The Neuron-specific K-Cl Cotransporter, KCC2

ANTIBODY DEVELOPMENT AND INITIAL CHARACTERIZATION OF THE PROTEIN

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The neuron-specific K-Cl cotransporter (KCC2) is hypothesized to function as an active Cl- extrusion pathway important in postsynaptic inhibition mediated by ligand-gated anion channels, like γ-aminobutyric acid type A (GABA_A) and glycine receptors. To understand better the functional role of KCC2 in the nervous system, we developed polyclonal antibodies to a KCC2 fusion protein and used these antibodies to characterize and localize KCC2 in the rat cerebellum. The antibodies specifically recognized the KCC2 protein which is an ~140-kDa glycoprotein detectable only within the central nervous system. The KCC2 protein displayed a robust and punctate distribution in primary cultured retinal amacrine cells known to form exclusively GABA_A-ergic synapses in culture. In immunolocalization studies, KCC2 was absent from axons and glia but was highly expressed at neuronal somata and dendrites, indicating a specific postsynaptic distribution of the protein. In the granule cell layer, KCC2 exhibited a distinct colocalization with the β3/β2-subunits of the GABA_A receptor at the plasma membrane of granule cell somata and at cerebellar glomeruli. KCC2 lightly labeled the plasma membrane of Purkinje cell somata. Within the molecular layer, KCC2 exhibited a distinctly punctate distribution along dendrites, indicating it may be highly localized at inhibitory synapses along these processes. The distinct postsynaptic localization of KCC2 and its colocalization with GABA_A receptor in the cerebellum are consistent with the putative role of KCC2 in neuronal Cl- extrusion and postsynaptic inhibition.

The K-Cl cotransporter mediates an obligatorily coupled, electroneutral movement of K+ and Cl- ions across the plasma membrane of many animal cells. The K-Cl cotransporter is normally a net efflux pathway, using the favorable K+ chemical gradient maintained by the Na+K+-ATPase, to drive Cl- out of the cell. The cotransporter, however, is bi-directional and can mediate a net efflux or influx, depending upon the prevailing K+ and Cl- chemical gradients. The predominant function of this transporter is cellular ion and water homeostasis.

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The abbreviations used are: GABA_A, γ-aminobutyric acid type A; ABC, avidin-biotin complex; DAB, diaminobenzidine; DMEM, Dulbecco’s modified Eagle’s medium; GFAP, glial fibrillary acidic protein; GS/PBS, goat serum phosphate-buffered saline; HEP, human embryonic kidney; [Cl-], intracellular Cl- concentration; [K+]o, extracellular [K+]; KCC1, ubiquitous K-Cl cotransporter; KCC2, neuron-specific K-Cl cotransporter; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVD, polyvinylidene fluoride; Tricine, N-(2-hydroxyethyl)glycine.

Example, in red blood cells a K-Cl cotransporter is involved in cell volume regulation (1). Additionally, a K-Cl cotransporter may play an important role in salt transport across certain epithelia (2, 3). In neurons, a K-Cl cotransporter appears to serve as the active Cl- extrusion mechanism responsible for maintaining the Cl- reversal potential (E_Cl) less than the membrane potential (E_m) as required for the proper function of GABA_A and glycine receptors in postsynaptic inhibition (for reviews see Refs. 4 and 5).

Recently, two isoforms of the K-Cl cotransporter have been identified and functionally characterized (KCC1, Ref. 6 and KCC2, Refs. 7 and 8). KCC1 has a ubiquitous tissue distribution, and functional expression studies demonstrated that it was activated by cell swelling as well as by application of N-ethylmaleimide. Both of these treatments are well known activators of the red blood cell K-Cl cotransport system (6). Furthermore, like the K-Cl cotransporter studied in red blood cells, exogenously expressed KCC1 exhibited low transport affinity for both external K+ ([K+]o >50 mM) and external Cl- ([Cl-]o >50 mM). These data support the hypothesis that KCC1 represents the “housekeeping” isoform of the K-Cl cotransporter involved in cell volume regulation (6). In contrast, KCC2 was determined to be neuron-specific and displayed unique functional characteristics, including the lack of swelling activation and a remarkably high apparent affinity for external K+ ([K+]o = 5 mM; Refs. 7 and 8). These functional characteristics indicated that KCC2 may have a novel function in the nervous system, distinct from the volume regulatory role of KCC1. As KCC2 appears to be a neuron-specific isoform, we have hypothesized that it is involved in active Cl- extrusion. Based on thermodynamic considerations, an electroneutral K-Cl cotransporter can function to maintain very low intracellular [Cl-] ([Cl-]i; 5–7 mM) and [K+]o more negative than E_m. However, with such a low [Cl-], the driving force for net K-Cl cotransport will be very close to thermodynamic equilibrium (8). Thus, KCC2 with its high affinity for external K+ can function as a very efficient neuronal K+ uptake system whenever external [K+]o ([K+]) becomes elevated (>5 mM) and reverses the driving force for net K-Cl cotransport from efflux to influx. With the driving force for net K-Cl cotransport poised at equilibrium, KCC2 will be very sensitive to subtle changes in both [Cl-] and [K+]o, and therefore, it may function as a “dynamic buffer” of these two ion concentrations. This hypothesis is unifying as it provides a
cellular mechanism to account for the functional link between increases in [K\(^+\)]\(_{\text{e}}\) and alterations in neuronal [Cl\(^-\)]\(_{\text{e}}\), and GABA\(_{A}\) receptor function, all of which occur after repetitive stimulation (i.e. activity-dependent disinhibition).

