Cloning and Functional Characterization of a Cation-Cl⁻ Cotransporter-interacting Protein*

Luc Caron†, François Rousseau‡, Édith Gagnon‡, and Paul Isenring¶

From the Groupe de Recherche en Néphrologie, ‡Department of Medicine, and the Unité de Recherche en Génétique Humaine et Moléculaire, ¶Department of Medical Biology, Faculty of Medicine, Laval University, Québec G1R 2J6, Canada

To date, the cation-Cl⁻ cotransporter (CCC) family comprises two branches of homologous membrane proteins. One branch includes the Na⁺-K⁺-Cl⁻ cotransporters (NKCCs) and the Na⁺-Cl⁻ cotransporter, and the other branch includes the K⁺-Cl⁻ cotransporters. Here, we have isolated the first member of a third CCC family branch. This member shares ~25% identity in amino acid sequence with each of the other known mammalian CCCs. The corresponding cDNA, obtained from a human heart library and initially termed WO₃.₃, encodes a 914-residue polypeptide of 96.2 kDa (calculated mass). Sequence analyses predict a 12-transmembrane domain (tm) region, two N-linked glycosylation sites between tm₂ and tm₅, and a large intracellular carboxyl terminus containing putative kinase C phosphorylation sites. Northern blot analysis uncovers an ~3.7-kilobase pair transcript present in muscle, placenta, brain, and kidney. With regard to function, WO₃.₃ expressed either in HEK-293 cells or Xenopus laevis oocytes does not increase Rb⁺-, Na⁺-, and Cl⁻-coupled transport during 5- or 6-h fluxes, respectively. In the oocyte, however, WO₃.₃ specifically inhibits human NKCC1-mediated ⁸⁶⁵Rb⁺ flux. In addition, communoprecipitation studies using lysates from WO₃.₃-transfected HEK-293 cells suggest a direct interaction of WO₃.₃ with endogenous NKCC. Thus, we have cloned and characterized the first putative heterologous CCC-interacting protein (CIP) known at present. CIP1 may be part of a novel family of proteins that modifies the activity or kinetics of CCCs through heterodimer formation.

Cation-Cl⁻ cotransporters (CCCs)³ mediate the coupled movement of Na⁺ and/or K⁺ to that of Cl⁻ across the plasma membrane of animal cells. In polarized tissues, cation-Cl⁻ cotransport is involved in net transepithelial water and salt movement (1–4). In non-polarized tissues, cation-Cl⁻ cotransport modulates the water and the electrolyte content of cells (1, 2, 5), and it may also prevent extracellular K⁺ accumulation (6).

In 1994, the molecular characterization of a protein responsible for Na⁺-K⁺-Cl⁻ cotransport (5), the shark Na⁺-K⁺-Cl⁻ cotransporter (sNKCC1), led to the identification of the CCC family (1, 7). In addition to the Na⁺-K⁺-Cl⁻ cotransporter, this family now includes the furosemide-sensitive Na⁺-independent K⁺-Cl⁻ cotransporter (KCC) and the thiazide-sensitive K⁺-independent Na⁺-Cl⁻ cotransporter (NCC; see Ref. 8). Within the family, the CCCs share 25–75% amino acid identity (see Fig. 2). Two types of NKCCs have been identified to date, the widely distributed NKCC1 (3, 4, 9) and the kidney-specific NKCC2 (10). Similarly, K⁺-Cl⁻ cotransport is mediated by at least four different isoforms as follows: KCC1 (7), KCC2 (11), KCC3 (12, 13), and KCC4 (12). Not surprisingly, several splice variants of the CCCs have been identified in recent years.

All of the CCCs exhibit similar hydrophathy profiles depicting three broad regions: an ~500-residue central domain predicted to contain 12-tm region, an amino terminal of variable length, and a long carboxyl terminus containing several α-helical and β-sheet structures (1, 7, 8). The central hydrophobic domain exhibits the highest levels of sequence conservation among the CCC family members; this is consistent with the presumed importance of the tms in ion movement (1, 15, 16).

Considerable indirect evidence indicates that the CCCs interact with other proteins. For example, changes in cell volume and in intracellular Cl⁻ (Cl⁻) affect cation-Cl⁻ cotransport (1, 2, 17, 18) by changes in the phosphorylation state of the CCCs distance error (17–20), thus implying that associations form between the carriers and kinases and/or phosphatases. Substantial data also support the notion of CCC regulation by phosphorylation-independent mechanisms, occurring in part through structural interactions with the cytoskeleton (21, 22). For instance, Matthews et al. (22) have found that NKCC1 was functionally linked to F-actin during cAMP-elicited electrogenic Cl⁻ secretion. Finally, recent studies (23, 24) suggesting that NKCCs form homo-oligomers at the cell surface of Xenopus laevis oocytes provide further evidence that the CCCs can assemble with other macromolecules.

Through BLAST searches of human and mouse expressed sequence tag (EST) data bases, we have identified novel overlapping sequences homologous to a region in the carboxyl terminus of the CCCs. Corresponding cDNAs were isolated from a human heart cDNA library. The full-length clone, termed

* This work was supported by grants from the Medical Research Council of Canada (characterization of hCIP1), the Heart & Stroke Foundation of Canada (studies on NKCC2). The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF284422.

¶ Medical Research Council Scientist.

† Medical Research Council Clinician Scientist II. To whom correspondence should be addressed: Research Center L'Hôtel-Dieu de Québec of the CHUQ, 10 Rue McMahon, Rm. 3852, Québec (Qué) G1R 2J6, Canada. Tel.: 418-691-5477; Fax: 418-691-5787; E-mail: paul.isenring@crhdq.ulaval.ca.

³ The abbreviations used are: CCC, cation-Cl⁻ cotransporter; UTR, untranslated region; PPES, 1,4-piperazinediethanesulfonic acid; NKCC, Na⁺-K⁺-Cl⁻ cotransporter; sNKCC1, shark Na⁺-K⁺-Cl⁻ cotransporters; tm, transmembrane; KCC, K⁺-Cl⁻ cotransporter; bp, base pair; h, human; rb, rabbit; PCR, polymerase chain reactions; NEM, N-ethylmaleimide; kb, kilobase; EST, expressed sequence tag; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DIDS, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
WO1.23 was found to encode a 914-residue polypeptide that is ~25% identical to each of the known CCCs. In heterologous expression systems, this novel CCC inhibits human (h) NKCC1-mediated transport, possibly through heterodimer formation at the cell surface. Hence, WO1.23 is denoted CCC-interacting protein type 1 (CIP1).

