Cloning, Characterization, and Chromosomal Location of a Novel Human K⁺-Cl⁻ Cotransporter*

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Differential display polymerase chain reaction has been used to isolate genes regulated in vascular endothelial cells by the angiogenic factor vascular endothelial cell growth factor (VEGF). Analysis of one of the bands consistently up-regulated by VEGF led us to the identification of a cDNA from a human umbilical vein endothelial cell library that is 77% identical to the human K⁺-Cl⁻ cotransporter1 (KCC1). We have referred to the predicted protein as K⁺-Cl⁻ cotransporter 3 (KCC3). Hydrophobicity analysis of the KCC3 amino acid sequence showed an almost identical pattern to KCC1, suggesting 12 membrane-spanning segments, a large extracellular loop with potential N-glycosylation sites, and cytoplasmic N- and C-terminal regions. The KCC3 mRNA was highly expressed in brain, heart, skeletal muscle, and kidney, showing a distinct pattern and size from KCC1 and KCC2. The KCC3 mRNA level in endothelial cells increased on treatment with VEGF and decreased with the proinflammatory cytokine tumor necrosis factor α, whereas KCC1 mRNA levels remained unchanged. Stable overexpression of KCC3 cDNA in HEK293 cells produced a glycoprotein of approximately 150 kDa, which was reduced to 120 kDa by glycosidase digestion. An increased initial uptake rate of ⁸⁶Rb⁺ was seen in clones with high KCC3 expression, which was dependent on extracellular Cl⁻ but not Na⁺ and was inhabitable by the loop diuretic agent furosemide. The KCC3 genomic localization was shown to be 15q13 by fluorescence in situ hybridization. Radiation hybrid analysis placed KCC3 within an area associated with juvenile myoclonic epilepsy. These results suggest KCC3 is a new member of the KCC family that is under distinct regulation from KCC1.

The cation chloride cotransporter (CCC) family is involved in the electroneutral movement of ions across the plasma membrane. There are three CCC subclasses identified thus far on the basis of their structures, ligands, and inhibitors. These are the thiazide-sensitive Na⁺-Cl⁻ cotransporters, the loop diuretics-sensitive Na⁺-K⁺-Cl⁻ (NKCC), and the K⁺-Cl⁻ cotransporters (KCC; Refs. 1–5). NKCC and KCC have two isoforms. NKCC1 shows ubiquitous distribution (3) among organs, whereas NKCC2 is restricted to kidney (2). KCC1 is ubiquitous (4), whereas KCC2 is only found in brain (5). In addition to the classical roles of transepithelial salt transport (6) and the regulation of cellular volume (7), Harling et al. (8) have recently shown that tobacco protoplast growth becomes independent of the plant hormone, auxin, when NKCC1 is overexpressed, suggesting the possible involvement of the CCC family in cell cycle regulation.

The physiological regulation of the NKCC and KCC family is complex. Other than the electrochemical gradient of their ligands, evidence suggests that activation of this passive transport system is regulated by phosphorylation (9), cytoskeletal rearrangement (10), change of intracellular Mg²⁺ concentration (6, 7), intracellular pH (11), oxygen concentration (12), and cellular ATP levels (13). In addition, some stimuli can mediate differential effects on various members of the CCC family. For example, cell swelling activates KCC, whereas cell shrinkage activates NKCC (6, 7). Phosphorylation activates NKCC, whereas KCC is activated by dephosphorylation (6, 7). In cultured endothelial cells, transcriptional regulation has been reported for NKCC1 in response to shear stress and proinflammatory cytokines (14). Cellular differentiation has also been shown to be associated with changes in NKCC and KCC gene expression. In the intestinal epithelial cell line HT29, a change of NKCC1 mRNA level during differentiation has been reported (15, 16), whereas the loss of K⁺-Cl⁻ flux during the maturation of sheep red blood cells is well known (7).

