Affinity-defining Domains in the Na-Cl Cotransporter

A DIFFERENT LOCATION FOR Cl– AND THIAZIDE BINDING

Received for publication, March 20, 2006, and in revised form, April 10, 2006 Published, JBC Papers in Press, April 19, 2006, DOI 10.1074/jbc.M602614200

Erika Moreno‡1, Pedro San Cristóbal‡1, Manuel Rivera, Norma Vázquez‡, Norma A. Bobadilla‡ and Gerardo Gamba‡2

From the Molecular Physiology Unit, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México Tlalpan 14000, Mexico City, Mexico and Instituto de Ciencias de la Salud, Universidad Autónoma del Estado de Hidalgo, Pachuca, Hidalgo 42160, México

The thiazide-sensitive Na+-Cl– cotransporter (NCC) is the major pathway for salt reabsorption in the distal convoluted tubule, serves as a receptor for thiazide-type diuretics, and is involved in inherited diseases associated with abnormal blood pressure. Little is known regarding the structure-function relationship in this cotransporter. Previous studies from our group reveal that mammalian NCC exhibits higher affinity for ions and thiazides than teleost NCC and suggest a role for glycosylation upon thiazide affinity. Here we have constructed a series of chimeric and mutant cDNAs between rat and flounder NCC to define the role of glycosylation status, the amino-terminal domain, the carboxyl-terminal domain, the extracellular glycosylated loop, and the transmembrane segments upon affinity for Na+, Cl–, and metolazone. Xenopus laevis oocytes were used as the heterologous expression system. We observed that elimination of glycosylation sites in flounder NCC did not affect the affinity of the cotransporter for metolazone. Also, swapping the amino-terminal domain, the carboxyl-terminal domain, the glycosylation sites, or the entire extracellular glycosylation loop between rat and flounder NCC had no effect upon ions or metolazone affinity. In contrast, interchanging transmembrane regions between rat and flounder NCC revealed that affinity-modifying residues for chloride are located within the transmembrane 1–7 region and for thiazides are located within the transmembrane 8–12 region, whereas both segments seem to be implicated in defining sodium affinity. These observations strongly suggest that binding sites for chloride and thiazide in NCC are different.

In the mammalian kidney, the apical thiazide-sensitive Na+-Cl– cotransporter (NCC) is the major pathway for salt reabsorption in the distal convoluted tubule (1, 2). NCC also serves as the target for the thiazide-type diuretics that are currently recommended by the Joint National Committee for the detection, evaluation, and treatment of high blood pressure as the first line pharmacological treatment of hypertension, either as the unique drug in patients with stage I hypertension or in combination with other anti-hypertensive agents for patients with stage II hypertension (3). The fundamental role for NCC in preserving the extracellular fluid volume and blood pressure homeostasis has been firmly established by the identification that Gitelman disease (4–6) (an inherited disorder featuring arterial hypotension, renal salt wasting, hypokalemic metabolic alkalosis, hypocalcinuria, and hypomagnesemia) is caused by inactivating mutations of the SLC12A3 gene that encodes NCC. In addition, a loss of the negative effect of the serine/threonine kinases WNK1 and WNK4 upon NCC activity has been implicated in the pathogenesis of a salt-dependent form of human hypertension known as pseudohypoaldosteronism type II (7, 8), which features a clinical picture that is a mirror image of Gitelman disease (9), with striking sensitivity to hydrochlorothiazide.

NCC belongs to the electroneutral cation Cl–-coupled cotransporter gene family (solute carrier family 12 (SLC12)) (10) and exhibits ~50% identity with the Na+-K+–2Cl– cotransporters NKCC1 and NKCC2 and ~25% identity with the KCl cotransporters KCC1 to KCC4 (for review see Ref. 11). Mammalian NCC is a protein of 1,002 amino acid residues featuring a central hydrophobic domain, which is flanked by a short amino- and a long carboxyl-terminal hydrophilic loops located within the cell (12)(Fig. 1). In the central hydrophobic domain, there are twelve putative transmembrane-spanning segments (TM 1–12) interconnected by six extracellular and five intracellular hydrophilic loops. The extracellular loop 4 (ECL4) is the longest one and exhibits two putative N-linked glycosylation sites. The proposed topology shown in Fig. 1 has been experimentally confirmed for NKCC1 (13), and glycosylation of the ECL4 has been confirmed for rat NCC (14) and rat NKCC2 (15).

Little is known about the structural requirements for ion translocation and thiazide binding in NCC. Tovar-Palacio et al. (16) provide evidence that the critical domains and/or residues that define the specificity for ion translocation and thiazide inhibition reside within the central hydrophobic domain. Hoover et al. (14) have observed that rNCC glycosylation status markedly affected NCC activity and the affinity for metolazone. Elimination of the ECL4 glycosylation sites reduce the cotransporter activity by 90% and increase the affinity for metolazone. Interestingly, NCC from flounder urinary bladder (fNCC) contains three putative N-linked glycosylation sites in ECL4 instead of two, and compared with rNCC, exhibits lower affinity for thiazide, as well as for both Na+ and Cl– ions (17).

The major goal of the present study was to determine the role of glycosylation sites and the different domains of the renal-specific apical sodium-chloride coupled cotransporter in defining the affinity of the transport process and diuretic interaction. We exploited the fact that rNCC and fNCC exhibit distinct affinities for the transported ions and thiazide diuretics together with extensive protein sequence similarities.
and differences. Accordingly, we constructed several point-mutated clones as well as chimeric proteins between rNCC and fNCC. The functional properties of the resultant proteins were determined by functional expression in Xenopus laevis oocytes. Our results show that affinity-defining regions for Cl− and thiazides are different, suggesting that Cl− and thiazide may bind to different regions of the cotransporter.

