Published in final edited form as:

Cell Physiol Biochem. 2008 ; 22(1-4): 31–44. doi:10.1159/000149781.

The RCC1 domain of Protein Associated with Myc (PAM) interacts with and regulates KCC2

Nicole Garbarini and Eric Delpire

Neuroscience Graduate Program and Department of Anesthesiology, Vanderbilt University Medical Center, Nashville, TN 37232

Abstract

GABAergic and glycinergic function is dependent on neuronal intracellular chloride. The neuron-specific electroneutral potassium (K^+) and chloride (Cl^-) cotransporter (KCC2), is a key regulator of neuronal Cl^- , yet little is known about KCC2 regulation. Using yeast two-hybrid, we identified Protein Associated with Myc (PAM) as a binding partner of KCC2. The RCC1 (Regulator of Chromatin Condensation) domain of PAM binds to the carboxyl terminus of KCC2, as demonstrated through yeast two-hybrid and GST-pull-down assays. RCC1/PAM and full-length KCC2 coimmunoprecipitate following heterologous co-expression in HEK293 cells. Additionally, $^{86}Rb/K^+$ uptake assays in this model system show that RCC1/PAM causes increased KCC2-mediated flux. After narrowing down RCC1/PAM binding to a 20 amino acid region on the KCC2 carboxyl terminus, we created a point mutant in this region to eliminate interaction between the KCC2 carboxyl terminus and RCC1/PAM. This same mutation abolishes N-ethylmaleimide activation of KCC2, suggesting that PAM plays a role in modulating KCC2 function.

Keywords

K-Cl cotransport; N-ethylmaleimide; Protein interaction; Heterologous expression; HEK293 cells

INTRODUCTION

Neuronal signaling via the ionotropic GABA_A and glycine receptors is dependent on the intracellular Cl^- level of the postsynaptic neuron. High internal Cl^- results in GABA eliciting depolarizing responses, as seen in immature central neurons [1-4] and in sensory neurons throughout life [5-7]. In contrast, most mature neurons have low internal Cl^- concentrations, facilitating the characteristic hyperpolarizing GABA response, and thus synaptic inhibition [4,8-10].

KCC2 is driven by the high intracellular K^+ concentration generated by the Na^+/K^+ pump, thus allowing the cotransporter to 'actively' extrude Cl^- in an electroneutral manner [10-12]. Our laboratory demonstrated that KCC2 activity maintains low intracellular Cl^- during synaptic activity, and is critical in establishing the large Cl^- gradient that favors GABA inhibition [4]. This function is absent in early postnatal development as KCC2 expression is low, but develops and strengthens during the first two weeks of postnatal life, concomitant to an increase in cotransporter expression [13-16].

The significance of KCC2 in regulating neuronal excitability is made evident by several animal models of cotransporter disruption. Mice completely lacking KCC2 die soon after birth due to respiratory distress and hypoxia [17]. Mice with a 95% reduction of total KCC2 expression exhibit tonic-clonic seizures and die approximately two weeks after birth, whereas mice with ~50% KCC2 reduction exhibit increased susceptibility to epileptic seizures [18]. Furthermore, KCC2 expression is altered in the rodent brain after epileptiform brain activity or stimulation [19]. This function is conserved in evolution, as mutations in the *Drosophila* orthologue of KCC2 also lead to a significant reduction in seizure threshold [20]. Additionally, KCC2 expression is altered in brain tissues isolated from human epilepsy patients [19,21-24].

Little is known about the regulation of KCC2 gene expression and the modulation of KCC2 function in neurons. Studies have shown that KCC2 function is in part influenced by Brain Derived Neurotrophic Factor (BDNF) activation of tyrosine kinase B (TrkB) receptors. The effect of BDNF on KCC2 may be dependent on development stage, as mice overexpressing the neurotrophic factor have been shown to express higher levels of KCC2, whereas exogenous application of BDNF to brain slices from adult mice causes decreases in KCC2 activity by increasing the rate of cell surface KCC2 degradation [25,26]. KCC2 function may also be linked to tyrosine phosphorylation pathways, as stimulation of insulin growth factor receptors and application of the cytosolic tyrosine kinase c-Src rapidly activates KCC2 transport in cultured hippocampal neurons [27].

Many membrane-bound ion transporters and channels are regulated through protein-protein interactions. Some studies indicate KCC2 forms homodimers and oligomers, but how this affects cotransporter activity is unknown [28-30]. Brain creatine kinase (CKB) has been shown to interact with KCC2 [31] and further study in mouse brain slices indicates it participates in activating KCC2 function [32]. The dendritic cytoskeletal protein 4.1N has been also shown to interact with KCC2, possibly linking KCC2 activity to coordination of synaptogenesis [33].

Here we report a novel interaction between KCC2 and Protein Associated with Myc, or PAM (also known as Phr1 [34] or MycBP2 in GenBank). Originally characterized as a myc binding protein [35], PAM is a large, widely expressed protein which is highly enriched in the brain and present throughout neurons [36]. We show that a portion of PAM within its RCC1 (Regulator of Chromatin Condensation) domain interacts with the extreme carboxyl terminus of KCC2, and that this interaction plays a role in modulating KCC2 function.

MATERIALS AND METHODS

Yeast Two-Hybrid

The carboxyl terminus of the rat KCC2 (KCC2-CT), from amino acid residue 635 to amino acid residue 1116, was moved from an existing KCC2 cDNA clone and fused to the Gal4 binding domain in the pGBDUC2 vector. KCC2-CT-pGBDUC2 was transformed into PJ69-4A yeast cells [37], and then used to screen an amplified mouse brain cDNA library in the pACT2 vector (Clontech, Mountain View, CA) as previously described [38]. Following small-scale yeast two-hybrid transformations, yeast was plated on double-dropout (-Uracil, -Leucine) plates to ensure both the pACT2 and pGBDUC2 clones were transformed. Growth of transformed yeast plated or re-streaked onto triple-dropout (-Uracil, -Leucine, -Histidine + 2 mM 3-amino-1,2,4-triazole) plates indicated positive protein-protein interactions. A total of 6×10^6 million clones were screened, as determined by library titers. After a second re-streak onto triple-dropout plates, surviving clones were tested using liquid Lac-Z/ β -galactosidase assays. Yeast clones with the highest β -galactosidase expression and activity were lysed with 10 μ l 5x Reporter Lysis Buffer (Promega, Madison, WI), and subjected to PCR using the

ExpandLong Template PCR kit (Roche, Indianapolis, IN). Amplified PCR fragments were sequenced with ABI Prism Big Dye Terminator (Applied Biosystems, Foster City, CA).

DNA constructs and point mutations

Truncations of the KCC2-CT were generated by PCR, Expand Long PCR (Roche, Indianapolis, IN) or annealed oligonucleotides (for sequences under 40 bp) with overhangs allowing for subcloning into the desired vectors. To generate the RXR KCC2 mutant, a 513 bp *SphI*-*ClaI* fragment consisting of the last 56 amino acids of rat KCC2, some 3'UTR and some polylinker were subcloned into a modified pBSK vector containing a *SphI* restriction site. The codon encoding the second arginine (CGC) of the RXR motif was mutated into an alanine (GCC) using QuikChange (Stratagene, La Jolla, CA). After sequencing, the *SphI*-*ClaI* fragment was re-introduced into the wild-type clone to produce the mutant KCC2 cDNA.

