replacing NMDG-Cl with NMDG-NO₃⁻, KCl and MgCl₂ with K₂SO₄ and MgSO₄, and CaCl₂ with calcium gluconate.

Cells were washed three times with 2 ml of flux medium and then incubated with 2 ml of flux medium at room temperature. After 15 min, the medium was replaced with 2 ml of flux medium containing 5 μCi/ml[^86Rb] and incubated for 10 min. Uptake was terminated by washing cells three times in ice-cold flux medium.[^86Rb] content was determined by liquid scintillation counting after digestion of cells in 2 ml of 0.1 N NaOH. Preliminary studies demonstrated that[^86Rb] uptake was linear for at least 30 min. For both oocyte and CHO cell flux measurements, it was assumed that[^86Rb] was a congener of K⁺. All flux data are therefore expressed as rates of K⁺ uptake.

Mutagenesis. Single amino acid mutations were generated using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutations were verified by automated DNA sequencing.

Immunolocalization. Oocytes were embedded in TissueTek OCT compound (Sakura Finetek, Torrance, CA), frozen, and sectioned by cryostat into 5-μm thick sections. Sections were fixed in ice-cold 100% acetone for 3 min and then washed four times for 5 min each in PBS containing 0.05%...
Tween 20. After washing, sections were treated with blocking solution (1% BSA and 4% goat serum in PBS) for 30 min and then incubated overnight at 4°C with purified polyclonal KCC2 antibodies (16). Antibody-treated sections were then washed four times for 5 min each in PBS/0.05% Tween 20, incubated for 30 min with blocking solution, treated for 1 h with a Cy3-conjugated mouse anti-rabbit secondary antibody, and washed in PBS/0.05% Tween 20.

Immunofluorescence was visualized using a Nikon Eclipse E800 equipped with a Nikon Plan Apo ×100 objective lens (1.4 NA) and an Optronics DEI-750 color charge-coupled device camera (Optronics Engineering, Goleta, CA). Montages were generated from digitized images using Adobe Photoshop 4.0 and printed with a Tektronix Phaser 450 color printer (Tektronix, Wilsonville, OR). Immunolocalization of KCC2 was performed in parallel in oocytes injected with either wild-type or mutant cotransporter cRNA. Images were acquired using identical camera settings to determine semiquantitatively whether mutagenesis altered KCC2 trafficking.

**Chemicals.** Tyrosine kinase and phosphatase inhibitors were purchased from Calbiochem (La Jolla, CA) and Biomol (Plymouth Meeting, PA). Pervanadate was prepared fresh before each experiment by mixing 1 part 500 mM H₂O₂ with 50 parts of 10 mM Na₃VO₄.

**Statistical analysis.** Data are presented as means ± SE. Statistical significance was determined using Student’s two-tailed t-test for unpaired, independent means. When comparing three or more groups, statistical significance was determined by one-way analysis of variance using the Tukey-Kramer Multiple Comparisons Test. *P* ≤ 0.05 was taken to indicate statistical significance.

**RESULTS AND DISCUSSION**

**Functional characterization of KCC2 expressed in Xenopus oocytes.** Figure 1A illustrates the basic properties of KCC2 expressed in oocytes. Injection of KCC2 cRNA increased Na+–independent K⁺ uptake nearly 20-fold compared with oocytes injected with water. Exposure to 1 mM NEM increased K⁺ uptake in KCC2 oocytes ~35% (*P* < 0.02). Both basal and NEM-stimulated K⁺ flux were inhibited 60–80% (*P* < 0.0001) by 2 mM furosemide. Swelling oocytes by exposure to a 100 mosmol/kgH₂O bath solution increased K⁺ uptake ~2.5-fold (*P* < 0.0001). Replacement of Cl⁻ with NO₃⁻ or exposure to 2 mM furosemide inhibited K⁺ uptake in hypotonic medium 80–90% (*P* < 0.0001).

Native K-Cl cotransport activity is inhibited by the serine/threonine protein phosphatase inhibitors calyculin A and okadaic acid, indicating that phosphorylation regulates cotransporter function (2, 11, 26). As shown in Fig. 1B, 1 μM calyculin A inhibited both basal and swelling-induced K⁺ uptake in KCC2-injected oocytes by 65–70% (*P* < 0.0008).

Basal KCC2 activity is inhibited by cell shrinkage (Figs. 1B and 2). Calyculin A had no significant (*P* > 0.5) effect on K⁺ uptake in KCC2-expressing oocytes exposed to hypertonic (270 mosmol/kgH₂O) medium (Fig. 1B).