EXPERIMENTAL PROCEDURES

Mutagenesis and Construction of Chimeric Cotransporters—For the present study, we used the rat and flounder NCC cDNAs that we previously cloned from rat kidney (rNCC) (12) and flounder urinary bladder (fNCC) (18), respectively. The wild-type rNCC cDNA contains a unique NsiI site on the base pairs 444–449 that encodes residues located at the middle of the first TM segment. To exchange regions or domains between rNCC and fNCC, we have engineered both cDNAs by introducing several silent restriction sites in exactly the same location. In fNCC, a silent NsiI site was introduced at the middle of the first TM segment, and in both rNCC and fNCC, silent MunI, SacII, and Hpal sites were created, MunI and SacII at the beginning and at the end of ECL4, respectively, and Hpal at the beginning of the carboxyl-terminal domain. Thereafter, the unique EcoRI (in the 5′ side of the polylinker) and NotI (in the 3′ side of the polylinker) together with the unique NsiI, MunI, SacII, and Hpal sites were used to exchange domains between rNCC and fNCC by gel purification and ligation of the appropriate cDNA bands. In addition, site-directed mutagenesis was used to add and/or eliminate N-glycosylation sites from rNCC, fNCC, and chimeric clones (see “Results”). The double mutant rNCC cDNA used in this study, in which both N-glycosylation sites were eliminated, was previously described (14). For fNCC, the oligonucleotides 5′–CTCTGTTAGTGGACAAGACAC CCTGCTCTAC-3′, 5′–CTCATCCAGCG AGCAATGTTGGGATTAGCTG-3′, and 5′–CGAGTGTATAAAG CAAAACATGCGAACCAC-3′, were used to mutate the asparagines at positions 403 (N403Q), 414 (N414Q), and 432 (N432Q), respectively, to glutamine. Thereafter, double or triple mutants were constructed using the same primers upon the previously mutated clones. Finally, by introducing several silent restriction sites in exactly the same location, in addition, site-directed mutagenesis was used to add and/or eliminate N-glycosylation sites from rNCC, fNCC, and chimeric clones (see “Results”). The double mutant rNCC cDNA used in this study, in which both N-glycosylation sites were eliminated, was previously described (14). For fNCC, the oligonucleotides 5′–CTCTGTTAGTGGACAAGACAC CCTGCTCTAC-3′, 5′–CTCATCCAGCG AGCAATGTTGGGATTAGCTG-3′, and 5′–CGAGTGTATAAAG CAAAACATGCGAACCAC-3′, were used to mutate the asparagines at positions 403 (N403Q), 414 (N414Q), and 432 (N432Q), respectively, to glutamine. Thereafter, double or triple mutants were constructed using the same primers upon the previously mutated clones. Finally, by means of double-step PCR, the epitope FLAG sequence DYYKDDDDK was added in-frame to fNCC following the first methionine. All silent or site-directed mutations were introduced using the QuikChange site-directed mutagenesis system ( Stratagene) following the manufacturer’s recommendations. Restriction analysis and automatic DNA sequencing was used to corroborate all of the mutations and in the switching place of each chimera. All primers used for mutagenesis were custom made ( Sigma).

In Vitro cRNA Translation—To prepare cRNA for microinjection, each wild-type, mutant, or chimeric cDNA was digested at the 3′-end using NotI from New England Biolabs (Carlsbad, CA), and cRNA was transcribed in vitro using the T7 RNA polymerase mMESSAGE mACHINE™ (Ambion) transcription system. cRNA product integrity was confirmed on agarose gels and concentration was determined by absorbance reading at 260 nm (DU 640, Beckman Instruments). cRNA was stored frozen in aliquots at −80 °C until used.

X. laevis Oocyte Preparation—Oocytes were harvested by surgery from adult female X. laevis frogs (Nasco) under 0.17% tricaine anesthetic and incubated in frog Ringer ND96 (in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 Heps/Tris, pH 7.4) in the presence of collagenase B (2 mg/ml) for 1 h. The oocytes were washed four times in ND96, defolliculated, and incubated overnight at 18 °C in ND96 supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml of gentamicin. Next day, mature oocytes (19) were injected with 50 nl of water alone or containing cRNA at 0.2–0.75 μg/μl (10–37.5 ng of cRNA/oocyte). Thereafter, the oocytes were incubated 4–6 days in ND96 with sodium pyruvate and gentamicin that was changed every 24 h. The night before the uptake experiments were performed, the oocytes were incubated in Cl−-free ND96 (in mM) 96 Na+ isethionate, 2 K+ gluconate, 1.8 Ca2+ gluconate, 1.0 Mg2+ gluconate, 5 mM Hepes, 2.5 sodium pyruvate, and 5 mg/100 ml gentamicin, pH 7.4) (18).

