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Chemical crosslinking studies with the mouse Kcc1 K–Cl cotransporter

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ABSTRACT

Oligomerization, function, and regulation of unmodified mouse Kcc1 K–Cl cotransporter were studied by chemical crosslinking. Treatment of *Xenopus* oocytes and 293T cells expressing K–Cl cotransporter Kcc1 with several types of chemical cross-linkers shifted Kcc1 polypeptide to higher molecular weight forms. More extensive studies were performed with the amine-reactive disuccinyl suberate (DSS) and with the sulfhydryl-reactive bis-maleimidohexane (BMH). Kcc1 cross-linking was time-dependent in intact oocytes, and was independent of protein concentration in detergent lysates from oocytes or 293T cells. Kcc1 cross-linking by the cleavable cross-linker DTME was reversible. The N-terminal and C-terminal cytoplasmic tails of Kcc1 were not essential for Kcc1 crosslinking. PFO-PAGE and gel filtration revealed oligomeric states of uncrosslinked KCC1 corresponding in mobility to that of cross-linked protein. DSS and BMH each inhibited KCC1-mediated $^{86}Rb^{+}$ uptake stimulated by hypotonicity or by N-ethylmaleimide (NEM) without reduction in nominal surface abundance of KCC1. These data add to evidence supporting the oligomeric state of KCC polypeptides.

Introduction

The Slc12/Ccc cation chloride cotransporter gene superfamily includes at least nine members in mammalian species, with homologs that are expressed in yeast, bacteria, and archaea. Mammalian Slc12 genes include four Kcc genes (Kcc1-4) encoding Na^{+}-independent K–Cl cotransporters, in addition to the Na–K–Cl cotransporters Nkcc1 and Nkcc2, and the Na–Cl cotransporter, Ncc [1]. K–Cl cotransporters regulate neuronal and glial electrochemical equilibrium potential for Cl^{-}, and so can determine the excitatory or inhibitory influences of GABA- and glycine-gated Cl^{-} channels [2]. The *Kcc2*$^{-/-}$ mouse dies perinatally due to respiratory failure consequent to loss of synaptic inhibitory input [3]. Human KCC3 mutations are associated with syndromic agenesis of the corpus callosum, and the mouse knockout phenocopies the human disease [4]. The *Kcc4*$^{-/-}$ mouse exhibits deafness and renal tubular acidosis [5].

The Kcc and Nkcc transporters play opposing roles in the acute and chronic regulation of cell volume through volume decrease and increase, respectively [2,6]. Kcc activating stimuli include hypotonic and isotonic swelling, sulfhydryl alkylation by N-ethylmaleimide (NEM), serine-threonine kinase inhibitors such as staurosporine, hypertonic urea (but not hypertonic NaCl), and acid pH. Kcc inhibitors include serine-threonine phosphatase inhibitors and loop diuretics [7]. These various regulatory stimuli are mediated in part by the kinases Osr1, Spak, Wnk3, and Wnk4 [2,8]. Elevated K–Cl cotransport activity in red cells genetically altered in cytoskeletal proteins [9] also suggests a tonic inhibitory role for cytoskeletal anchoring.

The important role of K–Cl cotransport in erythrocyte volume regulation is magnified in hemoglobinopathies characterized by red cell dehydration, such as HbS and HbC disease and β-thalassemia. Therapeutic inhibition of K–Cl cotransport has been studied as an adjunct treatment for these disorders [10,11]. Kcc3 and Kcc1 appear to be the predominant K–Cl cotransporter polypeptides of mouse erythrocytes [12]. Whereas Kcc3 mediates nearly all mouse erythroid K–Cl cotransport activity [12], KCC3 deficiency in human red cells selectively decreases cotransport stimulated by NEM, while leaving staurosporine-stimulated transport unaltered [13].