**EXPERIMENTAL PROCEDURES**

**Cloning of CIP1**—In June 1998, a BLAST search of the GenBank data base enabled the identification of ESTs that were 25% identical to known CCCs. These sequences appeared to cover the carboxyl terminus and the 3'-untranslated region (UTR) of a putative transporter corresponding to a member of a new CCC family branch. The cDNA of one of these ESTs (clone number aa104358) was obtained from the I.M.A.G.E. Consortium EST project (25).

A 500-bp BamHI fragment excised from aa104358 was gel-purified, random-labeled with 32P, and used to screen a XZAP II human heart cDNA library purchased from Stratagene (La Jolla, CA). Hybridization was performed in 2× PIPES buffer, 50% formamide, 0.5% SDS, and 10 μg/ml sonicated salmon sperm at 42 °C. The most stringent wash was in 0.5% SSC and 0.5% SDS at 55 °C. Height-positive plaques (out of 5 × 106 plated plaques) were carried through 2 or 3 rounds of screening and excised from XZAP as βBluescript phagemids using the Stratagene ExcAssist phage. Restriction analysis revealed that 6 of the 8 clones were identical.

The three different cDNAs (named WO1.23, WO1.49, and WO1.24) were sequenced using fluorescent dye terminators. The longest cDNA, WO1.23, was found to contain a 2472-open reading frame starting at base pair (bp) 128, with the first methionine downstream of a stop codon, and finishing at bp 2869; WO1.49 and WO1.24 were smaller overlapping clones. Sequence analyses and tree constructions were carried out with DNASTAR (Lasergene) and GCG programs. BLAST searches were performed with the NCBI BLAST program and structure predictions with the TMpred program. WO3.3 was eventually assigned to CIP1, based on additional characterizations.

**cDNA Construction and Vectors**—The WO1.23/cIP1 cDNA (3276 bp), originally in the vector pBluescript II SK (pBS), was subcloned in the mammalian expression vector pB200g as a Kpn-I-PesI fragment, and in the oocyte expression vector Pol1 as an EcoRI fragment. The vector pB200g (14–16, 26) is a cBS (27) derivative that contains intron sequences in the 3' linker and the AvrII and XhoI sites in the 5' linker. Pol1 is a modified pGEM vector containing the T7 promoter sequence, the T3 promoter sequence, the 5'-globin 5'-UTR, a poly(A) tract, and a 3'-UTR, a poly(A) tract, and linker. Pol1 is a modified pGEM vector containing the T7 promoter sequence, the T3 promoter sequence, the 5'-globin 5'-UTR, a poly(A) tract, and a 3'-UTR, a poly(A) tract, and linker.

The cIP1/pBS was c-Myctagged by inserting pairs of oligonucleotides3 at the 5'-end of the cDNA between the sites BstElI (bp 85) and NcoI (bp 126). This assembly adds a leader sequence, MEQLIKEEDL, in front of the first original methionine. The c-Myctagged inserts were then transferred from pBS to the expression vectors as described above.

To examine the function of other CCCs in the X. laevis expression system, we transferred full-length insert cDNAs from pBS to Pol1. The rabbit (rb) KCC1 insert (7) was subcloned as an XbaI-EcoRI fragment into XbaI-HindIII sites, the rabbit KCC2A insert (10) as an EcoRI fragment into phosphatase-treated EcoRI sites, and the human KCC1 insert (9) as an EcoRI-XhoI fragment into EcoRI-HindIII sites. To permit ligation between inserts and vectors, the XhoI and HindIII sites were pretreated with long-lane nucleases.

**Chromosomal Localization**—Chromosomal assignment of cIP1 was obtained with the Genebridge IV human/rodent somatic cell hybrid mapping panel (Research Genetics). Genomic DNA was amplified through PCRs with primers4 derived from the 3'-UTR of cIP1. The PCR products were analyzed by agarose gel electrophoresis and scored according to size correctness (310 bp) and amplification yield. Results were analyzed using the radiation hybrid server and the summary human gene maps of chromosome 7.

**Northern Blot**—A CLONTECH human Multiple Tissue Northern (MTN)5 Blot kit containing ~2 μg/lane poly(A) was hybridized with 32P-labeled cDNA probes synthesized by random primer extension from a gel-purified cIP1 BamHI fragment or from a commercial human β-actin fragment. Conditions for hybridization were as recommended by CLONTECH's user manuals.

**Expression of cIP1, c-Myctagged hCIP1, and Other CCCs in HEK-293 Cells**—Cell culture and the X. laevis Oocyte System—Approximately 100,000 HEK-293 cells were transfected with 40 μg of the hCIP1, the c-Myctagged hCIP1, or the hNKCC1 cDNA by calcium phosphate precipitation (0.75 mM NaHPO4, 125 mM CaCl2, pH 7.1). Starting 48 h after transfection, cells were collected for G418 resistance (950 μg/ml) in growth medium (see Refs. 14–16) during a 3-week period. For each type of cDNA, 12 individual, well-separated colonies were chosen for amplification. Protein expression was estimated by either 32P flux measurements for hNKCC1-transfected cells or by Western blotting for c-Myctagged hCIP1-transfected cells; in both cases, approximately half of the cell lines exhibited significant expression.

For expression in the X. laevis oocyte, cDNA inserts in the Pol1 vector were linearized and transcribed in vitro with T7 RNA polymerase using the mMESSAGE mACHINE T7 kit (Ambion). Defolliculated stage V–VI oocytes were injected with 50 nl of H2O or with ~5–25 ng of cDNA diluted in 50 nl of H2O. Functional expression was assessed 3–4 days after injection.

**Protein Analysis**—Transfected HEK-293 cells were grown to near-confluence and lysed in buffer X (10% glycerol, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors. The cell lysates were cleared by preclearing with 15–25% sodium peroxidase-activated anti-IgG. Bound antibody was visualized with the ECL assay.