Glutathione S-Transferase Pull-down Assay

In order to create Glutathione S-Transferase (GST) fusion proteins, KCC2-CT cDNA was cloned into the pGEX4T1 vector (Pharmacia, Piscataway, NJ) inserted after the vector's GST encoding sequence. KCC2-CT-pGEX4T1 or empty pGEX4T1 (for GST only controls) were transformed into a protease-deficient strain of *Escherichia coli*. During growth, protein production was induced with isopropyl β -D-1-thiogalactopyranoside (IPTG, 24mg/ml), and bacteria were lysed by freeze-thawing, sonication, and incubation with 20% Triton X-100. Lysates were centrifuged, and supernatants incubated with 50% Sepharose-Glutathione bead slurry. Following three spin/wash cycles, the pull-down assay was performed as previously described [38], incubating GST protein-bound beads with HEK cell lysates.

Cell culture and transfection

HEK 293 or HEK 293FT (Invitrogen, Carlsbad, CA) cells were maintained and routinely passaged in DMEM-F12 supplemented by 10% fetal bovine serum and 1% Penicillin/Streptomycin (Invitrogen). Transfections were performed with Fugene 6 (Roche) on cells that were approximately 50-70 percent confluent.

Coimmunoprecipitation

Transfected HEK 293FT cells (Invitrogen) were lysed with 1% Triton X-100 lysis buffer (150 mM NaCl, 10 mM Tris, 2 mM EDTA, 1% Triton X-100) containing protease inhibitors (Roche) for 30 min on ice. Lysates were scraped into 1.5 ml tubes and microcentrifuged for 10 min at 14,000 rpm at 4°C. KCC2 was immunoprecipitated from the supernatant with 7 μ l of polyclonal KCC2 antibody overnight at 4°C. Protein A-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA), pre-washed with lysis buffer, were incubated with the supernatant for 2 h at 4°C. Antibody-protein complexes were pulled down by centrifugation for 4 min at 4,000 rpm. Pelleted beads were subjected to three spin/washes with 1 ml lysis buffer, then resuspended in sample buffer containing 5% β -mercaptoethanol and denatured at 70°C for 20 min.

Western Blotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) using a semidry transfer apparatus. Membranes were blocked in 5% dry non-fat milk/TBST for 2 h at room temperature before incubation with antibody in milk/TBST overnight at 4°C. Antibody dilutions: KCC2, 1:1000 dilution; HRP-conjugated HA, 1:1000 (Roche, Indianapolis, IN), human transferrin receptor, 1:2500 (Invitrogen, Carlsbad, CA).

Microsome preparation

Cells grown in 100-mm dishes were scraped in sucrose solution (0.32 M sucrose, 2 mM EDTA, 2.5 mM β -mercaptoethanol, 5 mM Tris-Cl, pH 7.5, and protease inhibitors) and homogenized with a Teflon pestle for 20 strokes on ice. Cell debris was removed by centrifugation at $4,000 \times g$ for 15 min. The recovered supernatant was then centrifuged for 15 min at $9000 \times g$. The pellet from a final high-speed centrifugation ($100,000 \times g$) was resuspended in 500 μ l sucrose solution.

$^{86}\text{Rb}^+$ uptake

Transfected HEK293 cells were used two days post-transfection for $^{86}\text{Rb}^+$ uptake experiments. Cells were washed twice with room temperature isotonic Na^+ -free solution (150 mM NMDG-Cl, 5 mM KCl, 2 mM CaCl_2 , 0.8 mM MgSO_4 , 5 mM glucose, 5 mM HEPES, pH 7.4) then preincubated for 15 or 20 min with Na^+ -free solution plus 100 μ M ouabain. Following preincubation, cells were then incubated with Na^+ -free solution containing ouabain and $^{86}\text{Rb}^+$ at a concentration of 1 $\mu\text{Ci}/\mu\text{l}$. For N-ethylmaleimide (NEM) stimulation experiments, NEM was dissolved in DMSO and applied to cells in a final concentration of 1mM, for a total of 15 min. Vehicle (DMSO) alone was added as a control in non-stimulated flux conditions. Uptake was terminated by three washes with ice cold Na^+ -free solution. Cells were lysed with 500 μ l 1N NaOH for 1 h, and then neutralized with 250 μ l glacial acetic acid. $^{86}\text{Rb}^+$ uptake was measured from 150 μ l of cell lysate added to 5 ml Biosafe II scintillation cocktail (RPI, Mount Prospect, IL). Protein quantitation of lysates was determined by Bradford assay (BioRad, Hercules, CA). Final measurements were calculated as pmoles K^+ per μg protein. Statistical analyses were performed by One Way ANOVA and Tukey post-test using GraphPad Prism software.

RESULTS

Identification of a 323 amino acid portion of PAM which binds to the carboxyl terminus of KCC2

The carboxyl-terminal tail of rat KCC2 (KCC2-CT) was used to screen a two-hybrid mouse brain cDNA library. Among the positive clones, a portion of the RCC1 domain of Protein Associated with Myc (PAM, residues 551-874) was identified as a potential interactor of KCC2-CT (Figure 1, Panel A). RCC1 (Regulator of Chromatin Condensation) domains were first characterized in the RCC1 protein for cell cycle related effects in the nucleus, but have since been found in several cytoplasmic proteins, participating protein localization and protein-protein interactions [39-44]. A small scale yeast two-hybrid transformation to test this library clone against the empty bait vector shows that the library clone does not activate yeast reporter genes independent of interaction with KCC2-CT (Figure 1, Panel B).

The interaction between KCC2-CT and RCC1/PAM was confirmed outside of the yeast two-hybrid system using a GST pull-down assay. Lysates from HEK293 cells expressing HA-tagged RCC1/PAM were incubated with GST-KCC2-CT immobilized on GST-Sepharose beads. As seen in Figure 1, Panel C, only GST-KCC2-CT was able to pull-down RCC1/PAM (lane 2), whereas GST alone did not pull down RCC1/PAM.

We likewise used coimmunoprecipitation assays to test interaction between RCC1/PAM and full-length KCC2 *in vivo*. RCC1/PAM was tagged with an HA epitope, and cotransfected into HEK293FT cells with either empty pCDNA3 vector or full-length KCC2. Cell lysates were subjected to immunoprecipitation using the KCC2 antibody, and protein complexes were then isolated with Protein A-Sepharose beads. Immunoblotting with HA antibody shows that RCC1/PAM is immunoprecipitated with KCC2 (Figure 1, Panel D, lane 4). However, no RCC1/PAM was pulled-down in the absence of KCC2 (lane 2).

RCC1-PAM increases KCC2-mediated flux

To assess the effect of PAM on KCC2 function, we co-transfected the RCC1/PAM fragment with full length KCC2 in HEK293 cells. RT-PCR experiments showed that HEK293 cells express native PAM (data not shown). Function of the cotransporter was assessed using unidirectional $^{86}\text{Rb}^+/\text{K}^+$ uptakes. Cells were transfected with KCC2 and/or RCC1/PAM two days prior to functional assays, and uptakes were measured at 6 min intervals over 30 min. As seen in Figure 2, KCC2 expression in HEK cells resulted in large Na^+ -independent K^+ uptake, several fold greater than baseline (empty pCDNA3 transfected). Interestingly, co-expression of the partial PAM clone increased KCC2-mediated flux.

