Activity of the renal Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter is reduced by mutagenesis of N-glycosylation sites: role for protein surface charge in Cl\(^-\) transport

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Paredes, Anahí, Consuelo Plata, Manuel Rivera, Erika Moreno, Norma Vázquez, Rosario Muñoz-Clare, Steven C. Hebert, and Gerardo Gamba. Activity of the renal Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter is reduced by mutagenesis of N-glycosylation sites: role for protein surface charge in Cl\(^-\) transport. Am J Physiol Renal Physiol 290: F1094–F1102, 2006. First published November 15, 2005; doi:10.1152/ajprenal.00071.2005.—The renal-specific Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter NKCC2 belongs to the SLC12 gene family; it is the target for loop diuretics and the cause of type II Bartter’s syndrome. Because the NKCC2 sequence contains two putative N-linked glycosylation sites, one of which is conserved with the renal Na\(^+\)-Cl\(^-\) cotransporter in which glycosylation affects thiazide affinity, we assessed the role of glycosylation on NKCC2 functional properties. One (N442Q or N452Q) or both (N442,Q452Q) N-glycosylation sites were eliminated by site-directed mutagenesis. Wild-type NKCC2 and mutant clones were expressed in Xenopus laevis oocytes and analyzed by \(^{86}\)Rb\(^+\) influx, Western blotting, and confocal microscopy. Inhibition of glycosylation with tunicamycin in wild-type NKCC2-injected oocytes resulted in an 80% reduction of NKCC2 activity. Immunoblot of injected oocytes revealed that glycosylation of NKCC2 was completely prevented in N442,452Q-injected oocytes. Functional activity was reduced by 50% in N442Q- and N452Q-injected oocytes and by 80% in oocytes injected with N442,452Q, whereas confocal microscopy of oocytes injected with wild-type or mutant enhanced green fluorescent protein-tagged NKCC2 clones revealed that surface fluorescence intensity was reduced ~20% in single mutants and 50% in the double mutant. Ion transport kinetic analyses revealed no changes in cation affinity and a small increase in Cl\(^-\) affinity by N442Q and N442,452Q. However, a slight decrease in bumetanide affinity was observed. Our data demonstrate that NKCC2 is glycosylated and suggest that prevention of glycosylation reduces its functional expression by affecting insertion into the plasma membrane and the intrinsic activity of the cotransporter.

The apical bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter is the most important salt transport pathway in the mammalian thick ascending limb of Henle’s loop (TALH). This cotransporter is critical for salt reabsorption, countercurrent multiplication, acid-base regulation, and divalent mineral cation metabolism (10). The Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter protein in the TALH is the main pharmacological target of loop diuretics (19), which are extensively used in the treatment of edematous states. In addition, inactivating mutations of the Na\(^+\)-K\(^+\)-2Cl\(^-\)cotransporter gene in humans (36) and targeted disruption in mice (39) produce severe renal salt wasting or Bartter’s syndrome, an autosomal recessive disease that is characterized by metabolic alkalosis, hypokalemia, hypercalciuria, and severe volume depletion accompanied by reduction in arterial blood pressure.

The primary structure of the kidney-specific, bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, known as BSC1 or NKCC2, was elucidated by cloning corresponding cDNAs from rat (13), rabbit (32), mouse (21), and human kidney (36). NKCC2 belongs to the superfamily of electroneutral cation-coupled Cl\(^-\) cotransporters (SLC12A), for which 9 genes have been identified, two of which encode for Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporters: NKCC2 is the kidney-specific cotransporter that is expressed only at the apical membrane of the TALH, and NKCC1 is expressed in all tissues, either at the basolateral membrane of epithelial cells or in nonepithelial cells (11, 12). The degree of identity between both Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter genes is ~60 and 50% between these genes and the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter (NCC), the other Na\(^+\)-coupled to Cl\(^-\) transporter of the SLC12 family. The basic topology of the Na\(^+\)-K\(^+\)-2Cl\(^-\) and Na\(^+\)-Cl\(^-\) cotransporters has been deduced from hydrophathy analysis and features a central hydrophobic domain containing 12 transmembrane (TM)-spanning regions that are flanked by short NH\(_2\)- and long COOH-terminal domains, presumably located within the cell. A long loop between TM segments 7 and 8 faces the extracellular side of the protein. This topology has been experimentally confirmed only in NKCC1 (15).

