Transit Defect of Potassium-Chloride Co-transporter 3 Is a Major Pathogenic Mechanism in Hereditary Motor and Sensory Neuropathy with Agenesis of the Corpus Callosum*§

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Missense and protein-truncating mutations of the human potassium-chloride co-transporter 3 gene (KCC3) cause hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC), which is a severe neurodegenerative disease characterized by axonal dysfunction and neurodevelopmental defects. We previously reported that KCC3-truncating mutations disrupt brain-type creatine kinase-dependent activation of the co-transporter through the loss of its last 140 amino acids. Here, we report a novel and more distal HMSN/ACC-truncating mutation (3402C→T; R1134X) that eliminates only the last 17 residues of the protein. This small truncation disrupts the interaction with brain-type creatine kinase in mammalian cells but also affects plasma membrane localization of the mutant transporter. Although it is not truncated, the previously reported HMSN/ACC-causing 619C→T (R207C) missense mutation also leads to KCC3 loss of function in Xenopus oocyte flux assay. Immunodetection in Xenopus oocytes and in mammalian cultured cells revealed a decreased amount of R207C at the plasma membrane, with significant retention of the mutant proteins in the endoplasmic reticulum. In mammalian cells, cumin partially corrected these mutant protein mislocalizations, with more protein reaching the plasma membrane. These findings suggest that mis-trafficking of mutant protein is an important pathophysiological feature of HMSN/ACC causative KCC3 mutations.

Hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC‡; OMIM 218000) is a severe and progressive early onset motor and sensory neuropathy that is characterized by axonal swelling and is associated with neurodevelopmental defects in the central nervous system (CNS) (1). This autosomal recessive neuropathy is caused by mutations in the potassium-chloride co-transporter 3 (SLC12A6/) KCC3 (2) gene. However, cation chloride co-transporter-related diseases are mainly metabolic disorders that include Gitelman disease, which is caused by mutations in the SLC12A3/NCC gene (OMIM 2638000), and Bartter disease, which is caused by mutations in the SLC12A1/NKCC2 gene (OMIM 601678).

Cation chloride co-transporters share a structure of 12 membrane-spanning segments, which constitute a hydrophobic and glycosylated core, flanked by cytosolic amino- and carboxyl-terminal domains. Many pathomechanisms have already been proposed to explain the inactivation of NCC and NKCC2 in Gitelman and Bartter diseases, respectively; these include anomalies in the biosynthesis, processing, trafficking, conductance, regulation, and degradation of the mutant proteins (3); however, little is known about the pathomechanisms underlying KCC3 dysfunction in HMSN/ACC.

Ten HMSN/ACC-causing KCC3 mutations have been reported, which include six randomly distributed frameshift mutations, two carboxyl terminus-truncating nonsense mutations, and two missense mutations (2, 4, 5); thus, the most frequent alterations in HMSN/ACC are the total or partial elimination of KCC3 carboxyl-terminal regions. Data collected from heterologous expression studies in Xenopus oocytes using truncated KCC3 mutants revealed that the loss of the last 140 amino acids is sufficient to abolish the KCC3-driven flux of 86Rb⁺ (2, 4). We have recently demonstrated that this loss of function includes the failure of the transporter to interact with brain-type creatine kinase (CK-B) (6). However, the R207C missense mutation is predicted to result in a full-length protein bearing an amino acid exchange that should not impact the interaction of KCC3 with CK-B, which suggests that other mechanisms may be involved in KCC3 dysfunction in HMSN/ACC (5).

In this study, we report a novel and very distal KCC3 truncation mutation in an Algerian HMSN/ACC family. We found that the targeting of this and other mutant KCC3 proteins to the plasma membrane is perturbed. These results provide evidence that a trafficking defect may in part explain the KCC3 dysfunction found in HMSN/ACC.