The effects of furosemide, NEM, calyculin A, and Cl⁻ removal are signature characteristics of K-Cl cotransport (8, 14) and demonstrate that KCC2 is functionally...
expressed in oocytes. The inhibitory effect of calyculin A demonstrates that serine/threonine phosphorylation events regulate KCC2 activity. NEM and swelling also increased significantly (P < 0.0001, Fig. 1A) furosemide-sensitive and Cl<sup>-</sup>-dependent K<sup><sup>+</sup></sup> uptake in water-injected oocytes, suggesting the presence of an endogenous component of K-Cl cotransporter activity.

Payne (20) has demonstrated that cell swelling has no effect on KCC2 activity when the transporter is expressed in HEK-293 cells. One possibility for the discrepancy in our results and those of Payne (20) is the difference in the magnitude of hypotonic shocks used in the two studies. KCC2-transfected HEK cells were exposed to an ~20% reduction in bath osmolality (20). Experiments illustrated in Fig. 1 utilized an ~50% hypotonic shock. The differences in degree of hypotonicity suggest that KCC2 might require larger increases in volume for activation. We therefore examined the volume sensitivity of KCC2 in more detail and compared it to KCC1, a ubiquitous K-Cl cotransporter isoform shown previously to be swelling activated (8).

Figure 2 shows the relationship between bath osmolality and K<sup><sup>+</sup></sup> uptake in water-injected control oocytes and oocytes expressing KCC2 or KCC1. KCC1 activity under basal conditions (i.e., 195 mosmol/kgH<sub>2</sub>O) was not significantly different (P > 0.8) from control oocytes. Reduction of bath osmolality to 165, 130, or 100 mosmol/kgH<sub>2</sub>O increased K<sup><sup>+</sup></sup> uptake 413%, 568%, and 561%, respectively.

In contrast to KCC1, K<sup><sup>+</sup></sup> uptake under basal conditions in KCC2-injected oocytes was ~10-fold higher than water-injected controls. Hypertonicity inhibited basal uptake ~50%. Exposure to hypotonic bath solutions of 165, 130, or 100 mosmol/kgH<sub>2</sub>O increased K<sup><sup>+</sup></sup> uptake 240%, 335%, and 332%, respectively. These results indicate that the overall osmotic responsiveness of K<sup><sup>+</sup></sup> uptake in KCC1- and KCC2-injected oocytes is similar. However, the degree of swelling-induced stimulation of KCC1 is substantially higher than that of the KCC2 cotransporter.

Interestingly, we have observed that swelling has no effect on KCC2-mediated K<sup><sup>+</sup></sup> uptake when the cotransporter is transiently or permanently transfected into Chinese hamster ovary (CHO) cells (data not shown), a finding in agreement with those of Payne (20). Data shown in Figs. 1–2 demonstrate clearly that the KCC2 cotransporter possesses the molecular features necessary for swelling-induced activation. The difference in KCC2 volume sensitivity observed in various heterologous expression systems may be due to differences in the proteins that regulate the cotransporter. For example, in *Xenopus* oocytes, there may be “promiscuous” interactions between KCC2 and regulatory proteins, such as the putative volume-sensitive kinase (11) thought to play a role in swelling-induced signal transduction. Alternatively, regulatory proteins responsible for KCC2 volume sensitivity that are present in *Xenopus* oocytes may not be expressed in HEK-293 and CHO cells.

**Regulatory role of a carboxy terminal tyrosine residue.** As discussed in the Introduction, tyrosine phosphorylation has been implicated in the regulation of K-Cl cotransport (5, 6, 25). The carboxy terminus of KCC2 contains a canonical tyrosine kinase phosphorylation site at R1081–Y1087 (17, 21). To examine the possible role of this site in tyrosine phosphorylation-dependent regulation of KCC2, we replaced Y1087 with aspartate (Y1087D) to mimic phosphorylation (e.g., Ref. 28). As shown in Fig. 3, this mutation reduced K<sup><sup>+</sup></sup> uptake under isotonic conditions 70–80% and completely inhibited swelling-induced KCC2 activation (P < 0.0001 compared with wild type). Hypertonicity inhibited K<sup><sup>+</sup></sup> uptake 41% in oocytes expressing wild-type KCC2 (P < 0.0001). A similar degree of inhibition (42.5%, P < 0.002 compared with wild type) was seen with the Y1087D mutant. Replacement of Y1087 with phenylalanine (Y1087F), which differs from tyrosine by a single hydroxyl group, restored normal basal and swelling-induced cotransporter activity. Hypertonicity inhibited K<sup><sup>+</sup></sup> uptake 38% (P < 0.0001) in Y1087F-expressing oocytes.

The dramatic reduction in transport activity of the Y1087D mutant suggested that it may not be trafficked to the membrane properly. To address this issue, we immunolocalized KCC2. As shown in Fig. 4, immunofluorescence levels were qualitatively similar in the plasma membranes of oocytes expressing wild-type or...
Y1087D KCC2. These results indicate that the Y1087D mutant is trafficked normally to the cell surface.