Much remains to be learned about Kcc polypeptide structure. Chemical cross-linking data has suggested a homodimeric structure for NKCC1 [14], but a hetero-oligomeric structure was supported by the abilities of SLC12A9/CIP to co-immunoprecipitate NKCC1 and to suppress its function [15]. A hetero-oligomeric structure was also proposed to explain cAMP-sensitive dominant-negative functional inhibition of the NKCC2 variant mBSC1-A9 by mBSC1-A4 [16]. Both homo- and hetero-oligomeric structures were suggested by the ability of a dominant negative form of Kcc1 (Δ_N117) to co-immunoprecipitate

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wildtype Kcc1, and to inhibit function of coexpressed Kcc2, KCC3, and Kcc4 [17]. More recently, epitope-tagged rabbit Kcc1 was shown to undergo homo- and hetero-oligomeric interactions with other epitope-tagged Kcc isoforms as well as with Nkcc1 [18]. However, none of the cation chloride cotransporters have yet displayed a sigmoidal concentration vs. activity relationship that would support intersubunit interaction. Moreover, the possible regulation of KCC oligomerization state during acute regulation of ion transport rate remains unknown.

We hypothesized that chemical cross-linking might provide insight into the effect of Kcc1 oligomerization state on transporter function and regulation. We therefore have characterized the effect of chemical cross-linkers on recombinant, untagged, mouse Kcc1 polypeptides. The results provide independent evidence for a native oligomeric structure of mouse Kcc1 and other Kcc polypeptides.

Materials and methods

cRNA expression in Xenopus oocytes

Oocytes were harvested from female *Xenopus laevis*, and defolliculated as previously described [17,19]. On the same or next day, oocytes were microinjected with 50 nl water or cRNA (12.5 ng in water) transcribed with the T7 Megascript kit (Ambion, Austin, TX) from linearized cDNA templates encoding mouse Kcc1 [17,19], rat Kcc2, human Kcc3a, and mouse Kcc4 [17]. Oocytes were then incubated at 19 °C for 2–3 days in ND-96 containing (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 2.5 Na pyruvate, and gentamicin (5 mg%), titrated to a final pH of 7.40. Incubation medium was replaced daily.

Chemical cross-linking in intact Xenopus oocytes

Groups of 3–5 intact oocytes previously injected with cRNA or water were incubated 40 min at 20 °C in ND-96 lacking pyruvate and gentamicin (cross-linking buffer) and containing the following cross-linkers (from Pierce, Rockford, IL) at the noted concentrations (unless otherwise indicated): 5 mM bis(sulfosuccinimidyl) suberate (BS^3), or 2 mM disuccinyl suberate (DSS), dithio-bis-maleimidoethane (DTME), disuccinimidyl tartarate (DST), ethylene glycolbis (succinimidylsuccinate) (EGS), bis-maleimidohexane (BMH), succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB), or 2,2′-di-isothiocyanatostilbene-4,4′-disulfonate (DIDS, Calbiochem). The water-insoluble cross-linkers DSS, DST, BMH, DTME, EGS, and SMPB were made up in DMSO stock solutions. Final concentrations of DMSO in experimental solutions were less than 0.4%. The water-soluble cross-linkers BS^3 and DIDS were made up fresh at their final concentrations in ND96.

In some experiments, groups of 5–10 oocytes were preincubated at 20 °C in isotonic ND-96, in hypotonic medium (ND-72), in hypertonic medium (ND-200), or in ND-96 containing either 1 mM NEM or 200 μM (R(+)-[2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-indenyl-5-yl-)oxy]acetic acid (DIOA, Sigma). Some oocytes underwent 40 min additional incubation in the same solutions containing 2 mM DSS or BMH.

After rinsing, oocytes were homogenized in immunoprecipitation buffer (IP buffer, 10 μl/oocyte) of 1% Triton X-100 containing (in mM) 250 NaCl, 50 Tris–HCl, pH 8.0, pH 8.0, 1 EDTA, 1 phenylmethylsulfonylfluoride (PMSF), and Boehringer protease inhibitor tablets per manufacturer's recommendation. These detergent lysates were incubated with shaking for 30 min at 4 °C, then centrifuged 10 min in a microfuge. The resulting supernatants were subjected to SDS-PAGE, and the fractionated proteins were transferred to nitrocellulose membrane. KCC1 was detected by immunoblot as previously described, using affinity-purified rabbit polyclonal antibodies to the mouse KCC1 acetylated N-terminal aa 1–14 and to KCC1 C-terminal aa 1074–1086 [17,19]. Immunoblots were developed by enhanced chemiluminescence (Perkin-Elmer/NEN, Boston, MA).

