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Role of glycosylation in the renal electrogenic Na^+-HCO_3^- cotransporter (NBCe1)

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Choi, Inyeong, Lihui Hu, José D. Rojas, Bernhard M. Schmitt, and Walter F. Boron. Role of glycosylation in the renal electrogenic Na^+-HCO_3^- cotransporter (NBCe1). *Am J Physiol Renal Physiol* 284: F1199–F1206, 2003. First published February 25, 2003; 10.1152/ajprenal.00131.2002.—The electrogenic Na^+-HCO_3^- cotransporter NBCe1 is important for the regulation of intracellular pH (pH_i) and for epithelial HCO_3^- transport in many tissues, including kidney, pancreas, and brain. In the present study, we investigate glycosylation sites in NBCe1. Treatment of rat kidney membrane extracts with peptide N-glycosidase F (PNGase F) shifted the apparent molecular weight (MW) of NBCe1 from 130 to 116, the MW predicted from the deduced amino acid sequence. Treatment with endoglycosidase F_2 or H or O-glycosidase did not affect the MW of NBCe1. Lectin-binding studies, together with the enzyme data, suggest that the N-linked carbohydrates are of tri- or tetra-antennary type. To localize glycosylation sites, we individually mutated the seven consensus N-glycosylation sites by replacing asparagine (N) with glutamine (Q) and assessing mutant transporters in *Xenopus laevis* oocytes. Immunoblotting of oocyte membrane extracts treated with PNGase F indicates that NBCe1 is normally glycosylated at N597 and N617 (both on the third extracellular loop). However, N592 (on the same loop) is glycosylated when the other two sites are mutated. The triple mutant (N592Q/N597Q/N617Q) is completely unglycosylated but, based on microelectrode measurements of membrane potential and pH_i in oocytes, preserves the Na^+ and HCO_3^- dependence and electrogenicity of wild-type NBCe1.

transporter; pH measurement; acid-base mechanism

THE MOST IMPORTANT pH buffer system in the body is CO_2/HCO_3^-. Transporters that carry HCO_3^- not only have a tremendous impact on the regulation of intracellular pH (pH_i), but they also play an important role in the movement of $NaHCO_3$ and fluid across several epithelia. In particular, the electrogenic Na^+-HCO_3^- cotransporter plays key roles in both pH_i regulation and epithelial transport. In astrocytes, for example, electrogenic Na^+-HCO_3^- cotransporters are a major mechanism of HCO_3^- uptake (5, 11, 16). In the kidney, electrogenic Na^+-HCO_3^- cotransporters in the proximal tubule (7) are central in the reabsorption of ~85% of the HCO_3^- filtered in the glomeruli (4). In pancreatic ducts, electrogenic Na^+-HCO_3^- cotransporters are key components of the mechanism responsible for HCO_3^- secretion by the exocrine pancreas (21).

The key step in understanding the molecular physiology of electrogenic Na^+-HCO_3^- cotransporters was the expression cloning from the salamander kidney of the electrogenic Na^+-HCO_3^- cotransporter NBC (35). Together with the previously cloned $ClHCO_3^-$ exchangers or AEs (3, 12, 23), this first NBC defined a new bicarbonate transporter superfamily. Other closely related electrogenic NBCs were soon cloned from mammalian kidney (8, 34), pancreas (1), heart (10), and brain (6). Indeed, immunocytochemistry studies localize NBCe1 to the basolateral membrane of the proximal tubule (38), the basolateral membrane of pancreatic duct cells (27), and astrocytes and neurons in the brain (37). It is now clear that these NBC clones, which we will refer to as NBCe1, are alternative splice products of the same gene (2). NBCe1-A (also called kNBC1) is present mainly in the kidney, NBCe1-B (or pNBC1) is widely distributed but is present at particularly high levels in the pancreas, and NBCe1-C (or bNBC1) is present mainly in the brain.

The primary structure of rat NBCe1-A predicts a protein of 1,035 amino acids that is 30–40% identical to the AEs. Topological studies on AE1 suggest that the protein has cytoplasmic NH_2 and COOH termini and as many as 14 transmembrane (TM) segments (14, 32). AE1 is N-glycosylated once in the fourth extracellular loop, between TM7 and TM8, whereas AE2 and AE3 have consensus glycosylation sites in the third extracellular loop, between TM5 and TM6. The oligosaccharide on human AE1 takes two forms, a long-chain polylactosaminyl structure or a shorter complex structure (24). Mutation of the N-glycosylation site in AE1 reduces targeting the protein to the cell surface in *Xenopus* oocyte studies, but the nonglycosylated protein is still functional (19). One role of N-glycosylation in AE1 may be to permit the interaction of AE1 with calnexin, an endoplasmic reticulum chaperone; altering the N-glycosylation site by mutagenesis eliminates the interaction of AE1 with calnexin (31). AE2 is also glycosylated, but it lacks sialic acids (44). The glycosylation status of AE3 is unknown.