**Immunofluorescence Studies**—HEK-293 cells expressing c-Myctagged hCIP1 were conducted as described previously (1). Briefly, cells were fixed in 3% paraformaldehyde and incubated sequentially with c-Myc or anti-C1, and a rhodamine-conjugated secondary anti-IgG. Immunofluorescence studies with hNKCC1 and hCIP1 in oocytes were also performed as described previously (30). Here, egg cryosections (5 μm) were postfixed 3 min in acetone, incubated with T4 or anti-C1, and reincubated with horseradish peroxidase-activated anti-IgG. Bound antibody was visualized with the ECL assay.

**Functional Studies**—HEK-293 cells were grown to confluence in 96-well plates coated with poly-l-lysine, and oocytes were maintained 3–4 days in medium at 18 °C. Ion transport rates were determined by 36Cl-14C flux measurements, and all experiments were performed at ~22 °C. Different media were used for the functional studies (see Table 1, figure legends, and “Results” for details). In these studies, regular medium designates a physiological iso-osmolar solution with a pH of 7.4. When necessary, osmolality was adjusted with sucrose: cations were replaced by N-methylglucamine and anions by SO4− or gluconate, and solutions were supplemented with inhibitors, such as furosemide (125–250 μM), bumetanide (250 μM), N-ethylmaleimide (NEM; 1 mM), or ouabain (10 μM).

For the influx assays, cells were preincubated 15–60 min in tracer.

---

3 These primers are highly sensitive and specific anti-nNKCC1 antibody that recognizes an epitope localized in the amino terminus of the protein (see Refs. 1, 3, 28). The T4 antibody is highly specific to the NKCCs as previously shown (29). The sensitivity of T4 is usually increased with detergent detergent. However, in the presence of TGONCCs depicted in Fig. 5 with anti-c-Myc. This result indicates that T4 does not react with hCIP1 directly. For the anti-c-Myc antibody used in this study, we used the 9E10 antibody (from Sigma).
free media. Then, HEK-293 cells were incubated for 2–5 min and oocytes for 1–6 h in various media containing ouabain and 1–2 mCi/ml \( ^{86}\text{Rb} \), \( ^{22}\text{Na} \), or \( ^{36}\text{Cl} \). Fluxes were terminated by washing cells several times in regular medium without ouabain.

For efflux experiments, cells were loaded with the tracer (1–2 mCi/ml) for 30 min in Dulbecco’s modified Eagle’s medium (HEK-293 cells) or for 20 h in Barth medium containing 125 mM furosemide (oocytes). After the loading period, cells were reincubated for timed intervals in a tracer-free medium containing ouabain and NEM. Effluxes were terminated as for the influx assays.

After the final washes for both the influx and efflux studies, cells were solubilized in 2% SDS, and the activity of \( ^{86}\text{Rb} \), \( ^{22}\text{Na} \), or \( ^{36}\text{Cl} \) was detected by liquid scintillation counting using the TopCountNXT microplate counter (Packard). Flux rates from individual oocytes are presented as the means ± S.E. of 1–4 experiments.

RESULTS

Molecular Characterization of CIP1—Sequence alignments of four human CCCs including CIP1. The deduced primary structures of hCIP1, hKCC1, hNKCC1, and hNCC are aligned with each other using FILEUP (GCC). Penalties for gap creation and extension were 12 and 4, respectively. Low weighted pair-groups were realigned with DNAStar programs. Putative transmembrane segments are boxed in gray, and consensus sites for N-glycosylation, between tm5 and tm6, and between tm7 and tm8, are underlined.

Fig. 1. Sequence alignments of four human CCCs including CIP1. The deduced primary structures of hCIP1, hKCC1, hNKCC1, and hNCC are aligned with each other using FILEUP (GCC). Penalties for gap creation and extension were 12 and 4, respectively. Low weighted pair-groups were realigned with DNAStar programs. Putative transmembrane segments are boxed in gray, and consensus sites for N-glycosylation, between tm5 and tm6, and between tm7 and tm8, are underlined.
phylogenetic relationships between these various CCCs and two other members of the family (hNKCC2A and hKCC3) are illustrated in Fig. 2. It can be seen that the recently discovered hCIP1 corresponds to a member of a new CCC family branch, which would also include a putative C. elegans homologue.

As for previously characterized CCCs, topology analyses of hCIP1 (Fig. 3) predict a 12-tm region flanked by cytoplasmic amino and carboxyl termini. The greatest sequence conservatism is present in the membrane-associated domain (particularly in the intracellular loop following tm9 and in a short carboxyl-terminal domain ~140 residues downstream of tm12. Two N-linked glycosylation sites (Asn228 and Asn243) are found in an ~55-residue loop between tm8 and tm9 (Figs. 1 and 3), and 5 candidate sites for protein kinase C phosphorylation are present in the carboxyl terminus.

Chromosomal Localization—To localize CIP1 on the human chromosome, we screened the Genebridge IV somatic cell hybrid panel by PCR using cDNA-specific primers derived from the 3′-UTR. The gene of interest was assigned to locus 7q22 between markers D7S651 and D7S518 at approximately 112 centimorgans from top of the linkage group of chromosome 7.

Northern Blot—The level of expression and the tissue distribution of CIP1 were analyzed by Northern blot. A probe was derived from the 500-bp WO3.3 BamHI fragment, which includes ~225 bp of the carboxyl terminus coding sequences and ~275 bp of 3′-UTR. As shown in Fig. 4, ~3.8- kb transcripts are detected at high levels in several tissues, including placenta, brain, and kidney. A less prominent message is found in lung and a slightly smaller message (~3.6-kb) in heart, liver, and muscle. A shorter exposure time of the blot also revealed a muscle-specific ~5.1-kb transcript (results not shown).

Characterization of the Anti-C1 Antibody—Anti-C1 was derived from the synthetic peptide GPRDRLTPRPGPNG, which corresponds to a segment of hCIP1 between residues 215 and 229; this segment is in the putative third extracellular loop of hCIP1 where consensus sites for N-glycosylation are found. The anti-C1-specific signal is relatively weak, suggesting that the antibody has low affinity for hCIP1. Regarding anti-C1 specificity, it was assessed as follows: 1) positive anti-c-Myc detection of anti-C1-immunoprecipitated proteins from c-Myc-tagged hCIP1-transfected HEK-293 cells but not from hCIP1 or mock-transfected cells (see Fig. 10, right panel); 2) positive anti-C1 detection of anti-c-Myc-precipitated proteins from c-Myc-tagged hCIP1-transfected HEK-293 cells but not of anti-c-Myc-precipitated proteins from hCIP1-transfected HEK-293 cells (results not shown); 3) absent anti-C1 detection of J3-precipitated proteins from c-Myc-tagged hCIP1-transfected HEK-293 cells (results not shown); here, J3 was used as a non-related primary antibody to control for nonspecific sticking to immunobeads; 4) disappearance of the 90- to 100-kDa bands on Western blots of hCIP1-transfected HEK-293 cell lysates when anti-C1 is preincubated with the corresponding synthetic peptide or omitted before the incubation with the secondary antibody (results not shown); 5) immunofluorescence studies, which show cell surface delivery of hCIP1-transfected or hCIP1-injected cells (see additional results below).