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§ The abbreviations used are: HMSN/ACC, hereditary motor and sensory neuropathy; ANOVA, analysis of variance; CFTR, cystic fibrosis transmembrane conductance regulator; CK-B, creatine kinase brain-specific; DNFB, 1-fluoro-2,4-dinitrobenzene; ER, endoplasmic reticulum; NCC, sodium-chloride co-transporter; NGS, normal goat serum.
**EXPERIMENTAL PROCEDURES**

*Mutation Screening*—Blood samples were collected with informed consent from Algerian and Turkish families (Fig. 1). DNA was extracted from peripheral blood lymphocytes using standard methods. A clinical HMSN/ACC diagnosis was established by a neurologist according to the basic features of the disease (Table 1). The 26 exons of the KCC3 gene were amplified by PCR using intronic primers (4), and products were directly sequenced on an ABI 3700 sequencer, according to the manufacturer’s recommended protocol (Applied Biosystems, Foster City, CA).

*RT-PCR*—Total RNA was extracted from a lymphoblast cell line established from the blood of a Turkish patient bearing the exon 5 missense mutation (619C→T; R207C) using the RNasey kit (Qiagen), according to the manufacturer’s protocol. The extracted RNA was reverse-transcribed (RT) using a classic protocol (7). Different primers were design to flank the mutation site as follows: primers “4/4” hybridized at the beginning of exon 4 (5′-GAAATGGACACGACCGA-3′); primers “4/5” hybridized at the junction of exons 4 and 5 (5′-GGCCACCACAACCCAAA-3′); primers “5/6” hybridized at the junction of exons 5 and 6 (5′-TCAACATTGTACAGCAGCAG-3′), and primers “6/6” hybridized at the end of exon 6 (5′-TGCCACACCTCCAATGG-3′). The primers flanking exon 5 of the KCC3 gene were used in PCRs following a classic protocol (2), and products were loaded on agarose gel for observation. A DNA construct containing the wild-type KCC3 cDNA was used as a positive control in the PCR.

*Plasmid Constructs and Mutagenesis*—The wild-type KCC3 cDNA (in the pGEM vector), which was kindly provided by Dr. Mount (Harvard Medical School, Boston), was subcloned in pcDNA 3.1 vector (Invitrogen) to allow expression in mammalian cells. To generate the R207C and R1134X mutant trans-ports, the 619C→T transition mutation in exon 5 and the 3402C→T transition mutation in exon 25 of KCC3 were introduced in the pGEM and pcDNA constructs using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the lower primers 5′-TTTCTTCAGGTTTTTGCAA-3′ and 5′-GAAGTGATCACTATTTATTC-3′. DNA sequence accuracy was verified by sequencing.

* Xenopus laevis Flux Assay*—The activity of the co-transporter was determined by assaying radioluminescent agent furosemide. Dependence of the function of KCC3 on CK-B activity was determined by exposure to 200 μM 1-fluoro-2,4-dinitrobenzene (DNFB). The uptake experiment was stopped after 45 min by five washes in ice-cold uptake solution without the isotope to remove extracellular fluid tracer. The oocytes were lysed in 10% SDS, and tracer activity was measured for 2 min in a liquid scintillation counter.

*Plasma Membrane Fraction Preparation of Oocytes*—The oocytes were rinsed in MES-buffered saline solution (MBSS: 80 mM NaCl, 20 mM MES, pH 6.0) and incubated for 10 min at room temperature in the same solution with 0.005% subtilisin A (Sigma) to partially digest the vitelline membranes. The polymerizing steps were performed for 60 min with 1% ludox/MBSS and then 45 min with 0.1% polyacrylic acid (Sigma) at 4°C under mild agitation. Between each step, oocytes were rinsed three times in MBSS. The oocytes were then homogenized by pipetting in 1 ml of cold HbA (in mM: 5 MgCl₂, 5 NaH₂PO₄, 1 EDTA, 80 sucrose, and 20 Tris, pH 7.4) and then centrifuged at 16 × g, 25 × g, and 35 × g for 30 s at 4°C. Between each centrifugation, the supernatants were removed, and the pellet was resuspended in 1 ml of cold HbA. A final centrifugation at 16,000 × g for 20 min pelleted the purified plasma membranes, which were prepared for immunoblotting (8).