NKCC1 and NKCC2 form dimers, although each monomer is thought to be fully functional (28, 38). The predicted core molecular size of the NKCC2 monomer is ~120 kDa, whereas Western blot analysis of proteins extracted from rat kidney has shown an apparent molecular size of ~160 kDa (1, 6, 23, 24). In vitro translation experiments of rat NKCC2 protein revealed an increase in apparent molecular size in the presence of canine pancreatic microsomes, which are known to express functional oligosaccharyl transferase activity, and this effect was prevented by addition of the deglycosylating enzyme endoglycosidase H (13). Thus in vivo and in vitro observations suggest that the bumetanide-sensitive cotransporter BSC1/NKCC2 is a glycosylated protein. In addition, we recently showed that the rat NCC protein is glycosylated at two sites and that elimina-
tion of the N-glycosylation sites produced 1) a decrease in activity of the cotransporter secondary to a decrease in surface expression, 2) a slight increase in affinity for extracellular Cl⁻, and 3) a large increase in affinity for the thiazide-like diuretic metolazone (20). One of the sites at which NCC is glycosylated is conserved in NKCC2. Thus the present study was carried out to elucidate the specific glycosylation sites in the renal-specific Na⁺-K⁺-2Cl⁻ cotransporter NKCC2 and to determine the effects of glycosylation on transporter function.

METHODS

Extraction of crude membranes from renal kidney and Xenopus laevis oocytes. Total proteins were extracted from pooled rat renal medullas by homogenization using a Polytron homogenizer (Kinematica) in four volumes of lysis buffer (200 mM sucrose, 0.5 mM EDTA, 5 mM Tris-HCl, pH 7.0, and complete inhibitor protease cocktail). The homogenates were centrifuged at 4,000 g for 4 min at 4°C to remove tissue debris without precipitating plasma membrane fragments. For N-glycosidase F digestion, 100 μg (20 μl) of protein from kidney membranes were denatured in 0.5% SDS, 50 mM β-mercaptoethanol, 0.55 M Tris-HCl (pH 8.6), and 1 mM EDTA for 1–2 h at 4°C and diluted with Nonidet P-40 to a final concentration of 1.5% in the presence of 1 μg/ml leupeptin and 0.2 mM PMSF. After the samples were split into two aliquots, 2 μl of N-glycanase or water (control) were added and the samples were incubated for 12–15 h at 37°C. For analysis of proteins from oocytes (see below), groups of 15 oocytes injected with water or cRNA from wild-type or mutant clones were homogenized in homogenization buffer at 2 μl/oocyte and centrifuged at 100 g for 10 min at 4°C, the supernatant was collected and centrifuged again at 14,000 rpm for 20 min, and the pellet was resuspended in loading buffer (6% SDS, 15% glycerol, 150 mM Tris, 3% bromphenol blue, and 5% β-mercaptoethanol, pH 7.6). Protein concentrations were assessed in duplicate using the DC protein assay (Bio-Rad, Hercules CA).

Immunoblotting. For Western blot analysis, 50 μg of medulla protein or protein extracts from 1–10 oocytes, with or without N-glycosidase F or endoglycosidase digestion, were diluted in 10 μl of loading buffer and subsequently denatured by boiling for 5 min. Proteins were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech; 2 h at 400 mA). Prestained Rainbow markers (Amersham) were used as molecular size standards. Nonspecific binding sites were blocked for 60 min at 37°C in 500 mM NaCl-20 mM Tris-buffered saline containing 0.4% nonfat dry milk. Thereafter, membranes were incubated with 1:1,000 dilutions of the specific rabbit polyclonal antibody L320 diluted in blocking buffer (Tris-buffered saline + 0.1% Tween 20) overnight at 4°C. L320 antibody, which was raised against a peptide corresponding to residues 33–55 of the NH2-terminal domain of rat NKCC2, was generously provided by Mark Knepper (24) and Javier Alvarez-Leefmans. Membranes were subsequently washed three times in Tris-buffered saline + 0.1% Tween 20 for 10 min and incubated for 60 min at room temperature with alkaline phosphatase-conjugated secondary (anti-rabbit) antibody (Bio-Rad) diluted 1:2,000 in blocking buffer and washed again. Immunoreactive species were detected using Immuno-Star chemiluminescent protein detection systems (Bio-Rad).