Previous in situ hybridization studies supported a neuron-specific localization of the KCC2 transcript in rat brain (7). Cellular localization, however, of the KCC2 protein in the mammalian nervous system has not been performed. This may provide important information about the role of KCC2 in neuronal function. We hypothesized that if KCC2 is important in postsynaptic inhibition, it should colocalize with GABA\(_{A}\) receptors in the postsynaptic membrane. In the present report, we have prepared and characterized antibodies specific for the KCC2 protein and used these anti-KCC2 antibodies to characterize and localize the protein in the rat cerebellum. The rat cerebellum was chosen because of its very high expression of the KCC2 transcript (7). The KCC2 protein was restricted to neuronal somata and dendrites, and in the granule cell layer of the rat cerebellum it exhibited a distinct colocalization with the \(\beta_3\)/\(\beta_3\)-subunits of the GABA\(_{A}\) receptor. These data are fully consistent with KCC2 having an important role in neuronal Cl\(^-\) extrusion and postsynaptic inhibition.

**EXPERIMENTAL PROCEDURES**

**Production and Purification of KCC2 Fusion Protein and Anti-KCC2 Polyclonal Antibodies**—A KCC2 fusion protein (termed B22) was prepared, containing a 112-amino acid segment of the carboxyl terminus of rat KCC2 (amino acids 922–1043). The sequence encoding the fusion protein was amplified in a polymerase chain reaction (forward primer—GAATTCCAGATCAGATGAACTCTCG; reverse primer—CTCGAGTTAGTTCAAGTTTTCCACTCTCG). The reaction volume of 50 \(\mu\)l contained 5 \(\mu\)l of template cDNA (ERB10 clone at 2 ng/\(\mu\)l; see Ref. 7). 0.2 \(\mu\)g of each primer, 50 \(\mu\)M KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 0.1% Triton X-100, 2.5 mM MgCl\(_2\), 0.25 mM dNTPs, and 1 \(\mu\)l of Tq DNA polymerase (Promega, Madison, WI). Thirty cycles of PCR were performed (each consisting of incubation of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C). The PCR product was cloned into the plasmid pCR2.1 following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Using the restriction sites engineered into the forward (Ec\(\text{coRI}\)) and reverse (Xhol) PCR primers, the insert in the pCR2.1 vector was subcloned into pET28b+ (Novagen, Madison, WI). The B22 protein was very soluble in 1× binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9) containing 0.1% Triton X-100). It was purified using metal chelation chromatography following the manufacturer's instructions (His-Blue Resin; Novagen, Madison, WI). The B22 fusion protein was eluted from the column using a linear imidazole gradient (60 mM to 1 M). Four of the 1-ml fractions containing most of the eluted protein were combined and concentrated by centrifugation in microconcentrators (Amicon, Beverly, MA). This method of purification yields relatively pure B22 fusion protein with no other visible bands on Coomassie-stained gels (data not shown).

Rabbit polyclonal antibodies were generated against the purified B22 fusion protein by the Rabbit Antibody Production Program (Animal Resource Service of the School of Veterinary Medicine, University of California, Davis). Purified B22 fusion protein (250–300 \(\mu\)g) was injected subcutaneously with complete Freund's adjuvant. All subsequent boost injections were performed with incomplete Freund's adjuvant. Preimmune serum was obtained prior to first injection. Immune sera used in the present study was harvested >30 days after the second boost injection.

Immune antisera were purified using affinity chromatography. The B22 fusion protein was coupled to 1,1'-carbonyldimidazole-activated agarose beads following the manufacturer's instructions (Pierce). Immune antisera was incubated 2 days at 4 °C with B22 fusion protein-coupled agarose beads. Specific antibodies were eluted from the beads with 50 mM glycine (pH 2.7). Purified antibodies were stored in phosphate-buffered saline (PBS) containing 0.02% sodium azide at 4 °C.

**Tissue Culture**—The NG-108 and NE1-15 cells were maintained in growth medium, containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 5% hypoxanthine, aminopterin and thymidine (HT) supplement, and 2 mM L-glutamine. All other cultured cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 \(\mu\)g/ml). All cells were maintained in a humidified incubator with 5% CO\(_2\) at 37 °C.

A previous report described the development of HEK-293 cell lines stably expressing rat KCC2 protein (8). These stable HEK-293 cell lines (KCC2–9 and KCC2–22T) were maintained in growth medium containing 900 \(\mu\)g/ml geneticin (G418; Life Technologies, Inc.). A KCC1-KCC2 chimeric construct was produced by ligating rat KCC1 and rat KCC2 cDNA at a common BamHI site. This common BamHI site occurs 9 amino acids (i.e. 28 nucleotides) beyond the last predicted transmembrane segment of both proteins. The resulting construct had rat KCC2 encoding the hydrophilic amino-terminal region and transmembrane segment, and rat KCC1 encoding the remaining large carboxyl-terminal domain. This rat KCC1-KCC2 chimeric construct (termed KCC2–1C) also contained a 10 amino acid c-myc epitope (EQKLISEEDL) at the amino terminus of the protein. A stable HEK-293 cell line expressing the KCC2–1C construct was produced using previously described methods (9).