*Cell Culture and Transfection*—HeLa cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 units/ml penicillin and 50 mg/ml streptomycin) at 37°C with 5% CO₂ (all supplied by Invitrogen). PC12 cells were grown on poly-D-lysine-coated coverslips placed in DMEM/F-12 containing 10% heat-inactivated horse serum, 5% FBS, and antibiotics (50 units/ml penicillin and 50 mg/ml streptomycin) at 37°C with 5% CO₂.

The plasmids used in the transfection studies were prepared using the plasmid maxi kit (Qiagen), according to the manufacturer’s instructions. Transient transfections were performed on cells using the Lipofectamine transfection reagent (Invitrogen), according to the manufacturer’s protocol. Cells were incubated for 24 h with Lipofectamine before replacing the medium, and transgene expression was assayed at least 36 h post-transfection. Typically, a transfection efficiency of the transgene of 20–50% was achieved; equivalent transfection efficiency among the various transfection experiments was confirmed by Western blot. PC12 differentiation was induced by treating the cells with 50 ng/ml nerve growth factor (NGF) in a reduced serum environment (2.5% heat-inactivated horse serum) for 3 days.

Curcumin treatment was performed prior to the immunofluorescence experiment. The cells were incubated for 3 h in curcumin at a final concentration ranging from 5 to 40 μM. The cells were rinsed several times in PBS to get rid of the curcumin before the immunostaining experiment.

**Immunofluorescence Study**—Cells grown on coverslips were induced to differentiate if necessary (in the case of PC12 cells) and were then transiently transfected with the pcDNA 3.1 wild-type or mutant KCC3 constructs, fixed in 4% paraformaldehyde solution in PBS at room temperature for 10 min, and finally processed for immunofluorescence staining. For this, cells were permeabilized in very mild conditions (0.01% Triton X-100 for 3 min), and nonspecific site blocking was performed in 10%...
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normal goat serum (NGS) solution in PBS for 1 h at room temperature, which was directly followed by incubation with anti-KCC3 (1:500; Abnova), anti-CK-B (1:100; OEM concept), anti-pan cadherin (1:500; Abcam), or anti-calreticulin (1:250; Abcam) primary antibodies in 5% NGS/PBS overnight at 4 °C. Coverslips were then washed twice in PBS, incubated with the appropriate secondary antibodies (The Jackson Laboratory), and mounted on slides for confocal microscopy (Leica).

Paraffin-embedded fragments of brains from an HMSN/ACC patient were sectioned (5 μm) and placed on glass slides. The sections were processed for immunohistochemistry using the following protocol: after deparaffinization and rehydration, the slide-mounted tissue sections were incubated in an antigen retrieval solution (DAKO S1699) preheated to 85 °C for 1 h. Sections were cooled to room temperature and were then rinsed three times with PBS. After a 30-min permeabilization step in 0.2% Triton X-100/PBS, the sections were blocked in a 10% solution of NGS in 0.02% Triton X-100/PBS at room temperature for 1 h. Incubation of primary antibodies at suggested concentrations (diluted in 2% NGS in 0.02% Triton X-100/PBS) was then carried out overnight at room temperature. Sections were washed three times in PBS the following day. Secondary antibody incubation was carried out using the appropriate Alexa antibodies (1:1000 in 2% NGS, 0.02% Triton X-100/PBS) for 1 h at room temperature. Sections were again washed three times in PBS. The slides were then coverslip-mounted using Mowiol (Fluka, Polyvinyl alcohol 4–88) and were visualized using appropriate filters on a Leica DM6000 microscope.

Immunoblot—HeLa cells were plated onto 10-cm Petri dishes, grown to confluence, and then transfected with pcDNA-KCC3 wild-type or mutant constructs, as described previously (6). After transfection, cells were placed in fresh DMEM, 10% FBS for 48 h. Whole-cell lysates were harvested by washing cells in cold PBS and by scraping them into 0.1% Triton in PBS or SUB lysis buffer supplemented with Complete protease inhibitors (Roche Applied Science). The extracted proteins were separated by 8% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antisera to KCC3 (1:500; Abnova), anti-CK-B (1:100; OEM concept), anti-pan cadherin (1:500; Abcam), or anti-calreticulin (1:250; Abcam) primary antibodies (1:2500; Abcam) in 5% NGS/PBS overnight at 4 °C. The blots were stripped and re-probed with an anti-actin antibody (Chemicon) to verify equivalent loading of protein.