Reversible cross-linking of metabolically labeled Kcc1

Oocytes were co-injected with ^{35}S-methionine (1 mCi/ml) and either water or Kcc1 cRNA. 2–3 days later, groups of 10 labeled oocytes were incubated for 40 min at 37 °C in ND-96 containing DTME (2 mM), then washed and homogenized in IP buffer. Lysate supernatants prepared as above were diluted with an equal volume of NaCl-free IP buffer, precleared with 5% normal rabbit serum, then incubated overnight with 1:10 diluted affinity-purified rabbit polyclonal antibody raised against the acetylated N-terminal peptide of mouse Kcc1. Immune complexes precipitated with Protein A-agarose were washed 6 times in IP buffer, 6 more times in NaCl-free IP buffer without NaCl, subjected to SDS-PAGE in the absence or presence of 5% β-mercaptoethanol, then to ^{35}S-Met fluorography.

Cross-linking in lysates from oocytes and from 293T cells

Groups of 3–8 oocytes were solubilized in 1% Triton X-100 immunoprecipitation buffer (10 μl/oocyte) containing (in mM) 250 NaCl, 20 Na phosphate, pH 7.4, 1 EDTA, 1 PMSF. The resulting detergent lysate was incubated with shaking at 4 °C for 30 min, then centrifuged for 10 min in a microfuge. The lysate supernatant was incubated with 2 mM cross-linker for 3 h at 4 °C. Amine-reactive cross-linkers were then quenched with 20 mM Tris–Cl, and the samples were subjected to SDS-PAGE and immunoblot analysis.

HEK-293T cells were transfected with mouse Kcc1 cDNA in pcDNA3, using Lipofectamine 2000 (Invitrogen). Cells were subjected to G418 selection (200 μg/ml) in DMEM plus 10% calf serum containing 2 mM glutamine, 100 μM penicillin and 100 μM streptomycin. Resistant populations were isolated with cloning rings, expanded, and screened by immunoblot for Kcc1 expression.

A cell line overexpressing Kcc1 was subjected to detergent extraction, and the resulting lysate supernatant was prepared and incubated with cross-linker as described above. Lysates were subjected to SDS-PAGE and immunoblot analysis.

Gel filtration of oocyte lysates

A Superose 6HR 10/30 gel filtration column (Pharmacia) equilibrated with 0.2% reduced Triton X-100 buffer of pH 7.40 containing (in mM) 20 Na phosphate, 150 NaCl, and 1 EDTA was calibrated with globular proteins of the BioRad Gel Filtration Standards: thyroglobulin (670 kDa), immunoglobulin G (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and with cyanocobalamin (1355 Da). Oocyte detergent lysate supernatants were subjected to gel filtration at a flow rate of 0.2 ml/min using a Pharmacia FPLC system. Fractions of 0.5 ml were collected, and 7 μl samples of each fraction were analyzed by SDS-PAGE and immunoblot.

PFO-PAGE

Ten oocytes were homogenized in 100 μl buffer containing (in mM) 250 NaCl, 50 Tris–HCl pH 8.0, 1 EDTA, 1 PMSF, and 0.25–1.0% pentadecafluorooctanoic acid (PFO, Oakwood Products, W. Columbia, SC). To some samples, SDS was added after homogenization as indicated (Fig. 5). PFO-PAGE [20] was performed at 4 °C with 0–8% PFO in the 2× sample buffer, and 0.5% PFO in the running buffer, or as indicated with electrophoresis at 140 V. Crosslinked phosphorylase *b* and High-molecular-weight Rainbow markers (Sigma) served as M_r standards. Fractionated proteins were subjected to immunoblot analysis.

Kcc1 immunofluorescence detection in Xenopus oocytes

Confocal immunofluorescence microscopy was performed as described previously [17]. Five days after cRNA injection, 6–10 oocytes