Transport Assays—The function of the Na++Cl− cotransporter was determined by assessing tracer 22Na+ uptake (PerkinElmer Life Sciences) in groups of 10–15 oocytes following our protocol (20) of 30 min of incubation in a Cl−-free ND96 medium containing 1 mM ouabain, 0.1 mM amiloride, and 0.1 mM bumetanide followed by a 60-min uptake period in a K+−free, NaCl medium (in mM) 40 NaCl, 56 sodium-gluc- onate, 4.0 CaCl2, 1.0 MgCl2, and 5.0 Hepes/Tris, pH 7.4) containing ouabain, bumetanide, and 2 μCi of 22Na++/ml. We have previously demonstrated that X. laevis oocytes do not express a thiazide-sensitive Na++Cl− cotransporter (18, 20). Thus, in all experiments along the study, only one group of water-injected oocytes was included to determine the basal, unspecific tracer 22Na+ uptake. The sensitivity and kinetics for metolazone diuretics was assessed by exposing groups of cRNA-injected oocytes to metolazone at concentrations varying from 10−10 to 10−4 M. For these experiments, the desired concentration of the diuretic was present in both the incubation and uptake periods. All metolazone dependence curves were assessed twice for each clone. To determine the ion transport kinetics of the wild-type, mutant, or chimeric cotransporter, we performed experiments varying the concentrations of Na+ and Cl−. To maintain osmolarity and ionic strength, gluconate was used as a Cl− substitute and N-methyl-D-glucamine as a Na+ substitute. To minimize variations along the study due to ion concentrations, two sets of solutions for Na+ transport kinetic analysis and two sets for Cl− transport kinetic analysis were made. Thereafter, all clones were subjected to at least two different ion transport kinetic experiments with each set of solution.

All uptake experiments were performed at 32 °C. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without isotope to remove extracellular fluid tracer. After the oocytes were dissolved in 10% sodium dodecyl sulfate, tracer activity was determined for each oocyte by β-scintillation counting.

Western Blotting—Western blot analysis was used to analyze wild-type or N-glycosylation-mutant FLAG-fNCC proteins in corresponding cRNA-injected oocytes following our standard protocol (21). First, proteins extracted from 50 oocytes were immunoprecipitated using the FLAG-tagged protein immunoprecipitation kit following the manufacturer’s recommendations. Subsequently, proteins from 10 oocytes/lane were heated in sample buffer containing 6% SDS, 15% glycerol, 0.3% bromophenol blue, 150 mM Tris, pH 7.6, and 2% B-mercaptoethanol, resolved by Laemmli SDS-polyacrylamide (7.5%) gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. Immunode-tection was performed using an anti-FLAG monoclonal antibody ( Sigma). Membranes were exposed to the anti-FLAG antibody overnight at 4 °C, washed, incubated for 60 min at room temperature with alkaline phosphatase-conjugated secondary (anti-mouse) antibody (Bio-RAD) diluted 1:2000 in blocking buffer, and then washed again. Bands were detected by using the Immune-Star chemiluminescent protein detection system (Bio-Rad).

Data Analysis—All results presented are based on a minimum of two different experiments with at least 12 oocytes/group in each experiment. The significance of the differences between groups was evaluated by one-way analysis of variance, with multiple comparisons using Dunnett or Bonferroni correction as required. Results are presented as mean ± S.E.
Structure-functional Analysis of Renal NCC

RESULTS

Topology and Putative N-Glycosylation Site Comparison in the Na\textsuperscript{+}-Cl\textsuperscript{−} Cotransporter—Fig. 1A depicts the proposed topology of the Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporter in which a gray-black scale is used to highlight similarities and differences between rat and flounder NCC. The identity of the TMs -1, -3, -5, -6, -7, -8, and -10 is >80%, whereas TMs -2, -4, -9, and -12 are shown in black and the carboxy-terminal domain identity degree is between 60 and 80%. The more divergent segments are the amino-terminal domain and the extracellular connecting segments ECL3 and ECL4, highlighted in gray (Fig. 1). Because Hoover et al. (14) suggest that the glycosylation status of the ECL4 affects affinity for metolazone in rNCC, we studied ECL4 in greater detail. Fig. 1B shows an alignment of ECL4 among species from which NCC cDNA has been cloned and sequenced or deduced by genomic analysis. In all mammalian NCCs, two N-glycosylation consensus sequences are present (Asn-Xaa-(Ser/Thr), Xaa ≠ Pro (22)). We have previously shown in rNCC that both sites are glycosylated (14). In contrast, using the same algorithm, three N-glycosylation motifs can be found in flNCC ECL4. The first site, Asn-403, was conserved among all NCCs. The second site, Asn-414, was present only in teleost NCCs (flounder and zebrafish). Finally, a second site in mammalian NCC is absent in flNCC but present in all other NCCs, including zebrafish. * denotes second site of mammalian NCCs (NFT).

Mammalian and Flounder NCC Exhibit Different Affinity for Metolazone—We have previously shown that affinity for ions and thiazides is higher in rNCC than in flNCC (17, 20). However, these transport affinity analyses and thiazide inhibition curves were obtained from independent experiments in which rNCC and flNCC were expressed separately. Thus, for the present study, we first confirmed that differences in the affinity for ions and thiazide diuretics between mammalian and fish NCC were reproducible in simultaneous experiments in which X. laevis oocytes where microinjected with rNCC or flNCC cRNA, and 4 days later, the ion transport kinetics and metolazone dose-responses were determined using the same uptake solutions and metolazone dilutions. Therefore, the only difference between the rNCC and flNCC groups was the injected cRNA. The combined results of three different ion transport kinetic analyses and five curves for thiazide affinity are shown in Fig. 2. The $K_{m}$ value for Na\textsuperscript{+} transport in rNCC was 5.5 ± 1.0 mM, whereas in flNCC it was 30 ± 6.0 mM ($p < 0.05$). Similarly, the Cl\textsuperscript{−} transport kinetic analysis showed that apparent Cl\textsuperscript{−} $K_{m}$ for rNCC was 2.6 ± 0.6 mM, whereas for flNCC it was 15 ± 2.0 mM ($p < 0.05$). A similar situation was observed for thiazide affinity. As shown in Fig. 2C, the calculated IC\textsubscript{50} for metolazone inhibition in rNCC was 0.3 ± 0.005 μM, whereas in flNCC it was 4.0 ± 0.08 μM ($p < 0.01$). Thus, confirming our previous proposal, results in Fig. 2 show that rNCC exhibits a significantly higher affinity for ions as well as for metolazone than flNCC.