RCC1/PAM effect on KCC2 protein and RNA expression

To account for the increased KCC2 flux, we first considered the possibility that RCC1/PAM affects the amount of functional KCC2 protein. To quantitate the KCC2 protein levels, we prepared HEK293 cell microsomal fractions to enrich for membrane-associated proteins. Microsomes were subjected to Western blot analysis, and probed with anti-KCC2 antibody or with anti-human transferrin antibody to control for unspecific effects by RCC1/PAM. As shown in Figure 3, KCC2 levels increased significantly when the cotransporter was co-expressed with RCC1/PAM. This effect is RCC1-specific, as transfections were controlled for amount of DNA by using empty pcDNA3 vector. As an additional control, co-expression of a similarly sized non-relevant protein, GST, did not result in an increased in KCC2 flux (data not shown).

To determine whether the increased KCC2 protein expression by RCC1/PAM originates from increased transcription, we examined KCC2 RNA levels under the different transfection conditions. Using semi-quantitative RT-PCR with primers specific to KCC2, we showed that RCC1/PAM increases KCC2 RNA levels in HEK293 cells (Figure 4).

Location of RCC1/PAM binding on the KCC2-CT

The effect of RCC1/PAM on KCC2 transcription could account for the increased KCC2 flux observed in Figure 2. However, as RCC1/PAM also participates in protein-protein interaction with the carboxyl terminal tail of the cotransporter, this interaction could also affect cotransporter function, independent of the transcriptional effect. Thus, we decided to further characterize the site of RCC1/PAM binding on KCC2. In order to identify this site, we created truncation mutants of the KCC2-CT and tested them in small-scale yeast two-hybrid transformations against the PAM library clone (Figure 5A-C). Using this method, we were able to identify a 20 amino acid sequence of the KCC2-CT, which retains the ability to bind to this portion of PAM (Figure 5A-C, g). Since a tyrosine residue in the KCC2-CT had previously been shown to be critical for KCC2 function via a mechanism independent of phosphorylation [45], we considered that PAM binding to KCC2 might also involve this tyrosine residue. We found that PAM binding to the KCC2-CT was unaffected by mutation of this tyrosine residue into an alanine (Figure 5A-C, k).

KCC2 point mutant, KCC2-RXA, disrupts RCC1/PAM interaction

Since the 4 K-Cl cotransporters, KCC1-KCC4, have highly conserved carboxyl-terminal tails, we analyzed the sequence of the corresponding RCC1/PAM binding region in the 3 other cotransporters. As shown in Figure 6A, the PAM-binding region of KCC2 is very similar to that of the other potassium-chloride cotransporters. We took advantage of the few sequence differences to elucidate the specific amino acid residues responsible for this protein-protein interaction. Thus, these analogous regions were tested against RCC1/PAM in 'small scale' yeast two-hybrid experiments. Figure 6A shows that the RCC1/PAM fragment only interacts with this region in KCC2, but not with the corresponding regions in the other K-Cl

cotransporters. As highlighted by the sequence alignment, we identified an RXR motif within this 20 amino acid region of KCC2, while this motif is not present in the analogous sequences of the other cotransporters. This observation is particularly interesting as RXR motifs are known to function as ER retention signals. To explore the importance of the non-conserved residues and the RXR motif, we created single amino acid mutations in this region of both KCC2 and KCC3. As anticipated, disrupting the motif by substituting the second arginine residue into an alanine (RXA) prevented binding to partial RCC1/PAM (Figure 6B). However, introducing an RXR motif into this portion of KCC3 did not confer PAM binding (Figure 6B). Thus, this observation suggested that binding was not only dependent on the RXR motif, but also on neighboring amino acid residues. Indeed, introduction of a glutamic acid directly downstream of the RXR motif in KCC2 (mimicking KCC3), was sufficient to disrupt PAM interaction with KCC2 (Figure 6B).

Impact of KCC2-RXA mutant on ability of PAM to elevate RNA and protein levels, but not cotransport function

To assess the effect of preventing RCC1/PAM interaction with the C-terminal region, the RXA mutation was introduced into full-length cotransporter, thus referred to as KCC2-RXA. HEK293 cells were transfected with KCC2-RXA in the presence or absence of the RCC1/PAM fragment. As a control and point of comparison, transfection of wild-type KCC2, in the presence or absence of RCC1/PAM, was repeated alongside the KCC2-RXA transfections. Western blot and RT-PCR analysis showed that co-expressing RCC1/PAM with KCC2-RXA resulted in increased KCC2 RNA and protein levels, similar to those observed with wild-type KCC2 (Figure 7). Surprisingly, we also observed co-immunoprecipitation of KCC2-RXA and HA-tagged-RCC1/PAM (Figure 8), suggesting that the PAM fragment not only interacts with the carboxyl terminus, but also interacts with KCC2 somewhere else in addition to the 20 amino acid region identified by yeast 2-hybrid. Functional studies revealed that the RXA mutation prevented the increased cotransporter function by RCC1/PAM that we observed with wild-type KCC2. Furthermore, KCC2-RXA had no significant change in activity as compared to wild-type KCC2 (Figure 9).

Mutation of PAM binding site on KCC2 disrupts NEM stimulation of transport

N-ethylmaleimide (NEM) is a known activator of K-Cl cotransport in many cell types [46-49], including KCC2 flux in HEK293 cells [9,50]. In agreement with the literature, we show that NEM induced activation of KCC2-mediated K^+ uptake in HEK293 cells (Figure 10). Again, we observed in cells co-transfected with RCC1/PAM and wild-type KCC2 a significant increase in $^{86}Rb/K$ uptake. However, NEM did not further stimulate the KCC2-mediated flux induced by RCC1/PAM expression. Furthermore, KCC2-RXA function which was shown to be unaffected by RCC1/PAM expression, was also unaffected by NEM treatment of the cells.

DISCUSSION

Using a library-scale yeast two-hybrid screen, we have identified PAM as an interactor with the carboxyl-terminal tail of KCC2. PAM is a large 4264 amino acid protein expressed in many tissues, including in central and peripheral neurons. The interaction of PAM with KCC2 is significant when considering that 1) the two proteins are similarly up-regulated during postnatal neuronal maturation [14,51], 2) in the rodent brain, PAM is highly expressed in the pyramidal cells of the hippocampus, granule cells of the dentate gyrus and cerebellum, as well as Purkinje cells of the cerebellum — regions in which KCC2 likewise has high levels of expression [51, 52], 3) the two proteins are involved in spinal nociceptive processing [53-55,56 (review),57 (review)], and 4) disruption of both genes results in a similar phenotype of respiratory failure and postnatal death [17,18,34]. Furthermore, the PAM homologues in *C. elegans* (*rpm-1*), *D. melanogaster* (*highwire*), and *Danio rerio* (*Esrom*) have been shown to be involved in

synaptogenesis, synaptic growth, and presynaptic organization [58-61], indicating that PAM, like KCC2, plays an important role in neuronal physiology.

Since PAM is a large protein with several specialized domains, it is highly likely that it fulfills numerous functions in the cell. PAM contains several ubiquitin ligase domains on its extreme carboxyl terminal end, and a myc-binding domain proximal to these ubiquitin ligase regions [35]. The protein has been described to have alternative splice variants, indicating that it may further expand its repertoire of functions through post-translational processing [62]. Additionally, the RCC1 domain of PAM has been shown to function as an adenylyl cyclase inhibitor [63,64], and PAM protein has also been shown to interact with tubulin [36].