Dispersed retinal ganglia cells were prepared from embryonic chickens by methods previously described (10). Briefly, neurons from 8-day-old chick embryo retinas were dissociated in 0.1% trypsin. Cells were seeded into culture dishes containing poly-L-ornithine-coated glass coverslips. Cells were maintained in DMEM with 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 \(\mu\)g/ml), and 400 \(\mu\)M L-glutamine. Retinal cultures were incubated at 37 °C under a 5% CO\(_2\) humidified atmosphere. Cultured retinal cells were obtained for immunodetection after 16 days in culture.

**Protein Analysis**—Membranes were prepared from freshly isolated tissue and from cultured cell lines using differential centrifugation. Briefly, tissue was dissected or scraped off of culture plates and homogenized in 10–40 ml of homogenization buffer (250 mM sucrose, 10 mM Tris, 10 mM HEPES, 1 mM EDTA; pH adjusted to 7.2 at 24 °C) containing protease inhibitors. Following 10 strokes in a glass-Teflon homogenizer, the homogenate was centrifuged at 7,000 rpm for 10 min at 4 °C (Sorval RC5, SS-34 rotor). The supernatant was centrifuged at 20,000 rpm for 30 min at 4 °C. The final pellet was resuspended in ~100–500\(\mu\)l of homogenization buffer with protease inhibitors and stored at −80 °C. Protein concentration was determined using a Micro-BCA protein kit (Pierce). Membrane proteins were resolved by SDS-polyacrylamide gel electrophoresis using a 7.5% Tricine gel system. Gels were electrophoretically transferred from unstained gels to PVDF membranes (Immobilon; Millipore, Bedford, MA) in transfer buffer (192 mM Tris, 10 mM EDTA, 20 mM sodium phosphate buffer (pH 8.0), 50 mM EDTA, 1% Triton X-100) for 3 h at 200 mA. Membranes were blotted in PBS/milk (75% nonfat dried milk and 25% PBS) for 1.1 h and then incubated in PBS/milk containing 0.1% Tween 20 in PBS (pH 7.4) for 1 h and then incubated in PBS/milk with either preimmune serum, anti-KCC2 immune serum, affinity purified anti-KCC2 antibody, or c-myc monoclonal antibody overnight at 4 °C or 2 h at 24 °C. After three 10-min washes in PBS/milk, the PVDF membrane was incubated with secondary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG or anti-mouse IgG; Zymed Laboratories) for 2 h at room temperature. After three washes in PBS, 0.1% Tween 20, bound antibody was detected using an enhanced chemiluminescence assay (NEN Life Science Products).

Deglycosylation experiments were performed on membranes (20 \(\mu\)g) isolated from whole rat brain and HEK-293 stable cells. Membranes were incubated for 4 h at 37 °C in a medium containing 0.5% n-octyl glucoside, 20 mM sodium phosphate buffer (pH 8.0), 50 mM EDTA, protease inhibitors, and N-glycosidase F (20 units/ml; Boehringer Mannheim). Control samples were treated similarly, but incubation was carried out in the absence of N-glycosidase F. Enzymatic treatment was terminated by addition of electrophoresis sample buffer supplemented with 6 mM urea. Control and deglycosylated membrane proteins were separated on 7.5% Tricine gels, and KCC2 protein was identified by Western blotting.

**Immunolocalization of KCC2 Protein**—Immunocytochemical detection of KCC2, GABA\(_{A}\) receptor, and glial fibrillary acidic protein (GFAP) employed affinity purified rabbit anti-KCC2 polyclonal antibodies, mouse anti-GABA\(_{A}\) receptor \(\beta_3\)-subunit monoclonal antibody (Chemicon; Temecula, CA), and mouse anti-GFAP monoclonal antibody (Boehringer Mannheim). The anti-GABA\(_{A}\) receptor antibody is specific for \(\beta_3/\beta_3\) subunits (11, 12). Both an avidin-biotin complex (ABC) horseradish peroxidase technique and fluorescent labeling were used for immunocytochemical detection in rat cerebellum. Male Sprague-Dawley rats (200–275 g) were anesthetized with ketamine (20 mg/100 g intraperitoneally) and xylazine (0.1 mg/100 g intraperitoneally) and injected with heparin (10 units/100 g intraperitoneally). Their vasculature was immediately flushed with 200 ml of heparinized 0.9% saline solution.
and perfused with 500 ml of 4% paraformaldehyde in PBS via the ascending aorta. Brains were removed, postfixed in 4% paraformaldehyde for 2 h, frozen in 2-methyl butane on dry ice, embedded in TBS mounting media (Fisher), and stored at −80 °C. A Bright Instruments cryostat produced 15–30-μm frozen coronal sections.