The deduced amino acid sequence predicts that rat NBCe1 has seven putative consensus sites for N-linked

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glycosylation. On the basis of an AE1 topology model (14), three of the sites (N33, N199, and N208) are on the cytoplasmic NH_2 terminus, one (N497) is within TM3, and the remaining three (N592, N597, and N617) are on the third extracellular loop. These last three sites are thus candidates for glycosylation. Western blots show that NBCe1 migrates at 130 kDa in the kidney (38), whereas the deduced amino acid sequence predicts a 116-kDa protein. Thus at least one of the consensus sites may be glycosylated. In the present study, we investigated the oligosaccharide composition of NBCe1-A using a glycosidase enzyme assay and a lectin-binding assay. To locate the N-linked glycosylation sites, we individually mutated asparagine (N) to glutamine (Q) at all seven putative glycosylation sites and also created double and triple mutants. We analyzed these mutants by expressing them in *Xenopus* oocytes and examining the molecular weight (MW) of each mutant transporter after treatment of peptide N-glycosidase F (PNGase F). We also performed microelectrode experiments to assess the function of mutant transporters. We found that NBCe1-A is normally glycosylated at two sites on the third extracellular loop and that the unglycosylated protein has normal function.

MATERIALS AND METHODS

Extraction of crude membranes. A whole kidney from adult Sprague-Dawley rats or rabbits was removed under anesthesia (intraperitoneal injection, 100 mg/kg pentobarbital sodium). A frozen kidney from a cow was obtained commercially, and a kidney from the *Ambistoma tigrium* salamander was isolated as described previously (38). Slices of the kidney from each species were placed in an ice-cold homogenization buffer (HB)-I (250 mM sucrose, 20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin) and homogenized using a Polytron. For extraction of crude plasma membranes of oocytes expressing NBCe1, oocytes were homogenized in hypotonic HB-II (7.5 mM sodium phosphate, pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin) as described previously (10). Homogenates were centrifuged at 750 *g* in a microcentrifuge (Eppendorf, model 5415C) for 5 min at 4°C to remove cell debris; the supernatant was centrifuged for 30 min at 16,000 *g* at 4°C. The pellet, which contained most plasma and organellar membranes, was used for analysis.

Deglycosylation. Membrane fractions (200 μg) isolated from rat tissues were heat denatured at 70°C for 10 min in 0.2 M β-mercaptoethanol, 0.5% SDS and protease-inhibitor cocktail. Some samples were then incubated overnight at 37°C in an incubation solution (40 mM Na_2HPO_4, pH = 7.4, 10 mM EDTA, 1% β-mercaptoethanol, and 0.6% Triton X-100) containing PNGase F (8,000 mU/ml) or endoglycosidase (Endo) F_2 (3.2 mU/ml). Other samples were treated overnight at 37°C in 200 mM sodium acetate buffer (pH = 5) containing either Endo H (40 mU/ml) or O-glycosidase (8 mU/ml). For membrane fractions extracted from oocytes, pellets were dissolved in 20 μl of Na_2HPO_4 (40 mM, pH 7.0), and SDS was added to a final concentration of 1%. After the membrane fractions were denatured at 70°C for 10 min, they were incubated at 37°C for 6 h in the presence of 5 U of N-glycosidase F in the incubation solution. The reaction was stopped by adding the SDS gel-loading buffer.

Lectin binding. We used the following lectins: concanavalin A (Con A), wheat germ agglutinin (WGA), glycine max agglutinin (soy bean agglutinin; SBA), jacalin, *Galanthus nivalis* (GNA), *Lens culinaris* (LCA), *Ulex europaeus* (UEA-I), and *Tetragonobulys purpurea* (LTA). Lectins coupled to agarose beads (Sigma, St. Louis, MO) were equilibrated at room temperature with the lectin-binding buffer, which contained (in mM) 150 NaCl, 20 Tris, pH 7.5, 1 $MgCl_2$, 1 $MnCl_2$, 1 $CaCl_2$, and 1 $ZnSO_4$, 0.5% Triton X-100, and protease-inhibitor cocktail. Agarose beads were then preabsorbed with 1% BSA in the lectin-binding buffer for 5 min at RT, followed by three 10-min washes in the lectin-binding buffer. Crude membrane proteins (50 μg) were then added to the lectin-binding buffer containing lectin-agarose beads to a final volume of 200 μl. For competition experiments, sugars (methyl-α-D-mannopyranoside for Con A and GNA; galactose for SBA and jacalin; *N*-acetyl-D-glucosamine for WGA; and α-mannose for LCA) were added to a final concentration of 0.28 M. For UEA-1 and LTA, α-L-fucose was used to a final concentration of 1 M. The reaction mixture, with or without competing sugar, was incubated overnight at 4°C in a rotating shaker. Afterward, the beads were washed three times with the lectin-binding buffer for 10 min.

