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KCC2 transport activity requires the highly conserved L_{675} in the C-terminal β1 strand

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ABSTRACT

The activity of the neuron-specific K^+, Cl^- co-transporter 2 (KCC2) is required for hyperpolarizing action of GABA and glycine. KCC2-mediated transport therefore plays a pivotal role in neuronal inhibition. Few analyses have addressed the amino acid requirements for transport-competent conformation. KCC2 consists of 12 transmembrane domains flanked by two intracellular termini. Structural analyses of a related archaeal protein have identified an evolutionary extremely conserved β1 strand, which links the transmembrane domain to a C-terminal dimerization interface. Here, we focused on the sequence requirement of this linker. We mutated four highly conserved amino acids of the β1 strand ($_{673}$QLLV$_{676}$) to alanine and analyzed the functional consequences in mammalian cells. Flux measurements demonstrated that L_{675A} significantly reduced KCC2 transport activity by 41%, whereas the other three mutants displayed normal activity. Immunocytochemistry and cell surface labeling revealed normal trafficking of all four mutants. Altogether, our results identify L_{675} as a critical residue for KCC2 transport activity. Furthermore, in view of its evolutionary conservation, the data suggest a remarkable tolerance of the KCC2 transport activity to amino acid substitutions in the β1 strand.

1. Introduction

Cation-chloride-cotransporters (CCCs) are electro neutral secondary-active transporters, which participate in essential physiological processes such as epithelial salt transport, osmotic regulation, and Cl^--homeostasis [1]. CCCs are divided into Na^+, K^+, Cl^- inward transporters (NKCC1-2, NCC), K^+, Cl^- extruders (KCC1-4), a polyamine transporter (CCC9), and the cotransporter-interacting protein CIP1 [1,2]. Among the KCCs, the neuron-specific KCC2 plays an outstanding role, as its activity is required for the hyperpolarizing action of the inhibitory neurotransmitters GABA and glycine [3–8]. In accord with this essential role, $KCC2^{-/-}$ mice die perinatally due to respiratory failure in the absence of synaptic inhibition [5], and knocking-down of the transporter leads to generalized seizure [9,10].

The structural organization of transport-active CCCs is highly conserved. The functional units are oligomers [11,12] and the individual genes encode polypeptides consisting of 12 transmembrane domains (TMD), a large extracellular loop (LEL), and intracellular termini [1]. Evolutionary sequence conservation is highest in the TMDs, followed by the C-terminus, whereas the N-terminus is poorly conserved [1,13]. Functional analyses of NKCCs revealed that TMD 2, 5 and 7 are necessary for the ion binding and transport [14–16]. The termini and the LEL are involved in allosteric or regulatory effects [16–18]. Concerning KCC2, several sites critical for its transport-active conformation have been identified. Mutation of the four cysteines in the LEL [18] or mutation of the C-terminal Y_{1087} to aspartate drastically reduced transport activity [19], whereas mutations that mimicked the dephosphorylated state of T_{906}/T_{1007} increased transport activity [20]. Finally, a KCC2-specific ISO domain was identified, which is involved in constitutive activity under isotonic conditions [21].

Recently, the X-ray structure of the C-terminus of a prokaryotic CCC (*Methanosaccrina acetovirans*, *ma*CCC) was determined [13]. This analysis revealed that the C-terminus is organized into two antiparallel subdomains, each composed of five parallel β-sheets, connected by α-helices. Notably, the highest evolutionary sequence conservation in the entire C-terminus was observed for the β1 strand [13]. To analyze the importance of this evolutionary conservation, we performed mutational analyses in the rat KCC2 (*rn*KCC2). Four highly conserved amino acids ($_{673}$QLLV$_{676}$) of the β1 strand were replaced by alanine and the functional consequences studied in mammalian cells.