We report here the isolation and cloning of a new member of the KCC group of cotransporters, which we have named KCC3. KCC3 displays high homology to KCC1, and the characteristics of the ion flux mediated by KCC3 satisfies the criteria for a KCC. KCC3 is regulated at the mRNA level by the angiogenic factor VEGF and by the proinflammatory cytokine TNFα, neither of which has any effect on KCC1 mRNA levels. Finally, KCC3 has been localized to chromosome 15q13, a region linked to the inherited disease juvenile myoclonic epilepsy (17).

EXPERIMENTAL PROCEDURES

HUVECs—HUVECs were isolated as described previously (18). The cells were cultured on gelatin-coated culture flasks in medium 199 with

NKCC, sodium potassium chloride cotransporter; KCC, potassium chloride cotransporter; VEGF, vascular endothelial cell growth factor; HUVEC, human umbilical vein endothelial cell; PCR, polymerase chain reaction; TNFα, tumor necrosis factor α; kb, kilobase(s).

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Eagle’s salts supplemented with 20% fetal calf serum. After passage 1, the cells were grown in medium with 25 μg/ml endothelial cell growth factor (Collaborative Research) and 25 μg/ml heparin (Sigma). For the differential display, HUVECs were cultured in Opti-MEM medium (Life Technologies, Inc.) with 2% fetal calf serum for 2 days.

Cloning and Sequencing of KCC3—One of the bands consistently up-regulated by VEGF treatment encoded a product whose DNA sequence was 90% identical to human KCC1. We used this sequence as a probe (TAV1 probe) to screen a Agt10/HUVEC library (a gift from Dr. Sawamura, Kyoto University). A 2.7-kb phage clone (clone 3) showed significant homology to KCC1 (U55054 in GenBankTM/EBI data bank). We further screened the library with a 5′ sequence of clone 3 (3R probe, a PCR product using primers 5′-CATGGACGTTTGCTCTAAGACC and 5′-GTGTGATCCAGCCATGATC, see Fig. 2A) to obtain a 2.1-kb clone (clone 9) that spanned a putative initiating ATG signal. Clone 3 and clone 9 shared an overlap of 1 kb including a BstB1 site, which enabled us to construct a 3.7-kb cDNA with an open reading frame for a 1099-amino-acid protein (see Fig. 2, A and B). This cDNA sequence was deposited in GenBank® (GenBank accession number AF108831). Analysis of the nucleic acid and amino acid sequences was carried out using the programs provided by ANGIS (Australian National Genomic Information Service).

Northern Blot Analysis—Total RNA was extracted from HUVECs of primary and passed cultures using Trizol reagent. Twenty μg of total RNA was separated and separated by electrophoresis in 1.3% agarose containing formaldehyde. RNA was transferred to Hybond N (Amersham Pharmacia Biotech) membrane and UV-cross-linked. To produce a specific KCC3 probe, we generated a 942-base pair PCR product using primers (GTCTCATCAAGATTAG and GCAATGCT- TGTAGCAGCCTCG, corresponding to amino acids 349–548). This segment of cDNA was chosen because it had low homology to KCC1. After purification of this product from agarose gel using Bresa Clean Kit (Bresatec), the fragment was labeled with [32P]dATP (Giga label kit, Pharmacia Biotech) membrane and UV-cross-linked. To produce a KCC1-specific probe, we generated a 1-kb PCR product that was digested with CTCCCTTGGGTAGGTAATTA (corresponding to amino acids 1–403). The PCR generated a 1-kb PCR product that was digested with XhoI and linked to the rest of the KCC3 sequence. The construct was introduced into the pCDNA3.1 mammalian expression vector (Invitrogen), which was introduced into HEK293 cells using Lipofectamine® (Life Technologies, Inc.). HEK293 cells were cultured with 100 μg/ml Geneticin (Invitrogen), and colonies were picked to generate clonal populations.