Immunoblotting. Membrane extracts treated with glycosidases or material from lectin-binding assays were mixed with Laemmli loading buffer and heated for 10 min at 70°C. The proteins were then separated on a 7.5% SDS-polyacrylamide gel and transferred overnight to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Blots were preincubated for 1 h in a blocking buffer containing 5% nonfat dry milk and 0.1% Tween 20 in Tris-saline (TBS; 50 mM Tris, pH 7.4; 150 mM NaCl) and then incubated with an antibody (1:500 dilution), either NBC-5 (38) or K1A (6), which recognizes the COOH terminus of NBCe1-A. After several washes with TBS containing 0.1% Tween 20, blots were incubated with anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Chemicon) for 1 h (1:5,000 dilution). Blots were washed three times and developed by an HRP/hydrogen peroxide-catalyzed oxidation of luminol under alkaline conditions (Pierce, Rockford, IL).

Site-directed mutagenesis. The plasmid pTLN-NBCe1-A (35) served as a template for site-specific mutagenesis as described by Pusch et al. (33). Seven mutagenic primers were designed to replace the codons for Asn (N) at each of the putative glycosylation sites (N33, N199, N208, N497, N592, N597, and N617). We created a double (or triple) mutant with the same approach, but using a single (or double) mutant as the template. The final constructs were sequenced to verify the desired sequences.

Measurement of V_m and pH_i. Wild-type and mutant cDNA were transcribed in vitro using a mMessage mMachine kit (Ambion, Austin, TX) with SP6 RNA polymerase. Defolliculated *Xenopus* oocytes (Stage V-VI) were prepared as described previously and injected with 20 ng of cRNA or 50 nl water and incubated in OR3 media (50% Leibovitz L-15 media with L-glutamine, 5 mM HEPES, pH 7.5) supplemented with 5 U/ml penicillin/streptomycin (35). Injected oocytes were maintained for 3–7 days at 18°C before use. Voltage and pH microelectrodes were prepared as described previously (17). The pH electrode tip was filled with proton ionophore 1 cocktail B (Fluka Chemical, Ronkonkoma, NY) and back filled with a pH 7 phosphate buffer. Electrodes were connected to high-impedance electrometers (model FD-223; WPI, Sarasota, FL), which in turn were connected to the analog-to-digital converter of a computer. In electrophysiological experiments, the CO_2/HCO_3^--free ND-96 solution contained (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5

HEPES (pH 7.5); osmolality was adjusted to 196–200 mosmol/kgH_2O. In solutions equilibrated with 1.5% CO_2 (pH 7.5), we replaced 10 mM NaCl with 10 mM $NaHCO_3$.

Statistics. Data are reported as means ± SE. Levels of significance were assessed using the unpaired, two-tailed Student's *t*-test. A P value of <0.05 was considered significant. Rates of pH_i change (dpH_i/dt) were fitted by a line using a least-square method.

RESULTS

N-glycosylation of native NBCe1 in different species. To test whether the N-glycosylation of NBCe1 is a general phenomenon among various species, we first prepared membrane extracts prepared from the kidneys of rabbit, rat, bovine, and salamander and then treated these extracts with PNGase F. This enzyme digests the amide bond between the reducing end of *N*-acetylglucosamine and the β-amide group of asparagine. We examined the effect of PNGase F treatment on the MW of the NBCe1 protein by immunoblot analysis, using an antibody specific to the COOH terminus of the protein. Figure 1 shows that PNGase F treatment reduced the apparent MW of NBCe1 in all species examined. The immunoreactive bands in rabbit, rat, and bovine decreased from ~130 to ~116, which is the size expected from the deduced amino acid sequence. Not shown are data demonstrating that PNGase F treatment also reduces the apparent MW of NBCe1 from ~130 to ~116 in mouse, human, and guinea pig kidneys. These results suggest that N-glycosylation of NBCe1 is a general phenomenon in mammals. As noted previously, salamander NBCe1 has an apparent MW of ~160 (38), which is 30 higher than in mammalian species. Because PNGase F treatment reduced the apparent MW of salamander NBCe1 to ~130, rather than the predicted MW of 116, it is possible that salamander kidney NBCe1 has an additional O-glycosylation site(s).

Previous work on preparations not treated with PNGase F showed that the antibody recognizes other lower MW bands in some preparations (37, 38) but not

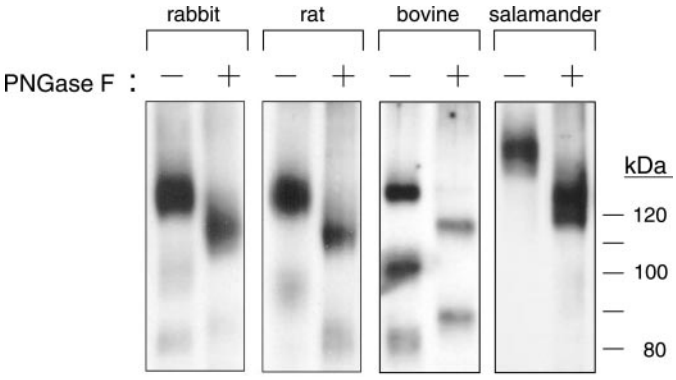

Fig. 1. Immunoblot showing N-linked glycosylation of renal electrogenic Na^+-HCO_3^- cotransporter (NBCe1) from rabbit, rat, bovine, and salamander. The crude membranes extracted from kidneys were incubated in the absence (−) or presence (+) of peptide N-glycosidase F (PNGase F). Membrane proteins were separated on a 7.5% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with an antibody to the COOH terminus of rat NBCe1.

