Neuronal Restrictive Silencing Element Is Found in the KCC2 Gene: Molecular Basis for KCC2-Specific Expression in Neurons

MICHAEL F. KARADSHEH AND E. DELPIRE

Departments of Anesthesiology and Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee 37232-2520

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KCC2 is one of four known isoforms of the K-Cl cotransporter with an expression pattern restricted to neurons. It mediates efflux of Cl\(^{-}\) across neuronal membranes and plays an important role in GABAergic and glycineric neurotransmission. To understand the molecular basis for neuronal specificity of KCC2 expression, we isolated and sequenced portions of the KCC2 gene, including some of its 5′ flanking (control) region. We found a 21-bp sequence, within intron 1, that shares 80% homology to the consensus site for neuronal-restrictive silencing factor binding. We demonstrated that this specific sequence of the KCC2 gene promotes transcriptional regulation by showing that nuclear proteins isolated from a mouse neural progenitor cell line interact with this 21-bp element and by establishing that this element silences reporter gene expression in nonneuronal cells.

INTRODUCTION

GABA and glycine are inhibitory neurotransmitters in the adult CNS. However, they produce excitatory responses in young immature brain (Ben-Ari et al. 1989; Ehrlich et al. 1999). The conversion of the GABA effect from excitatory to inhibitory occurs shortly after birth and is related to the intracellular Cl\(^{-}\) concentration in neurons (Ehrlich et al. 1999; Owens et al. 1996). Immature neurons have an intracellular Cl\(^{-}\) concentration higher than predicted for a passive distribution (Ehrlich et al. 1999; Owens et al. 1996). On activation of GABA\(_{\alpha}\) receptor, there is an outward movement of Cl\(^{-}\) that leads to membrane depolarization (excitation). During postnatal development, the intracellular Cl\(^{-}\) concentration decreases to values below equilibrium and GABA induces Cl\(^{-}\) influx resulting in membrane hyperpolarization (inhibition).

The expression of the Na-K-2Cl cotransporter, NKCC1, decreases (Plotkin et al. 1997), whereas expression of KCC2, an isoform of the K-Cl cotransporter, increases (Clayton et al. 1998; Lu et al. 1999) during postnatal development. These changes in cotransporter expression are consistent with a decrease in intracellular Cl\(^{-}\) concentration and with the switch from depolarizing to hyperpolarizing GABA currents.

Analysis of KCC2 expression by Northern blot, in situ hybridization, and immunofluorescence has revealed that KCC2 expression is restricted to neurons (Lu et al. 1999; Payne et al. 1996). To study the neuronal specificity of KCC2 expression, we isolated and examined the gene encoding this cotransporter. In the course of identifying and isolating exon 1 and the upstream regulatory region of the gene, we discovered a 21-bp motif downstream of exon 1 that resembles the consensus sequence of the known neuronal-restrictive silencing element (NRSE). We report here that this KCC2 element binds to nuclear proteins and inhibits the transcription of a reporter gene in C17 nonneuronal cells.

METHODS

Genomic library

A Lambda FIXII mouse genomic library (Stratagene, La Jolla, CA) was screened using the first 280-bp fragment of the rat KCC2 cDNA (Payne et al. 1996). Five positive clones were isolated. After secondary screening, two single positive clones were grown in liquid culture, and phage DNA was isolated and mapped with a panel of restriction enzymes. Southern blot analysis was performed to identify exon 1. A 12-kb EcoRI-NorI fragment was subcloned into the vector Bluescript, and 1.6 kb at the 5′ end was sequenced.

Electromobility shift assay

C17 progenitor cells from eight confluent 10-cm culture dishes were trypsinized, washed, and spun for 5 min at 1,500 rpm. Nuclear proteins were isolated as previously described (Shelton et al. 1992) and quantitated using the Bradford assay (Bradford 1976). Two complementary neuronal-restrictive silencing element (NRSE) oligonucleotides were end-labeled with \(^{32}\)P-dATP using T4 polynucleotide kinase, annealed, and purified by phenol:chloroform extraction. Protein-DNA binding was achieved by incubating 7 μg nuclear protein with 2 μl probe (4 pmols) in a 25-μl reaction containing (in mM) 100 KCl, 5 MgCl\(_2\), 1 EDTA, 0.5 DTT, 0.1 ZnCl\(_2\), 10% glycerol, 0.05% Nonidet P-40, and 10 HEPES pH 7.7 for 15 min at room temperature. The reactions were then separated on 4% polyacrylamide gel.

PGL3 constructs and luciferase assays

A 1.5-kb EcoRI-Xhol fragment of the mouse KCC2 gene containing some 5′ flanking sequence, the putative minimal promoter and some 5′ untranslated region was ligated at the EcoRI and Xhol sites of the promoterless luciferase reporter gene vector PGL3-basic (Promega). This new vector was designed to test the activity of the KCC2 promoter. Two synthetic complementary oligonucleotides containing the 21-bp KCC2 NRSE sequence flanked by EcoRI sites were then-ligated at the EcoRI site upstream of the KCC2 promoter. The three
and 4.4 followed by the addition of 334 C17 neural progenitor cells were incubated with 32 P-end-labeled NRSE oligonucleotide (TCCAGAACCGTGAGCAGCCG) for 15 min at room temperature. The reactions were separated on 4% acrylamide gels and exposed to autoradiography. The position corresponding to the 5′ end of the rat cDNA, the 5′ untranslated region (UTR), the ATG start of the KCC2 protein (origin of arrow, M, methionine; L, leucine; N, asparagine), and the exon/intron boundary (5′ splice junction). The position of the KCC2 NRSE sequence, depicted as a star, is observed downstream of exon 1. The alignment of the KCC2 putative neuronal-restrictive silencing element (NRSE) sequence with the NRSE consensus sequence is shown with identical residues placed in boxes and with the residues, likely subject to modifications (asterisk). M and S in the consensus sequence represents (A, C) and (C, G), respectively. Selected restriction sites are shown as landmarks: X, XhoI; E, EcoRI. The thick black line starting at the EcoRI site indicates the fragment which was sequenced, and the thick gray line represents a 10.2-kb genomic fragment released in Genbank under Accession No. AJ011033.