Site-directed mutagenesis. Sequence analysis of the rat NKCC2 protein (Lasargue protein) revealed six asparagine residues that fit the consensus sequence for an N-glycosylation motif: Asn-Xaa-Ser/Thr,Xaa ≠ Pro (Fig. 1) (25). Only two (N442 and N452) were considered putative N-glycosylation sites, because they are located within an extracellular loop of the protein that has been shown to be glycosylated in NCC (20). The consensus sites N395 and N579 are located within TM domains, and the consensus sites N868 and N875 are located within the cytoplasmic COOH-terminal domain. One or both N-glycosylation sites located at the extracellular loop was eliminated by site-directed mutagenesis (Quickchange, Stratagene) according to the manufacturer’s recommendations. The custom-made (Sigma) oligonucleotides 5’-CAGTCGAGGAGATGCTAAAGCAGAAGAGACT-GTCTTCTCTG-3’ and 5’-GGATGAAATTGCAGAAATGTTCCGCAGCGTCCTGCG-3’ were used to mutate the asparagine at positions 442 (N442Q) and 452 (N452Q), respectively, to glutamine in wild-type and enhanced green fluorescent protein (EGFP)-tagged NKCC2 clones. The latter primer was also used to create double mutants (N442Q,452Q). Automatic DNA sequencing was used to corroborate all mutations.

Heterologous expression in X. laevis oocytes. Stage V–VI oocytes were harvested from adult female X. laevis (Nasco, Fort Atkinson, WI) by surgery under anesthesia with 0.17% tricaine. All protocols were approved by the Institutional Animal Care and Use Committee. Oocytes were incubated for 1 h in frog Ringer ND96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES-Tris, pH 7.4) in the presence of collagenase B (2 mg/ml), washed four times in ND96, manually defolliculated, and incubated overnight at 18°C in the same medium supplemented with 2.5 mM sodium pyruvate and gentamicin (5 mg/100 ml). Oocytes (5) were injected with 50 nl of water (control) or cRNA from wild-type or mutant clones at 0.5 μg/μl (25 ng cRNA per oocyte). cRNA was in vitro transcribed from corresponding clones using the T7 RNA polymerase mMESSAGE kit (Ambion). After injection, oocytes were incubated for 3–4 days in ND96 with sodium pyruvate and gentamicin. The incubation medium was changed every 24 h. On the night before the uptake experiments were performed, oocytes were incubated in Cl⁻-free ND96 (in mM: 96 sodium isethionate, 2 potassium gluconate, 1.8 calcium gluconate, 1.0 magnesium gluconate, 5 mM HEPES, 2.5 sodium pyruvate, and 5 mg/100 ml gentamicin, pH 7.4) (14).

Assessment of Na⁺-K⁺-2Cl⁻ cotransporter function. The function of the Na⁺-K⁺-2Cl⁻ cotransporter was assessed by measurement of tracer ⁸⁸Rb⁺ uptake (New England Nuclear) in groups of ≥15 oocytes following our general protocol (27, 33, 34): 30 min of incubation in isotonic K⁺⁻ and Cl⁻⁻-free medium (mM: 96 sodium gluconate, 6.0 calcium gluconate, 1.8 magnesium gluconate, 5.0 HEPES-Tris, pH 7.4) with 1 mM ouabain followed by a 60-min uptake period in the presence of Na⁺, K⁺, and Cl⁻. For most experiments, the isotonic medium contained (mM) 96 NaCl, 10 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES, pH 7.4, and was supplemented with 1 mM ouabain and 2.0...
The transport kinetics for a single ion (Na\(^{+}\)) of groups of wild-type NKCC2 or mutant cRNA-injected oocytes to the sensitivity and kinetics for bumetanide were assessed by exposure of NKCC2 antibody (1:1,000 dilution).

Digestion. Proteins were separated by SDS-PAGE (6% polyacrylamide gel), transferred to polyvinylidene membranes, and incubated with polyclonal anti-NKCC2 antibody (1:1,000 dilution). Lane 1, liver untreated proteins. Lane 2, renal medulla untreated proteins. Lane 3, renal medulla proteins treated with N-glycanase for 15 h at 37°C. Molecular size markers (in kDa) are shown by arrows at left; calculated mass of NKCC2 proteins with or without N-glycanase treatment are shown by arrows at right.

muCi of \(^{86}\)Rb\(^{+}\). Because *X. laevis* oocytes express an endogenous NKCC1-type Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (13), every experiment included control water-injected oocytes.

