Na^+/H^+ Exchanger-2 Is an O-Linked but Not an N-Linked Sialoglycoprotein†

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ABSTRACT: A polyclonal antibody (Ab597) was produced in rabbit against a fusion protein of glutathione-*S*-transferase and the last 87 amino acids of the Na^+/H^+ exchanger isoform, NHE2. By Western blotting, Ab597 recognized proteins of 75 and 85 kDa in PS120/NHE2 membranes (PS120 cells stably transfected with NHE2), and this antibody did not cross-react with NHE1 and NHE3. When Ab597 was used to immunocytochemically stain PS120/NHE2 cells, permeabilization of the cells was required for staining, confirming the putative membrane topology of NHE2 that the C-terminus is cytoplasmic. NHE1 is N-glycosylated. NHE2 was predicted to be N-glycosylated as it contains one potential N-linked glycosylation site ($N^{350}VS$), which is conserved among NHE1, NHE3, and NHE4. However, NHE2 was resistant to peptide:*N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H) digestion, suggesting that NHE2 is not N-glycosylated. In contrast, neuraminidase shifted the mobility of the 85 kDa NHE2 protein in PS120/NHE2 membranes into an 81 kDa band, and *O*-glycanase further shifted the mobility of the neuraminidase-treated 81 kDa protein to 75 kDa. Incubation of PS120/NHE2 cells with benzyl *N*-acetyl-α-D-galactosaminide (BzαGalNAc), an O-glycosylation inhibitor, decreased the size of the 85 kDa protein to 81 kDa. This treatment had no effect on the initial rate of Na^+/H^+ exchange of PS120/NHE2 cells. The 75 kDa protein was not affected by the glycosidase treatment of PS120/NHE2 membranes or the BzαGalNAc treatment of PS120/NHE2 cells. These results suggest that the 85 kDa protein is an O-glycosylated form of NHE2, while the 75 kDa protein is an unglycosylated form. Thus, unlike NHE1, which is N- and O-glycosylated, NHE2 has only O-linked glycosylation.

Na^+/H^+ exchangers (NHEs[1]) are plasma membrane proteins that catalyze the electroneutral exchange of extracellular Na^+ for intracellular H^+ and are found in all mammalian cells (Grinstein et al., 1989; Wakabayashi et al., 1992a; Tse et al., 1993a). Intestinal and kidney epithelial cells have NHEs on both apical and basolateral membranes (Knickelbein et al., 1983, Haggerty et al., 1988). In these cells, the apical membrane NHE is involved in Na^+ absorption and is relatively resistant to amiloride inhibition compared to the basolateral housekeeping isoform.

The first identified mammalian NHE, now called NHE1, was cloned by Sardet et al. (1989, 1990). Since then, three additional isoforms have been identified (NHE2, NHE3, and NHE4) (Tse et al., 1992, 1993b; Wang et al., 1993; Orlowski et al., 19993; Collins et al., 1993). All NHEs identified have similar sizes estimated from the deduced amino acid composition, ranging from 81 (NHE4) to 93 kDa (NHE3), and similar predicted membrane topology, that is, 10–12 membrane-spanning domains that are involved in ion exchange and a hydrophilic C-terminus that is involved in growth-factor regulation (Wakabayashi, 1992b). Unlike NHE1, which is ubiquitously expressed in tissues and cells, message expression of NHE2, NHE3, and NHE4 has tissue specificity (Tse et al., 1992, 1993b; Wang et al., 1993; Orlowski et al., 1992; Collins et al., 1993). The NHE3 message is expressed only in intestine, kidney, and stomach. Messages of NHE2 and NHE4 are predominantly expressed in, but not restricted to, intestine, kidney, and stomach. The NHE2 message is also found in adrenal gland and in a lesser amount in skeletal muscle and trachea, whereas NHE4 is also found in brain, uterus, and skeletal muscle.