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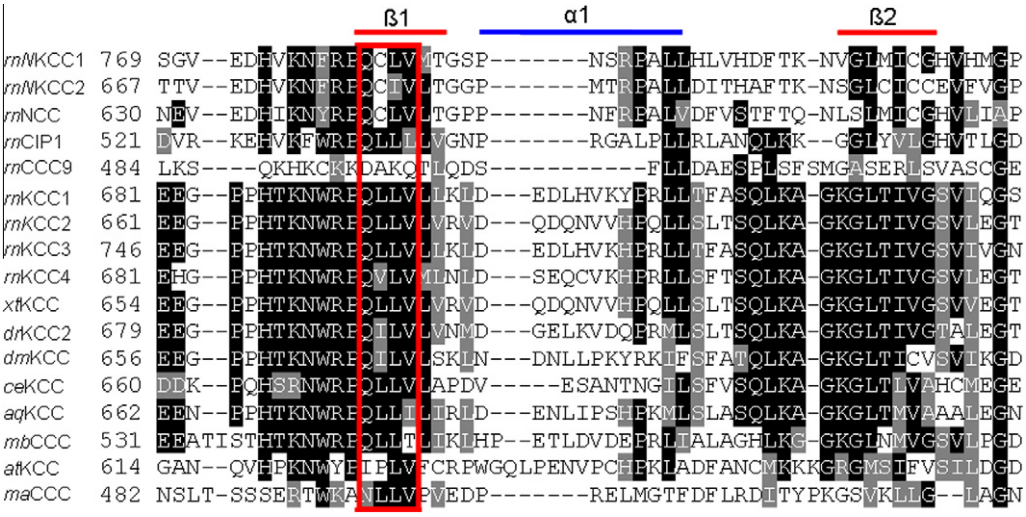

Fig. 1. QLLV residues are highly conserved in the β1 strand structure. A multiple sequence alignment of the C-terminal region of different CCC transporter was made with ClustalW [29]. Secondary structure data were derived from the crystal structure of the C-terminus of a CCC from *M. acetovirans* [13] and are shown above the alignment. QLLV residues are displayed in the box. The beginning of the C-terminus is indicated by an arrow. *Rn*, *rattus norvegicus*; *dm*, *drosophila melanogaster*; *aq*, *amphimedon queenslandica*; *mb*, *monosiga brevicollis*; *dr*, *daneo rerio*; *xt*, *xenopus tropicalis*; *ce*, *caenorhabtidis elegans*; *ma*, *methanosaccrina acetovirans*; *at*, *arabidopsis thaliana.* Amino acid sequences are: *rn*NKCC1 (GenBank ID: NP_113986.1), *rn*NKCC2 (GenBank ID: NP_062007.2), *rn*NCC (GenBank ID: NP_062218.3), *rn*KCC1 (GenBank ID: NP_062102.1), *rn*KCC2 (GenBank ID: NP_599190.1), *rn*KCC3 (GenBank ID: NP_001103100.1), *rn*KCC4 (GenBank ID: NP_001013162.2), *rn*CIP1 (GenBank ID: Q66HR0.1), *rn*CCC9 (GenBank ID: EDM11357.1), *xt*KCC (GenBank ID: NP_001072306.2), *dr*KCC (GenBank ID: XP_701000.4), *dm*KCC (GenBank ID: NP_726378.1), *ce*KCC (GenBank ID: ACN62948.1), *aq*KCC (GenBank ID: XP_003384645.1), *mb*KCC (GenBank ID: XP_001743661.1), *at*KCC (GenBank ID: AAF19744.1), and *ma*CCC (GenBank ID: NP_619366.1).

2. Materials and methods

2.1. Site-directed mutagenesis

Site directed mutagenesis was performed according to the Quick-Change mutagenesis system (Stratagene, Heidelberg, Germany), using a previously reported rat KCC2b (GenBank ID: NM_134363) expression clone [18]. Oligonucleotides for the generation of the mutations were as follows (only forward primers are given): $KCC2_{Q673A}$ 5′-CCAGCACCAGTAGCGCGGGCCTCCAGTTCT-3′, $KCC2_{L674A}$ 5′CTGGAGGCCCCAGGCACTGGTGCTGGTG-3′, $KCC2_{L675A}$ 5′TGGAGGCCCCAGCTAGCGGTGCTGGTGC-3′; $KCC2_{V676A}$ 5′-CCCCAG CTACTGGCGCTGGTGCGTGTG-3′. All generated clones used in this study were confirmed by sequencing.