A critical requirement for the validity of the protein-protein interaction is that both proteins be expressed in the same cellular compartment. Since KCC2 subcellular localization in neurons is predominantly in the postsynaptic areas and in cell soma [65,66], it is important that PAM be also expressed in these compartments. Despite the fact that non-mammalian homologues of PAM indicate a presynaptic role of the protein, mammalian PAM was shown to be present throughout neurons [36,67].

Through deletions and small-scale yeast two-hybrid transformations, we were able to narrow down the interacting region to rat KCC2 residues 1069-1088, a 20 amino acid domain located between the extreme C-terminus and the region unique to KCC2 which confers constitutive basal activity in isosmotic conditions [68]. The region of KCC2 encompassing this domain and the extreme C-terminus is known to be essential for cotransporter function. The 20 amino acid peptide interacting with PAM contains a putative non-canonical type II Src homology domain 3 (SH3) binding sequence ($\Phi P x \Phi P x +$) [69] that is directly adjacent to a putative RXR motif [70]. SH3 domains are ubiquitous protein interaction modules that are involved in many types of protein-protein interactions: (i) they participate in linking membrane transporters and channels to the cytoskeleton [71,72]; (ii) they promote protein dimerization [73]; and (iii) they play a central role in multi-protein scaffolding [74-76]. On the other hand, the RXR motif may serve as an endoplasmic reticulum retention signal. This motif was originally identified in the ATP-sensitive potassium channel, and is commonly located in cytoplasmic tails of membrane proteins [70,77-79]. The masking of the motif by homomeric or heteromeric subunits, or by ER chaperone proteins [80-82], allows protein release from the ER for trafficking to the cell membrane. This trafficking regulation mechanism is utilized by several membrane-bound transport proteins, such as potassium channels, ionotropic NMDA receptors, and kainate receptors [70,77,79,83-86].

Despite the high degree of conservation among the four K-Cl cotransporters, it is striking that only the 20 amino acids from KCC2 interacted with the RCC1 domain of PAM in our yeast two-hybrid assay. RCC1/PAM did not promote yeast survival in small-scale yeast two-hybrid tests against the highly homologous region in KCC1, KCC3, and KCC4. In fact, amino acid alignment revealed that only two residues differed between KCC3 and KCC2, and only KCC2 contained an RXR motif. All four sequences were identical for the MPGPP[R/K] SH3 putative binding sequence. Our mutagenesis analysis (Figure 6) revealed that mutating the RXR motif in KCC2 to RXA disrupted RCC1/PAM binding. However, mutating the KCC3 sequence to contain an RXR motif did not confer binding to RCC1/PAM, indicating a role for surrounding residues. Indeed, mutating the asparagine (N) residue directly after the RXR motif in KCC2 also disrupted RCC1/PAM binding. Our functional experiments with full-length RXA KCC2 mutant did not show increased cotransporter function similar to what would be expected if unmasking an RXR motif. Therefore it is questionable whether the RCC1/PAM binding is related to the known function of a bona-fide RXR motif. It is clear, however, that the RXR sequence and surrounding residues participate in the RCC1/PAM binding. It is also clear that this binding has functional significance since our unidirectional ion flux experiments show that

RCC1/PAM co-expression increases K^+ uptake in cells transfected with wild-type KCC2, but not in cells transfected with the KCC2 -RXA mutant.

Our experiments demonstrate that RCC1/PAM affects KCC2 in multiple ways. First, it seems that RCC1/PAM increases KCC2 transcription and consequently protein upregulation, although at this point, we do not know if this effect is necessarily specific to KCC2. As KCC2 is expressed in these cells in the pCDNA3 vector without its native promoter sequence, we do not expect that this effect would carry over to the physiological setting. Additionally, since both KCC2 and KCC2/RXA exhibit increased expression due to RCC1/PAM coexpression, we did not further explore this effect, and set this aspect aside in order to focus on differences between KCC2 and KCC2/RXA and the RCC1-PAM effects which can be attributed to protein-protein interaction. Second, when KCC2 is in the membrane, binding of RCC1-PAM potentially causes a conformational change leading to increase in cotransporter activity. Absence of RCC1 binding to the RXA mutant thus results in lack of activation. Finally, RCC1-PAM likely competes with wild-type PAM as indicated by our experiments with N-ethylmaleimide.

NEM is a thiol reactive agent that is known to maximally stimulate KCC function. Our data demonstrating that NEM does not further activate KCC2 in the presence of RCC1/PAM and that NEM does not activate the KCC2-RXA mutant indicates that endogenous PAM binding is required for NEM activation. The most convincing evidence suggests that NEM primarily exerts its effect on KCC2 through a phosphorylation/dephosphorylation pathway. Indeed, NEM is thought to cause net dephosphorylation of the cotransporter through kinase inhibition [87]. Although this evidence does not conclusively rule out a direct effect of NEM on KCC2, the fact that NEM has been shown to have opposing effects on NKCCs and KCCs would indicate regulation via upstream signaling pathways as opposed to direct action on the cotransporters (for review, see [47]).

How PAM is intimately involved in modulating the activity of the cotransporter, apart from binding to it and affecting NEM stimulation, is yet to be determined. The effect of RCC1/PAM on NEM stimulation of the wild-type cotransporter and absence of effect on the RXA mutant also suggests that the effect is not related to an increased number of transporters in the plasma membrane. This is important to stress as RCC1/PAM also increases overall KCC2 expression. Indeed, as NEM is believed to maximally stimulate KCC2 activity by promoting dephosphorylation of the cotransporter, one would have expected to observe an additive effect of NEM and RCC1/PAM. To the contrary, we observed that RCC1/PAM interferes with the NEM stimulation. As HEK293 cells express endogenous PAM (data not shown), it is possible that the transfected RCC1/PAM fragment competes with endogenous PAM for binding on the KCC2 carboxyl terminus. In the absence of endogenous PAM binding, as in the case of KCC2-RXA or when the RCC1/PAM fragment is present, NEM stimulation has no effect. This suggests that PAM functions as an integral part of the same kinase/phosphatase pathway through which NEM exerts its effect. One possibility is that PAM functions as a scaffolding protein. When PAM is bound to KCC2, it could bring PP1 to the cotransporter for dephosphorylation and activation. It could also serve as a scaffold for the inhibitory kinase which is functionally inhibited by NEM alkylation. Alternatively, PAM could instead directly affect kinase activity in the vicinity of the cotransporter, as PAM has been shown to inhibit adenylyl cyclase activity [63,64]. The possibility that PAM overlaps with the phosphoregulatory pathway of KCC2 is enhanced by reports that PAM, as well as PAM homologues, can regulate double leucine zipper bearing MAPKKs in *Drosophila*, *C. elegans*, and mouse [88-90].

Future studies are needed to address the role of PAM on KCC2 function in neurons. One possibility involves examining the effect of PAM deletion on KCC2 function and Cl^-

homeostasis. As mentioned above, at the animal level, deletion of PAM results in a phenotype similar to the KCC2 knockout phenotype. A viable mouse expressing PAM without the RCC1 domain (or inactive/mutated RCC1 domain) could represent a useful model for studying the role of RCC1/PAM-KCC2 interaction in central neurons. Another possibility would involve altering PAM expression at the level of the single neuron using RNA interference, and assessing the effect of a lack of PAM expression on chloride regulation. Since PAM is a large protein with many domains, it is likely that its down-regulation by RNAi affects neurons well beyond effects related to KCC2. Indeed, a recently published study of mice showing axon projection deficits not only identified the C' terminal of PAM as important for axon projection, but moreover, the same study showed that RNAi knockdown of PAM in neurons caused a looping of microtubules in the growth cones [90]. Regardless of whether using cultured cells or whole animal models, based on the present studies, we believe that PAM plays a role in modulating KCC2 function in neurons via the phosphoregulatory pathway of KCC2.