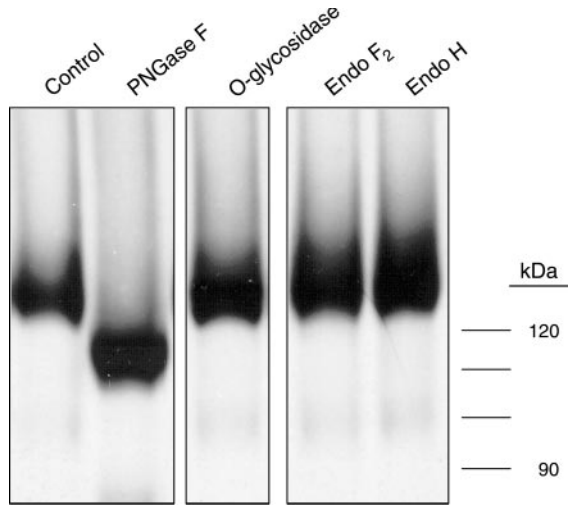

Fig. 2. Sensitivity of rat renal NBCe1 to various glycosidases. The enzymes used were PNGase F, O-glycosidase, endoglycosidase (Endo) F_2, and Endo H. The O-glycosidase preparation also contained sialidase, which by itself had no effect on apparent molecular weight.

in others (22, 42). It is interesting to note that PNGase F treatment caused MWs of the faint 101 band of rabbit kidney to fall to 86, the 97 band of rat kidney to fall to 82, and the darker 103 band of bovine kidney to fall to 89, decreases of ~15 in each case. These results support the hypothesis that the lower MW bands represent COOH-terminal proteolytic products.

Glycosidase treatment of native rat NBCe1. To investigate carbohydrate components of the protein, we focused on rat kidney NBCe1, which has been characterized in terms of both its functional properties (18, 34, 39) and its cellular localization (6, 28, 37, 38). In Fig. 2, *lane 1* (no enzyme treatment) and *lane 2* (PNGase F) confirm the rat data of Fig. 1, which indicate that kidney NBCe1 has an N-glycosylated oligosaccharide. To verify directly the absence of an O-glycosylated carbohydrate-peptide linkage, we next treated rat kidney membrane extracts with an O-glycosidase, which digests the bond between the reducing end of *N*-acetylgalactosamine (GalNAc) and the hydroxyl group of serine or threonine. We found that treatment with a combination of O-glycosidase and sialidase (separate experiments showed that sialidase had no effect by itself) did not change the MW of the protein, indicating absence of O-glycosylation (Fig. 2, compare *lanes 2* and *3*). To further characterize the carbohydrate component of NBCe1, we treated the protein with Endo F_2, which cleaves biantennary complex-type oligosaccharides, or Endo H, which cleaves oligosaccharides of the high mannose and hybrid types. Because neither treatment shifted the MW of NBCe1 (Fig. 2, *lanes 4* and *5*), we can conclude that NBCe1 has an N-linked oligosaccharide that is probably of the tri- or tetra-antennary type.

Lectin binding of native rat NBCe1. Because a lectin affinity profile can provide some insight into the nature of the linked oligosaccharide, we performed lectin affinity chromatography with a series of lectins immobi-

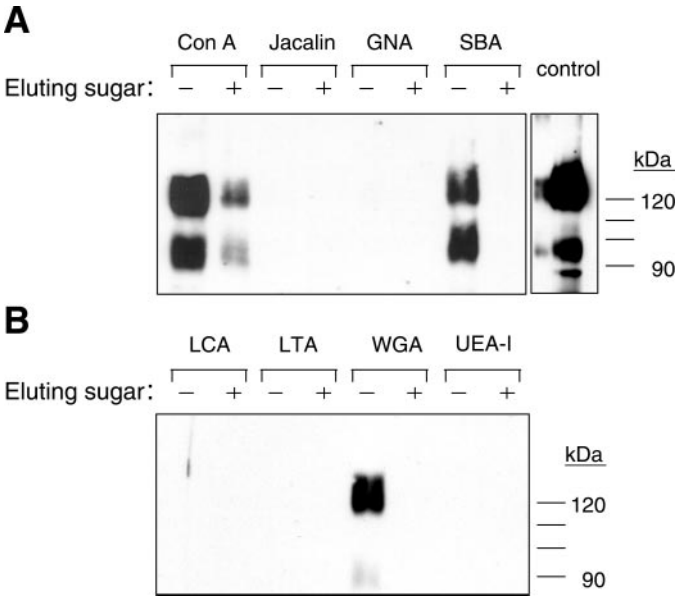

Fig. 3. Lectin binding of rat kidney NBCe1. Lectins coupled to agarose beads were incubated with crude membrane proteins from rat kidney. Afterward, either nothing was added (−), or a competing monosaccharide was added (+) to elute bound glycoprotein from the beads. Immunoblotting was performed with an antibody to the COOH terminus of NBCe1. Lectins used in the experiments are concanavalin A (Con A), jacalin, lectins from *Galanthus nivalis* (GNA), soy bean agglutinin (SBA), *Lens culinaris* (LCA), *Tetragonobulys purpurea* (LTA), wheat germ agglutinin (WGA), and *Ulex europaeus* (UEA-I).

lized to agarose beads. We incubated crude membrane extracts with lectin-agarose beads, allowed glycoprotein to bind to the lectin, added either nothing or a competing monosaccharide (see MATERIALS AND METHODS) to elute bound glycoprotein from the beads, isolated the beads, eluted bound glycoprotein (if present) from the beads, and then performed an immunoblot on the supernatant.