Immunofluorescence Studies—As illustrated in Fig. 5, B and C, immunofluorescence studies with the anti-c-Myc antibody demonstrate that in HEK-293 cells, c-Myc-tagged hCIP1 accumulates at the cell surface. The observed concomitant intracellular staining is a common finding when membrane proteins are overexpressed in mammalian cell lines (1, 7). Similar results are obtained with the anti-C1 antibody (see Fig. 5F), whereas no signal is detected in untransfected HEK-293 cells (results not shown). In the X. laevis oocytes, immunofluorescence localization studies of hCIP1 using the anti-C1 antisera (Fig. 5E) and of hNKCC1 using the T4 antibody (Fig. 5H) show cell surface delivery of both proteins. Importantly, the surface expression of hNKCC1 is not grossly decreased or modified with coexpression of hCIP1 (Fig. 5F). In these studies, no immunostaining is observed at the cell surface of H2O-injected oocytes with either anti-C1 (Fig. 5D) or T4 (Fig. 5G).

Flux Studies—To determine whether CIP1 is functionally a CCC, we measured ion transport rates in the plasma membranes of both hCIP1- and c-Myc-tagged hCIP1-transfected HEK-293 cells (up to 5-min fluxes) and of hCIP1-injected X. laevis oocytes (1-h fluxes). These measurements were obtained using 1 of 3 tracers (86Rb+, 22Na+, or 36Cl−) after incubating cells in different media (Table I): regular, hyperosmolar, hypo-
osmolar, hypo-osmolar + NEM, hypo-osmolar low Cl−; as discussed previously, changes in external Cl− and osmolality are associated with changes in K+−Cl− and Na+−K+−Cl− cotransporter activity (1, 7, 11–16). Alteration of the regular medium was generated by the following: 1) changing the pH with NaOH or HCl; 2) replacing Rb+ with NH4+, SO42−, PO43−, or Cl− with gluconate or Na+, Rb+, Ca2+, or Mg2+ with N-methylglucamine; 3) by adding amino acids at 1 mM; or 4) by increasing the concentrations of either SO42−, PO43−, Ca2+, or Mg2+; 13-, 5-, 3-, and 3-fold, respectively. Surprisingly, none of the various preincubating or flux media detailed above supported Na+, Rb+, or Cl− transport above background levels in either of the hCIP1-injected oocytes or hCIP1-transfected HEK-293 cells. By way of illustration, Fig. 6 shows that hCIP1-injected oocytes accumulate 86Rb+ at rates that are not higher than those of H2O-injected oocytes, regardless of the conditions tested, whereas rbNKCC2A-injected oocytes have an increased 86Rb+ content that is maximal with prior incubation in hypo-osmolar low Cl− medium.

In the X. laevis oocyte, 1-h fluxes may have been insufficient to detect activity mediated by a low capacity, low affinity Cl−-coupled carrier. To test this possibility, we incubated hCIP1-injected oocytes for longer periods (1–6 h and overnight). Data are illustrated in Fig. 7 for tracer content (86Rb+ , 36Cl−, or 22Na+) versus time, using hNKCC1 and rbNKCC2A as positive controls. After incubating oocytes with the tracer for up to 6 h in the absence of furosemide or bumetanide (Fig. 7, A–D), the flux by hCIP1-injected oocytes is not above that by H2O-injected oocytes, whereas fluxes by the CCC-injected controls are always higher (at least after 2 h) than that by the H2O-injected oocytes. In the presence of bumetanide (results only shown for Fig. 7B), fluxes decrease substantially for both H2O-injected cells (>30% regardless of the isotope used) and CCC-injected cells (>50% reduction of the background-subtracted flux), indicating prominent CCC activity in both the positive and the H2O controls. On the other hand, this degree of bumetanide-sensitivity also indicates that there are apparent discrepancies in the magnitude of 86Rb+ and 22Na+ fluxes, at least for NKCC1 (compare Fig. 7, A and C), that are not directly due to the activity of a CCC transporter moving ions in the Na+-K+-2Cl− mode of operation (see “Discussion”). Interestingly, preincubation of hCIP1-injected oocytes in hyperosmolar (285 mOsm) medium (Fig. 7B) or in hypo-osmolar (100 mOsm) medium (Fig. 8, left bars) is associated with 86Rb+ fluxes that are lower compared with H2O-injected controls; in the latter, the decrease in 86Rb+ transport corresponds to a

|  |  |  |
|---|---|---|
| **A** | Mock-HEK | anti-c-Myc | anti-C1 | T4 |
| **B** | c-Myc-hCIP1-HEK |  |  |
| **C** | hNKCC1-HEK | | | |

**Fig. 4. Northern blot analyses of the hCIP1.** A CLONTECH human multiple tissue Northern blot was incubated with a 500-bp cDNA probe derived from the carboxyl terminus coding sequence and the 3′-UTR of hCIP1. A 3.6-kb transcript is seen in heart, liver, and kidney, and a less prominent message in lung and liver. The blot was reprobed with a β-actin cDNA probe to assess RNA integrity.

**Fig. 5. Immunofluorescence micrographs of HEK-293 cells and X. laevis oocytes using anti-c-Myc (left panel), anti-C1 (middle panel), and T4 (right panel).** A, mock-transfected HEK-293 cells. B, C, and F, c-Myc-hCIP1-transfected HEK-293 cells. D and G, H2O-injected oocytes. E, hCIP1-injected oocytes. H, hNKCC1-injected oocyte. I, oocytes coinjected with hCIP1 and hNKCC1. White arrows delineate plasma membrane of the oocytes. Immunofluorescence micrographs of hCIP1-transfected HEK-293 cells and of c-Myc-hCIP1-transfected HEK-293 cells using the anti-C1 antibody were identical to that shown in A. The pictures in D, E, and G–I are from a representative membrane section among ≥3 oocytes; the exposure times were similar between each panel. The results shown in A–C and F are from representative cells grown at ~50–75% confluence on 20 × 20-mm coverslips.