To examine further the role of tyrosine phosphorylation in KCC2 regulation, we treated oocytes with 100 μM dephostatin, a broadly selective tyrosine phosphatase inhibitor (27). As shown in Fig. 5, dephostatin had no significant \( P > 0.05 \) effect on basal KCC2 cotransporter activity. However, swelling-induced cotransporter activation was inhibited \( P < 0.05 \) nearly completely by the drug.

The inhibitory effect of dephostatin could be due to changes in the activity of tyrosine phosphatases that directly alter the cotransporter phosphorylation state. Alternatively, dephostatin may indirectly alter KCC2 function by disrupting the activity of other signaling and/or regulatory pathways. To test for this possibility, we examined the effects of dephostatin on the Y1087F mutant. As observed with wild-type KCC2, the drug had no effect on basal transport activity. However, swelling-induced cotransporter activity was inhibited \( P < 0.003 \), (Fig. 5).

The inhibitory effect of dephostatin on swelling-induced \( K^+ \) uptake in oocytes expressing the Y1087F mutant implies that direct phosphorylation of Y1087 does not downregulate KCC2 cotransporter activity. Instead, these findings are more consistent with those

Fig. 4. The Y1087D KCC2 mutant is trafficked normally to the plasma membrane. KCC2 was immunolocalized using anti-KCC2 purified polyclonal antibodies and a Cy3-labeled secondary antibody in water-injected (H2O) oocytes and in oocytes expressing wild-type (WT) KCC2 or the Y1087D mutant.

Fig. 5. Effect of the tyrosine phosphatase inhibitor dephostatin on basal (isotonic, Iso) and swelling-induced (hypotonic, Hypo) cotransporter activity in oocytes expressing wild-type or Y1087F KCC2. Oocytes were incubated with 100 μM dephostatin for 20 min prior to performing flux measurements and throughout the 1-h uptake period. Values are means ± SE \( (n = 36–56 \text{ oocytes}) \).

Fig. 6. Functional properties of KCC2 transiently expressed in Chinese hamster ovary (CHO) cells. A: transient transfection of CHO cells with KCC2 cDNA induces Cl\(^-\)-dependent and furosemide (Furo)-sensitive \( K^+ \) uptake. CHO cells were transiently transfected with KCC2 cDNA or empty vector (mock transfection). Potassium uptake was measured over a 10-min period in Na\(^+\)-free medium containing 0.5 mM ouabain. In Cl\(^-\)-free medium, Cl\(^-\) was replaced with NO\(_3\). Values are means ± SE \( (n = 6–11) \). B: effects of tyrosine phosphatase and kinase inhibitors on \( K^+ \) uptake in CHO cells transfected with KCC2 cDNA. Results are presented as rates relative to those observed in mock transfected cells treated with the same agents. Cells were exposed to the drugs during the 15-min equilibration period and throughout the 10-min uptake period. No significant \( P > 0.05 \) inhibitory effect was observed for any of the drugs tested. Dephostatin appeared to induce a small but significant \( P < 0.001 \) stimulation of \( K^+ \) uptake. Values are means ± SE \( (n = 4–12) \).
of De Franceschi et al. (5). These investigators proposed that tyrosine phosphorylation mediated by Fgr and Hck tyrosine kinase activity negatively regulates serine/threonine protein phosphatase 1 (PP-1). Increased phosphorylation of substrate proteins brought about by inhibition of PP-1 leads to reduced cotransport activity. Inhibition of tyrosine phosphatase activity by dephostatin could have a similar negative regulatory effect on PP-1, thereby reducing KCC2 function.

The results obtained using oocytes treated with dephostatin were extremely variable. For example, in one experiment we observed that dephostatin completely inhibited swelling-induced activation of the Y1087F mutant, whereas in two other experiments it had little or no effect.

In our hands, we have observed that certain types of pharmacological experiments in oocytes are often difficult to reproduce and interpret. We therefore developed a KCC2 flux assay using CHO cells. As shown in Fig. 6A, transient transfection of CHO cells with KCC2 cDNA increased K+ uptake approximately threefold \((P < 0.001)\). Replacement of Cl\(^-\) with NO\(_3\) or application of 2 mM furosemide completely inhibited K+ uptake in KCC2-expressing cells \((P < 0.001)\) but had no inhibitory effect \((P > 0.05)\) on mock transfected cells. These studies demonstrate clearly that 1) CHO cells do not normally express Cl\(^-\)-dependent and furosemide-sensitive K+ transport and 2) that transient transfection with KCC2 cDNA induces robust K-Cl cotransporter activity.