Effect of Elimination of N-Glycosylation Sites in flNCC—We have previously shown that elimination of the N-glycosylation sites in rat NCC is associated with a dramatic increase in affinity for metolazone (14) and a smaller, although significant, increase in Cl\textsuperscript{−} affinity. Because flNCC exhibits lower affinity for metolazone and contains three
N-linked glycosylation sites, we reasoned that the presence of three sites in flNCC could be responsible, at least in part, for the difference in metolazone affinity between mammalian and teleost NCC. Thus, to define the effect of N-glycosylation on the flounder Na\(^+\)-Cl\(^-\) cotransporter functional properties, single, double, and triple N-glycosylation mutants were constructed by site-directed mutagenesis. The mutations were performed upon the FLAG-flNCC cDNA to allow immunodetection of the cotransporter in proteins extracted from injected oocytes using monoclonal anti-FLAG antibodies (Sigma). Oocytes were injected with water or with similar amounts of wild-type FLAG-rNCC cRNA, FLAG-flNCC cRNA, or with cRNA from each of the single, double, and triple FLAG-flNCC N-glycosylation mutants. Proteins were extracted from oocytes and immunoprecipitated by means of anti-FLAG antibodies. As shown in Fig. 3A, RNCC and flNCC resulted in the expression of a thick band of \(\sim 110\) kDa with a broad smear above it. This band is not present in proteins extracted from water-injected oocytes. The smear was not changed or was slightly reduced by the elimination of one or two N-glycosylation sites. However, the triple mutation in which the asparagines 403, 414, and 432 were changed to glutamine resulted in a clear reduction of the smear. These observations suggest that in flNCC, as occurred with rNCC (14) and rat NKCC2 (15), elimination of all of the ECLA N-glycosylation sites is required to affect glycosylation of the protein. Fig. 3B shows the effect of the elimination of one, two, or the three N-glycosylation sites upon the activity of flNCC. X. laevis oocytes were injected with similar amounts of cRNA from each clone. Four days later, the tracer \(\text{\textsuperscript{22}}\text{Na}\) uptake was assessed in groups injected with water, wild-type flNCC cRNA, or with any of the single, double, or triple mutant cRNAs. Elimination of one or two N-glycosylation sites had no effect upon the flounder cotransporter activity. In contrast, although \(\text{\textsuperscript{22}}\text{Na}\) uptake in wild-type flNCC-injected oocytes was \(11,738 \pm 1,293\) pmol/oocyte/h, \(\text{\textsuperscript{22}}\text{Na}\) uptake in the triple mutant-injected oocytes was \(6576 \pm 919\) pmol/oocyte/h \((p < 0.01)\). Thus, elimination of the three N-glycosylation sites resulted in a significant reduction of flNCC activity. We have shown previously that elimination of ECLA glycosylation sites in RNCC or NKCC2 results in the reduction of the cotransporter activity by 90 and 80%, respectively, whereas the observed reduction in transport activity of the triple mutant flNCC was \(\sim 50\%\). Thus, although the effect is qualitatively similar to our previous results, with rNCC (14) or rNKCC2 (15), the effect is quantitatively different.

As discussed above, the elimination of N-glycosylation sites in rNCC resulted in a significant increase in metolazone affinity (14), whereas in rat, NKCC2 resulted in a slight decrease in bumetanide affinity (15).
Thus, it was of interest to define the metolazone dose-response behavior in the wild-type and mutant flNCC cotransporters. The metolazone dose-response for flNCC single mutants N403Q, N414Q, and N432Q are shown in Fig. 4A, for the flNCC double mutants N403Q/N414Q, N403Q/N432Q, and N414Q/N432Q, Fig. 4B, and for the triple mutant, Fig. 4C. For comparison purposes, in all graphs, the dose-response observed for the wild-type flNCC is included. In striking contrast to our previous observations in rNCC, elimination of one, two, or all three N-glycosylation sites resulted in no significant changes in the flNCC affinity for metolazone. Thus, the effect of elimination of ECL4 N-glycosylation sites upon diuretic affinity in flNCC resembles what was observed in mammalian NKCC2 (15) rather than in mammalian NCC (14). This observation indicated that the difference in glycosylation sites cannot explain the differences in affinity for ions or metolazone between rNCC and flNCC.

Because rat and flounder exhibit such strikingly different effects of glycosylation upon thiazide affinity, together with the fact that the long extracellular loop is one of the most divergent domains between these proteins (Fig. 1B), we decided to construct and analyze the functional properties of several mutant and chimeric proteins between rat and flounder NCC to explore the role of ECL4, the amino- and carboxyl-terminal domains, TM regions, and glycosylation sites upon NCC activity and metolazone affinity.