To analyze the ion transport kinetics of the wild-type NKCC2 or glycosylation mutants, the concentrations of each ion were varied: for Na\(^{+}\) kinetics, the extracellular K\(^{+}\) and Cl\(^{-}\) concentrations were fixed at 90 and 10 mM, respectively. Ionic strength and osmolarity were maintained by substitution of N-methyl-

\[\text{glycosidase F (0.25 U)}\]

Fig. 2. Western blot analysis of proteins from liver and rat renal medulla and effect of enzymatic deglycosylation. Membrane proteins extracted from liver and rat renal medulla were isolated and exposed to N-glycosidase F (0.25 U) digestion. Proteins were separated by SDS-PAGE (6% polyacrylamide gel), transferred to polyvinylidene membranes, and incubated with polyclonal anti-

**RESULTS**

Western blot analysis of proteins extracted from renal medulla. Immunodetecion of rat NKCC2 protein from renal medulla membrane proteins revealed a broad band at ~160 kDa, similar to our previous observations and those of others with anti-NKCC2 antibodies raised against different epitopes in the NH\(_2\)- or COOH-terminal domain (Fig. 2) (1, 6, 23). After enzymatic deglycosylation with N-glycosidase F, the broad band was reduced to a lower band of ~120 kDa, which represents the expected core molecular size of rat NKCC2. Thus the rat renal-specific Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter is an N-glycosylated protein.

Effect of tunicamycin on glycosylation and functional expression of NKCC2. To begin to define the role of glycosylation on Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter function, NKCC2 cRNA-microinjected X. laevis oocytes were coinjected with vehicle or the N-glycosylation inhibitor tunicamycin (50 \(\mu\)g/ml) (3, 7). After 4 days, the glycosylation status and functional expression were assessed by immunoblotting and \(^{86}\)Rb\(^{+}\) uptake, respectively, and the results are depicted in Fig. 3. As we showed previously (13, 27, 34), microinjection of X. laevis oocytes with 25 ng of NKCC2 cRNA resulted in a significant increase in \(^{86}\)Rb\(^{+}\) uptake over that of water-injected oocytes (14,336 ±

\[\text{Statistical analysis. The significance of the differences between groups was tested by one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks, with Dunn’s method for multiple comparison procedures as needed. Values are means ± SE.}

**Fig. 3. Effect of tunicamycin on rat NKCC2 expression and activity in oocytes. Xenopus laevis oocytes were injected with water or NKCC2 cRNA alone or co.injected with tunicamycin (dissolved in DMSO). At 4 days after incubation, functional expression was determined by assessment of \(^{86}\)Rb\(^{+}\) uptake in the absence (open bars) or presence (solid bars) of \(10^{-4}\) M bumetanide in groups of 15 oocytes each. \#P < 0.01 vs. absence of tunicamycin. Inset: results from 10 oocytes used for protein extraction and immunoblot with anti-NKCC2 antibodies. Lane 1, protein from NKCC2-injected control oocytes; ~120 kDa band, which represents molecular core size of NKCC2 protein, and ~160 kDa band, which represents glycosylated fraction of NKCC2 protein. Lane 2, protein from tunicamycin-co-injected oocytes in which only 120-kDa band was expressed. Lane 3, protein from water-injected control oocytes.**
Characterization of wild-type and mutant NKCC2 glycosylation. To confirm that NKCC2 expressed in oocytes is also glycosylated, proteins were extracted from oocytes injected with wild-type NKCC2 cRNA and treated with N-glycosidase F. Similar to observations with renal medulla proteins (Fig. 2), the upper broad band observed in the absence of N-glycanase was reduced to a lower band of ~120 kDa, which correspond with NKCC2 core molecular size (Fig. 4A). Thus NKCC2 expressed in oocytes is also glycosylated. As shown in Fig. 3, treatment of NKCC2-injected oocytes with tunicamycin not only prevented the N-glycosylation of the cotransporter but also decreased the core molecular size by ~1–3 kDa. This slight decrease was also observed after N-glycanase treatment of proteins extracted from NKCC2-injected oocytes (Fig. 4A). The reduction in the molecular size with tunicamycin treatment suggested that the lower band may represent a high-mannose glycoprotein. It is known that N-glycosidase F removes core and N-oligosaccharide side chains of high-mannose and complex glycoproteins, whereas endoglycosidase H hydrolyzes only the high-mannose glycoproteins. Therefore to confirm that the lower band is indeed a high-mannose glycoprotein, we exposed NKCC2-injected oocyte proteins to endoglycosidase H. Endoglycosidase treatment resulted in a similar small decrease in the size of the lower band without a change in the upper band (Fig. 4B), confirming that the upper band is a complex glycoprotein and the lower band is a high-mannose glycoprotein.