As shown in Fig. 3, the NBCe1 protein bound to Con A, glycine max agglutinin (i.e., SBA), and WGA. Con A has an affinity for D-glucopyranose or D-mannopyranose with unmodified hydroxyl groups at the C3, C4, and C6 positions. Elution with methyl-α-D-mannopyranoside markedly reduced the binding of NBC to Con A; the small residual signal probably reflects incomplete washing of the beads with the hapten sugar. In other experiments (not shown), we saw complete dissociation of the NBCe1 protein from the Con A-agarose beads. SBA preferentially binds to oligosaccharide structures with terminal GalNAc and, to a lesser extent, terminal galactose residues. Because NBCe1 did not bind to jacalin (Fig. 3), it is unlikely that SBA interacted with NBCe1 via a terminal nonreducing α-linked galactose. Therefore, SBA probably interacted with a terminal GalNAc residue on NBCe1. WGA has specificity for internal GlcNAc residues and, to a lesser extent, sialic acid residues.

In addition to jacalin, rat kidney NBCe1 does not bind to lectins from GNA, LCA, UEA-I, or LTA. The insensitivity to GNA indicates the absence of a terminal, nonreducing α-linked mannose, which suggests that the NBCe1 oligosaccharides are neither of the high mannose nor of the hybrid type. This interpretation is consistent with the insensitivity of NBCe1 to Endo F_2 or Endo H. The absence of binding to LCA, LTA, and UEA-I indicates an absence of core and outer fucose residues. It is interesting to note that all three of these fucose-dependent lectins did interact with NBCe1 isolated from rat brain (not shown).

Mutational analysis of consensus N-glycosylation sites. As noted before, the amino acid sequence of rat kidney NBCe1-A predicts seven consensus N-glycosylation sites (N-X-S/T): N33, N199, N208, N497, N592, N597, and N617 (Fig. 4). To test whether these consensus sites are responsible for N-glycosylation, we individually mutated each asparagine (N) to glutamine (Q). We then heterologously expressed each mutant transporter in *Xenopus* oocytes, which are known to functionally express wild-type rat kidney NBCe1-A (34). After extracting crude oocyte plasma membranes, and treating some of them with PNGase F, we analyzed the mutant proteins by immunoblotting. As shown in Fig. 5, wild-type NBCe1-A expressed in oocytes and then either not treated or treated with PNGase F, had the same apparent MWs (i.e., ~130 and ~116, respectively) as native NBCe1 in mammalian tissues (see Figs. 1 and 2). The same is true for each of the first five N-to-Q mutants. It is interesting to note that N592 (predicted to be on the third extracellular loop, 28 amino acids downstream from the end of TM5) does not appear to be glycosylated significantly in the wild-type NBCe1 protein.

For both N597Q (which expressed only modestly in this experiment) and N617Q, the MW in the absence of PNGase F treatment was only ~122, consistent with the hypothesis that N597 and N617 contribute about equally to glycosylation in the wild-type protein. Treatment with PNGase F reduced the MW to ~116 for each of these two mutants.

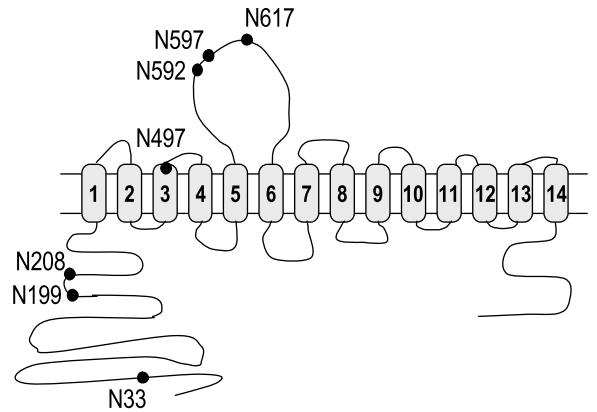

Fig. 4. Consensus sites for N-glycosylation in rat NBCe1. The membrane topology is based on the topology of AE1 (14) and NBCe2 (41). The first 3 sites (N33, N199, and N208) are in the predicted cytoplasmic NH_2 terminus, the fourth site (N497) is in the predicted transmembrane *segment 3*, and the last 3 sites (N592, N597, and N617) are in the third extracellular loop, between transmembrane *segment 5* and *6*.