Nomenclature and Construction of Mutant and Chimeric Proteins—We used the silent restriction sites described under “Experimental Procedures” to cut the cotransporters into five different pieces, the amino-terminal domain, TM segment 1–7, the ECL4, TM segments 8–12, and the carboxyl-terminal domain. Thereafter, CDA fragments were ligated to obtain several chimeric proteins. All chimeras are denoted by five letters. The first corresponds to the amino-terminal, the second to the TM segment 1–7, the third to ECL4, the fourth to the TM segment 8–12, and the fifth to the entire carboxyl-terminal domain. In addition, color is used to denote the origin of each fragment. Thus, the letter R and blue color denote when the domain belongs to the RAT NCC, and the letter F and red color denote when domains belong to flounder NCC. The nomenclature of the studied constructs is as follows (see Fig. 5): (i) rNCC without glycosylation sites (RG−) and flNCC without glycosylation sites (FG−), (ii) six chimeras in which the amino-terminal, the carboxyl-terminal, or both domains were interchanged between rNCC and flNCC (FRRRR, RRFR, FRRF, FFFFR, and FFRRF), (iii) four chimeras in which ECL4 was swapped between rNCC and flNCC, with or without glycosylation sites (RRFRR, FRRF, FFRRF/G−, and FFRRF/G−), (iv) rNCC containing the three flounder glycosylation sites (rNCC-fl-G-like) and flNCC containing the two rat glycosylation sites (flNCC-r-G-like), (v) RRFRR with the rat glycosylation site (RRFRR-r-G-like), (vi) FFRF with the flounder glycosylation site (FFRF-fl-G-like), and (vii) four chimeras in which the TM segments 1–7 or 8–12 were swapped between rat and flounder NCC (RRFRR, FFRRF, FFRR, and FFFFR).

Effect of Glycosylation Sites upon Thiazide Affinity—The results of several experiments in which the activity and the metolazone affinity were assessed in X. laevis oocytes microinjected with cRNA, transcribed from the different clones described above, are depicted in Figs. 6 and 7. Most of the constructs described in Fig. 5 exhibited enough activity to allow us to perform consistent metolazone dose-response curves and ion transport kinetic analyses. The exceptions were RRFRR/G−, RRFRr-G-like, RRFRF, FRRF, and FFRRF, which were considered non-functional.

Fig. 6 shows the metolazone dose curves of each functional construct. For comparison purposes, in all cases, the average dose-response curves for rNCC (IC50 = 0.3 ± 0.005 μM) and flNCC IC50 = 12.5 ± 1.8 μM) are shown in blue and red, respectively. The observed curve for each construct is shown in black. The graphs in Fig. 6, A–D, depict the dose-response curve in four constructs based upon rNCC backbone, whereas Fig. 6, E–J, shows the curves in constructs based upon flNCC. When compared with rNCC, the IC50 for metolazone was shifted to the left in the clones RRFRR and RG− (IC50 = 0.01 ± 0.008 and 0.03 ± 0.003 μM, respectively) and was not changed in clones rNCC-fl-G-like and FRRR (IC50 = 0.28 ± 0.003 and 0.3 ± 0.005 μM, respectively). This observation in the RG− clone confirmed our previous report of increased affinity for metolazone when both N-glycosylation sites of rNCC were eliminated (14) and suggested that RRFRR protein is probably not properly glycosylated. When contrasted with flNCC, no change in metolazone IC50 was observed in RFFRR, FFRR, FFRF-fl-G-like, and FG− (IC50 = 12.5 ± 2.0, 12.6 ± 1.9, 20 ± 0.42, and 20 ± 0.4 μM, respectively), and a small shift to right was observed in FFRF-fl/G− and FFRF-fl-r-G-like (IC50 = 25 ± 4.9 and 50 ± 2.9 μM, respectively). Fig. 7 shows both the activity and mean IC50 observed in each construct. The groups are ordered from the construct that exhibited the highest to the one with the lowest affinity (from left to right). The level of uptake observed in wild-type rNCC and flNCC was 18873 ± 203/0.28 pmol/oocyte/h, 15825 ± 1171 pmol/oocyte/h, respectively. Most of the mutant and chimeric proteins were functional and exhibited activity that was at least 50% of that shown in the corresponding wild type. The constructs that induced the lowest degree of activity were RG− and RRFRR, with uptake values of 2177 ± 393 and 2710 ± 285 pmol/oocyte/h, respectively. This level of uptake was ~10-fold higher than the uptake observed in water-injected oocytes (204 ± 31 pmol/oocyte/h, p < 0.01). Because the level of activity in the studied clones varied from 2,177 ± 393 to
31,052 ± 1,021 pmol/oocyte/h, we wanted to know whether the metolazone IC$_{50}$ could be affected by the level of expression observed in each clone. In a single experiment, several groups of *X. laevis* oocytes were injected with increased concentrations of rNCC cRNA that are known to increase rNCC activity, either alone or together with cRNA in vitro-transcribed from the serine/threonine kinase WNK3 (23). Subsequently, a metolazone dose-response curve for each group was assessed. As expected and shown in Fig. 8A, the more rNCC cRNA injected, the greater the thiazide-sensitive tracer $^{22}$Na uptake. The activity in all groups was increased by the presence of WNK3. However, as shown in Fig. 8B, dose-response curves to metolazone were similar among all groups. In addition, we have previously shown, in rNCC-injected oocytes, metolazone IC$_{50}$ of $\sim 1$ M with $^{22}$Na$^+$ uptakes of $\sim 3,000$ pmol/oocyte/h (20), similar to that shown for RG$^-$ or RRFRR in the present study. Thus, the metolazone IC$_{50}$ was not affected by different levels of activity, suggesting that differences observed in metolazone affinity in chimeric and mutant constructs are not due to the different degree of activity.