Because N-glycosylation on one site can account for 4–20 kDa (25), the 40-kDa reduction in NKCC2 migration with deglycosylation (Figs. 2 and 3) requires participation of at least two different glycosylation sites. Of the six asparagines in NKCC2 that are contained within a glycosylation motif (Fig. 1), only N442 and N452 fit the requirements known to be associated with true glycosylation; i.e., the motif is located in a loop longer than 30 amino acid residues and is exposed to the extracellular side of the protein. To determine the effects of these two predicted glycosylation sites, N442 and N452 were mutated to glutamine, and the effects of these mutations on NKCC2 protein abundance and glycosylation in X. laevis oocytes were assessed by Western blotting. Oocytes were injected with similar amounts of wild-type NKCC2 cRNA or with cRNA from N442Q or N452Q mutants or the double mutant N442,452Q cRNA. Mutation of N442 or N452 resulted in expression of only the lower 120-kDa band, with a faint broad band midway between the two bands shown for wild-type NKCC2 (Fig. 4C). Elimination of N442 and N452 resulted in complete loss of the upper band, with only the lower 120-kDa band remaining. No difference was observed in the amount of NKCC2 protein among the four groups. Thus elimination of both sites located in the extracellular loop between TM segments 7 and 8 (Fig. 1) completely prevented glycosylation of the NKCC2 protein, apparently without affecting the synthesis or degradation rate of the protein, suggesting that the other N-glycosylation sites observed in the computational analysis of NKCC2 (N395, N579, N868, and N875) are not functional glycosylation sites. Because in the present study we analyzed the cell surface expression of the

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**Fig. 4.** Western blot analysis of membranes from oocytes injected with water, wild-type (WT) NKCC2 cRNA, N442Q cRNA, N452Q cRNA, or N442Q/N452Q cRNA, with or without enhanced green fluorescent protein (EGFP) tag. Oocytes were injected with water or 25 ng of cRNA in vitro transcribed from regular or EGFP-tagged wild-type NKCC2 and mutant cRNA. At 4 days after injection, proteins were isolated and separated by SDS-PAGE (7% polyacrylamide gel), transferred to polyvinylidene membranes, and incubated with anti-NKCC2 immune serum (1:500 dilution), and immunoreactive bands were visualized. A: effect of N-glycosidase F (PNG-F) treatment of proteins extracted from oocytes injected with wild-type NKCC2. B: effect of endoglycosidase (Endo H) treatment of proteins extracted from oocytes injected with wild-type NKCC2. C: immunoblot of proteins extracted from oocytes injected with wild-type or mutant NKCC2 cRNA. D: immunoblot of proteins extracted from oocytes injected with EGFP-tagged wild-type or mutant NKCC2 cRNA.
EGFP-tagged wild-type and mutant NKCC2 proteins (see below), we also assessed whether the amount of EGFP-tagged proteins was also similar among the wild-type and mutant constructs. After injection of similar concentrations of EGFP-tagged wild-type and mutant NKCC2 cRNA, we detected similar amounts of proteins by Western blot of oocyte proteins using the anti-NKCC2 antibody (Fig. 4D) or the anti-EGFP monoclonal antibody (data not shown).