6 Bumetanide-sensitive fluxes (using the inhibitor at 250 μM) were measured in a number of experiments as follows. 1) For 86Rb+ using the conditions of Fig. 7A, these measures were obtained for H2O-, hCIP-, and hNKCC1-injected oocytes after 3-h fluxes (5–6 oocytes from 1 and 2 experiments). 2) For 22Na+ using the conditions in Fig. 7C, bumetanide-sensitive measures were obtained for H2O-, hCIP-, and hNKCC1-injected oocytes after 1-h fluxes (4–6 oocytes from 1 and 2 experiments) and 3-h fluxes (3–4 oocytes from 1 experiment). 3) For 36Cl− using the conditions in Fig. 7D, bumetanide-sensitive measures were obtained for H2O-, hCIP-, and rbNKCC2A-injected oocytes after 3-h fluxes (4–6 oocytes from 1 and 2 experiments).
bumetanide-sensitive flux (250 μM bumetanide), as suggested by the data shown in Fig. 7B. In a separate experiment, already presented in Fig. 6, it is seen that 86Rb+ fluxes by hCIP1-injected oocytes are also slightly lower than those by water-injected oocytes after hypo-osmolar or hyperosmolar preincubations. Similar findings are obtained with HEK-293 cells overexpressing hCIP1; indeed, the bumetanide-sensitive 86Rb+ fluxes by these hCIP1-transfected cells are lower than those by the untransfected cells (see Fig. 8, right bars). Taken together, the data suggest that hCIP1 inhibits the activity of an endogenous KCl-coupled CCC.

Longer (20-h) incubations in Barth medium (Table I) produce a furosemide-insensitive 36Cl− and 86Rb+ accumulation in hCIP1-injected oocytes that is higher than that in H2O-injected oocytes (see Fig. 7, E and F). 22Na+ accumulation was not increased compared with the negative controls (results not shown). It is important to note, however, that ion fluxes by the positive controls are not completely inhibited with 250 μM furosemide (see Fig. 7, E and F), as is often the case (in our hands) using an oocyte expression system to measure heterologous CCC activity. Nonetheless, the data presented here suggest that hCIP1, similar to the NKCCs, alter steady-state K+ and Cl− fluxes at the cell surface of the oocyte. It is also pertinent to note that following the 20-h incubations in isotope-supplemented Barth medium, rates of tracer efflux (from 1 to 6 h) in furosemide- and isotope-free media (regular 6NEM) or hypo-osmolar low Cl− 6NEM) were comparable for H2O-injected controls and hCIP1-expressing oocytes (results not shown).

CIP1 may require a subunit to transport ions at higher turnover rates. It is possible that this subunit consists of an unknown protein, but it could also correspond to a known CCC, including NKCC2 or KCC1. Recent data (23, 24) demonstrating

---

**TABLE I**

Composition of flux solutions

| Na+ | Rb+ | Cl− | Ca2+ | Mg2+ | PO4 3− | SO4 2− | HEP | GLU | SUC | osm |
|-----|-----|-----|------|------|--------|--------|-----|-----|-----|-----|
|     |     |     |      |      |        |        |     |     |     |     |
| Oocytes |
| Hypo-osmolar low Cl− | 43.5 | 2.5 | 4 | 1 | 1 | 1 | 5 | 39 | 0 | 100 |
| Hypo-osmolar | 43.5 | 2.5 | 43 | 1 | 1 | 1 | 5 | 0 | 0 | 100 |
| Regular medium | 87 | 5 | 86 | 2 | 2 | 2 | 2 | 10 | 0 | 200 |
| Modified regular medium (low K+) | 87 | 0.1 to 1 | 86 | 2 | 2 | 2 | 2 | 10 | 0 | 200 |
| Hyperosmolar | 87 | 5 | 86 | 2 | 2 | 2 | 2 | 10 | 0 | 200 |
| Barth medium | 90.4 | 0 | 89.6 | 0.74 | 0.82 | 0 | 0.82 | 10 | 0 | 0 |
| HEK-293 |
| Hypo-osmolar low Cl− | 77 | 2.5 | 2 | 0.5 | 0.5 | 1 | 1 | 7.5 | 70 | 0 |
| Hypo-osmolar | 77 | 2.5 | 72 | 0.5 | 0.5 | 1 | 1 | 7.5 | 0 | 163 |
| Regular medium | 154 | 5 | 144 | 1 | 1 | 1 | 2 | 15 | 0 | 326 |
| Hyperosmolar | 154 | 5 | 144 | 1 | 1 | 1 | 2 | 15 | 0 | 100 |

---

**Fig. 6.** 86Rb+ fluxes of *X. laevis* oocytes injected with H2O, hCIP1, and rbNKCC2A. The bars express S.E. of 3–15 oocytes from 3 to 15 determinations. Cells were first incubated 45 min in 1 of 4 media (see Table I): hypo-osmolar low Cl−, regular, hyperosmolar, and regular 1 mM NEM). Then, cells were assayed for 1-h 86Rb+ fluxes in regular medium ([Rb+]=5 mM) + 10 μM ouabain.

**Fig. 7.** Six-h and overnight fluxes of *X. leavis* oocytes. A, C, and D, oocytes are preincubated 45 min in a regular medium and assayed for 86Rb+ content in a regular medium + 10 μM ouabain (see Table I). B, oocytes are preincubated 45 min in a hyperosmolar medium and assayed as in A. E and F, oocytes are incubated 20 h with 86Rb+ or 36Cl− in Barth medium + 250 μM furosemide, washed several times in regular medium, and counted by liquid β-scintillation (note, [K+] in Barth medium = 1 mM). The data are shown as the average ± S.E. of 3–13 oocytes from 1 to 4 experiments. Black circles, hCIP1-injected oocytes.
that NKCC2 can form homo-oligomers in oocytes support the latter hypothesis; conserved domains within the CCC termini would therefore have to exist to enable CCC-CCC interactions. To explore the possibility of heterodimer formation, we first determined whether any functional relationships existed between CIP1 and other CCCs by conducting flux experiments using oocytes coinjected with cRNAs from different sources (hCIP1, rbKCC1, hNKCC1, or rbNKCC2A). Data from 86Rb+ flux assays using a regular or a hypo-osmolar preincubating medium are shown in Fig. 9, A and B (data with hyperosmolar preincubation were similar). Whereas the transport activities of rbKCC1 or rbNKCC2A are not modified by coexpression of hCIP1 in either of the preincubation conditions, that of hNKCC1 is greatly reduced after the incubation in hypo-osmolar medium; similar results are observed using different [Rb+] in the flux medium (100 μM in Fig. 9A and 1 mM in Fig. 9B). These data, thus, indicate that the end result of expression of hCIP1 in oocytes is inhibition of the hNKCC1-specific transport activity.