At the protein level, NHE1 is a glycoprotein of 110 kDa and can be phosphorylated *in vivo* (Sardet et al., 1990). The amount of phosphorylation on NHE1 increases in response to growth-factor activation. In addition to phosphorylation, an accessory protein has been implicated in regulating NHE1 (Wakabayashi et al., 1994). NHE1 is found in both polarized epithelial cells and nonpolarized cells (Sardet et al., 1990). In intestinal and renal epithelial cells, NHE1 is the basolateral isoform (Tse et al., 1991; Biemesderfer et al., 1992). NHE3 is the brush border isoform, based on functional characterization and immunohistochemical localization (Biemesderfer et al., 1993; Bookstein et al., 1994; Tse et al., 1993c; Hoogerwerf et al., 1994). NHE2 is also localized to the brush border in rabbit and human ileum and ascending colon (Hoogerwerf et al., 1994).

Recently, we and Shull et al. cloned and functionally expressed NHE2 (Tse et al., 1993b; Wang et al., 1993; Levine et al., 1993; Yu et al., 1993). From the rabbit NHE2 cDNA, NHE2 is predicted to have 809 amino acids with a calculated size of 90 787. To further study the biochemical, physiological, and molecular properties of NHE2, we prepared a polyclonal antibody against NHE2. In this paper, we report that NHE2

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[1] Abbreviations: NHE, Na^+/H^+ exchanger; PNGase, F, peptide:*N*-glycosidase F; Endo H, endoglycosidase H; IPTG, isopropyl β-D-thiogalactoside; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; FITC, fluorescein isothiocyanate; GST, glutathione-*S*-transferase; IgG, immunoglobulin G; NEB, New England Biolabs; bp, base pair; BzαGalNAc, benzyl *N*-acetyl-α-D-galactosaminide.

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is made up of 85 and 75 kDa proteins. NHE2 contains one potential N-linked glycosylation site ($N^{350}VS$), which is conserved among all cloned NHEs, and thus NHE2 was predicted to be N-glycosylated (Tse et al., 1993b; Wang et al., 1993). NHE2 is an intestinal epithelial brush broader NHE (Hoogerwerf et al., 1994), and N-linked glycosylation has been suggested to be important for brush border Na^+/H^+ exchange, at least by the kidney brush border NHE (Yusufi et al., 1988). Therefore, in the present study, we characterized and studied the functional consequences of the glycosylation of NHE2.

EXPERIMENTAL PROCEDURES

Induction and Affinity Purification of Glutathione S-Transferase/NHE2-C87 (GST/E2-C87) Fusion Protein. A *Bam*HI linker was ligated to the 714 bp *Hind*III and *Eco*RI fragment of NHE2. This cDNA fragment contains nucleotides 2167–2870, which correspond to amino acids 723–809; this region of NHE2 does not have any homology with other known NHEs. The linker-ligated cDNA fragment was then subcloned into the *Bam*HI site of the pGEX-3X vector to produce the fusion protein construct (pGEX/E2-C87), which was transformed into *E. coli* (NM522).

Fusion protein was produced in transformed bacteria by induction with isopropyl β-D-thiogalactoside (IPTG) and was affinity-purified from bacterial lysates by batch absorption with glutathione Sepharose 4B, as described by Smith and Johnson (1988). Briefly, 1 L of transformed bacteria, which was propagated in 2XYT medium and had been induced with 0.1 mM IPTG for 3 h for fusion protein production, was pelleted and resuspended in 20 mL of phosphate-buffered saline (PBS) [150 mM NaCl and 20 mM Na_2HPO_4 (pH 7.3)]. The cells were then lysed by sonication. Triton X-100 (1%) was added, and the lysed cell suspension was then centrifuged at 1000*g* for 5 min at 4 °C. The fusion protein was then affinity-purified from supernatant by mixing for 20 min at room temperature with 1 mL of 50% glutathione Sepharose 4B that had been preequilibrated with PBS containing 1% Triton X-100. Bacterial proteins that were not bound to the glutathione Sepharose beads were washed extensively with PBS with 1% Triton X-100 and then with PBS. Bound fusion protein was eluted with 5 mM glutathione in 50 mM Tris-HCl (pH 8.0) and then dialyzed against PBS.