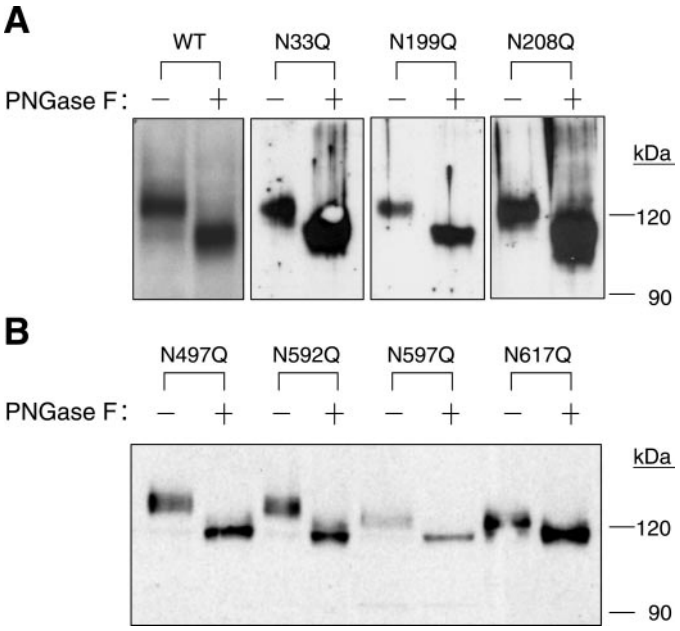

Fig. 5. PNGase F treatment of the membrane extracts from oocytes expressing the mutant transporters. Mutant transporters were individually generated by replacing asparagine (N) with glutamine (Q) at each glycosylation site and then expressed in oocytes. The crude membranes extracted from oocytes were incubated in the absence (−) or presence (+) of PNGase F. WT, wild type.

If N597 and N617 both normally contribute to the glycosylation of wild-type NBCe1-A, then one might predict that the double mutant N597Q/N617Q should have a MW of 116, and PNGase F should fail to produce a MW shift. However, as shown in Fig. 6, in the absence of PNGase F, N597Q/N617Q exhibited two bands, one at a MW of ~116 (presumably representing the predicted, nonglycosylated protein) and another at ~120 (presumably representing glycosylation at a residue other than N597 or N617). Treatment with PNGase F yielded only the band at ~116. The presence of the doublet in the absence of PNGase F implies that an alternative glycosylation site may be used when the two normal glycosylation sites are eliminated. To test the hypothesis that N592 is the alternative glycosylation site, we created the triple mutant N592Q/N597Q/N617. Indeed, this triple mutant yielded a single band at ~116, the expected MW of the nonglycosylated protein, both in the absence and the presence of PNGase F. Thus, in the absence of all three consensus N-glycosylation sites on the third extracellular loop, *Xenopus* oocytes failed to glycosylate NBCe1 measurably. We conclude that the oocyte can glycosylate N592 when bulky sugar chains are not present at positions 597 and 617. The oocyte may also glycosylate N592 to a lesser extent when glycosylation occurs at position 617 but not 597: on the original films for Fig. 5*B*, *bottom*, as well as for a similar experiment (not shown), it is possible to see a faint doublet at ~122 kDa.

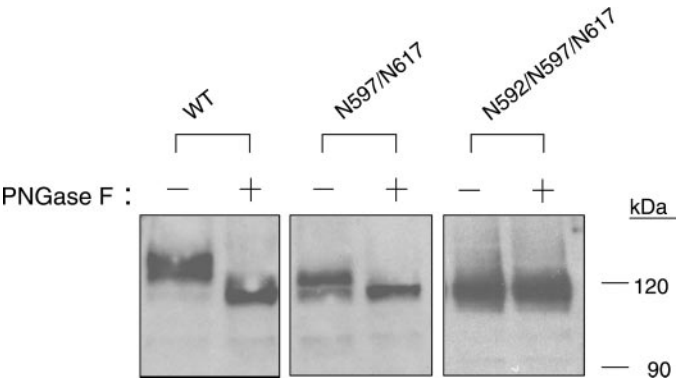

Fig. 6. PNGase F treatment of the membrane extracts from oocytes expressing the double (N597Q/N617Q) or triple mutant (N592/N597Q/N617Q). The crude membranes extracted from oocytes were incubated in the absence (−) or presence (+) of PNGase F.