Coimmunoprecipitation Studies—It is noteworthy that in hCIP1-transfected HEK-293 cells, the activity of the endogenous NKCC (hNKCCHEK), which is probably mediated at least in part by hNKCC1 or an hNKCC1 splice-variant (see Ref. 14), was found to be reduced compared with untransfected HEK-293 cells (see Fig. 8). This phenomenon, which we had also observed on numerous occasions in cells expressing inactive NKCC1 mutants (1),7 is consistent with the above suggestion that the CCCs can form homo-oligomers (23, 24). This phenomenon also suggests that the CCCs have conserved domains that could lead to interactions between homologous proteins.

To determine whether hNKCCHEK can physically interact with hCIP1, we performed coimmunoprecipitation studies using mock-, hCIP1-, and c-Myc-tagged hCIP1-transfected HEK-293 cells, and we analyzed the results by Western blotting. In Fig. 10, for example, it can be seen that anti-c-Myc detects c-Myc-tagged hCIP1 exclusively, and that detection occurs whether the c-Myc-tagged is preimmunoprecipitated with the anti-c-Myc (Fig. 10, left panel), the T4 (Fig. 10, middle panel), or the anti-C1 (Fig. 10, right panel) antibody. In conjunction with results presented above indicating that anti-C1 is specific to hCIP1, these data suggest that hNKCCHEK does interact with heterologous hCIP1. It is important to note, however, that anti-c-Myc detects different sizes of the carrier depending on

7 R. D. Behnke, P. Isenring, and B. Forbush, personal observations.

![Figure 8](image1.png) **Effect of hCIP1 on endogenous NKCC activity in oocytes and in HEK-293 cells.** Oocytes (left panel) and HEK-293 cells (right panel) are preincubated 45 min in a hypo-osmolar medium and assayed for 86Rb+ content in a modified regular medium containing 0.1 mM Rb+ (oocytes) or in a regular medium containing 5 mM Rb+ (HEK-293 cells). For the oocytes, the data are shown as the average ± S.E. of 12–16 oocytes from three representative experiments. For HEK-293 cells, the data are shown as the average ± S.E. of 12–16 individual wells from 3 to 4 experiments.

![Figure 9](image2.png) **Coinjection studies in X. laevis oocytes.** Oocytes are injected with ~5–25 ng of hCIP1 cRNA + equimolar amounts of rbKCC1, hNKCC1, or rbNKCC2A cRNA. Eggs are preincubated 45 min in a hypo-osmolar medium or in a regular flux medium (see Table I) and assayed for 86Rb+ content in modified regular media + 10 μM ouabain (A, Rb+ = 100 μM, and B, Rb+ = 1 mM); at these concentrations of cold Rb+, the CCCs still produce above-background fluxes (see right panels in both A and B) that are furosemide-sensitive (data not shown). The results presented in this Fig. 9 are from a representative experiment among ≥4 determinations (A) and among 2 determinations (B). In both A and B, bars express S.E. of 5–10 oocytes. With modified regular medium containing 1 mM [Rb+] (B), higher Vmax are observed, but differences between conditions are maintained, and hCIP1 inhibition in the presence of NKCC1 still occurs.
similar hydropathy profile (see Fig. 1). Phylogenetically, hCIP1 intracellular loop (see Fig. 1) between tm2 and tm3. Accordingly, and because tm2 has been implicated in cation transport and because tm2 has been implicated in cation transport, this region could play a role in protein-protein interactions; candidate CCC-interacting proteins include cytoskeletal elements, regulatory enzymes, and the CCCs themselves.

The overall hydropathy structure of CIP1 is closer to that of K+–coupled CCCs (7, 12, 13) than to that of Na+–coupled CCCs (3, 8–10). For example, the CIP1 protein and all of the KCC isoforms have a large (>55-residue) extracellular loop between predicted tm2 and tm3 that contains at least two potential sites for N-linked glycosylation. As for the Na+–coupled CCCs, there is also a large extracellular loop containing glycosylation sites, but it is found between predicted tm2 and tm3. Another apparent structural similarity shared by CIP1 and the KCCs is their relatively short amino terminus compared with that of the NCC or NKCC proteins. The functional significance of these similarities is unknown.

Extensive analyses in this work did not allow determining the precise function of CIP. Lack of substantial transport activity by hCIP1-expressing cells (after 6-h fluxes for oocytes and 5-min fluxes for HEK-293 cells) was not due to deficient protein synthesis or lack of cell surface delivery, as shown by Western blot and immunofluorescence studies (see Figs. 5 and 10). It was also not due to inappropriate stimulation methods or to the omission of potentially key cosubstrates in the flux solutions. Although these possibilities cannot be entirely excluded, CIP1 could require unusual factors for activation or transport unanticipated substrates, they appear unlikely considering that multiple conditions were tested all of which resulted in hNKCC1-, rbKCC1-, or rbNKCC2A-mediated activity. It is noteworthy, however, that we observed a slight increase in furosemide-insensitive 86Rb+ and 36Cl– fluxes by hCIP1-expressing oocytes after overnight incubations with the ion tracer. Presumably, this modest increase in net isotope accumulation was due to hCIP1-mediated ion translocation.