ACKNOWLEDGMENTS

This work was supported by an American Heart Association pre-doctoral fellowship to N.J.G., and by a National Institutes of Health grant (NS36758) to E.D.

REFERENCES

1. Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL. Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J. Physiol. (London)* 1989;416:303–325. [PubMed: 2575165]
2. Luhmann HJ, Prince DA. Postnatal maturation of the GABAergic system in rat neocortex. *J. Neurophysiol* 1991;65:247–263. [PubMed: 1673153]
3. Owens DF, Boyce LH, Davis MBE, Kriegstein AR. Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. *J. Neurosci* 1996;16:6414–6423. [PubMed: 8815920]
4. Zhu L, Lovinger D, Delpire E. Cortical neurons lacking KCC2 expression show impaired regulation of intracellular chloride. *J Neurophysiol* 2005;93:1557–1568. [PubMed: 15469961]
5. Alvarez-Leefmans, FJ.; Nani, A.; Marquez, S. Chloride transport, osmotic balance, and presynaptic inhibition. In: Rudomin, P.; Romo, R.; Mendell, LM., editors. *Presynaptic inhibition and neural control*. Oxford University Press; New York: 1998. p. 50-79.
6. Sung K-W, Kirby M, McDonald MP, Lovinger DM, Delpire E. Abnormal GABA_A-receptor mediated currents in dorsal root ganglion neurons isolated from Na-K-2Cl cotransporter null mice. *J. Neurosci* 2000;20:7531–7538. [PubMed: 11027211]
7. Reisert J, Lai J, Yau KW, Bradley J. Mechanism of the excitatory Cl[−] response in mouse olfactory receptor neurons. *Neuron* 2005;45:553–561. [PubMed: 15721241]
8. Thompson SM, Deisz RA, Prince DA. Outward chloride/cation co-transport in mammalian cortical neurons. *Neurosci. Lett* 1988;89:49–54. [PubMed: 3399141]
9. Payne JA. Functional characterization of the neuronal-specific K-Cl cotransporter: implications for [K⁺]_o regulation. *Am J Physiol* 1997;273:C1516–1525. [PubMed: 9374636]
10. Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. The K⁺/Cl[−] co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 1999;397:251–255. [PubMed: 9930699]
11. Delpire E. Cation-Chloride Cotransporters in Neuronal Communication. *News Physiol Sci* 2000;15:309–312. [PubMed: 11390932]
12. Ganguly K, Schinder AF, Wong ST, Poo M. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell* 2001;105:521–532. [PubMed: 11371348]
13. Chudotvorova I, Ivanov A, Rama S, Hubner CA, Pellegrino C, Ben-Ari Y, Medina I. Early expression of KCC2 in rat hippocampal cultures augments expression of functional GABA synapses. *J Physiol* 2005;566:671–679. [PubMed: 15961425]

14. Lu J, Karadsheh M, Delpire E. Developmental regulation of the neuronal-specific isoform of K-Cl cotransporter KCC2 in postnatal rat brains. *J Neurobiol* 1999;39:558–568. [PubMed: 10380077]
15. Zhang LL, Fina ME, Vardi N. Regulation of KCC2 and NKCC during development: membrane insertion and differences between cell types. *J Comp Neurol* 2006;499:132–143. [PubMed: 16958091]
16. Akerman CJ, Cline HT. Depolarizing GABAergic conductances regulate the balance of excitation to inhibition in the developing retinotectal circuit in vivo. *J Neurosci* 2006;26:5117–5130. [PubMed: 16687503]
17. Hubner CA, Stein V, Hermans-Borgmeyer I, Meyer T, Ballanyi K, Jentsch TJ. Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron* 2001;30:515–524. [PubMed: 11395011]
18. Woo NS, Lu J, England R, McClellan R, Dufour S, Mount DB, Deutch AY, Lovinger DM, Delpire E. Hyperexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. *Hippocampus* 2002;12:258–268. [PubMed: 12000122]
19. Palma E, Amici M, Sobrero F, Spinelli G, Di Angelantonio S, Ragozzino D, Mascia A, Scoppetta C, Esposito V, Milei R, Eusebi F. Anomalous levels of Cl[−] transporters in the hippocampal subiculum from temporal lobe epilepsy patients make GABA excitatory. *Proc Natl Acad Sci U S A* 2006;103:8465–8468. [PubMed: 16709666]
20. Hekmat-Scafe DS, Lundy MY, Ranga R, Tanouye MA. Mutations in the K⁺/Cl[−] cotransporter gene *kazachoc (kcc)* increase seizure susceptibility in *Drosophila*. *J Neurosci* 2006;26:8943–8954. [PubMed: 16943550]
21. Aronica E, Boer K, Redeker S, Spliet WG, van Rijen PC, Troost D, Gorter JA. Differential expression patterns of chloride transporters, Na⁽⁺⁾-K⁽⁺⁾-2Cl^(−)-cotransporter and K⁽⁺⁾-Cl^(−)-cotransporter, in epilepsy-associated malformations of cortical development. *Neuroscience* 2007;145:185–196. [PubMed: 17207578]
22. Deisz RA. Cellular mechanisms of pharmacoresistance in slices from epilepsy surgery. *Novartis Found Symp* 2002;243:186–199. [PubMed: 11990776] discussion 199–206, 231–185
23. Jin X, Huguenard JR, Prince DA. Impaired Cl[−] extrusion in layer V pyramidal neurons of chronically injured epileptogenic neocortex. *J Neurophysiol* 2005;93:2117–2126. [PubMed: 15774713]
24. Munakata M, Watanabe M, Otsuki T, Nakama H, Arima K, Itoh M, Nabekura J, Inuma K, Tsuchiya S. Altered Distribution of KCC2 in Cortical Dysplasia in Patients with Intractable Epilepsy. *Epilepsia*. 2007
25. Rivera C, Li H, Thomas-Crusells J, Lahtinen H, Viitanen T, Nanobashvili A, Kokaia Z, Airaksinen MS, Voipio J, Kaila K, Saarma M. BDNF-induced TrkB activation down-regulates the K⁺-Cl[−] cotransporter KCC2 and impairs neuronal Cl[−] extrusion. *J Cell Biol* 2002;159:747–752. [PubMed: 12473684]
26. Rivera C, Voipio J, Thomas-Crusells J, Li H, Emri Z, Sipila S, Payne JA, Minichiello L, Saarma M, Kaila K. Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2. *J Neurosci* 2004;24:4683–4691. [PubMed: 15140939]
27. Kelsch W, Hormuzdi S, Straube E, Lewen A, Monyer H, Misgeld U. Insulin-like growth factor 1 and a cytosolic tyrosine kinase activate chloride outward transport during maturation of hippocampal neurons. *J Neurosci* 2001;21:8339–8347. [PubMed: 11606621]
28. Blaesse P, Guillemain I, Schindler J, Schweizer M, Delpire E, Khiroug L, Friauf E, Nothwang HG. Oligomerization of KCC2 correlates with development of inhibitory neurotransmission. *J Neurosci* 2006;26:10407–10419. [PubMed: 17035525]
29. Casula S, Shmukler BE, Wilhelm S, Stuart-Tilley AK, Su W, Chernova MN, Brugnara C, Alper SL. A dominant negative mutant of the KCC1 K-Cl cotransporter: both N- and C-terminal cytoplasmic domains are required for K-Cl cotransport activity. *J Biol Chem* 2001;276:41870–41878. [PubMed: 11551954]
30. Simard CF, Bergeron MJ, Cotton-Frenette R, Carpentier GA, Pelchat ME, Caron L, Isenring P. Homooligomeric and heterooligomeric associations between K-Cl cotransporter isoforms and between K-Cl and Na-K-Cl cotransporters. *J Biol Chem*. 2007
31. Inoue K, Ueno S, Fukuda A. Interaction of neuron-specific K⁺-Cl[−] cotransporter, KCC2, with brain-type creatine kinase. *FEBS Lett* 2004;564:131–135. [PubMed: 15094054]