Statistics—Protein co-localization rates were evaluated using Pearson’s coefficient on confocal microscopy, using the LAS AF software (Leica). Significance was set at a two-tailed Student’s t test with a p value of <0.01. Results were presented as means ± S.E. The significance of the differences between Xenopus oocyte groups in the flux assays was tested by one-way analysis of variance (ANOVA).

RESULTS

R1134X Is a Novel and Most Distal KCC3-truncating Mutation—Sequencing of the SLC12A6/KCC3 gene in two HMSN/ACC cases from an Algerian consanguineous family revealed a homozygous cysteine-to-thymine transition (3402C→T) in exon 25 (Fig. 1a). The mutation creates a premature stop codon that leads to the loss of the last 17 amino acids of KCC3, which corresponds to only ~2% of the total protein sequence (R1134X mutant); thus, R1134X represents the least truncated KCC3 mutant. In comparison, the exon 22 mutation, which was the smallest previously identified KCC3 truncation, causes the elimination of the last 140 amino acids of the protein, which correspond to ~12% of the whole protein. In all, 9 of the 11 HMSN/ACC mutations result in a net loss of amino acids within the carboxyl-terminal domain of KCC3.

Truncated R1134X Fails to Interact with CK-B—The brain-type creatine kinase (CK-B), which is an ATP-generating protein, is a potent activator of KCC3 via a direct interaction with the carboxyl-terminal domain of the co-transporter (6). We have previously used a yeast two-hybrid system to demonstrate that the sequence encompassing the last 18 amino acids of KCC3 is necessary for its interaction with human CK-B (6), which suggests that the physical interaction between CK-B and R1134X (which lacks the last 17 amino acids) may be impaired in vivo (Fig. 1b). To confirm this hypothesis, we performed dual immunofluorescence studies with CK-B- and KCC3-specific antibodies using human HeLa cells that were transfected with R1134X construct and that express CK-B endogenously (Fig. 2a). Although we observed the interaction between wild-type KCC3 and CK-B as reported previously, we failed to observe a co-localization between CK-B and R1134X (Fig. 2b).

To confirm that the disruption of R1134X and CK-B interaction has an impact on the co-transport activity, wild-type KCC3 and the nonsense mutant R1134X were expressed in Xenopus oocytes, and 86Rb+ uptake upon swelling activation of KCC3 was measured. As expected, the wild-type transporter induced a significant flux (p = 0.027 ANOVA compared with the control), which was abolished by chloride substitution or by treat-
ment with 200 μM DNFB, a specific creatine kinase inhibitor. In contrast, R1134X did not induce a significant flux in Xenopus oocytes (Fig. 2c). This further associates the loss of interaction with CK-B with the loss of function of truncated KCC3.

It is noteworthy that the last 17 amino acids missing in R1134X contain the following two highly conserved sequences probably involved in the protein trafficking: a hydrophobic tetrad that participates in the transit of other proteins of the SLC12 family (9) and a carboxyl-terminal tyrosine sorting motif probably involved in the protein trafficking (Fig. 1b). When we performed immunoblots of plasma membrane fractions from Xenopus oocytes expressing R1134X, we observed only small amounts of the mutant protein in the purified extract (Fig. 2d). In addition, co-staining of R1134X and CK-B in mammalian cells revealed that the most abundant staining of the truncated protein was intracellular and not at the cell periphery. These data suggest that the loss of interaction with CK-B could be a pathomechanism leading to KCC3 dysfunction even without a truncation.