Affinity-purified GTS/E2-C87 fusion protein in PBS was then sent to HRP Inc. (Denver, PA) for custom antibody production in rabbit. A non-affinity-purified antiserum, called Ab597, was used in this study.

Preparation of Crude Cell Membranes. Cells [PS120 cells and its derivatives, LAP1 (Franchi et al., 1986), TP3, a rabbit lymphoblast cell line, and rabbit skin fibroblasts (Tse et al., 1992, 1993b)] grown to confluency on 10 cm Petri dishes were lysed with 10 mM Tris and 1 mM EDTA (pH 7.5) containing 0.1 mM PMSF, 1 mM iodoacetamide, and 1 mM *o*-phenanthroline. Lysed cells were then scraped and homogenized with a 26G needle and centrifuged at 1000*g* to remove cell debris, mitochondria, and nuclei. The supernatant was then subjected to high-speed centrifugation for 30 min at 40000*g* to obtain a crude cell membrane fraction. The crude cell membranes were resuspended in 10 mM Tris and 1 mM EDTA (pH 7.5) for SDS–PAGE and Western blotting. For preparation of the PS120/NHE2 membrane for *O*-glycanase experiments, the 10 mM Tris/1 mM EDTA buffer was replaced by 5 mM sodium phosphate (pH 8). This was to avoid the possibility of inhibiting the *O*-glycanase activity by Cl^- from the Tris buffer.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blotting. Crude cell membrane proteins (10–30 μg) were solubilized in Laemmli sample buffer and resolved by SDS–PAGE by the method of Laemmli (1970). Proteins were electrotransferred onto nitrocellulose membranes. Western blotting was performed by blocking nitrocellulose membranes with solution containing 5% nonfat dry milk in Tris-buffered saline [TBS: 150 mM NaCl and 13 mM Tris (pH 7.5)] for 1 h, followed by incubation with primary antibody for 1 h [1:1000–2000 dilution in blocking solution (Sardet et al., 1990)] at room temperature. Nitrocellulose membranes were then washed extensively with washing buffer (0.02% Triton X-100 in TBS). After washing, the nitrocellulose membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h at 1:5000 dilution in blocking solution. Excess secondary antibody was again washed, and the bound secondary antibody was detected by enhanced chemiluminescence.

Immunocytochemistry. Cells (untransfected PS120 and PS120/NHE2) were fixed in 2% paraformaldehyde, 1.4% lysine, and 0.2% sodium periodate and permeabilized with 0.06% digitonin. After quenching with 0.25% NH_4Cl and blocking with 0.2% gelatin, cells were then incubated sequentially with the primary antibody, Ab597 (1:100 dilution), and the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G(IgG), each for 30 min at room temperature. The cells were washed extensively with PBS after each incubation with antibodies and were then mounted in 25% glycerol in PBS containing 0.1% *p*-phenylenediamine. The immunostained cells were examined by both phase contrast and fluorescence microscopy.

Endoglycosidase Treatment. In all endoglycosidase experiments, membranes (20–30 μg) were denatured in 0.5% SDS and 1% β-mercaptoethanol by boiling for 10 min before incubation with endoglycosidase(s) for 6 h at 37 °C, except in studies with Endo F and Endo H in which membranes were incubated for 18 h. The specific conditions for each endoglycosidase were as suggested by the manufacturer, as follows: (i) Endo H: Denatured membranes were incubated with 500–1500 units of Endo H in 50 mM sodium citrate (pH 5.5). (ii) PNGase F: Denatured membranes were incubated with 1000-5000 units of PNGase F in 1% NP-40 and 50 mM sodium citrate (pH 7.5). (iii) Neuraminidase: Denatured membranes were incubated with 20 munits of neuraminidase in 50 mM sodium citrate (pH 5.5) and 1% NP-40. (iv) *O*-Glycanase and the combination of *O*-glycanase and neuraminidase: Denatured membranes were incubated with 4 units of *O*-glycanase alone or in combination with neuraminidase (20 munits) in 20 mM sodium phosphate (pH 6.0) and 1% NP-40.