32. Inoue K, Yamada J, Ueno S, Fukuda A. Brain-type creatine kinase activates neuron-specific K⁺-Cl⁻ co-transporter KCC2. *J Neurochem* 2006;96:598–608. [PubMed: 16336223]
33. Li H, Khirug S, Cai C, Ludwig A, Blaesse P, Kolikova J, Afzalov R, Coleman SK, Lauri S, Airaksinen MS, Keinänen K, Khiroug L, Saarna M, Kaila K, Rivera C. KCC2 Interacts with the Dendritic Cytoskeleton to Promote Spine Development. *Neuron* 2007;56:1019–1033. [PubMed: 18093524]
34. Burgess RW, Peterson KA, Johnson MJ, Roix JJ, Welsh IC, O'Brien TP. Evidence for a conserved function in synapse formation reveals *Phr1* as a candidate gene for respiratory failure in newborn mice. *Mol Cell Biol* 2004;24:1096–1105. [PubMed: 14729956]
35. Guo Q, Xie J, Dang CV, Liu ET, Bishop JM. Identification of a large Myc-binding protein that contains RCC1-like repeats. *Proc Natl Acad Sci U S A* 1998;95:9172–9177. [PubMed: 9689053]
36. Murthy V, Han S, Beauchamp RL, Smith N, Haddad LA, Ito N, Ramesh V. Pam and its ortholog highwire interact with and may negatively regulate the TSC1.TSC2 complex. *J Biol Chem* 2004;279:1351–1358. [PubMed: 14559897]
37. James P, Halladay J, Craig EA. Genomic libraries and host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* 1996;144:1425–1436. [PubMed: 8978031]
38. Piechotta K, Lu J, Delpire E. Cation chloride cotransporters interact with the stress-related kinases Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). *J Biol Chem* 2002;277:50812–50819. [PubMed: 12386165]
39. Ohtsubo M, Okazaki H, Nishimoto T. The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. *J Cell Biol* 1989;109:1389–1397. [PubMed: 2677018]
40. Bischoff FR, Ponstingl H. Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature* 1991;354:80–82. [PubMed: 1944575]
41. Yamanaka K, Vande Velde C, Eymard-Pierre E, Bertini E, Boespflug-Tanguy O, Cleveland DW. Unstable mutants in the peripheral endosomal membrane component ALS2 cause early-onset motor neuron disease. *Proc Natl Acad Sci U S A* 2003;100:16041–16046. [PubMed: 14668431]
42. Lai C, Xie C, McCormack SG, Chiang HC, Michalak MK, Lin X, Chandran J, Shim H, Shimoji M, Cookson MR, Haganir RL, Rothstein JD, Price DL, Wong PC, Martin LJ, Zhu JJ, Cai H. Amyotrophic lateral sclerosis 2-deficiency leads to neuronal degeneration in amyotrophic lateral sclerosis through altered AMPA receptor trafficking. *J Neurosci* 2006;26:11798–11806. [PubMed: 17093100]
43. Hadano S, Benn SC, Kakuta S, Otomo A, Sudo K, Kunita R, Suzuki-Utsunomiya K, Mizumura H, Shefner JM, Cox GA, Iwakura Y, Brown RH Jr, Ikeda JE. Mice deficient in the Rab5 guanine nucleotide exchange factor ALS2/alsin exhibit age-dependent neurological deficits and altered endosome trafficking. *Hum Mol Genet* 2006;15:233–250. [PubMed: 16321985]
44. He S, Parapuram SK, Hurd TW, Behnam B, Margolis B, Swaroop A, Khanna H. Retinitis Pigmentosa GTPase Regulator (RPGR) protein isoforms in mammalian retina: insights into X-linked Retinitis Pigmentosa and associated ciliopathies. *Vision Res* 2008;48:366–376. [PubMed: 17904189]
45. Strange K, Singer TD, Morrison R, Delpire E. Dependence of KCC2 K-Cl cotransporter activity on a conserved carboxy terminus tyrosine residue. *Am J Physiol Cell Physiol* 2000;279:C860–867. [PubMed: 10942735]
46. Lauf PK, Theg BE. A chloride dependent K⁺ flux induced by N-ethylmaleimide in genetically low K⁺ sheep and goat erythrocytes. *Biochem. Biophys. Res. Comm* 1980;70:221–242.
47. Adragna NC, Fulvio MD, Lauf PK. Regulation of K-Cl cotransport: from function to genes. *J Membr Biol* 2004;201:109–137. [PubMed: 15711773]
48. Misri S, Chimote AA, Adragna NC, Warwar R, Brown TL, Lauf PK. KCC isoforms in a human lens epithelial cell line (B3) and lens tissue extracts. *Exp Eye Res* 2006;83:1287–1294. [PubMed: 16949074]
49. Gagnon KB, Adragna NC, Fyffe RE, Lauf PK. Characterization of glial cell K-Cl cotransport. *Cell Physiol Biochem* 2007;20:121–130. [PubMed: 17595522]
50. Gillen CM, Brill S, Payne JA, Forbush B 3rd. Molecular cloning and functional expression of the K-Cl cotransporter from rabbit, rat, and human. A new member of the cation-chloride cotransporter family. *J Biol Chem* 1996;271:16237–16244. [PubMed: 8663127]
51. Yang H, Scholich K, Poser S, Storm DR, Patel TB, Goldowitz D. Developmental expression of PAM (protein associated with MYC) in the rodent brain. *Brain Res Dev Brain Res* 2002;136:35–42.