Isotopic Flux Assays—Transfected HEK 293 cells were grown to confluency in 24-well dishes coated with poly-n-lysine. Cells were washed twice with flux medium (135 mM NaCl, 5 mM glucose, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM NaHPO4, 2 mM Na2SO4, 20 mM HEPES, pH 7.4, and 0.1 mM ouabain) and then incubated for 15 min at room temperature with 400 μl of flux medium containing 1 mM Na+ ethyleneglycolbismaleimide (ICN). One hundred μl of flux medium containing 10 μCi/ml 86RbCl (Amersham Pharmacia Biotech) was quickly added. Cells were incubated for 3 min before washing 3 times with ice-cold phosphate-buffered saline. For sodium-free experiments, sodium was replaced by N-methyl-N-glycine (ICN), and for chloride-free experiments, it was replaced by gluconate. Bumetanide (Sigma) and furosemide (Sigma) were administered at the indicated concentration at the start of the preincubation with Na+-ethyleneglycolbismaleimide. Cells were lysed with 2% SDS and assayed for protein content using a BCA protein assay kit (Pierce) and for 86Rb using Cerenkov radiation in a scintillation counter.

Preparation of Anti-KCC3 Antibody and Protein Detection—Synthetic Kcc peptide 1 (SQNSITGEHSSLQD) and peptide 2 (AIFHSDKALKESAA) were linked to chicken albumin (Sigma) to immunize rabbits, and anti-KCC3 peptide antibodies (P1 antibody by peptide 1 and P2 antibody by peptide 2) were prepared as described previously (19). To digest KCC3 protein by N-glycanase F (Boehringer Mannheim), immunoprecipitated KCC3 was incubated with 250 milliunits/ml glycosidase overnight at 30 °C in the presence of 10% Nonidet P-40.

Genomic Localization—The KCC3 coding sequence in the pGEM4Z vector was nick-translated with biotin-14-dATP and hybridized in situ at a final concentration of 15 ng/ml to metaphases from two normal males. The fluorescence in situ hybridization method was modified from that previously described (20). Chromosomes were stained before analysis with both propidium iodide (as counterstain) and 4′,6-diamino-2-phenylindole dihydrochloride (for chromosome identification). For the radiation hybrid analysis, we performed a screen of a medium resolution (G5 panel of 83 clones) to refine the map position of the KCC3 gene. PCR amplification was carried out on this panel using primers g5 (TGCACATTTTTACTGTCG) and g6 (TCACTGAACTC-GAATCC), both of which lie in the 5′ region of KCC3 gene. PCR results were analyzed using the radiation hybrid mapping facility at the Stanford Human Genome Center.

RESULTS

Isoisotopic Analysis and Isolation of a KCC3 cDNA—In the differential display PCR using total RNA extracted from 4-h VEGF-treated HUVECs, a band was consistently up-regulated in experiments using three independent pools of primary and secondary treated HUVECs as RNA sources. A representative example of these differential displays is shown in Fig. 1. We have characterized this product and generated a full-length cDNA from two overlapping clones (Fig. 2A). The cDNA sequence shows high homology to KCC1 and encodes a predicted protein of 1099 amino acids. The primary amino acid sequence of this protein, which we have named KCC3, is 77% identical to KCC1 and 73% identical to KCC2 (Fig. 3). Five N-glycosylation consensus sites are found in the large extracellular domain between the 5th and 6th membrane-spanning regions (Fig. 3). The hydrophobic profile (by Kyte-Doolittle analysis) of KCC3 was almost identical to that of KCC1, predicting a protein with 12 membrane-spanning segments and large intracellular N- and C-terminal

**Fig. 1.** Differential display PCR comparing VEGF-stimulated and -unstimulated RNA populations. HT1A anchor primer and AP7 arbitrary primer (RNA image kit, GenHunter) were used to amplify cDNAs derived from primary HUVECs treated with VEGF (VEGF) or without VEGF (ctrl). Amplons from each group were applied in duplicate. Consistently up-regulated bands are indicated by an arrow. Similar up-regulation was seen in three other independent experiments.
domains (Fig. 2B). Considerable diversity is seen, relative to KCC1, in the N-terminal portion, the extracellular domain between the 3rd and 4th membrane-spanning segments and the 5th and 6th membrane-spanning segments and in the area near the C terminus. KCC3 does not have a glutamic acid residue at the beginning of the transmembrane domain 2. This residue has been suggested to be important for the enhanced extracellular potassium binding in KCC2 (21).