For immunoperoxidase labeling, cerebellar sections were washed 3 times in PBS and then placed in 2% goat serum/PBS (GS/PBS; PBS containing 2% goat serum, 0.2% Triton X-100, 0.1% bovine serum albumin) for 2 h at room temperature. Sections were incubated at 4 °C for 48 h with primary antibody diluted 1:200 in GS/PBS. After three PBS washes, sections were incubated for 2 h at room temperature with either a biotin-conjugated goat anti-rabbit IgG (1:200 dilution; Vector Laboratories, Burlingame, CA) or goat anti-mouse IgG (1:200 dilution; Vector Laboratories, Burlingame, CA). Sections were washed three times in PBS and incubated for 3 h in an avidin/horseradish peroxidase solution prepared from an ABC kit (Vector Laboratories, Burlingame, CA). Following three washes in PBS, the horseradish peroxidase reaction was carried out using diaminobenzidine (DAB; 0.015% in PBS, Sigma) and 0.001% hydrogen peroxide. Sections were triple washed in PBS and mounted on silanized slides. Some sections were counterstained with hematoxylin. Sections stained with DAB were taken through an ethanol/xylene dehydration series and coverslipped with Permount (Fisher). Photographs of DAB-stained sections were taken with a Zeiss 135 microscope.

Double-labeled fluorescent immunocytochemical detection of KCC2 with either the μ2/δ2-subunits of GABA A receptor or glial fibrillary acidic protein (GFAP) was conducted on 10–15-μm cryostat sections that were free-floating in 24-well tissue culture plates or mounted on slides. Sections were blocked in 20% GS/PBS (PBS containing 20% goat serum, 0.2% Triton X-100, 0.1% bovine serum albumin) for 3 h at 24 °C and then incubated for 3 h at 24 °C with either anti-GABA A receptor antibody or anti-GFAP antibody. After 3 washes in PBS, sections were incubated for 2 h at 24 °C with a fluorescein (FITC)-conjugated goat anti-mouse IgG secondary antibody (1:200; Jackson Labs, West Grove, PA). Sections were washed 3 times in PBS and stored overnight in PBS at 4 °C. Sections were again blocked for 3 h in 20% GS/PBS and then incubated with anti-KCC2 antibodies (1:200) for 3 h at 24 °C. Following three PBS washes, sections were incubated for 2 h with Cy3-conjugated goat anti-rabbit IgG (1:200, Jackson Laboratories). Sections were then washed 3 times in PBS, once in distilled water, and mounted on acid-washed slides with Gel Mount (Biomedia, Foster City, CA). Cerebellar sections were examined, and digital images were obtained using laser scanning confocal microscopy (Zeiss 510 or Leica TCS-NT).

Immunoperoxidase labeling of KCC2 in retinal cultures employed the same specific procedures outlined above with the following modifications. Retinal cultures grown on coverslips were fixed in 4% paraformaldehyde in PBS for 30 min at 24 °C. They were washed 3 times in PBS and blocked with GS/PBS for 3 h at 24 °C. Cell cultures were incubated with anti-KCC2 antibodies for 3 h, washed 3 times in PBS, and then incubated at 24 °C for 2 h with biotin-conjugated goat anti-rabbit (Vector Laboratories). After 3 PBS washes, cells were incubated for 3 h in the avidin/horseradish peroxidase solution (ABC kit), and the DAB reaction was carried out as outlined above. Cultured cells were mounted on slides and then examined and photographed with a Zeiss 135 microscope.

Two types of controls were performed for immunocytochemistry. For each experiment, every third slide was incubated without primary antibody. None of the control slides run without primary antibody showed significant staining or fluorescent signal. A second control was tested further in an immunoadsorption experiment. B22 fusion protein to agarose beads in an affinity column (see “Experimental Procedures”). Affinity purified antisera as anti-KCC2 antibodies. The specificity of the antibodies for the KCC2 protein. In all subsequent experiments, we refer to affinity purified antisera as anti-KCC2 antibodies. The specificity of the anti-KCC2 antibodies for the KCC2 protein was confirmed using the same techniques as described above.

**RESULTS**

Development and Characterization of Anti-KCC2 Antibodies—We targeted the development of KCC2 antibodies to the predicted intracellular carboxyl-terminal domain, as this represents a large hydrophilic region of the protein containing a number of areas with high antigenicity (PEPTIDESTRUCTURE program, Genetics Computer Group, Madison, WI). Since both KCC1 and KCC2 are known to be present in the rat brain (6, 7), our goal was to develop antibodies that were specific for the KCC2 protein. To this end, we generated a fusion protein (B22) that contained a small segment of the carboxyl terminus of KCC2. The 112 amino acids contained within the B22 fusion protein are very poorly conserved between the KCC1 and KCC2 isoforms as follows: 1) only 61 of the 112 amino acids align with KCC1 and they display very low identity to KCC1 (31%), and 2) the remaining 51 amino acids represent residues unique to the KCC2 sequence (i.e. deleted from KCC1). Therefore, use of this region of KCC2 as antigen had a high likelihood of producing KCC2-specific antibodies.