**R207C Mutation Does Not Result in KCC3 Truncation**—The screening of the KCC3 gene for mutations in a Turkish patient and his/her parents revealed the presence of a 619C→T missense mutation in exon 5 (Fig. 3a). This mutation was previously reported and contrasted with the contention that KCC3 truncation mutations alone can lead to disease (5). Given that the vast majority of known HMSN/ACC mutations truncate the co-transporter, we investigated whether the nucleotide transition in exon 5 might lead to aberrant RNA splicing and result in a “classic” case of HMSN/ACC truncation. To do so, total RNA extracted from lymphoblasts of the patient bearing the 619C→T mutation was reverse-transcribed to the corresponding cDNA. Three primer sets that flank the mutated exon 5 were used in a PCR to assess exon splicing. We observed that all predicted PCR fragments from the patient were present and were equal in size to those of the control sample, which implies a normal exon splicing of the mutant RNA (Fig. 3b). This overall suggests that a full-length KCC3 mutant protein (R207C) can be produced from this mutated KCC3 mRNA.

**R207C Mutation Affects KCC3 Function through Transit Defect to the Cell Surface**—So far, KCC3 loss of function was associated with disruption of truncated KCC3 interaction with CK-B. However, the missense KCC3 mutant R207C is not truncated but is a full-length protein that still co-localizes with CK-B in mammalian cells (supplemental Fig. S1). This suggests that disruption of KCC3 interaction with CK-B is not the mechanism of dysfunction of the R207C mutation. Given that the KCC3 R207C mutant interacts with CK-B and is likely trans-
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FIGURE 3. **R207C is not a truncated protein.** a, sequencing results for exon 5 of SLC12A6/KCC3 in a Turkish patient bearing the 619C→T transition; the mutation is expected to exchange Arg<sup>619</sup> for Cys<sup>619</sup> (R207C). b, total RNA extracted from lymphoblasts of the patient bearing the R207C mutation was analyzed by RT-PCR to assess the structure of the mutant RNA. PCR primers encompassing the junction of exons 4 and 5 (referred to as 4/5) and exons 5 and 6 (referred to as 5/6) and at the beginning of exon 4 (referred to as 4) and at the end of exon 6 (referred to as 6) were used to assess the structure of KCC3 RNA. The control corresponds to the wild-type cDNA. c, representation of the protein sequence encompassing the R207C mutation and amino acid conservation in various species. Highly conserved residues are indicated by an asterisk.

lated into an intact protein, we wondered if it may in fact be a benign variant and not a causative mutation. To assess its activity, R207C was expressed in Xenopus oocytes, and <sup>86</sup>Rb<sup>+</sup> uptake upon swelling activation of KCC3 was measured. Similarly to the truncated mutants, full-length R207C expression did not result in a significant flux in Xenopus oocytes (Fig. 4a), confirming that this mutation impairs KCC3 function and is likely causative in HMSN/ACC. Although R207C co-localized with CK-B in HeLa cells, we observed a strong perinuclear localization of the mutant protein in these cells, suggesting a deficiency in the transit of the R207C protein to the plasma membrane in this system (supplemental Fig. S1). KCC3 loss of function could therefore be caused by the absence of the mutant co-transporter at the plasma membrane. To investigate this hypothesis, we first performed Western blot analysis of plasma membrane proteins isolated from Xenopus oocytes expressing the wild-type and the mutant transporters. Similarly to R1134X, little R207C protein was observed in purified plasma membrane extracts of Xenopus oocytes (Fig. 4b), suggesting defective KCC3 transport to the cell membrane. To further support this hypothesis, we sought to measure how much of the mutant forms of KCC3 reached the plasma membrane in mammalian cells. Wild-type and mutant KCC3 were expressed in HeLa cells (Fig. 5a), and cell surface localization was quantified by measuring the rate of co-localization between KCC3 and pan-cadherin, which is a plasma membrane marker, using Pearson’s coefficient (Fig. 5b). The wild-type protein was mainly found at the plasma membrane of transfected HeLa cells (Pearson’s coefficient = 0.84). In contrast, most of the mutant R207C did not reach the plasma membrane (Pearson’s coefficient = 0.285; p < 0.0001 when compared with wild-type-transfected cells). For the R1134X mutation, plasma membrane localization was also impaired (Pearson’s coefficient = 0.57; p = 0.003 when compared with wild-type-transfected cells). Instead, R207C and R1134X accumulated inside the cytosol. This shows that the transit of these mutant co-transporters is deficient.