52. Williams JR, Sharp JW, Kumari VG, Wilson M, Payne JA. The neuron-specific K-Cl cotransporter, KCC2. Antibody development and initial characterization of the protein. *J Biol Chem* 1999;274:12656–12664. [PubMed: 10212246]
53. Ehnert C, Tegeder I, Pierre S, Birod K, Nguyen HV, Schmidt A, Geisslinger G, Scholich K. Protein associated with Myc (PAM) is involved in spinal nociceptive processing. *J Neurochem* 2004;88:948–957. [PubMed: 14756816]
54. Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De Koninck P, De Koninck Y. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* 2003;424:938–942. [PubMed: 12931188]
55. Morales-Aza BM, Chillingworth NL, Payne JA, Donaldson LF. Inflammation alters cation chloride cotransporter expression in sensory neurons. *Neurobiol Dis* 2004;17:62–69. [PubMed: 15350966]
56. Price TJ, Cervero F, de Koninck Y. Role of cation-chloride-cotransporters (CCC) in pain and hyperalgesia. *Curr Top Med Chem* 2005;5:547–555. [PubMed: 16022677]
57. Mantyh PW, Hunt SP. Setting the tone: superficial dorsal horn projection neurons regulate pain sensitivity. *Trends Neurosci* 2004;27:582–584. [PubMed: 15374667]
58. Schaefer AM, Hadwiger GD, Nonet ML. rpm-1, a conserved neuronal gene that regulates targeting and synaptogenesis in *C. elegans*. *Neuron* 2000;26:345–356. [PubMed: 10839354]
59. Zhen M, Huang X, Bamber B, Jin Y. Regulation of presynaptic terminal organization by *C. elegans* RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. *Neuron* 2000;26:331–343. [PubMed: 10839353]
60. Wan H, DiAntonio A, Fetter RD, Bergstrom K, Strauss R, Goodman CS. Highwire regulates synaptic growth in *Drosophila*. *Neuron* 2000;26:313–329. [PubMed: 10839352]
61. D'Souza J, Hendricks M, Le Guyader S, Subburaju S, Grunewald B, Scholich K, Jesuthasan S. Formation of the retinotectal projection requires Esrom, an ortholog of PAM (protein associated with Myc). *Development* 2005;132:247–256. [PubMed: 15590740]
62. Santos TM, Han S, Bowser M, Sazani K, Beauchamp RL, Murthy V, Bhide PG, Ramesh V. Alternative splicing in protein associated with Myc (Pam) influences its binding to c-Myc. *J Neurosci Res* 2006;83:222–232. [PubMed: 16342205]
63. Gao X, Patel TB. Histidine residues 912 and 913 in protein associated with Myc are necessary for the inhibition of adenylyl cyclase activity. *Mol Pharmacol* 2005;67:42–49. [PubMed: 15470080]
64. Scholich K, Pierre S, Patel TB. Protein associated with Myc (PAM) is a potent inhibitor of adenylyl cyclases. *J Biol Chem* 2001;276:47583–47589. [PubMed: 11590159]
65. Gulyas AI, Sik A, Payne JA, Kaila K, Freund TF. The KCl cotransporter, KCC2, is highly expressed in the vicinity of excitatory synapses in the rat hippocampus. *Eur J Neurosci* 2001;13:2205–2217. [PubMed: 11454023]
66. Bartho P, Payne JA, Freund TF, Acsady L. Differential distribution of the KCl cotransporter KCC2 in thalamic relay and reticular nuclei. *Eur J Neurosci* 2004;20:965–975. [PubMed: 15305865]
67. Szabadics J, Varga C, Molnar G, Olah S, Barzo P, Tamas G. Excitatory effect of GABAergic axo-axonic cells in cortical microcircuits. *Science* 2006;311:233–235. [PubMed: 16410524]
68. Mercado A, Broumand V, Zandi-Nejad K, Enck AH, Mount DB. A C-terminal domain in KCC2 confers constitutive K⁺-Cl⁻ cotransport. *J Biol Chem* 2006;281:1016–1026. [PubMed: 16291749]
69. Li SS-C. Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction. *Biochem. J* 2005;390:641–653. [PubMed: 16134966]
70. Zerangue N, Schwappach B, Jan YN, Jan LY. A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron* 1999;22:537–548. [PubMed: 10197533]
71. Choi WS, Khurana A, Mathur R, Viswanathan V, Steele DF, Fedida D. Kv1.5 surface expression is modulated by retrograde trafficking of newly endocytosed channels by the dynein motor. *Circ. Res* 2005;97:363–371. [PubMed: 16051887]
72. Tian L, Chen L, McClafferty H, Sailer CA, Ruth P, Knaus HG, Shipston MJ. A noncanonical SH3 domain binding motif links BK channels to the actin cytoskeleton via the SH3 adapter cortactin. *FASEB J* 2006;20:2588–2590. [PubMed: 17065230]

73. Kristensen O, Guenat S, Dar I, Allaman-Pillet N, Abderrahmani A, Ferdaoussi M, Roduit R, Maurer F, Beckmann JS, Kastrup JS, Gajhede M, Bonny C. A unique set of SH3-SH3 interactions controls IB1 homodimerization. *EMBO J* 2006;25:785–797. [PubMed: 16456539]
74. Roos J, Kelly RB. Dap160, a neural-specific Eps15 homology and multiple SH3 domain-containing protein that interacts with *Drosophila* dynamin. *J Biol Chem* 1998;273:19108–19119. [PubMed: 9668096]
75. Sheng M, Kim E. The Shank family of scaffold proteins. *J Cell Sci* 2000;113(Pt 11):1851–1856. [PubMed: 10806096]
76. Xu Z, Kukekov NV, Greene LA. POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis. *Embo J* 2003;22:252–261. [PubMed: 12514131]
77. Shikano S, Li M. Membrane receptor trafficking: evidence of proximal and distal zones conferred by two independent endoplasmic reticulum localization signals. *Proc Natl Acad Sci U S A* 2003;100:5783–5788. [PubMed: 12724521]
78. Margeta-Mitrovic M, Jan YN, Jan LY. A trafficking checkpoint controls GABA(B) receptor heterodimerization. *Neuron* 2000;27:97–106. [PubMed: 10939334]
79. Standley S, Roche KW, McCallum J, Sans N, Wenthold RJ. PDZ domain suppression of an ER retention signal in NMDA receptor NR1 splice variants. *Neuron* 2000;28:887–898. [PubMed: 11163274]
80. Vivithanaporn P, Yan S, Swanson GT. Intracellular trafficking of KA2 kainate receptors mediated by interactions with coatamer protein complex I (COPI) and 14-3-3 chaperone systems. *J Biol Chem* 2006;281:15475–15484. [PubMed: 16595684]
81. Hermosilla R, Oueslati M, Donalies U, Schonenberger E, Krause E, Oksche A, Rosenthal W, Schulein R. Disease-causing V(2) vasopressin receptors are retained in different compartments of the early secretory pathway. *Traffic* 2004;5:993–1005. [PubMed: 15522100]
82. Yuan H, Michelsen K, Schwappach B. 14-3-3 dimers probe the assembly status of multimeric membrane proteins. *Curr Biol* 2003;13:638–646. [PubMed: 12699619]
83. Coussen F, Mulle C. Kainate receptor-interacting proteins and membrane trafficking. *Biochem Soc Trans* 2006;34:927–930. [PubMed: 17052229]
84. Jaskolski F, Normand E, Mulle C, Coussen F. Differential trafficking of GluR7 kainate receptor subunit splice variants. *J Biol Chem* 2005;280:22968–22976. [PubMed: 15805114]
85. Scott DB, Blanpied TA, Swanson GT, Zhang C, Ehlers MD. An NMDA receptor ER retention signal regulated by phosphorylation and alternative splicing. *J Neurosci* 2001;21:3063–3072. [PubMed: 11312291]
86. Scott DB, Blanpied TA, Ehlers MD. Coordinated PKA and PKC phosphorylation suppresses RXR-mediated ER retention and regulates the surface delivery of NMDA receptors. *Neuropharmacology* 2003;45:755–767. [PubMed: 14529714]
87. Jennings ML, Schulz RK. Okadaic acid inhibition of KCl cotransport. Evidence that protein dephosphorylation is necessary for activation of transport by either cell swelling or N-ethylmaleimide. *J Gen Physiol* 1991;97:799–817. [PubMed: 1647439]
88. Nakata K, Abrams B, Grill B, Goncharov A, Huang X, Chisholm AD, Jin Y. Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. *Cell* 2005;120:407–420. [PubMed: 15707898]
89. Collins CA, Wairkar YP, Johnson SL, DiAntonio A. Highwire restrains synaptic growth by attenuating a MAP kinase signal. *Neuron* 2006;51:57–69. [PubMed: 16815332]
90. Lewcock JW, Genoud N, Lettieri K, Pfaff SL. The Ubiquitin Ligase Phr1 Regulates Axon Outgrowth through Modulation of Microtubule Dynamics. *Neuron* 2007;56:604–620. [PubMed: 18031680]