Expression and Regulation of KCC3 mRNA— Fig. 4A shows the tissue-specific expression of KCC3. Unlike ubiquitously expressed KCC1 and brain-restricted KCC2, strong expression of KCC3 was observed in brain, heart, skeletal muscle, and kidney. Transcripts of approximately 9, 7.5, and 4.5 kb were detected (lanes 2, 3, 7, and 8 in Fig. 4A), and these showed tissue-specific differences in abundance. KCC3 mRNA level increased from as early as 1.5 h after VEGF administration, whereas KCC1 levels remained unchanged (Fig. 4B). This was true not only in primary HUVECs but also in passaged cells (data not shown). We have also used semiquantitative PCR to analyze the VEGF responsiveness and have obtained results (data not shown). We have also used semiquantitative PCR to analyze the VEGF responsiveness and have obtained results (data not shown). We have also used semiquantitative PCR to analyze the VEGF responsiveness and have obtained results (data not shown). We have also used semiquantitative PCR to analyze the VEGF responsiveness and have obtained results (data not shown). We have also used semiquantitative PCR to analyze the VEGF responsiveness and have obtained results (data not shown). We have also used semiquantitative PCR to analyze the VEGF responsiveness and have obtained results (data not shown).

Detection of KCC3 Protein in HUVECs and in HEK293 Cells—To further analyze the KCC3 gene product, we have generated a KCC3 cDNA incorporating an N-terminal FLAG epitope (N-FLAG KCC3). We have produced stable HEK293 cells overexpressing N-FLAG KCC3. The FLAG-tagged protein, when immunoprecipitated with anti-FLAG antibody (M2 antibody, Eastman Kodak Co.), was approximately 150 kDa (Fig. 5A, lanes 1 and 2) and reduced to 120 kDa by digestion with glycosidase treatment (Fig. 5B). Immunoprecipitation using M2 antibody followed by Western blotting with anti-KCC3 synthetic peptide 1 antibody (P1 antibody, Fig. 5A, lanes 3 and 4) and immunoprecipitation using P1 antibody followed by Western blotting with M2 antibody (Fig. 5A, lanes 5 and 6) gave the same results. These results were also reproduced when we used P2 antibody instead of P1 antibody (data not shown). KCC3 protein was also immunoprecipitated and blotted from cultured HUVECs using P1 antibody (Fig. 5C).

Functional Characterization of KCC3—We used a 86Rb uptake assay as a measure of K+ flux as described elsewhere (4). When the FLAG sequence was added to the C terminus of KCC3, there was no measurable difference in 86Rb uptake between KCC3 clones and control populations (data not shown). Therefore the N-FLAG KCC3 construct was used for the functional analysis of KCC3. Expression of KCC3 in HEK293 cells was confirmed after selection in zeocin by Western blotting using M2 antibody. The results for 1 clone (clone 847) are shown in Fig. 6, although similar results were seen in 5 other independent clones (data not shown). A 3-min assay was used because in both control and transfectants, 86Rb uptake was linear at least for the initial 15 min (data not shown). The results shown in Fig. 6B demonstrate a significantly increased furosemide-sensitive 86Rb uptake in clone 847 that expresses a high level of KCC3 (Fig. 6A). The magnitude of furosemide-sensitive 86Rb uptake was similar to that reported for KCC1 (4), because our value of 10 cpm/µg of protein/3 min is equal to 3.2 nmol Rb/µg of protein/min. Such an increase was not seen in clones that were zeocin-resistant but expressed a low level of KCC3 (data not shown). The uptake was dependent on extracellular Cl− with little dependence on extracellular Na+ (Fig. 6C). The loop diuretics furosemide and bumetanide showed a dose-dependent inhibition of uptake with furosemide slightly more effective than bumetanide (K, of approximately 10 µM versus 40 µM, Fig. 6D). The KCC3 transfectants did not show a significant increase in 86Rb uptake in response to hypotonic treatment (data not shown). These results show that KCC3 satisfies the functional criteria of the KCC class of cotransporters.