Functional expression of unglycosylated NBCe1. To test the functional significance of the glycosylation of rat kidney NBCe1, we expressed the triple mutant N592Q/N597Q/N617Q in *Xenopus* oocytes and then used microelectrodes to monitor pH_i and membrane potential (V_m). Previous work showed that, as expressed in *Xenopus* oocytes, wild-type NBCe1-A is electrogenic, Na^+ dependent, and HCO_3^- dependent (34). As shown in Fig. 7*A*, for oocytes expressing the triple mutant, introducing 1.5% CO_2/10 mM HCO_3^- into the extracellular solution elicited a rapid fall in pH_i (due to

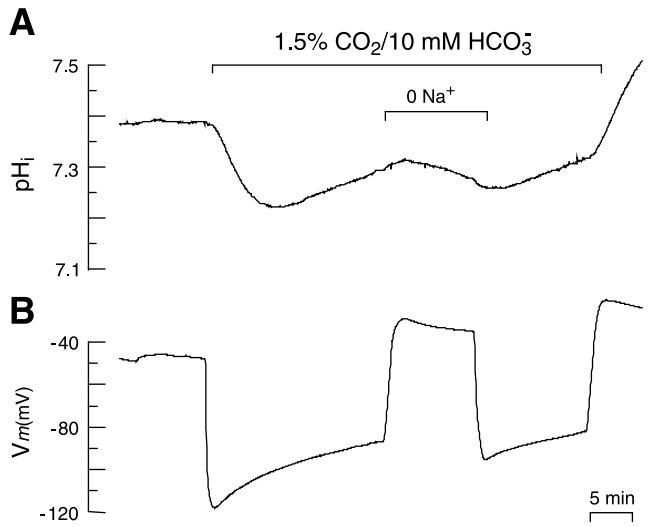

Fig. 7. Microelectrode measurements of the intracellular pH (pH_i) and membrane potential (V_m) in an oocyte expressing the triple mutant (N592/N597Q/N617Q). The oocyte was initially bathed with CO_2/HCO_3^--free ND-96 solution. During the indicated period, the oocyte was superfused with 1.5% CO_2/10 mM HCO_3^-. The mean rate of pH_i change (dpH_i/dt) was $13.8 \pm 1.7 \times 10^{-5}$ pH U/s during the pH_i recovery from the CO_2-induced acid load and $-25.3 \pm 7.3 \times 10^{-5}$ pH U/s during the acidification elicited by removing Na^+ ($n = 6$). These values are not significantly different from those in oocytes expressing wild-type NBCe1. For these, the mean dpH_i/dt was $14.8 \pm 1.8 \times 10^{-5}$ pH U/s during the pH_i recovery ($n = 5$; $P = 0.35$ in unpaired, 2-tailed t-test with comparable value for the triple mutant) and $-27.6 \pm 4.9 \times 10^{-5}$ pH U/s in the absence of Na^+ ($n = 5$; $P = 0.41$). In oocytes expressing the mutant transporter, applying CO_2/HCO_3^- caused a mean hyperpolarization of 63.4 ± 2.5 mV, whereas removing Na^+ caused a mean depolarization of 53.4 ± 1.8 mV ($n = 6$). In oocytes expressing wild-type NBCe1 ($n = 5$), applying CO_2/HCO_3^- caused a mean hyperpolarization of 45.2 ± 7.7 mV ($n = 5$; $P = 0.02$), whereas removing Na^+ caused a mean depolarization of 31.8 ± 5.1 mV ($n = 5$; $P = 0.001$).

CO_2 influx), followed by an increase that reflects the uptake of HCO_3^- or a related ion such as CO_3^{2-}. The introduction of CO_2/HCO_3^- also caused an immediate hyperpolarization (Fig. 7*B*), indicative of the electrogenic influx of Na^+ with two or more HCO_3^- ions (or an equivalent species, such as CO_3^{2-}). The negative shift in V_m was followed by a decay, the time course of which approximately followed the time course of the pH_i recovery.

Removing extracellular Na^+ reversibly converted the pH_i recovery to a very slow acidification, reflecting reversal of the mutant transporter. Simultaneously, Na^+ removal caused a rapid depolarization, consistent with the electrogenic efflux of Na^+ plus two or more HCO_3^- ions (or their equivalent). A statistical analysis (Fig. 7 legend) shows that the rates of pH_i change, both during the pH_i recovery from the CO_2-induced acid and the pH_i decline elicited by Na^+ removal, were not significantly different between oocytes expressing the triple mutants and those expressing wild-type NBCe1-A. The magnitudes of the V_m changes elicited by applying CO_2/HCO_3^- or removing Na^+ were significantly greater for oocytes expressing the triple mutant. However, this latter observation could reflect differences in several other parameters (e.g., membrane resistance) not related to the transport rate. We conclude that, similar to wild type, the triple mutant is electrogenic and Na^+ dependent and activated by applying CO_2/HCO_3^-. These results indicate that glycosylation is not required for the basic function of the protein. We obtained similar results (not shown) in mutants lacking one or both of the two natural glycosylation sites (i.e., N597Q, N617Q, and N597Q/N617Q).

DISCUSSION

Carbohydrate structure of rat kidney NBCe1. In this study, we provide the first direct evidence for the glycosylation of any of the Na^+-coupled HCO_3^- transporters. Specifically, the MW shift produced by PNGase F indicates that the electrogenic Na^+-HCO_3^- cotransporter from rat kidney NBCe1 is a glycoprotein in which the carbohydrate moiety is linked to the protein by an N-glycosidic bond. The insensitivity of rat kidney NBCe1 to either Endo F_2 or Endo H suggests that oligosaccharide structure of rat kidney NBCe1 is neither of the biantennary nor of the high mannose/hybrid type. Thus the carbohydrate is probably of the tri- or tetra-antennary/complex type. The lectin-binding assays further suggest that the carbohydrate components of rat kidney NBCe1 probably do not include a terminal nonreducing α-linked galactose (insensitivity to jacalin); a terminal nonreducing α-linked mannose (insensitivity to GNA); or either a core or outer fucose (insensitivity to LCA, LTA, and UEA-I). On the other hand, the carbohydrate component may include a terminal GalNAc (sensitivity to SBA), either an internal GlcNAc or a terminal sialic acid (WGA sensitivity), and a D-glucopyranose or D-mannopyranose with unmodified C3, C4, and C6 hydroxyl groups (Con A sensitivity).