Western blots of rat brain membranes using sera from animals immunized with the B22 fusion protein showed strong reactivity to a broad ~140-kDa band that was not observed with preimmune sera (Fig. 1). A number of additional bands were observed at high membrane loads; however, these bands were present at much lower intensity than the ~140-kDa band. In order to purify the B22 antisera, we cross-linked the B22 fusion protein to agarose beads in an affinity column (see “Experimental Procedures”). Affinity purification of the B22 antisera completely removed all nonspecific bands on the Western blot of rat brain membranes, demonstrating the specificity of the antibodies for the ~140-kDa band (Fig 2A). These data provide strong evidence that the ~140-kDa band represents the KCC2 protein. In all subsequent experiments, we refer to affinity purified antisera as anti-KCC2 antibodies. The specificity of the anti-KCC2 antibodies for the ~140-kDa band was tested further in an immunoadsorption experiment. B22 fusion protein was incubated at increasing amounts with a fixed amount of antibody. The B22 fusion protein at levels ≥1 μg was able to prevent completely any reactivity of the anti-KCC2

**FIG. 1.** Western blot analysis of the KCC2 protein in rat brain membranes using antisera of rabbits immunized with the B22 fusion protein. Rat brain membranes were loaded onto a 7.5% Tricine-SDS gel at 10–200 μg per lane as noted. The separated proteins were transferred to PVDF membranes and probed with either immune sera (antisera) or preimmune (Pre) sera. Both preimmune and immune sera were used at a 1:1,000 dilution.

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![Western blot analysis of the KCC2 protein in rat brain membranes using antisera of rabbits immunized with the B22 fusion protein.](image-url)
antibodies when tested against rat brain membranes on strip blots (Fig. 2B).

In order to provide further evidence of antibody specificity and to test potential antibody cross-reactivity with the KCC1 protein, we used the anti-KCC2 antibodies on Western blots of membranes prepared from various HEK-293 cell lines. Membranes were prepared from control untransfected HEK-293 cells and HEK-293 cells stably expressing either a full-length KCC2 construct (KCC2–22T) or a chimeraic construct of KCC1 and KCC2 (KCC2–1C). We have previously described the development and functional expression of the KCC2–22T cell line that expresses the full-length KCC2 protein (8). The chimeric protein expressed by the KCC2–1C cell line contains the amino-terminal and transmembrane domains of KCC2 and the carboxy-terminal domain of KCC1. In this chimera, the region over which the B22 fusion protein was prepared has now been replaced with KCC1 sequence. The KCC2–1C chimera is functional as the KCC2–1C cell line expressed a significantly elevated furosemide-sensitive $^{86}$Rb influx relative to control cells (data not shown). Both the KCC2 and KCC2–1C constructs were epitope-tagged with an amino-terminal c-myc peptide. The c-myc peptide monoclonal antibody displayed no reactivity in control HEK-293 cells but recognized a broad ~150-kDa protein in the two stably transfected cell lines (Fig. 3A). The exogenously expressed KCC2 protein consistently ran ~150 kDa following treatment with N-glycosidase F. Significantly, this core ~125-kDa protein migrates at ~10-kDa larger than from native tissue. The deglycosylation experiment presented in Fig. 4 demonstrates that this is the result of differences in N-linked glycosylation of the protein as both native and exogenously expressed KCC2 protein were epitope-tagged with the 10-amino acid c-myc peptide. Western blot panels were probed with either the c-myc peptide monoclonal antibody (1:2,000) or the anti-KCC2 antibodies (1:2,000).

Characterization of the KCC2 Protein—In the following studies, we used the anti-KCC2 antibodies purified from the B22 antiserum to characterize the KCC2 protein in the rat nervous system. We have previously demonstrated that KCC2 is a glycoprotein when it is exogenously expressed in stably transfected HEK-293 cells (8). Deglycosylation experiments confirmed this post-translational modification of the native KCC2 protein in rat brain (Fig. 4). As noted above, exogenously expressed KCC2 protein migrated on SDS-polyacrylamide gel electrophoresis ~10-kDa larger than from native tissue. The deglycosylation experiment presented in Fig. 4 demonstrates that this is the result of differences in N-linked glycosylation of the protein as both native and exogenously expressed KCC2 protein migrate at ~125 kDa following treatment with N-glycosidase F. Significantly, this core ~125-kDa KCC2 protein is similar in size to that predicted from the cDNA (7).

Previous studies examining the KCC2 transcript by Northern blot and in situ hybridization indicated that KCC2 was restricted to but widely distributed throughout the central nervous system (7). Western blot analysis with the anti-KCC2 antibodies was fully consistent with these findings. KCC2 protein was undetectable outside the central nervous system but was found throughout all regions of the brain and spinal cord (Fig. 5). Although retinal tissue was not present on this blot, there are numerous expressed sequence tags in the GenBank™ data base derived from mammalian retinal cDNA libraries, indicating high abundance of KCC2 in this tissue. KCC2 may be distributed largely on neuronal somata and dendrites, as it was not observed in the sciatic nerve by West-
The KCC2 antibodies displayed a very broad cross-reactivity among vertebrates. Although the anti-KCC2 antibodies were developed against protein deduced from the rat cDNA sequence, they recognized a 140-kDa protein in whole brain membranes prepared from every vertebrate species tested, including spiny dogfish, winter flounder, leopard frog, chicken, rabbit, rat, and mouse. This indicates that the B22 fusion protein represents a very well conserved region of the KCC2 protein.