**R207C Amino Acid Exchange Stabilizes the Homodimeric Structure of KCC3**—It was reported that cation chloride co-transporters form transient homodimers and heterodimeric structures in Xenopus oocytes (10). As enhanced dimerization can alter the membrane targeting process through organelle retention (11), we investigated whether this mechanism was at the origin of the deficient transit of the mutant proteins to the plasma membrane. Wild-type and mutant KCC3 proteins were transiently expressed in mammalian cells, and total protein lysates were prepared for immunodetection by Western blots. The immunoblots for wild-type KCC3 revealed an abundant band below 150 kDa (KCC3 expected size) but also a faint band at ~300 kDa, which corresponds to double the size expected for KCC3 monomers. In the R207C protein extract, immunoblots revealed an abundant band at ~300 kDa (Fig. 5c). Increased stringency or additional sonication was required to disrupt some of these structures and restore a migration profile closer to the wild-type protein (Fig. 5, d and e). This suggests that the arginine to cysteine exchange in R207C favors the stabilization of homodimeric structures. This may affect the transit of R207C to the plasma membrane, which results in the intracellular accumulation of the mutant protein.

**Curcumin Treatment Partially Rescues the Transit Defect of Mutant KCC3**—To determine the predominant site of mutant protein accumulation, we used a panel of organelle markers, which included markers for the Golgi, proteasome, clathrin-coated vesicles (data not shown), lamellipodia (e.g. cortactin), ER (e.g. calreticulin), and plasma membrane (e.g. pan-cadherin) in co-immunofluorescence labeling experiments. No specific localization of R1134X was detected. However, we observed a
strong co-localization signal between R207C and the ER markers (Fig. 6b). Aberrant distribution of the mutant proteins was also observed in PC12 cells that were induced to differentiate by NGF treatment (Fig. 6c). This overall suggests that R207C dimers may be retained in the ER and thus be kept from reaching the plasma membrane.

Chemical compounds have been shown to favor the release of mutant transmembrane proteins that are aberrantly retained in the ER. Curcumin is one of the most promising compounds that help to relieve this retention, as it is harmless, eatable, and works in mouse models (12, 13). We sought to determine whether R207C retention in cultured cells could be lessened by curcumin treatment by incubating cells expressing the mutant protein with 5–40 μM curcumin at 37 °C. The localization of the proteins at the plasma membrane was evaluated using pan-cadherin as a specific plasma membrane marker in immunofluorescence studies. A concentration of curcumin of 10 μM was sufficient to rescue some of the transit to the plasma membrane (data not shown); however, the best results were observed with 20 μM curcumin (Fig. 6, d and e). This result further supports the hypothesis that R207C dimers are retained within the ER.

Aberrant Mutant KCC3 Localization in HMSN/ACC Brain—HMSN/ACC is most frequent in the French-Canadian population and is mainly caused by the T813X truncation mutation. We have previously shown that the T813X mutation leads to the loss of function of the co-transporter using a Xenopus oocyte flux assay. Although T813X was detected at the oocyte membrane (2), it has been reported that mutant channels or transporters can behave differently in the Xenopus oocyte system, where mutant proteins are less prone to traffic defects compared with mammalian cells and in vivo scenarios (11). In addition, when we expressed mutant proteins R1011X, L808X (4), or R1134X in mammalian cells, we observed aberrant intracellular accumulation of the mutant proteins. Finally, all truncated transporters, including the French-Canadian mutant protein T813X, lack a carboxyl-terminal hydrophobic tetrad involved in the trafficking of other SLC12 co-transporters. All these data suggest that T813X and the other mutant transporters might have some traffic defects in vivo.