FIG. 1. Structure of a 18-kb genomic fragment isolated from a mouse genomic library. Magnification of exon 1 displays the position corresponding to the 5′ end of the rat cDNA, the 5′ untranslated region (UTR), the ATG start of the KCC2 protein (origin of arrow, M, methionine; L, leucine; N, asparagine), and the exon/intron boundary (5′ splice junction). The position of the KCC2 NRSE sequence, depicted as a star, is observed downstream of exon 1. The alignment of the KCC2 putative neuronal-restrictive silencing element (NRSE) sequence with the NRSE consensus sequence is shown with identical residues placed in boxes and with the residues, likely subject to modifications (asterisk). M and S in the consensus sequence represents (A, C) and (C, G), respectively. Selected restriction sites are shown as landmarks: X, XhoI; E, EcoRI. The thick black line starting at the EcoRI site indicates the fragment which was sequenced, and the thick gray line represents a 10.2-kb genomic fragment released in Genbank under Accession No. AJ011033.

RESULTS AND DISCUSSION

Negative transcriptional regulation of neuronal genes in nonneuronal cells was first described for the SCG10 and type II Na+ channel genes (Kraner et al. 1992; Mori et al. 1990). Since these original reports, transcription of many genes expressed specifically in neurons have been shown to be regulated through this mechanism. These genes contain in their promoter or introns a specific sequence NRSE that constitutes the binding site for a protein expressed in nonneuronal cells (NRSF or REST) and inhibits their transcription. This negative transcriptional regulation is also critical in the development of the nervous system. Most genes expressed in differentiated or mature neurons are not found in precursor and immature cells. Up-regulation of these neuronal specific genes coincides with the down-regulation of NRSF expression (Schoenherr and Anderson 1995). Importance of NRSF regulation is also evidenced by the multiple malformations observed by Chen and coworkers in different nonneural tissues and by the embryonic lethality of complete inactivation of NRSF in the knockout mouse (Jones and Meech 1999).

Out of four genes that encode K-Cl cotransporter, KCC2 has been shown to be neuronal specific with potential relevance in CNS development and intracellular Cl− homeostasis (Clayton et al. 1998; Lu et al. 1999). Previous work has determined that KCC2 expression is low at birth and increases during postnatal development (Clayton et al. 1998; Lu et al. 1999). Previous work has determined that KCC2 expression is low at birth and increases during postnatal development (Clayton et al. 1998; Lu et al. 1999). The KCC2 gene is therefore a very good candidate for regulation by NRSF. To understand the molecular basis for the restrictive expression of KCC2 in neurons, we isolated the promoter region of the KCC2 gene. A mouse lambda phage genomic library was screened with a probe consisting of the first 280-bp fragment of the rat KCC2 cDNA (Payne et al. 1996). A ~18-kb genomic clone was obtained and analyzed by restriction digest and Southern blot analysis. This genomic clone contains ~7 kb of 5′ flanking region, exon 1, and ~11 kb of the downstream sequence. The 5′ flanking region (1.5 kb of it), exon 1, and a short portion of intron 1 were sequenced. We did
not characterize the 5′ boundary nor the 3′ end of the gene. The entire sequenced fragment was analyzed using the transcription factor database available at http://www@genome.ad.jp. As expected, we found in intron 1 a 21-bp sequence with high sequence homology (81%) to the known consensus sequence of NRSE (Fig. 1). This consensus was derived from 19 sequences for which NRSF binding was experimentally determined (Schoenherr et al. 1996). Out of the four mismatched nucleotides, three are located at positions most frequently modified in functional NRSEs and subsequently demonstrated to be nonessential of the NRSF binding (Schoenherr et al. 1996).

To demonstrate that the 21-bp fragment of the KCC2 gene is involved in the regulation of KCC2 expression, we first examined possible interaction of this fragment with nuclear proteins isolated from C17 nonneuronal cells. Two complementary synthetic oligonucleotide primers were annealed, labeled with 32-P, and incubated with nuclear proteins isolated from mouse progenitor C17 neural cells. Interaction of the probe with nuclear proteins was demonstrated by acrylamide gel electrophoresis. As demonstrated in Fig. 2A, the mobility of the probe (lane 1) was retarded in the presence of nuclear proteins (lane 2), indicating protein-DNA interaction. The protein-DNA complex was completely displaced by a cold NRSE fragment (Fig. 2B, lane 2) but not by an unrelated DNA fragment of the same size and same G + C content (Fig. 2B, lane 3).

Using a luciferase gene reporter assay, we examined the effect of the 21-bp KCC2 NRSE fragment on gene transcription. A 1,500-bp EcoRI-XhoI fragment consisting of the most proximal 5′ flanking region of the KCC2 gene, and possibly containing the minimal promoter (see Fig. 1), was inserted upstream of the promoterless luciferase gene. Two complementary synthetic oligonucleotides consisting of the 21-bp sequence for which NRSF binding was experimentally determined completely the KCC2 promoter-induced luciferase signal as indicated by a return to baseline levels. These results demonstrate that the KCC2 NRSE-like 21-bp sequence is capable of silencing transcription of the luciferase reporter gene in C17 nonneuronal cells. These are pluripotent, undifferentiated, neuronal or glial-precursor cells. Taken together, these experiments suggest an important role for this putative element in regulating KCC2 gene expression.

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