By using Western blot analysis, we examined KCC2 expression in membranes prepared from a number of cultured neuronal cell lines derived from mammalian central or peripheral nervous system. The cell lines tested included two mammalian neuroblastomas (human SH-SY5Y cells and murine N1E-115 cells), a murine neuroblastoma-glioma hybrid (NG-108), a clonal murine hypothalamic neuron (GT1-7 cells), and a pheochromocytoma cell derived from rat adrenal (PC12 cells). None of these cultured cells expressed detectable amounts of the KCC2 protein. Also, as expected for a neuron-specific protein, KCC2 was not detectable in membranes from primary cultured rat astrocytes or rat glioma C6 cells. The failure to detect KCC2 in cultured neuronal cell lines indicated that neuronal differentiation might be required for KCC2 expression. In support of this hypothesis, we have identified two expressed sequence tags derived from a differentiated postmitotic human neuron (hNT) that clearly represent sequence from KCC2 (GenBank™ accession numbers AA16760 and AA166885). Furthermore, we examined the expression of KCC2 in a number of primary cultured neurons. Low levels of KCC2 protein were detected by immunocytochemistry in both rat hippocampal neurons and rat cerebellar granule cells during the first 7 days in culture (data not shown). In contrast, we observed significant KCC2 protein expression in retinal neurons grown 16 days in a dispersed culture system prepared from chicken. This dispersed retinal culture has been well characterized and is composed of amacrine cells, photoreceptors, bipolar cells, and glial cells, all of which are distinguishable on the basis of morphology and antibody staining. Amacrine cells in these cultures have been shown to form synapses from one cell to another (10) as well as autapses from one cell to itself (14). Significantly, these synapses and autapses are exclusively GABAergic (10). As shown in Fig. 8, amacrine cells in the retinal cultures abundantly expressed the KCC2 protein. In contrast, glial cells that form a monolayer upon which neurons were often found were never seen to express KCC2, although antibodies to the Na-K-Cl cotransporter stained glial cells strongly (data not shown). Among the neurons within cultures examined at embryonic equivalent day 16 or older, many although not all labeled for KCC2. Amacrine cells and some apparent bipolar cells showed the heaviest KCC2 staining. In those cells that stained, weak and homogeneous staining of the cell body cytoplasm was observed together with punctate staining of the plasma membrane both at the cell body and along the dendrites (arrows in Fig. 8). In general, the greatest density of stained spots occurred at the places where cell bodies or dendrites touched each other, consistent with the idea that KCC2 is found predominantly at synapses.

Immunocytochemical Localization in Rat Cerebellum—Further confirmation of the neuron-specific nature of the KCC2 protein was obtained by immunocytochemical analysis of sagittal sections of rat cerebellum. The B22 fusion protein was detected in the Purkinje cells, granule cells, and stellate cells of the cerebellum. In Purkinje cells, KCC2 staining was observed in the cell body, with punctate staining of the plasma membrane both at the cell body and along the dendrites (arrows in Fig. 8). In general, the greatest density of stained spots occurred at the places where cell bodies or dendrites touched each other, consistent with the idea that KCC2 is found predominantly at synapses.

### Deglycosylation of the KCC2 protein

**Fig. 4.** Deglycosylation of the KCC2 protein. Membranes prepared from whole rat brain and stable HEK cells expressing KCC2 (KCC2-22T) were incubated with (+) or without (−) N-glycosidase F for 4 h at 37 °C. Western blot was probed with anti-KCC2 antibodies (1:2,000).

### Western blot analysis of membranes prepared from rat brain regions

**Fig. 5.** Western blot analysis of membranes prepared from rat brain regions (100 µg), spinal cord (100 µg), sciatic nerve (200 µg), and various rat tissues (200 µg). KCC2 protein was detected with anti-KCC2 antibodies (1:2,000).

### Western blot analysis of membranes prepared from whole brains of shark, winter flounder, teeleost fish, amphibia, bird, and mammals

**Fig. 6.** Western blot analysis of membranes prepared from whole brains of shark (spiny dogfish, *Squalus acanthias*), teeleost fish (winter flounder, *Pseudopleuronectes americanus*), amphibian (leopard frog, *Rana pipiens*), bird (chicken, *Gallus domesticus*), and various mammals, rabbit (New Zealand White), rat (Sprague-Dawley), and mouse (BALB/c). All membranes were loaded at 100 µg. KCC2 protein was detected with anti-KCC2 antibodies (1:2,000).
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FIG. 7. Western blot analysis of membranes prepared from whole rat brain and various cultured cells (100 µg) with anti-KCC2 antibodies (upper panels, 12,000) and anti-β-actin antibodies (lower panels, 12,000). The cell lines include the following: A, rat primary astrocytes, rat C6 glioma cells, human neuroblastoma SH-SY5Y cells, murine hypothalamic GT1-7 neurons, murine neuroblastoma N1E-115 cells, murine neuroblastoma-glioma hybrid NG-108 cells, and rat pheochromocytoma PC12 cells; B, primary cultured retinal cells prepared from chicken.