To test this hypothesis, we focused on KCC3 localization in brain. First, both the wild-type KCC3 and the mutant T813X could be detected by Western blot using protein extracted from brain tissue of a control individual and a French-Canadian HMSN/ACC patient (Fig. 7a). Then to evaluate if defective transit participates in T813X pathogenesis, we observed the distribution of T813X-truncated protein in brain histological
sections by immunofluorescent labeling of KCC3. Using confocal microscopy, we found particularly abundant staining of T813X around the nucleus, and KCC3 distribution in unaffected neurons was more peripheral (Fig. 7b). In addition, swollen axons of the HMSN/ACC patient lacked KCC3 staining (data not shown). This suggests that a transit defect of T813X may occur in HMSN/ACC brains as well.

**DISCUSSION**

Potassium/chloride co-transporters participate in cell volume regulation, but they also control neuronal activity by transporting $K^+$ and $Cl^-$ ions across the plasma membrane.

Here, we confirmed that the loss of KCC3 activity is systematic in HMSN/ACC but that different pathogenic mechanisms can lead to the inactivation of the co-transporter. To participate in the electroneutral transport of ions, KCC3 needs to be translated and targeted to the plasma membrane, where it has to be properly activated. Some mutations have been associated with abrogated or defective protein biosynthesis. Because of their altered structure, the mutant mRNAs tend to be unstable and are efficiently cleared from the cell. As a result, virtually no protein is expressed. However, we did not observe anomalies in mutant KCC3 biosynthesis in this study (supplemental Fig. S2). Many variants, including those expressed in heterologous systems, fail to be properly processed to a mature glycosylated form and/or to be transported to the plasma membrane. In Gitelman disease, the majority of the missense mutations affects the glycosylation of NCC; however, nonglycosylated forms can still be processed to the cell surface. Alternatively, an apparently normal processing of a mutant protein with normal functional and kinetic properties can be associated with an impaired insertion into the plasma membrane. The most common mutation in cystic fibrosis ($i.e.$ $F508$) belongs in this category and, if correctly processed to the membrane, possesses...
residual activity and leads to a sustained normal or only mildly affected phenotype. For this reason, mutations in this group are promising targets for therapies aimed at correcting the processing and delivery of the mutant protein to the membrane. Mutations in channels/co-transporters, which produce abnormal protein variants, can also affect the regulation of the function of the protein eventually by preventing physical binding to partners that are required for its activation. In type I Bartter disease, the mutated co-transporters are normally synthesized, glycosylated, and inserted into the plasma membrane, which implies a defect in the functional properties or in the intrinsic activity of the protein. Finally, some mutations accelerate the removal from the plasma membrane and/or degradation of the mutant protein. Truncation of the carboxyl-terminal domain of CFTR reportedly leads to a marked instability of an otherwise fully processed and functional variant.

Our biochemical and cellular data demonstrate the presence of at least two distinct mechanisms that lead to KCC3 dysfunction in HMSN/ACC. We confirmed that the functional interaction between CK-B and truncated KCC3 is systematically disrupted. We also provide evidence that indicates that defective transit to the plasma membrane is involved in the loss of function of mutant KCC3 in HMSN/ACC. In addition, we showed that enhanced dimerization of cation chloride co-transporters seems to disturb their transit and activity. The R207C missense mutation results in the replacement of a highly conserved arginine residue with a cysteine residue, within or close to the first transmembrane domains of R207C (Fig. 3c). Moreover, cysteines are prone to form intramolecular and intermolecular disulfide bonds in proteins to promote the formation of tertiary and quaternary structures; thus the introduction of novel cysteines might be at the origin of these abnormal structures. Interestingly, several similar arginine/glycine to cysteine substitutions in NCC (R399C, G496C, R642C, R852C, and R919C) have been reported as being causative in Gitelman disease (3).