To define the effect of N-glycosylation on Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter functional properties, we assessed \textsuperscript{86}Rb\textsuperscript{+} uptake in \textit{X. laevis} oocytes microinjected with wild-type NKCC2 cRNA or with N442Q, N452Q, or N442,452Q cRNA. Wild-type NKCC2-injected oocytes exhibited the expected 13-fold increase in \textsuperscript{86}Rb\textsuperscript{+} uptake over water-injected oocytes: 17,314 \pm 814 pmol-oocyte\textsuperscript{-1}.h\textsuperscript{-1} (Fig. 5). Whereas bumetanide-sensitive \textsuperscript{86}Rb\textsuperscript{+} uptake was also observed in oocytes injected with cRNA from each mutant clone, transport activity was significantly lower than in wild-type NKCC2: uptake was 7,294 \pm 308, 8,140 \pm 331, and 3,777 \pm 150 pmol-oocyte\textsuperscript{-1}.h\textsuperscript{-1} (all \(P < 0.01\)) in N442Q-, N452Q-, and double mutant-injected oocytes, respectively. The reduction in \textsuperscript{86}Rb\textsuperscript{+} uptake in mutant clones was 58%, 53%, and 80%, respectively. Figure 5 depicts the results of a representative experiment; however, similar observations were obtained in \(\geq 10\) different experiments involving different batches of oocytes. In all experiments, activity of each single mutant and activity of the double mutant were \(-50\) and \(20\)%, respectively, of that shown for the wild-type NKCC2-injected oocytes. Thus elimination of the N-glycosylation sites in NKCC2 cDNA results in a reduction of the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter activity.

Role of glycosylation in kinetic properties of NKCC2. One mechanism for the reduction in NKCC2 functional activity in mutant clones is the effect of elimination of N-glycosylation sites on affinity for the cotransported ions. In a previous study, we observed that elimination of one of the N-glycosylation sites in the renal thiazide-sensitive Na\textsuperscript{+}-Cl\textsuperscript{-} cotransporter resulted in a slight, but significant, increase in affinity for Cl\textsuperscript{-} (20), suggesting a role for glycosylation in Cl\textsuperscript{-} transport kinetics. Thus we assessed the kinetic properties of Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{-} transport simultaneously in wild-type NKCC2 and mutant clones. The renal Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter gene encodes for three alternatively spliced variants (A, B, and F), exhibiting different affinities for the three ions (34). Because the F isoform has the lowest Cl\textsuperscript{-} affinity, we used this isoform to study the effects of glycosylation on ion affinities. Results from two different experiments are shown in Table 1. The \(K_m\) values for Na\textsuperscript{+} and K\textsuperscript{+} uptakes in oocytes injected with mutant clones were not altered. Thus a reduced affinity for these ions cannot explain the reduced activity of the cotransporters in which the N-glycosylation sites were eliminated. Interestingly, however, the \(K_m\) for Cl\textsuperscript{-} significantly decreased when the N442 site was eliminated by itself or in the context of the double mutant (Table 1). In contrast, elimination of the second N-glycosylation site, N452, had no effect on Cl\textsuperscript{-} affinity. This finding is similar to our observation of the thiazide-sensitive Na\textsuperscript{+}-Cl\textsuperscript{-} cotransporter (20), in which elimination of the first N-glycosylation site also marginally increased the affinity for extracellular Cl\textsuperscript{-}. Thus changes in ion affinities cannot account for the reduction in transport activity of the N-glycosylation mutants.

We had also observed that elimination of the N-glycosylation sites in the thiazide-sensitive Na\textsuperscript{+}-Cl\textsuperscript{-} cotransporter increased affinity for the thiazide diuretic metolazone (20). Thus we also assessed the affinity of the wild-type NKCC2 and mutant clones for bumetanide. The experiment was performed in duplicate, and the dose response to bumetanide was assessed simultaneously for all control and experimental groups to obtain the kinetics of bumetanide inhibition with use of the

Table 1. Ion transport and bumetanide-inhibitory kinetic analysis in wild-type NKCC2 and glycosylation mutants

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<th>WT-NKCC2</th>
<th>N442Q</th>
<th>N452Q</th>
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Values are means \pm SE. WT, wild type; DM, double mutant. *\(P < 0.05\) vs. WT-NKCC2.