Figure 1. RCC1 PAM/Phr1 interacts with the KCC2 carboxyl terminus

A: Schematic of full-length PAM protein, and the 323 amino acid region of PAM (referred to as RCC1/PAM) pulled out of a yeast two-hybrid screen using KCC2-CT. **B:** RCC1/PAM tested in a small scale yeast two-hybrid transformation against the empty bait vector, pGBDUC2. Left, re-streak on -Uracil, -Leucine media (control plate); Right, -Uracil, -Leucine, -Histidine +AT media (experimental plate). Yeast growth on the experimental plate indicates a positive identification of protein-protein interaction, whereas no yeast growth is a negative result/ indicates no protein-protein interaction. **C:** Immunoblot using HRP-conjugated anti-HA antibody. Lanes 1-4: GST pull-down assay using either purified GST or GST-KCC2-CT, incubated with HEK293 cell lysate transfected with either pCDNA3 alone or HA-tagged RCC1/PAM. Lanes 5 and 6: whole cell lysates of transfected HEK293 cells. **D:** Representative Western Blot of coimmunoprecipitation experiments. HEK293 cells were transfected with either RCC1/PAM alone, or KCC2 and RCC1/PAM. Anti-KCC2 antibody was used for immunoprecipitation, and HRPconjugated anti-HA antibody was used for immunoblotting.

Figure 2. RCC1/PAM increases KCC2-mediated flux in HEK293 cells

Unidirectional $^{86}\text{Rb}^+$ uptake in HEK293 cells measured at 6 min intervals over 30 min. Cells were transfected with either: KCC2 and RCC1/PAM clone (filled circles, dotted line), KCC2 only (open diamonds, solid line), RCC1/PAM only (open circles/dashed line) or empty pCDNA3 vector only (filled squares, dashed/dotted line). Graph shown is representative of three experiments.

Figure 3. RCC1/PAM increases KCC2 protein in HEK293 cells

HEK293 cells were transfected with rKCC2-pCDNA3 plus empty pCDNA vector or rKCC2 plus RCC1/PAM. Western blot was probed with anti-KCC2 antibody. Representative of three separate experiments.

Figure 4. RCC1/PAM increases KCC2 RNA levels in HEK293 cells

RNA was isolated from transfected HEK293 cells, and RNA yield used for reverse transcription. Resulting cDNA was then used for semi-quantitative PCR with reactions taken out at indicated cycles and run on agarose-TAE gels.

Figure 5. RCC1/PAM interaction narrowed down to 20 amino acids in the carboxyl terminus
 A-C: Truncation mutants of KCC2-CT were used in small scale yeast two-hybrid transformations with RCC1/PAM clone. A: Re-streak on -Uracil, -Leucine media (control plate). B: Re-streak on -Uracil, -Leucine, -Histidine + AT media (experimental plate). C: Schematic/sequences of regions of KCC2-CT used for the truncation mutants. Solid bar/plus sign represents a positive interaction; open bar/minus sign represents a negative interaction.

A.

a: LVLLNMPGPPKNSEG D E N Y M E (**KCC1**)
b: LVLLNMPGPPRN R N G D E N Y M E (**KCC2**)
c: LVLLNMPGPPRN P E G D E N Y M E (**KCC3**)
d: LVLLNMPGPPKSRQGDENYME (**KCC4**)

B.

a: LVLLNMPGPPRN R N G D E N Y M E (**KCC2 w.t.**)
b: LVLLNMPGPPRN **A** N G D E N Y M E (**KCC2-RXA**)
c: LVLLNMPGPPRN P E G D E N Y M E (**KCC3 w.t.**)
d: LVLLNMPGPPRN **R** E G D E N Y M E (**KCC3, RXR**)
e: LVLLNMPGPPRN **R E** G D E N Y M E (**KCC2, RXRE**)
f: LVLLNMPGPPRN **P N** G D E N Y M E (**KCC3, RXPN**)

Figure 6. KCC2 point mutant, KCC2-RXA, disrupts RCC1/PAM binding with the minimum 20 amino acid sequence

A: Small-scale yeast two-hybrid transformation testing interaction between RCC1/PAM against portions of other KCCs homologous to the minimum binding region in KCC2. Top, amino acid sequence alignment. Left, re-streak on -Uracil, -Leucine media (control plate). Right, restreak on -Uracil, -Leucine, -Histidine + AT media (experimental plate). B: Small-scale yeast two hybrid transformation testing interaction between RCC1/PAM in pACT2, and various mutants of the minimum binding site in KCC2 and homologous region in KCC3. Top, amino acid sequence alignment. Rectangle denotes nonconserved residues between KCC2 and KCC3 in the 20 amino acid region. Bold and underlined residues represent introduced point mutations. Left, re-streak on -Uracil, -Leucine media (control plate). Right, re-streak on -Uracil, -Leucine, -Histidine + AT media (experimental plate).

A.

B.

Figure 7. KCC2-RXA, like KCC2, has increased protein and RNA levels when cotransfected with RCC1/PAM

A: Microsome preparations were made from HEK293 cells transfected with rKCC2-pCDNA3 plus empty pCDNA vector (lane 1), rKCC2 plus RCC1/PAM pCDNA3 (lane 2), KCC2-RXA-pCDNA3 plus empty pCDNA vector (lane 3), and KCC2-RXA plus RCC1/PAM pCDNA3 (lane 4). Western blot was probed with anti-KCC2 antibody and anti-human transferrin receptor antibody. Representative of three separate experiments. B: Semi-quantitative PCR was performed as described earlier, with the indicated HEK cell transfection conditions.

Figure 8. Coimmunoprecipitation of RCC1/PAM with KCC2 and KCC2-RXA

A: Representative Western blot of co-immunoprecipitation experiments. HEK293FT cells were transfected with either RCC1/PAM alone, KCC2 and RCC1/PAM, or KCC2-RXA and RCC1. KCC2 antibody was used for immunoprecipitation and HRP-conjugated anti-HA antibody was used for immunoblotting. B: Co-immunoprecipitation experiments were quantitated by measuring pixel intensity using ImageJ (NIH). Data is normalized to the negative control (RCC1/PAM only condition).

Figure 9. KCC2, but not the mutant KCC2-RXA, has increased activity in the presence of RCC1/PAM

Unidirectional $^{86}\text{Rb}^+$ uptake in HEK293 cells were measured after 10 min of incubation with $^{86}\text{Rb}^+$. $P < 0.001$ (Tukey post-test) except where indicated.

Figure 10. KCC2 mediated flux in the presence and absence of RCC1/PAM and/or Nethylmaleimide

HEK293 cells were transfected with KCC2 alone or KCC2 and RCC1/PAM. KCC2 uptake activity was measured using unidirectional $^{86}\text{Rb}^+$ uptake after 10 min of incubation with $^{86}\text{Rb}^+$. In +NEM conditions, stimulation was performed for 15 min total (5 min prior to $^{86}\text{Rb}^+$ incubation, and throughout the 10 min $^{86}\text{Rb}^+$ incubation). *** = $P < 0.001$ (ANOVA, Tukey post-test).