**Role of Amino- or Carboxyl-terminal Domain and ECL4 upon Ion and Metolazone Affinity**—Fig. 9 shows the $K_m$ values for sodium and chloride of the chimeras FRRRR, RRFRR, RFFFR, and FFRFF. The $K_m$ values for Na$^+$ in the chimeras containing the central hydrophobic domain from rNCC were 8.1 ± 1.1 and 5.0 ± 0.6 M for FRRRR and RRFRR, respectively, whereas in the chimeras containing the central hydrophobic domain from flNCC, the $K_m$ values for Na$^+$ were 28 ± 6.0 and 87 ± 45 M for RFFFR and FFRFF, respectively (Fig. 9A). The apparent Cl$^-$ $K_m$ values in the same chimeras were 2.1 ± 1.1, 1.8 ± 0.5, 17 ± 2.8, and 11 ± 4.1 M, respectively (Fig. 9B). Thus, ion transport kinetics in these chimeras followed the central hydrophobic domain. Taken together, the results shown in Figs. 6–9 strongly suggest that ion transport kinetics and metolazone affinity-modifying residues are not located in the amino-terminal domain, the carboxyl-terminal domain, or in ECL4.

**Role of Transmembrane Segments 1–7 and 8–12 in Ion and Metolazone Affinity**—We then constructed four chimeric proteins in which the TM 1–7 or TM 8–12 regions were swapped between rNCC and flNCC to
create the chimeras RFRRR, RRRFR, FFFRF, and FRFFF (Fig. 6, lower panel). As shown in Fig. 10A, microinjection of X. laevis oocytes with 25 ng of cRNA from wild-type rNCC, fNCC, or each chimera revealed transport activity in the first three chimeras. FRFFF chimera was not functional. Fig. 10B shows the metolazone dose-response curves obtained simultaneously in groups of oocytes injected with each construct. The IC₅₀ values in chimeras RFRRR and FFFRF were 0.4 ± 0.002 and 0.6 ± 0.006 μM, respectively. These values are similar to that of rNCC (0.3 ± 0.005; p = NS) but different from fNCC (12.5 ± 1.8 μM; p < 0.05). In contrast, the RRRFR IC₅₀ was 4.0 ± 0.08 μM. This value was different from that shown in rNCC (p < 0.05) but similar to fNCC (p = NS). Thus, metolazone affinity in chimeras containing the rNCC TM 8–12 region was similar to rNCC, whereas the chimera with the same region from fNCC behaved as fNCC. These observations suggest that the metolazone affinity modifier residues are located within the TM 8–12 region.

The results of Na⁺ and Cl⁻ transport kinetic analysis of these chimeras are shown in Fig. 10, C and D, respectively. The Kₘ values for Na⁺ transport were (in mM) 22 ± 2.8 in RFRRR, 14.5 ± 0.7 in RRRFR, and 42.3 ± 12.6 in FFFRF. These values are significantly different from rNCC Kₘ value for Na⁺ transport (5.5 ± 1.0 mM observed in rNCC (p < 0.05) but similar to the Kₘ value of 30 ± 6.0 mM fNCC (p = NS). A different situation occurred for Cl⁻ transport kinetics. The apparent Kₘ value for Cl⁻ transport was 17 ± 4.6 mM in RFRRR and 12.8 ± 3.0 mM in FFFRF. These values are significantly different from rNCC Kₘ values for Cl⁻

FIGURE 6. Kinetic analyses of inhibition of cotransporter function by metolazone. Each graph shows the dose-response curves of the mutant or chimeric protein in black, as stated. Each point represents the mean ± S.E. of 20 oocytes from two different experiments. For comparison purposes, in all panels, the results of rNCC and fNCC are shown in blue and red lines, respectively. For rNCC and fNCC, each point represents the mean ± S.E. of 50 oocytes from five different experiments.
transport (2.6 ± 0.7 mM; p < 0.05) but similar to fNCC (15 ± 2.0 mM; p = NS). In contrast, the apparent Cl\(^{-}\) \(K_m\) value in RRRFR was 2.4 ± 0.7 mM. This value was similar to rNCC (2.6 ± 0.6 mM; p = NS) but different from fNCC (15 ± 2.0 mM; p > 0.05). Thus, Cl\(^{-}\) affinity in chimeras containing the \(\text{fNCC-TM 1–7}\) region was similar to rNCC, whereas the chimera with the same region from rNCC behaved as rNCC. These observations suggest that chloride affinity-modifying residues are located within the TM 1–7 region.

**DISCUSSION**

In the present study, chimeric proteins between rNCC and fNCC were used to define ions and diuretic affinity-modifying regions. A similar approach has been successfully used by Isenring et al. (24–26) (for a review, see Ref. 27) between the human and shark basolateral isoform of the \(\text{Na}^+\text{-K}^+\text{-2Cl}^-\) cotransporter NKCC1.