The MW of the glycosylated renal NBCe1 is ~130 in rabbit, rat, and bovine. In rat, NBCe1 bands of similar MW appear in other tissues, including brain (6) and parotid gland (36). On the other hand, the MW of NBCe1 is ~160 in rat epididymis (22), and our unpublished data show that NBCe1 is mostly present in an unglycosylated form in rat stomach. Therefore, the carbohydrate component of NBCe1 is organ specific.

Our data indicate that the N-glycosylation of NBCe1 is common among various species. A sequence comparison reveals that three consensus glycosylation sites on the third extracellular loop of NBCe1 (i.e., N592, N597, and N617) are conserved among bovine, rabbit, human, mouse, and salamander. Salamander NBCe1 also has a fourth N-glycosylation on this loop. In addition, salamander NBCe1 has an additional carbohydrate linkage, probably an O-glycosylation because PNGase F treatment reduced the apparent MW of salamander NBCe1 to ~130, rather than the predicted MW of 116. It is interesting to note that the third extracellular loop of salamander NBCe1 has a cluster of threonines that are flanked by strongly hydrophobic residues, reminiscent of a mucin-type O-glycosylation site (13).

Glycosylation sites. Our data indicate that the N-linked glycosylation in NBCe1 normally occurs at N597 and N617 and thus prove that both of these residues, which are located between putative TM5 and TM6, face the extracellular fluid.

Glycosylation at N597 and N617 probably contributes about equally to the total glycosylation of the wild-type protein because *1*) disrupting either N597 or N617 reduces the apparent MW of the mutant protein from ~130 to ~122, about one-half of the way to the MW of the unglycosylated protein; and *2*) treatment of either mutant with PNGase F reduces the MW to ~116, the other one-half of the way. The N597Q/N617Q double mutant exhibits a pair of bands (MW of 116 and 120 in Fig. 6) that presumably represent the protein with or without glycosylation of N592. However, in the absence of PNGase F treatment, the N617Q single mutant exhibited only a single band (MW of 122), suggesting that glycosylation at N597 prevents glycosylation at N592.

By steric hindrance, the bulky carbohydrate moiety at N597 may contribute to the absence of glycosylation at N592 in wild-type NBCe1. However, steric hindrance is not the entire explanation because, as noted above, glycosylation at N592 is optional even for single and double mutants in which we disrupt glycosylation at N597. One possibility is that N592, which is a 28-amino acid residue from the putative end of TM5, is too close to the membrane for efficient glycosylation. However, the N-glycosylation site on the fourth extracellular loop of AE1 is only 15 residues from the putative end of TM5. Alternatively, folding of the extracellular loop near N592 may make this asparagine an intrinsically poor substrate for the oligosaccharyl transferase in the endoplasmic reticulum.

Retention of function in unglycosylated NBCe1. The *Xenopus* oocyte expression system has been very useful for analyzing the effect of glycosylation on the function of membrane proteins (25). In most of the membrane proteins studied to date, such as the glutamate transporter GLT1 (40), the γ-aminobutyric acid transporter GAT1 (26), and the nicotinic acetylcholine receptor AChR (15), the naturally occurring N-glycosylation is essential for functional expression of the glycoprotein. Nonetheless, in other cases, such as the Na^+-dicarboxylate cotransporter NaDC-1 (29), the Na-Pi cotransporter NaPi-2 (20), the water channel aquaporin-1 (43), and the Na^+ channel ENaC (9), the N-glycosylation is not essential for protein function. We found that mutations at one of the two natural sites (N597Q or N617Q), at both of the two natural sites (N597Q/N617Q), or at the two natural sites plus the one alternative site (N592Q/N597Q/N617Q) yield transporters, as expressed in *Xenopus* oocytes, functionally comparable to the wild-type NBCe1 in terms of electrogenicity as well as Na^+ and HCO_3^- dependence.

The unglycosylated AE1 mutant is also functional in the *Xenopus* expression system (30). However, the oocytes expressing the unglycosylated AE1 mutant exhibit a lower exchanger activity than those expressing wild-type AE1, possibly due to incorrect protein folding in the endoplasmic reticulum. It is also reported that removing oligosaccharides from AE2 increases the susceptibility to proteolytic degradation (44). We cannot rule out the possibility that lack of a natural glycosylation pattern in our NBCe1 mutants might have reduced their folding efficiency and/or stability. However, if so, these deleterious effects must have been overcome by a very high level of expression.

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