During analysis of the flux data described above, certain inconsistencies were noted. For example, there is an ~5-fold difference between hNKCC1-induced 86Rb+ fluxes (Fig. 7A) and hNKCC1-induced 22Na+ fluxes (Fig. 7C). This unexpected difference, which was not due to hematine-insensitive CCC activity, may have occurred as a result of substantial 22Na+ movement through NKCC1 taking place as Na+-Na+ exchange. Alternatively, higher than expected 22Na+ fluxes may have been due to the activity of various Na+-dependent pathways triggered by CCC-mediated changes in [ion]. Apparent inconsistencies were also observed in regard to the 36Cl– flux data. For instance, equilibration of the Cl– isotope in rbNKCC2A-injected oocytes (see Fig. 7D) is surprisingly rapid, whereas Rb+ is maintained at a lower concentration. Here, conceivably, equilibration of Cl– may have been more complete than that of Rb+ because of concomitant Cl–/HCO3– exchange. Consistent with this hypothesis is a recent experiment by our group in which 36Cl– fluxes by hNKCC1-injected oocytes were found to be partially attenuated after adding DIDS in the preincubation medium (results not shown).

Coexpression of hNKCC1 and hCIP1 in oocytes showed that the activity of hNKCC1 was abolished in the presence of hCIP1 at the cell surface. This functional relationship was also observed in hCIP1-transfected HEK-293 cells where endogenous CCC activity appeared to be slightly decreased compared with untransfected cells. The effect of hCIP1 on NKCC1 activity was abolished in the presence of hCIP1 (see Fig. 9) nor was the gross pattern of hNKCC1 distribution at the cell surface of the oocyte (see Fig. 5J).
The coimmunoprecipitation experiments presented in Fig. 10 indicate that a plausible explanation for the hCIP1-hNKCC1 functional connection is a physical association between the two proteins; these experiments were performed using hCIP1- and c-Myc-tagged hCIP1-transfected HEK-293 cells. However, it is important to recognize that it was only possible to coimmunoprecipitate c-Myc-hCIP1 and hNKCC1HEK when T4 was used for immunoprecipitation and anti-c-Myc for detection, and not when anti-c-Myc was used for immunoprecipitation and T4 for detection. Consequently, and although the absence of reciprocity in these studies could be due to differences in relative antibody efficiency for detection and immunoprecipitation, and/or to relative differences in antigen accessibility, the conclusion that hCIP1 and hNKCC1HEK directly interact with each other remains to some extent hypothetical.

In most cases, the formation of quaternary structures probably requires correct folding of the interacting proteins. In the present study, Western analyses (see Fig. 10) suggest that if physical association between hNKCC1 and CIP1 does occur, only the processed forms of the proteins would assemble with each other. For example, T4-immunoprecipitated c-Myc-tagged hCIP1 detected with anti-c-Myc appears on Western blots as an upper band only (Fig. 10, middle panel); hypothetically, this band represents the processed form of the carrier. On the other hand, anti-C1 primarily recognizes a lower band (Fig. 10, right panel) and is not able to detect T4-immunoprecipitated c-Myc-tagged hCIP1 nor is it able to precipitate a protein complex detectable by T4 (results not shown). Thus, anti-C1 may not have been useful to show an hCIP1-hNKCC1HEK interaction because the antibody binds mainly to the incompletely processed hCIP1. However, it is important to point out that the anti-C1 antibody has relatively low sensitivity in Western analyses (see “Results”), which could also explain some of the negative results.

The results of biochemical characterizations of hCIP1 with anti-C1 seem to indicate that the behavior of the antibody differs in immunofluorescence studies compared with that in Western analyses; indeed, anti-C1 is able to detect hCIP1 at the cell surface of oocytes and of HEK-293 cells, despite its inability to recognize an upper (presumably processed) band in Western analyses. However, discrepancies in antibody behavior could also be fictitious; for example, a fraction of immature (deglycosylated?) hCIP1 molecules may be capable of assuming some form of tertiary conformation and of reaching the plasma membrane. Consistent with this possibility is a recent study by Karpa et al. (32) showing that N-glycosylation is not required for plasma membrane localization of D1 dopamine receptors in transfected mammalian cells. Nonetheless, and even if the data presented here demonstrate that anti-C1 is specific for CIP1, further studies are required to determine how post-translational modifications of the carrier in HEK-293 cells affect electrophoretic mobility.

Based on coimmunoprecipitation studies in HEK-293 cells and on the effect of hCIP1 on hNKCC1-mediated activity in oocytes, it is tempting to conclude that CIP1 is an NKCC1 inhibitor. However, because CIP1 may have intrinsic transport capabilities, and because it is highly homologous to members of the CCC family, alternative possibilities should also be considered. For example, CIP1 could correspond to a subunit of a multimeric transport system; then the failure of the hCIP1 monomer or of an hNKCC1-hCIP1 heterodimer to bring about substantial fluxes would indicate that additional subunits are required for function. In support of this hypothesis, the demonstration in recent studies (23, 24) that NKCC2 may form cell surface homo-oligomers points to the existence of conserved protein domains that would make CCC-CCC interactions possible. We have also noted that in HEK-293 cells expressing inactive mutants, the hNKCC1HEK activity was often reduced (1); this observation also suggests that the NKCC1s can self-dimerize.

As a general rule, multimeric formation leads to diversity in the kinetic and in the pharmacological properties of channels. For the CCC proteins, homo- or hetero-oligomerization could also play an important role in transport capacitiation and in substrate specificity. Thus, reorganization of higher order structures could represent a mechanism by which various conditions affect the kinetics of cation-Cl− cotransport at the cell surface. Such a mechanism, for instance, could account for loop diuretic-sensitive cotransport that becomes K+−dependent in the presence of vasopressin (33). Similarly, alternative splicing in the NKCC2 carboxyl terminus, which results in K+−independent furomeside-sensitive cotransport (24), could have prevented or promoted heteromeric assembly with CCC subunits that play a role in K+ translocation.

To elucidate further the role played by CIP1, chromosomal assignment studies were carried out using radiation hybrid panels. These studies allowed localizing the CIP1 gene to chromosome 7q22 between markers D7S651 and D7S518; these markers flank a region that contains at least 8 genes. CIP1 was found to be an unlikely candidate for genetic disorders or susceptibilities mapping to the 7q22 region. However, some cases of Bartter’s (34) and Gordon’s syndromes (see Ref. 35), both of which demonstrate locus heterogeneity (36), could be due to abnormal NH4+-Cl− and/or K+-Cl− secretion in the outer medullary collecting duct. In this nephron segment, α-intercalated cells have been shown to express NKCC1 (37). If CIP1 was also expressed in this cell type, it would correspond to a functional candidate gene for cases of Bartter’s or Gordon’s syndromes that have not been linked to a specific gene.