2.2. Determination of K^+–Cl^- cotransport

Transport activity was determined by measuring Cl^--dependent uptake of $^{86}Rb^+$ (PerkinElmer Life Sciences Life Sciences) in HEK-293 cells [22]. Cells were cultured in DMEM (Invitrogen) and transfected using TurboFect (Fermentas, St. Leon-Roth, Germany). Cells were harvested 40 h after transfection and transferred into poly-L-lysine-coated wells of a six well culture dish and incubated for 3 h. After removal of the medium, cells were incubated in 1 ml preincubation buffer (100 mM N-methyl-D-glucamine-chloride, 5 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 5 mM HEPES, pH 7.4, 0.1 mM ouabain) for 15 min at room temperature. A 10 min uptake period in preincubation buffer supplemented with 1 μCi/ml $^{86}Rb^+$ at room temperature followed. At the end of the uptake period, cells were washed three times in 1 ml ice-cold preincubation buffer without ouabain to remove extracellular tracer. Cells were lysed in 500 μl 0.25 M NaOH for 1 h and then neutralized with 250 μl pure acetic acid. $^{86}Rb^+$ uptake was assayed by Cerenkov radiation, and the protein amount was determined by BCA (Thermo Fisher Scientific, Bonn, Germany).

In some experiments, non-radioactive flux measurements based on thallium (Tl^+)-mediated Fluozin-2 fluorescence were performed [18,23]. 24 h after transfection, HEK-293 cells were plated in poly-L-lysine-coated wells of a 96-well culture dish, black-walled with clear bottom (Greiner Bio-One) at a concentration of 100,000 cells/well. The next day, the medium was replaced by 80 μl of preincubation buffer (100 mM *N*-methyl-D-glucamine chloride, 5 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 5 mM HEPES, pH 7.4) containing 2 μM Flouzin-2 dye (Invitrogen) plus 0.2% (w/v) Pluronic F-127 (Invitrogen). Cells were incubated at room temperature for 48 min. Afterwards, the cells were washed three times with 80 μl of preincubation buffer and incubated for 15 min with 80 μl of preincubation buffer plus 0.1 mM ouabain. The cell plate was inserted into a fluorometer (Fluoroskan Accent, Thermo Scientific, Bremen, Germany), and the wells were injected with 40 μl of thallium stimulation buffer (12 mM Tl_2SO_4, 100 mM NMDG, 5 mM Hepes, 2 mM $CaSO_4$, 0.8 mM $MgSO_4$, 5 mM glucose, pH 7.4). The fluorescence was measured in a kinetic-dependent manner (excitation, 485 nm; emission, 538 nm; 1 frame in 5 s in a 200-s time span). The activity was calculated with the initial values of the slope of Tl^+-stimulated fluorescence increase by using linear regression.

In addition, expression of the respective construct was determined for each flux measurement by immunoblot analysis or immunocytochemistry. At least three biological and three technical replicas were performed for each experiment. Data are given as mean ± standard deviation. Significant differences between the groups were analyzed by a Student's *t*-test.

2.3. Immunocytochemistry

For immunocytochemistry, transfected cells were seeded on 0.1 mg/ml poly-L-lysine-coated coverslips. After 36 h, cells were fixed with 4% paraformaldehyde in 0.2 M phosphate buffer for 10 min. After fixation, cells were washed three times with phosphate-buffered saline (PBS) and incubated with blocking solution (0.3% Triton X-100, 3% bovine serum albumin, 11% goat serum in PBS) for 30 min at room temperature. Cells were then incubated with primary antibody N1/12 (NeuroMab, Davis, USA), diluted 1:500 in carrier solution (0.3% Triton X-100, 1% bovine serum albumin, 1% goat serum in PBS) for 1 h and washed three times with PBS for 5 min. After transfer in carrier solution, cells were treated