Hoover et al. (14) make the striking observation that elimination of N-glycosylation sites in the rat \(\text{Na}^+\text{-Cl}^-\) cotransporter is associated with increased affinity for thiazide-type diuretics. rNCC contains two N-glycosylation sites in ECL4. Elimination of one site only (Asn-404...
or -424) reduced the activity of the cotransporter by -50% and increased the affinity for metolazone in a one order of magnitude from $I_C_{50} \approx 1$ to $\sim 100$ nM. Elimination of both sites reduces the activity by 90–95% and increases the affinity for metolazone to an $I_C_{50}$ of $\sim 10$ nM. Confocal image analysis of an enhanced green fluorescent protein-tagged NCC has revealed that most of the reduction in rNCC activity, when both N-glycosylation sites were eliminated, is because of decreased surface expression of the cotransporter. In the present study, however, we observed that the consequences of N-glycosylation site elimination from fNCC were not similar to rNCC (14). Elimination of one or two N-glycosylation sites had no effect on fNCC activity, and elimination of the three sites reduced the activity of the cotransporter but only by -50% (Fig. 3). Thiazide affinity was not affected in single, double, or triple fNCC mutants (Fig. 4). Therefore, the consequences of the N-glycosylation site elimination are different between rNCC and fNCC. In rNCC, the activity was critically reduced, and the affinity for thiazides was increased, whereas in fNCC, the activity was mildly reduced and the affinity for thiazide was not affected. In addition, any change of ECL4 in fNCC had no critical functional effects (Fig. 6). Thus, increased affinity for thiazides in rNCC, when the N-glycosylation sites are eliminated, is a unique feature of rNCC. The mechanism is not clear and will need further studies to be clarified.

In the present study, we also showed (Figs. 6 and 7) that swapping the amino- or carboxyl-terminal domains also had no effect upon thiazide affinity. In this regard, we have previously shown that interchanging these domains between NKCC2 and NCC had no effect upon bumetanide or thiazide sensitivity (16). All of these data together suggest that affinity-modifying residues for thiazides are unlikely to be located within ECL4 or the intracellular amino- or carboxyl-terminal domain. Instead, these residues must be located within the TM segments. The same conclusion can be reached for ion transport affinity-defining domains following our observations, shown in Fig. 9, that interchanging ECL4 or the amino- or carboxyl-terminal domains between rNCC and fNCC had no significant effect upon the $K_m$ values for Na$^+$ or Cl$^-$ transport.

Following our observations in the present study, we propose the model shown in Fig. 11. There is no role for amino-terminal, carboxyl-terminal, and ECL4 in defining ions or thiazide affinity constants. The affinity-modifying regions for Cl$^-$ are probably located within TM segments 1–7, whereas for thiazides, within TM segments 8–12. Both regions contain affinity-modifying residues for Na$^+$. These conclusions are inconsistent with a previous proposal by Tran et al. (28) that thiazide and chloride ions may bind to the same site on the cotransporter. These investigators observed in membrane preparations from rat renal cortex that increased concentrations of chloride in the extracellular medium decreased the affinity binding for $[^{3}H]$metolazone. In a study in which the functional properties of rNCC expressed in X. laevis oocytes were analyzed with certain detail (20), we have also observed a relationship between ion concentrations and thiazide affinity, not only for chloride ions, but also for sodium ions. We observed that the lower the sodium or chloride concentration in the uptake medium, the higher the affinity for thiazides. Thus, it is possible that ion binding to the cotransporter may induce a conformational change on the protein that reduces the affinity for thiazides. This, however, does not imply that ions and diuretics share the same binding site.

In the basolateral isoform of the Na$^+$-K$^+$-2Cl$^-$ cotransporter NKCC1, Issing et al. (27) took advantage of the kinetic differences in apparent affinity for ions and bumetanide between the shark and human isoforms to reveal that the TM 1–7 segment was the only segment defining ion transport kinetics and bumetanide affinity. No role was observed for the TM 8–12 region. Specifically, they observed that TM 2 is implicated in Na$^+$, K$^+$, and bumetanide affinity (24, 25), TM 4 in K$^+$ and Cl$^-$ affinity (26), and TM 7 in Na$^+$, K$^+$, and Cl$^-$ affinity (26). The role of TM 2 in defining kinetic properties has also been studied in NKCC2. Three alternative spliced variants known as A, B, and F, which differ in the sequence of TM 2 and the interconnecting segment...
between TMs 2 and 3 exhibit different kinetic properties for Na\(^+\), K\(^+\), and Cl\(^-\), as well as affinity for bumetanide (29–31). Therefore, no role for Cl\(^-\) or bumetanide affinity was observed in TM 2 from NKCC1, whereas a clear role was observed in NKCC2. Here we have shown, for the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter, that Cl\(^-\) affinity-modifying residues reside only in the TM 1–7 region, whereas Na\(^+\) affinity-modifying residues reside in both the TM 1–7 and 8–12 regions. Supporting this conclusion, we have previously shown that a single nucleotide polymorphism changing the glycine residue 264 within the long glycosylated loop (ECL4) are not involved in defining affinity for ions and thiazides. Affinity-modifying residues for chloride are located within TM segments 1–7 and for thiazides within TM segments 8–12, whereas both segments seem to be implicated in defining sodium affinity.

In summary, the present study shows that, in rat and flounder NCC, the amino-terminal domain, the carboxyl-terminal domain, and the long glycosylated loop (ECL4) are not involved in defining affinity for ions and thiazides. Affinity-modifying residues for chloride are located within TM segments 1–7 and for thiazides within TM segments 8–12, whereas both segments seem to be implicated in defining sodium affinity.

Acknowledgments—We are grateful to members of the Molecular Physiology Unit for their suggestions and assistance.