In conclusion, this study reports the cloning and the functional characterization of a first member of a new CCC family branch. This protein was termed CCC-interacting protein type 1 (CIP1) based on the demonstration that it blocks the activity of hNKCC1 in the X. laevis oocytes and that it may coimmunoprecipitate with hNKCC1HEK. These results suggest that the formation of higher order structures containing homologous and heterologous subunits may be an important feature in the mode of operation of CCC proteins. Further studies are needed to discover the precise role of CIP1 and to determine if other subunits are required for function.

Acknowledgments—We thank Claude Villeneuve and Dominique Heitz for their superb technical assistance and Vincent Gaudreau, and Dr. John H. Grose for their careful reading of our manuscript. We are also very grateful to Dr. Biff Forbush for providing clones and reagents; these include the T4 antibodies, which were originally produced by Dr. C. Y. Lytle.

REFERENCES

1. Isenring, P., and Forbush, B., III (1997) J. Biol. Chem. 272, 24556–24562
2. Haas, M., and Forbush, B. (1998) J. Bioenerg. Biomembr. 30, 161–172
3. Xu, J., Lytle, C., Zhu, T., Payne, J. A., Benz, E., and Forbush, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2201–2205
4. Delpire, E., Rauchman, M. I., Beier, D. R., Hebert, S. C., and Gullans, S. R. (1994) J. Biol. Chem. 269, 25677–25683
5. Lafuente, J., Adraga, N. C., Fajadet, J., and Delpire, E. (1992) Exp. Cell. Res. 203, 91–97
6. Mitani, A., and Shattock, M. J. (1995) J. Biol. Chem. 270, 16237–16244
7. Gillen, C. M., Brilli, S., Payne, J. A., and Forbush, B., III (1996) J. Biol. Chem. 271, 16237–16244
8. Gamba, G., Saltzberg, S. N., Lombardi, M., Miyashita, A., Lytton, J., Hediger, M. A., Brenner, B. M., and Hebert, S. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2749–2753
9. Payne, J. A., Xu, J.-C., Haas, M., Lytle, C. Y., Ward, D., and Forbush, B., III (1995) J. Biol. Chem. 270, 17977–17985
10. Payne, J. A., and Forbush, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4544–4548
11. Payne, J. A., Stevenson, T. J., and Donaldson, L. F. (1996) J. Biol. Chem. 271, 16245–16252
12. Mount, D. B., Mercado, A., Song, L., Xu, J., George, A. L., Jr., Delpire, E., and...
Cloning and Characterization of a CIP

Gamba, G. (1999) J. Biol. Chem. 274, 16355–16362
13. Hiki, K., D’Andrea, R. J., Furze, J., Crawford, J., Woollatt, E., Sutherland, G. R., Vadas, M. A., and Gamble, J. R. (1999) J. Biol. Chem. 274, 10661–10667
14. Isenring, P., Jacoby, S. C., Payne, J. A., and Forbush, B., III (1998) J. Biol. Chem. 273, 11285–11301
15. Isenring, P., Jacoby, S. C., and Forbush, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7179–7184
16. Isenring, P., Jacoby, S. C., Chang, J., and Forbush, B. (1998) J. Gen. Physiol. 112, 549–558
17. Lytle, C. (1997) J. Biol. Chem. 272, 15069–15077
18. Gillen, C. M., and Forbush, B. (1999) Am. J. Physiol. 276, C328–C336
19. Lytle, C., and Forbush, B. (1996) Am. J. Physiol. 270, C1122–C1130
20. Kracke, G. R., and Dunham, P. B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8575–8579
21. Kelley, S. J., Dunham, P. B. (1996) Am. J. Physiol. 270, C1122–C1130
22. Matthews, J. B., Smith, J. A., Tally, K. J., Awtry, C. S., Nguyen, H., Rich, J., and Madara, J. L. (1994) J. Biol. Chem. 269, 15703–15709
23. Ichinose, M., Hall, A. E., Cheng, S., Xu, J. Z., and Hebert, S. C. (1999) J. Am. Soc. Nephrol. 10, 34 (Abstr. A0176)
24. Plata, C., Mount, D. B., Rubio, V., Hebert, S. C., and Gamba, G. (1999) Am. J. Physiol. 276, F359–F366
25. Lennon, G. G., Auffray, C., Polymeropoulos, M., and Soares, M. B. (1996) Genomics 33, 151–152
26. Jacoby, S. C., Gagnon, E., Caron, L., Chang, J., and Isenring, P. (1999) Am. J. Physiol. 277, C684–C692
27. Beck, P. J., Orlean, P., Albright, C., Robbins, P. W., Gething, M. J., and Sambrook, J. F. (1990) Mol. Cell. Biol. 10, 4612–4622
28. Lytle, C. Y., Xu, J. C., Biemesderfer, D., Haas, M., and Forbush, B. (1992) J. Biol. Chem. 267, 25428–25437
29. Lytle, C. Y., Xu, J. C., Biemesderfer, D., and Forbush, B., III (1995) Am. J. Physiol. 269, C1496–C1505
30. Tanphaichitr, V. S., Sunheesnanonda, A., Ideguchi, H., Shyakul, C., Brugnara, C., Takao, M., Veerakul, G., and Alper, S. L. (1998) J. Clin. Invest. 102, 2173–2179
31. Payne, J. A., and Forbush, B. (1995) Curr. Opin. Cell Biol. 7, 493–503
32. Karpa, K. D., Lidelew, M. S., Pickering, M. T., Levenson, R., and Bergson, C. (1999) Mol. Pharmacol. 56, 1071–1078
33. Sun, A., Grossman, E. B., Lombardi, M., and Hebert, S. C. (1991) J. Membr. Biol. 120, 83–94
34. Barter, F. C., Pronove, P., Gill, J. R., and MacCardle, R. C. (1998) J. Am. Soc. Nephrol. 9, 516–528
35. Isenring, P., Lebel, M., and Grose, J. H. (1992) Hypertension 19, 371–377
36. Karet, F. E., and Lifton, R. P. (1997) Recent Prog. Horm. Res. 52, 263–277
37. Ecelbarger, C. A., Terris, J., Hoyer, J. R., Nielsen, S., Wade, J. B., and Knepper, M. A. (1996) Am. J. Physiol. 271, F619–F628