Fig. 5. Functional expression of wild-type NKCC2 and mutant cotransporters in \textit{X. laevis} oocytes. Oocytes were injected with 25 ng of wild-type NKCC2 cRNA, N442Q cRNA, N452Q cRNA, or N442,452Q cRNA and analyzed for \textsuperscript{86}Rb\textsuperscript{+} uptake after 4 days of incubation. Water-injected oocytes were used as controls. Uptake was assessed in the absence (open bars) or presence (solid bars) of 10\textsuperscript{-4} M bumetanide in uptake medium. Values are means \pm SE of 15 oocytes. *\(P < 0.01\) vs. WT-NKCC2 and \(H_2O\).
same batch of oocytes, solutions, and bumetanide dilutions. As shown in Fig. 6 and Table 1, the affinity for bumetanide was not increased but, rather, reduced by elimination of the \( N \)-glycosylation sites.

Surface expression of wild-type and mutant EGFP-NKCC2 constructs in \( X. \) laevis oocytes. Another potential mechanism accounting for a reduction in the NKCC2 activity of \( N \)-glycosylation site mutants is a reduction in the steady-state expression of protein in the plasma membrane. Thus, to quantitatively compare the plasma membrane expression of wild-type NKCC2 vs. N442Q, N452Q, and N442,452Q NKCC2 mutants, we assessed NKCC2 surface expression by confocal fluorescence microscopy using the EGFP-tagged wild-type and mutant constructs. We previously showed (27) surface colocalization of the EGFP-NKCC2 fusion construct with plasma membrane marker FM 4-64 fluorescence in all tested oocytes, indicating that the EGFP-NKCC2 fluorescence measured at equatorial confocal sections in oocytes is indicative of expression on the plasma membrane. Images from oocytes injected with wild-type EGFP-NKCC2 or each of the EGFP-NKCC2 \( N \)-glycosylation mutant clones are shown in Fig. 7A, and the results of densitometric analyses are shown in Fig. 7B. Control oocytes injected with water or NKCC2 cRNA showed no significant fluorescence at the emission and excitation wavelengths for EGFP (data not shown). Fluorescence intensity in the single mutants EGFP-N442Q and EGFP-N452Q was 86 and 81%, respectively, of that observed in wild-type EGFP-NKCC2 oocytes. The difference, however, was not statistically significant. In contrast, in the double-mutant-injected oocytes, fluorescence intensity was 48% of that shown for the wild-type EGFP-NKCC2 group (\( P < 0.05 \)).

DISCUSSION

In the present study, we show that the renal-specific \( \text{Na}^+ \)-\( \text{K}^+ \)-\( 2\text{Cl}^- \) cotransporter NKCC2 is a glycosylated protein in vivo and that N442 and N452, located in the putative extracellular domain between TM segments 7 and 8, are used as the...
N-glycosylation sites. This observation verifies that the loop between TM segments 7 and 8 in NKCC2 is indeed extracellular, as has been observed in NCC (20) and in the basolateral Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter NKCC1 (15). We also demonstrate that glycosylation at N442 and N452 in NKCC2 is essential for normal activity, such that elimination of one site reduces the activity of the cotransporter by \(\sim 50\%\), whereas elimination of both sites reduces activity by 80%. This lowered cotransporter activity in the mutant constructs was not due to nonspecific effects of the N \(\rightarrow\) Q mutations per se, because prevention of glycosylation with the inhibitor tunicamycin also decreased the activity of the wild-type NKCC2.