Genomic Localization of KCC3—Twenty-five metaphases from a normal male were examined for fluorescent signal. All of these metaphases showed a signal on one or both chromatinids of chromosome 15 in the region 15q13 (Fig. 7). There was a total of 2 nonspecific background dots observed in these 25 metaphases. A similar result was obtained from hybridization of the probe to 15 metaphases from a second normal male (data not shown). Radiation hybrid analysis indicated that KCC3 is most closely associated with the chromosome 15 marker SHGC-33497 with a LOD score of 1000. Assessment of flanking markers D15S1010 and D15S1040, using the integrated gene maps available at NCBI, gave a result consistent with the localization of KCC3 by fluorescence in situ hybridization analysis.
![Fig. 4. Distribution and regulation of the KCC3 mRNA. Panel A, tissue-specific expression of KCC3 mRNA. A multiple tissue Northern blot membrane (CLONTECH) was probed with a KCC3-specific probe. Lane 1, pancreas; lane 2, kidney; lane 3, skeletal muscle; lane 4, liver; lane 5, lung; lane 6, placenta; lane 7, brain; lane 8, heart. Molecular size (kb) is indicated on the left. Panel B, regulation of KCC3 mRNA level by VEGF and TNFα treatment. Primary culture HUVECs were treated for 1.5 h with (VEGF) or without (ctrl) VEGF. Passaged HUVECs were treated for 4 h with (TNFα) or without (ctrl) TNFα. Each membrane was probed with KCC3, KCC1, and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe in that order. The increase of the KCC3 mRNA observed by treatment with VEGF is 1.5-fold, and the decrease by TNFα is 52%. Two other experiments showed similar results.

DISCUSSION

We describe here the cloning of a new member of the CCC family that is structurally closely related to the potassium chloride cotransporter KCC1 and that we therefore have named KCC3. The amino acid sequence shows significant homology to other CCC family members with overall amino acid identity between KCC1 and KCC3 being 77%. There is a large predicted extracellular domain between the 5th and 6th putative membrane-spanning regions that is common to the KCC family but not observed in the NKCC family. Considerable diversity is observed in the N-terminal portion and in the extracellular domains between the 3rd and 4th and 5th and 6th putative membrane-spanning segments (Fig. 3). It is also notable that there are four deletions in the C-terminal region common to KCC1 and KCC3 that are not present in KCC2 (Fig. 3). The C-terminal conserved portion appears important in its function as addition of a FLAG epitope to the C terminus of KCC3 abolished the uptake of 86Rb in our assay (data not shown). Furthermore, Harling et al. (8) find that the C-terminal fragment of AXI 4, a plant CCC, is sufficient for establishing auxin-independent growth of tobacco protoplasts.

Overexpression of KCC3 in HEK293 cells allowed functional analysis and demonstrated that KCC3 exhibits characteristics expected of a KCC. 86Rb flux was significantly increased in KCC3 transfectants. This increase was independent of extracellular Na⁺ but dependent on extracellular Cl⁻. This and the fact that the 86Rb flux was measured in the presence of the SH-reactive reagent N-ethylmaleimide (22), which inhibits NKCC1 but activates KCC (4), suggest that NKCC1 is not a major contributor in our assay. It is also considered unlikely that KCC1 is responsible for all the 86Rb uptake observed in the transfectants, because increased uptake was only seen in clones expressing high levels of KCC3. Furthermore, in both KCC3-expressing transfectants and control transflectants, the mRNA levels of KCC1 were equivalent (data not shown).

Analysis of multiple tissue blot Northern filters probed with a KCC3-specific probe showed a tissue-specific expression pattern with highest levels observed in kidney, skeletal muscle, heart, and brain. This contrasted with the expression of KCC1, which is ubiquitous (4), and KCC2, which is restricted to brain (5). Although the reason for the selective tissue distribution is unknown, it suggests that KCC3 does not serve a general housekeeping function such as cell volume regulation as has been proposed for KCC1 (4). This is further supported by the lack of detectable regulation of KCC3 activity in response to alterations in osmolarity (data not shown).