We tested the effects of tyrosine phosphatase (dephostatin and pervanadate) and kinase (genistein) inhibitors on KCC2-mediated K+ uptake in transiently transfected cells. As shown in Fig. 6B, 50 \(\mu\)M dephostatin, 100 \(\mu\)M pervanadate, or 100 \(\mu\)M genistein had no significant \((P > 0.05)\) inhibitory effect on K+ uptake. Taken together with results obtained in Xenopus oocytes (Fig. 5), these findings indicate that tyrosine phosphorylation of the KCC2 protein does not play an important role in regulating its activity.

Y1087, as well as a number of other amino acid residues located in the carboxy terminus of KCC2, are conserved in all of the identified mammalian and nonmammalian K-Cl cotransporters (8–10, 17, 21) (Fig. 7). This conservation of primary structure indicates that the carboxy terminus likely plays an important regulatory role in KCC function.

The unambiguous inhibitory effect of the Y1087D mutation (Fig. 3), coupled with the conservation of the equivalent tyrosine residue in all identified KCC isoforms of KCC2, is further highlighted by the red box. Gaps are added to obtain best alignment. Alignments were performed using AlignX from VectorNTI 5 Suite (Informax, North Bethesda, MD).

### Table 1. Effect of various amino acid substitutions at Y1087 on KCC2-mediated K+ uptake in Xenopus oocytes

<table>
<thead>
<tr>
<th>Injection</th>
<th>195 mosmol/ (\text{kg}H_2O)</th>
<th>100 mosmol/ (\text{kg}H_2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(H_2O)</td>
<td>92 ± 5 (17)</td>
<td>99 ± 6 (17)</td>
</tr>
<tr>
<td>Wild type</td>
<td>831 ± 43 (19)</td>
<td>1,801 ± 53 (23)</td>
</tr>
<tr>
<td>Y1087A</td>
<td>500 ± 31 (16)</td>
<td>939 ± 107 (15)</td>
</tr>
<tr>
<td>Y1087I</td>
<td>522 ± 22 (26)</td>
<td>1,348 ± 36 (25)</td>
</tr>
<tr>
<td>Y1087R</td>
<td>116 ± 9 (26)</td>
<td>160 ± 7 (27)</td>
</tr>
</tbody>
</table>

Values are means ± SE; no. of oocytes is in parentheses. KCC2, K-Cl cotransporter.
forms and across widely divergent species (Fig. 7), suggested that this amino acid plays a key functional role in K-Cl cotransport. To test this hypothesis, we mutated the equivalent tyrosine residue, Y1056, to aspartate in KCC1. Mutant and wild-type KCC1 were expressed in Xenopus oocytes. As shown in Fig. 8, the Y1056D mutation completely inhibited swelling-induced activation of KCC1 (P < 0.001). When Y1056 was replaced with phenylalanine, swelling-induced activation of KCC1 was observed, albeit at a rate ~50% lower (P < 0.001) than that of the wild-type transporter. As with the Y1087D form of KCC2, we observed by immunofluorescence that the Y1056D KCC1 mutant appeared to be trafficked to the cell surface normally (data not shown).

On the basis of the data shown in Figs. 5, 6, and 8, we conclude that 1) KCC2 Y1087 does not regulate cotransporter activity by functioning as a site for regulatory tyrosine phosphorylation and 2) that this conserved residue (see Fig. 7) plays an important functional role in all identified KCC isoforms. Given this conclusion, it was of interest to determine the functional consequences of other amino acid substitutions at Y1087. As shown in Table 1, near-normal K-Cl uptake is observed in oocytes injected with KCC2 mutants in which Y1087 was substituted with amino acids possessing either short (alanine) or long (isoleucine) aliphatic side chains. These results are similar to those observed when tyrosine is substituted with the aromatic side chain amino acid phenylalanine (Figs. 3 and 8). However, replacement of tyrosine with the positively charged arginine caused nearly complete loss of cotransporter activity under both basal and swelling conditions similar to that of aspartate (Figs. 3 and 8). These results indicate that the presence of a charged residue at Y1087 disrupts the conformation of the carboxy terminus and/or protein-protein interactions that are essential for normal cotransporter activity.

Conclusions. In summary, our studies have identified a conserved tyrosine residue located in the carboxy terminus of K-Cl cotransporters that plays an important role in normal cotransporter function. Mutation of this tyrosine to charged residues dramatically inhibits cotransporter activity. We postulate that this conserved tyrosine residue, as well as other highly conserved amino acids in the carboxy termini of all identified K-Cl cotransporters (see Fig. 7), are required to maintain the active conformational state of the proteins and/or that they function as sites for essential regulatory protein-protein interactions.

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