The possible mechanisms to explain a reduction in \(^{86}\)Rb\(^{+}\) uptake in glycosylation mutant clones include a change in the ion affinities, a decrease in the protein synthesis and/or protein expression in the plasma membrane, or a defect in the turnover rate of the cotransporter. For example, elimination of glycosylation sites reduced the activity of the GLUT-1 transporter by increasing the \(K_{m}\) for glucose (2). However, ion transport kinetic analyses of NKCC2 revealed no significant increases in \(K_{m}\) values for Na\(^{+}\), K\(^{+}\), or Cl\(^{-}\), suggesting that a decrease in ion affinity is not responsible for the reduction of activity in glycosylation mutant proteins. Western blot analysis revealed that, when constructs with and without EGFP were used, elimination of the N-glycosylation sites had no effect on the amount of detected proteins, suggesting that protein synthesis and degradation rates were not affected. In contrast, confocal imaging of X. laevis oocytes injected with cRNA transcribed from EGFP-tagged wild-type and mutant clones revealed a decrease in surface expression of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter only when both N-glycosylation sites were eliminated. No significant decrease in plasma membrane fluorescence signal was seen for the single N442Q and N452Q mutants, nor can the signal account for the 50\% reduction in \(^{86}\)Rb\(^{+}\) uptake observed for these mutant constructs. Moreover, the 50\% reduction in surface expression of the double mutant (N442,452Q) is less than the 80\% decrease in \(^{86}\)Rb\(^{+}\) uptake (Fig. 5). Thus elimination of glycosylation sites on NKCC2 appears to reduce the intrinsic activity or turnover rate of this cotransporter. This latter possibility was also suggested for the appearance of glycosylation sites on NKCC2 (N442,452Q) is less than the 80\% decrease in \(^{86}\)Rb\(^{+}\) uptake by 50\%, there was no effect on the affinities for cations or anions. Given these arguments, our results suggest that the Cl\(^{-}\) binding site is near or associated with the glycosylation site N442 or that glycosylation partially restricts the access of Cl\(^{-}\) to its binding site. Because complex oligosaccharides can contain sialic acid, glycosylation can contribute to the net negative external surface charge of proteins. For example, Cronin et al. (4) recently demonstrated that the shift of the conductance-voltage curves observed in deglycosylated voltage-gated Na\(^{+}\) channels appears to be secondary to a change in the electrostatic charge that occurs with loss of a large negative charge, rather than a conformational change in the protein. Thus a decrease in the net negative charge at the external face of NKCC2 due to the absence or reduction of N442 glycosylation could potentially enhance access of Cl\(^{-}\) to one of its binding sites.

In the present study, the affinity for the loop diuretic bumetanide was decreased in N442Q and the double mutant N442,452Q. This finding is opposed to our observations in NCC, in which a large increase in the affinity for thiazides was observed (20). In this regard, on the basis of the competition between Cl\(^{-}\) and tracer \(^{3}H\text{bumetanide or }^{3}H\text{metolazone in membrane preparations from renal medulla (17) or renal cortex (41), respectively, it has been proposed that in the electroneutral cotransporters the diuretic binding site could be the same as, or part of, the Cl\(^{-}\) binding site. Site-directed mutagenesis used to substitute single amino acid residues in NCC to eliminate one or two glycosylation sites (20), to introduce a single nucleotide polymorphism that switches glycine 264 to alanine (29), or to introduce a partially functional Gitelman-type mutation (35) have shown that an increase in affinity for Cl\(^{-}\) was accompanied in all cases by a simultaneous increase in affinity for metolazone. In contrast, chimera studies between the human colonic and shark rectal gland NKCC1 cotransporter revealed that chimeras exhibiting changes in affinity for Cl\(^{-}\) do not exhibit changes in affinity for bumetanide, and vice versa (22). In the present study, we observed that elimination of the N442 glycosylation site resulted in an NKCC2 cotransporter with increased affinity for Cl\(^{-}\), but with decreased affinity for bumetanide. In other words, elimination of the N442 glycosylation site has an opposite effect on Cl\(^{-}\) and bumetanide affinity. Thus functional analyses of native, chimeric, or mutant cotransporters in heterologous expression systems have provided evidence supporting the hypothesis of Cl\(^{-}\) and diuretic competition in NCC, but not NKCC1 or NKCC2, cotransporters.

In the present study, the functional characteristics of wild-type and glycosylation mutant NKCC2 were assessed using the heterologous expression system in X. laevis oocytes. This expression system has been shown to be an excellent tool for

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a robust and reproducible expression of NKCC2 in our hands (13, 27, 33, 34, 40) and in other laboratories (8, 9, 16, 37). Although pharmacology determined in oocytes may not be completely applicable to proteins in mammalian systems and NKCC2 functional analysis in mammalian cells will be preferred, NKCC2 expression in transfected mammalian cells has not been successful in any laboratories, including our own.

In summary, the present study demonstrates that the renal apical bumetanide-sensitive Na+/K+-2Cl− cotransporter NKCC2 is a glycosylated protein and that N442 and N452, located in the extracellular loop between TM segments 7 and 8, are the sites used for glycosylation. This finding provides compelling evidence that the loop between TM segments 7 and 8 in NKCC2 is indeed extracellular. Glycosylation appears to be important for normal surface expression and maximal turnover of the transporter. In addition, the change in surface charge due to glycosylation may modulate Cl− affinity but only slightly affect the affinity for bumetanide. Thus our results provide further evidence of a role for glycosylation in transport kinetics.

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