At present we do not know the role of KCC3 in endothelial...
cells. However, the responsiveness of this gene in HUVECs to VEGF suggests an involvement in angiogenesis. It is tempting to speculate that the up-regulation of KCC3 mRNA levels mediated by VEGF may be through modulation of the cytoskeleton, which results in changes in cell shape (24). Such changes have been reported to be one of the initial events upon angiogenic stimulation (24), and changes of this type can also affect the expression of the CCC members (7). Recently Edwards et al. (25) reported that K⁺ released from endothelial cells in response to acetylcholine stimulation caused hyperpolarization and relaxation of smooth muscle cells through activation of the Na-K-ATPase and Ba²⁺-sensitive K⁺ channel. Thus K⁺ may also be important in the control of blood pressure. Because the Na-K-ATPase generates the chemical gradient that drives CCCs, it also implies that KCC3 may be involved in modulating the local K⁺ concentration.

We also observed a down-regulation of the KCC3 mRNA level in response to TNFα. Because NKCC1 is up-regulated by TNFα (14), our results suggest that KCC3 may be a functional counterpart of NKCC1. Certainly KCC1 mRNA levels showed no change in response to TNFα, supporting the idea that KCC3 and NKCC1 are coregulated in response to TNFα. NKCC1 has been reported to play a role downstream of the growth hormone auxin in tobacco protoplasts (8), where it is involved in cell cycle progression; therefore we speculate that KCC3 may also be involved in cell cycle regulation. Interestingly, the tissue-specific expression pattern of KCC3 resembles that of cyclin G1 (26), a cyclin involved in cell cycle arrest.

KCC3 has been localized to 15q13 and between the genetic markers D15S1010 and D15S1040. This region has recently been linked to juvenile myoclonic epilepsy (17), raising the possibility that KCC3 is a candidate gene for this disease.

Fig. 6. Functional analysis of KCC3. Panel A, expression of the KCC3 protein in clone 847 cells. KCC3 was immunoprecipitated from either clone 847 (lane 1) or control cells (lane 2) by M2 antibody followed by Western blotting with P1 antibody. Panel B, demonstration of enhanced ⁶⁷⁴Rb uptake by KCC3-transfected HEK293 cells. HEK293 cells, HEK293 cells transfected with blank vector (CTRL), and clone 847 were assayed for their ⁶⁷⁴Rb uptake after preincubation with 1 mM N-ethylmaleimide. Values after subtracting furosemide-insensitive ⁶⁷⁴Rb uptake are shown. Values are shown as mean ± S.D. (n = 3). Two other experiments showed similar results. Panel C, extracellular Na⁺ and Cl⁻ dependence of KCC3. Clone 847 cells were assayed for furosemide-sensitive ⁶⁷⁴Rb uptake in the absence of extracellular Na⁺ (Na⁻) or Cl⁻ (Cl⁻), n = 3. See “Experimental Procedures” for details. Values are shown as mean ± S.D. Two other experiments showed similar results. Panel D, sensitivity for loop diuretic agents. Percent decrease of ⁶⁷⁴Rb uptake of clone 847 cells by incremental concentration of furosemide (closed circle) and bumetanide (open circle) are shown (n = 3). Values are shown as mean ± S.D. Two other experiments showed similar results.
Normal male chromosomes were stained with propidium iodide and 4',6-diamino-2-phenylindole dihydrochloride before hybridization with a KCC3 probe. This is a single metaphase spread of 40 that were analyzed. All showed a similar hybridization pattern. Hybridization sites on chromosome 15 are indicated by arrows. Payne (21) has postulated that KCC2, a brain-restricted KCC, acts as a neuronal Cl⁻ pump, complementing other systems in regulating K⁺ homeostasis. The concept is now emerging that the idiopathic epilepsies may represent ion channel disorders (27) based on certain inherited forms of epilepsy in mice (28), mutations in the α4 subunit of neuronal nicotinic acetylcholine receptor responsible for autosomal dominant nocturnal frontal lobe epilepsy (29), and benign familial neonatal convulsions because of mutations of potassium channel gene (30–32). Thus, we suggest that KCC3 may be a candidate gene for juvenile myoclonic epilepsy worthy of further investigation.

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