protein was obtained from localization studies with the anti-KCC2 antibodies in rat cerebellum. Immunocytochemical staining for the KCC2 protein in the cerebellum was distinct and positive relative to controls (Fig. 9, A and B; data not shown for controls). KCC2 staining occurred principally in the granule cell and the molecular layers of the cerebellar cortex. There was no apparent difference in the distribution or intensity of staining among the lobules or along the length of the folia. No detectable immunocytochemical KCC2 staining was observed in axons or in glial cells (Fig. 9, A and B). In experi-

ments using double-labeled fluorescent microscopy, the KCC2 protein and a glial-specific marker, GFAP, exhibited distinctly different localization patterns (Fig. 10, A–C). The anti-KCC2 antibodies (in red) brightly labeled the plasma membranes of neuronal somata and dendrites throughout the rat cerebellum, indicating that KCC2 is largely postsynaptic in its distribution. In contrast, the anti-GFAP antibody (in green) labeled Bergmann fibers that predominate in the molecular layer as well as some glial processes that occur within the granule cell layer. These data are consistent with the neuron-specific nature of the KCC2 protein and confirm our earlier findings based on in situ hybridization experiments (7).

The granule cell layer of the cerebellum exhibited the most intense immunocytochemical staining for KCC2 as well as for the GABA_A receptor β/β-subunits (Fig. 9, A–D). Using double-labeled fluorescent microscopy, these two proteins exhibited a distinct colocalization in the granule cell layer (Fig. 10, D–F). There was no stratification of KCC2 staining through the depth of the granule cell layer. Fluorescent labeling for KCC2 and β/β-subunits of the GABA_A receptor brightly outlined the plasma membranes of granule cell somata (Fig. 10, D–F). Structures that stained most intensely for KCC2 and the β/β-subunits of the GABA_A receptor did not show nuclear counterstaining with hematoxylin, indicating they were cerebellar glomeruli.

In sections stained by the immunoperoxidase method, there was little overt concentration of KCC2 around the cell body or axon hillock of Purkinje cells (Fig. 9B). Immunofluorescent labeling for KCC2, however, showed clear labeling on the plasma membrane of Purkinje cell somata (Fig. 10, A and D). As observed in earlier studies, the β/β-subunits of the GABA_A receptor exhibited little reactivity with the Purkinje cell somata using either immunoperoxidase or immunofluorescent labeling (Figs. 9D and 10E; Refs. 11 and 15).

Immunostaining for KCC2 in the molecular layer of the rat cerebellum was distinctly punctate and oriented mostly along neuronal processes (Figs. 9B and 10, A and D). The anti-GABA_A receptor antibodies exhibited very low level staining in the molecular layer (Figs. 9, C and D, and 10E), confirming previous reports for the β/β-subunits of the GABA_A receptor in this region (11, 15). Purkinje cell dendrites alone may not account for all of the KCC2 immunostaining in the molecular layer. Golgi and basket cells have significant numbers of small dendrites running perpendicularly and obliquely through the molecular layer. Staining in the molecular layer may be associated with the dendritic processes of all these neurons. The plasma membranes of neuronal somata in the molecular layer were also distinctly outlined with KCC2 (Figs. 9B, and 10, A and D). These neurons likely included both basket and stellate cells. There was little KCC2 staining of neuronal processes along the long axis of the folia in transverse sections, indicating the absence of significant KCC2 protein in the parallel fibers of the granule cells.

DISCUSSION

Intracellular [Cl⁻] is an important component in determining the direction of Cl⁻ movement through conductive pathways. In order for conductive Cl⁻ movement to take place, Cl⁻ must be maintained away from equilibrium through active transport mechanisms. An emerging hypothesis is that the cation chloride cotransporters (i.e. Na-K-Cl and K-Cl cotransport) of various cell types are important regulators of [Cl⁻], and, therefore, are important determinants of the direction and driving force for net Cl⁻ movement through anion channels (16–19). In neurons, a K-Cl cotransport system has been implicated as the active Cl⁻ extrusion mechanism that maintains low [Cl⁻], and an inwardly directed Cl⁻ electrochemical gradi-
ent necessary for the inhibitory function of GABA<sub>A</sub> receptors (e.g. Refs. 20–24). In the present study, we have developed antibodies against the neuron-specific K-Cl cotransporter, KCC2. These immunological probes were used to characterize and localize the protein to understand better the role of KCC2 in ion homeostasis in the nervous system.

The anti-KCC2 antibodies were generated against a purified KCC2 fusion protein, and they specifically recognized the 140-kDa KCC2 protein from a number of vertebrate species. Importantly, the antibodies displayed no cross-reactivity with the KCC1 isoform. Like the exogenously expressed KCC2 protein in stable HEK-293 cells (8), the native KCC2 protein is glycosylated. Following treatment with N-glycosidase F, the apparent molecular mass of the rat brain KCC2 protein decreased from 140- to 125-kDa. The fact that the protein, following treatment with N-glycosidase F, displayed a molecular mass very close to that predicted from the cDNA (123.6-kDa) indicates that the predominant glycans are N-linked. Other members of the cation chloride cotransporter gene family have been shown to be adorned predominantly with N-linked oligosaccharides (9, 25, 26). The region of the KCC2 protein likely harboring the N-linked glycans is the large predicted extracellular loop between putative transmembrane segments 5 and 6 where four consensus sites for N-glycosylation are located.