REFERENCES

1. Ellison, D. H., Velazquez, H., and Wright, F. S. (1987) Am. J. Physiol. (Renal Fluid Electrolyte Physiol.) 253, F546–F554
2. Plotkin, M. D., Kaplan, M. R., Verlander, J. M., Lee, W.-S., Brown, D., Poch, E., Guillans, S. R., and Hebert, S. C. (1996) Kidney Int. 50, 174–183
3. Chobanian, A. V., Bakris, G. L., Black, H. R., Cushman, W. C., Green, L. A., Izzo, J. L., Jr., Jones, D. W., Materson, B. J., Oparil, S., Wright, J. T., Jr., and Roccella, E. J. (2003) JAMA 289, 2560–2571
4. Simon, D. B., Nelson-Williams, C., Johnson-Bia, M., Ellison, D., Karet, F. E., Morey-Molina, A., Vaara, I., Ivata, F., Cusmer, H. M., Koolen, M., Gainza, F. J., Gitelman, H. J., and Lifton, R. P. (1999) Nature Genetics 12, 24–30
5. Mastroianni, N., DeFusco, M., Zollo, M., Arrigo, G., Zaffardi, O., Bettinelli, A., Ballabio, A., and Carsi, G. (1996) Genomics 35, 486–493
6. Mastroianni, N., Bettinelli, A., Bianchetti, M., Colussi, G., de Fusco, M., Sereni, F., Ballabio, A., and Carsi, G. (1996) Am. J. Hum. Genet. 59, 1019–1026
7. Wilson, F. H., Kahle, K. T., Sabath, E., Lalioti, M. D., Rapan, A. K., Hoover, R. S., Hebert, S. C., Gamba, G., and Lifton, R. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 680–684
8. Yang, C. L., Angell, J., Mitchell, R., and Ellison, D. H. (2003) J. Clin. Invest 111, 1039–1045
9. Mayan, H., Veder, I., Mouzlem, M., Tzadok-Witkon, M., Pauzner, R., and Farfel, Z. (2002) J. Clin. Endocrinol. Metab 87, 3248–3254
10. Gamba, G. (2000) Curr. Opin. Nephrol. Hypertens. 9, 535–540
11. Gamba, G. (2005) Physiol. Rev. 85, 423–493
12. Gamba, G., Miyashita, A., Lombardi, M., Lytton, J., Lee, W. S., Hediger, M. A., and Hebert, S. C. (1994) J. Biol. Chem. 269, 17713–17722
13. Gerike et al. (2000) J. Biol. Chem. 275, 40471–40477
14. Hoover, R. S., Poch, E., Monroy, A., Vazquez, N., Nishio, T., Gamba, G., and Hebert, S. C. (2003) J. Am. Soc. Nephrol. 14, 271–282
15. Pavet, D., Plata, C., Rivera, M., Moreno, E., Vazquez, N., Munoz-Clares, R., Hebert, S. C., and Gamba, G. (2006) Am. J. Physiol. Renal Physiol. 290, F1094-F1102
16. Tovar-Palacio, C., Bobadilla, N. A., Cortes, P., Plata, C., De Los, H. P., Vazquez, N., and Gamba, G. (2004) Am. J. Physiol. Renal Physiol 287, F570-F577
17. Vazquez, N., Monroy, A., Dorantes, E., Munoz-Clares, R., Gamba, G. (2002) Am. J. Physiol. Renal Physiol. 282, F599-F607
18. 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
19. Dumont, J. N. (1970) J. Morph. 136, 153–180
20. Monroy, A., Plata, C., Hebert, S. C., and Gamba, G. (2000) Am. J. Physiol. Renal Physiol 279, F161-F169
21. Meade, P., Hoover, R. S., Plata, C., Vazquez, N., Bobadilla, N. A., Gamba, G., and Hebert, S. C. (2003) Am. J. Physiol. Renal Physiol. 284, F1145-F1154
22. Landlott-Marticorena, C., and Reithmeier, R. A. F. (1994) Biochem. J. 302, 253–260
23. Rinehart, J., Kahle, K. T., De Los, H. P., Vazquez, N., Meade, P., Wilson, F. H., Hebert, S. C., Gimenez, I., Gamba, G., and Lifton, R. P. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 16777–16782
24. Isenring, P., and Forbush, III B. (1997) J. Biol. Chem. 272, 24556–24562
25. Isenring, P., and Forbush, III B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7179–7184
26. Isenring, P., Jacoby, S. C., and Forbush, III B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7179–7184
27. Isenring, P., Jacoby, S. C., Chang, J., and Forbush, III B. (1998) J. Gen. Physiol. 112, 549–558
28. Tran, J. M., Farrell, M. A., and Fanestil, D. D. (1998) Am. J. Physiol. (Renal Fluid Electrolyte Physiol.) 258, F908–F915
29. Plata, C., Moutet, D., Rubio, V., Hebert, S. C., and Gamba, G. (1999) Am. J. Physiol. (Renal Physiol.) 276, F359–F366
30. Plata, C., Meade, P., Vazquez, N., Hebert, S. C., and Gamba, G. (2002) J. Biol. Chem. 277, 11004–11012
31. Gimenez, I., Isenring, P., and Forbush, III B. (2000) J. Biol. Chem. 275, 8767–8770
32. Moreno, E., Tovar-Palacio, C., De Los, H. P., Guzman, B., Bobadilla, N. A., Vazquez, N., Ricciardi, D., Poch, E., and Gamba, G. (2004) J. Biol. Chem. 279, 16553–16560