Cation chloride co-transporters show self-interacting properties when expressed in Xenopus oocytes (10). Here, we provide evidence that the R207C mutation induced the stabilization of dimeric structures, which demonstrates the occurrence of KCC3 dimerization in mammalian cells. To allow the formation of intermolecular disulfide bonds, the cysteine residues involved need to be physically close in the quaternary structure, which suggests that the first transmembrane domains directly allow the association between the KCC3 subunits. However, the failure of the aberrant dimers to reach the plasma membrane suggests that, in normal physiological conditions, the
dimerization is a regulated process that is not constitutive but occurs transiently. A similar mechanism leads to the mislocalization of the G480C form of CFTR in cystic fibrosis. In this case, the substitution of glycine with cysteine results in a protein that is not fully glycosylated and fails to reach the plasma membrane in mammalian cells. However, the mutant CFTR protein retained normal chloride channel activity in Xenopus oocytes, as channels and transporters are less likely to experience intracellular processing and trafficking disturbance in this system (11). Unfortunately, we were not able to detect a significant activity of R207C in Xenopus oocytes, maybe because the number of mutant proteins that reached the oocyte plasma membrane was not sufficient to allow the detection of co-transport activity.

The R1134X protein was under-represented on Western blot from mammalian cells when compared with the wild-type protein (Figs. 2a and 5, c–e, and supplemental Fig. S1). This under-representation of R1134X on Western blot may indicate a failure of cultured cells transfected with the mutant construct to proliferate at the rate of the cells expressing the wild-type construct. This hypothesis could be supported by the fact that KCC3 has been implicated in cell proliferation in normal and pathological instances (14). However, no obvious differences in cell density were observed in the nervous system of this HMSN/ACC patient when compared with the control sample. It is worth noting that the mutant co-transporter T813X also seemed to be under-expressed in the brain of an HMSN/ACC patient (Fig. 7a). Therefore, these observations may indicate an increased instability of the truncated proteins, which could either be due to the aberrant structure of the mutants and recognition by the cellular misfolding/degradation pathway or to their accelerated recycling from the plasma membrane to the cytoplasmic degradation machinery.

HMSN/ACC is a severe and progressive neurodegenerative disease that exhibits an early onset of symptoms. Signs of HMSN/ACC, such as hypotonia and delays in motor development skills, are noticed before 1 year of age. However, the motor abilities of patients progress slowly to 4–6 years of age, and these children are able to stand and walk with some help. This is followed by a motor deterioration that generally renders affected subjects wheelchair-dependent by adolescence. The patients generally die at the average age of 33. Interestingly, the mutant protein that exhibits a truncation of the last 17 amino acids is associated with one of the worst clinical manifestations of HMSN/ACC, whereas the patient bearing the missense mutation presents with one of the milder phenotypes reported.

| Observations               | Case 1 (5)       | Case 2                        | Case 3
|----------------------------|------------------|-------------------------------|------------------|
| Mutation                   | R207C            | R1134X                        | R1134X           |
| Reflexes                   | Present tendon reflex | Absent                       | Absent           |
| Tonicity                   | Normal tonus in upper extremities | Absent                     | Absent           |
| Motor milestones           | Walk with support at 3 y/o | Never achieved walking       | Never achieved walking |
| Mental retardation          | Mild             | Moderate                      | Moderate         |
| Seizures                   | None             | Partial and generalized       | Generalized      |
| ACC                        | Complete (MRI)   | Complete (MRI)                | Complete (MRI)   |

3 M. Shekarabi and G. A. Rouleau, unpublished data.
to date (Table 1) (5). Further investigations will be required to allow the clear establishment of genotype-phenotype correlations, as nonfunctional truncated transporters worsen the HMSN/ACC phenotype, and potentially functional but aberrant KCC3 dimers may attenuate some aspects of the disease presentation.

The distinct pathomechanisms uncovered here suggest novel therapeutic approaches for the management of the disorder or for the modulation of the progression of the disease. The structural similarities between KCC3 and CFTR suggest that efficient therapies identified for cystic fibrosis may be applicable to HMSN/ACC, at least at the molecular and cellular levels.

For example, incubation with curcumin restores the transit of CFTR units that are functional but are trapped in the ER by altering the function of resident ER chaperones as well as other mechanisms (15–17). This treatment yielded promising results in mouse models of cystic fibrosis (11). Accordingly, we found that curcumin relieved the ER retention of dimerized R207C in mammalian cultured cells. A diet enriched in curcumin may therefore be beneficial for the relief or delay of some of the HMSN/ACC symptoms in patients bearing the R207C mutation, including the Turkish patient described in this study (as patient has not yet reached puberty).

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