Expression of KCC2 appears to require neuronal differentiation. We failed to detect KCC2 protein in a number of undifferentiated neuronal cell lines. However, we did observe KCC2 protein in primary cultured neurons, especially in a dispersed retinal culture. In these retinal cultures, KCC2 protein exhibited a distinctly punctate distribution at the plasma membrane of amacrine cell somata and dendrites, and the highest expression was observed in regions where cells contacted each other. As amacrine cells in culture form synapses and autapses that are exclusively GABA<sub>A</sub>ergic (10), these findings indicate that KCC2 might be highly localized at GABA<sub>A</sub>ergic synapses. Such a model would allow KCC2 to effectively control [Cl<sup>-</sup>] immediately at the inhibitory synapse. The fact that KCC2 was observed only in differentiated cultured neurons indicates that synaptic formation may be an important requirement for KCC2 expression.

In an earlier report, we demonstrated that KCC2 transcript was specifically localized in neurons throughout the rat central nervous system and was especially abundant within the cerebellum (7). Western blot analysis and immunolocalization studies conducted with the anti-KCC2 antibodies fully support these findings. Distinct KCC2 immunostaining was observed in most cerebellar neurons, and it was found predominantly if not exclusively at the plasma membranes of neuronal somata and dendrites. No significant KCC2 staining was evident along axons or in glial cells of the cerebellum (e.g. Bergmann glial cells). The lack of glial cell staining with the anti-KCC2 antibodies was confirmed in a double label fluorescent experiment with GFAP, which is a specific marker for glial cells (Fig. 10, A–C). These data are consistent with a neuron-specific localization of KCC2 and also provide strong support for a postsynaptic function of KCC2.

The most intense immunostaining for KCC2 in the cerebell-
lum was observed in discrete areas within the granule cell layer that appeared to be cerebellar glomeruli. Importantly, KCC2 exhibited a distinct colocalization with the β2/β3-subunits of the GABA A receptor at the cerebellar glomeruli. Golgi cells are GABAergic, and their axons synapse with granule cells at the cerebellar glomeruli where granule cell dendrites form complex synaptic contacts with the terminals of afferent mossy fibers. The cerebellar glomeruli are very rich in inhibitory synapses and exhibit significant immunoreactivity for glutamic acid decarboxylase, GABA, and GABAA receptor (Fig. 9, C and D, Refs. 15 and 27–29). We propose that the intense KCC2 immunostaining associated with the cerebellar glomeruli is likely at the postsynaptic membrane of the granule cell dendrites making up these synaptic structures. Interestingly, we clearly observed plasma membrane staining of KCC2 around the cell bodies of granule cells. The plasma membranes of granule cell somata also stained for the GABA A receptor β2/β3-subunits (Fig. 9, C and D; Refs. 15, 29, and 30). The somatic localization of GABA A receptors in granule cells is believed to be extrasynaptic as granule cells are not innervated at their cell body (31). This extrasynaptic colocalization of GABA A receptor and KCC2 at the plasma membrane of granule cell somata is consistent with an hypothesis of inhibitory transmission by GABA spillover which may be important in controlling granule cell excitability (32, 33).

We observed a lower level of KCC2 immunoreactivity within the molecular layer of the cerebellum. The plasma membranes of neuronal somata within the molecular layer clearly stained for KCC2, and these likely included both basket and stellate cells. The KCC2 immunoreactivity of stellate and basket cell bodies is consistent with the GABAergic inhibition these cells receive from other stellate and basket cells as well as reciprocal GABAergic inhibition from Purkinje cells (34). Much of the KCC2 immunostaining within the molecular layer was punctate, indicating that KCC2 may localize at specific sites along the numerous dendrites that occur in this region, especially from Purkinje cells. Although β2/β3-subunits of the GABA A receptor only weakly stain the dendritic tree of Purkinje cells (Fig. 9, C and D, and see Refs. 15 and 29), significant immunoreactivity of Purkinje cell dendrites has been observed with anti-GABA A receptor antibodies prepared to the γ2-subunit (35). Purkinje cell dendrites receive GABAergic synapses predominantly from stellate cells. As proposed for KCC2 expression in cultured amacrine cells, we hypothesize that KCC2 is highly localized at GABAergic inhibitory synapses along Purkinje cell dendrites. GABAergic inhibition is known to occur at the soma of Purkinje cells (36, 37). The axons of basket cells surround the soma and axon hillock of Purkinje cells, forming a synaptic structure called pinceaux (38, 39). Basket cells are GABAergic inhibitory interneurons as their axons are strongly associated with both GABA and glutamic acid decarboxylase immunoreactivity (15). Significantly, we observed distinct but low level immunostaining for KCC2 at the plasma membranes of Purkinje cell somata. A weak staining pattern was also observed for the GABA A receptor β2/β3-subunit antibodies (Fig. 9, C and D, and see Refs. 15, 29, and 30). The plasma membranes of Purkinje cell somata have been reported to exhibit strong immunoreactivity for the α1- and γ2-subunits of the
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