Na Uptake Studies. ^{22}Na uptake into PS120/NHE2 cells acidified with NH_4Cl was measured as previously described (Tse et al., 1993b).

Materials. pGEX-3X vector and glutathione Sepharose 4B were obtained from Pharmacia; ECL Western blotting detection reagents from Amersham; Endo H and PNGase F were from New England Biolabs; neuraminidase was from Boehringer Mannheim; *O*-glycanase was from Genzyme; FITC-conjugated goat anti-rabbit IgG was from Kirkegaard and Perry Inc.; horseradish peroxidase-conjugated goat anti-rabbit IgG was from Jackson Immuno Research; benzyl *N*-acetyl-α-D-galactosaminide was from Sigma; and nitrocellulose membranes were from Costar. All other reagents were of the highest available grades.

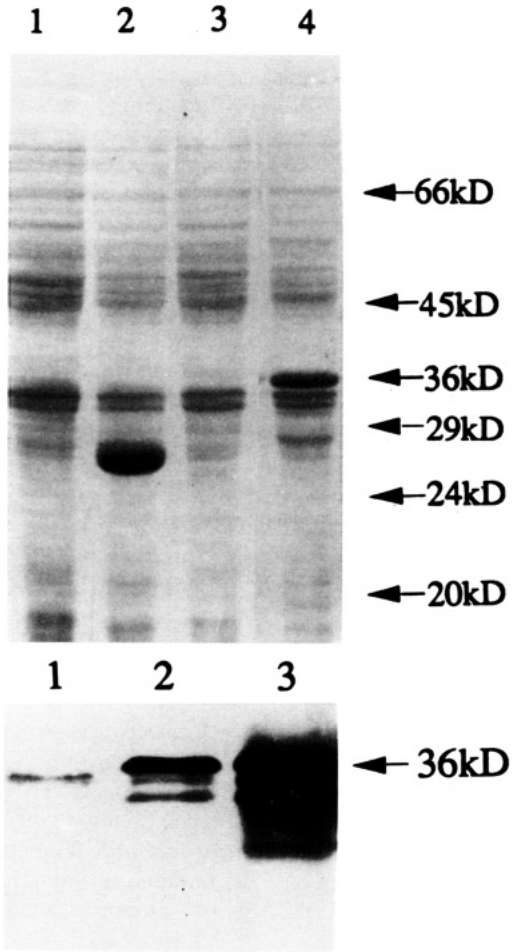

FIGURE 1: Coomassie Blue staining and Western blot analysis of the GST/E2-C87 fusion protein expressed in bacteria. (A, top) Coomassie Blue staining of SDS–PAGE of bacterial lysates isolated from bacteria transformed with either the pGEX vector (lanes 1 and 2) or the construct, pGEX/E2-C87 (lanes 3 and 4). The bacteria were grown in 2XYT medium to an OD_{600} of 2.0 and were induced with 0.1 mM IPTG (lanes 2 and 4) or remained growing without induction (lanes 1 and 3) for an additional 2 h. Cells were pelleted and resuspended in Laemmli sample buffer and were analyzed by 12% SDS–PAGE. Each lane contained bacterial lysates from 20 μL of bacteria at an OD_{600} of 2.6. Molecular weight markers are shown on the right side. In the presence of IPTG, the 36 kDa GST/E2-C87 fusion protein and the 26 kDa glutathione transferase were produced in bacteria transformed with pGEX/E2-C87 and the pGEX vector, respectively. (B, bottom) Western blotting. Bacterial lysates were separated by 12% SDS–PAGE, electrotransferred onto nitrocellulose membrane, and immunoblotted with Ab597 (1:1000 dilution): lane 1, lysate from untransformed bacteria; lanes 2 and 3, lysates from bacteria transformed with pGEX/E2-C87 that were not induced (lane 2) or were induced (lane 3) with IPTG. Each lane contained lysates from 0.01 μL of bacteria at an OD_{600} of 2.6. The arrow points to the 36 kDa protein recognized by Ab597.

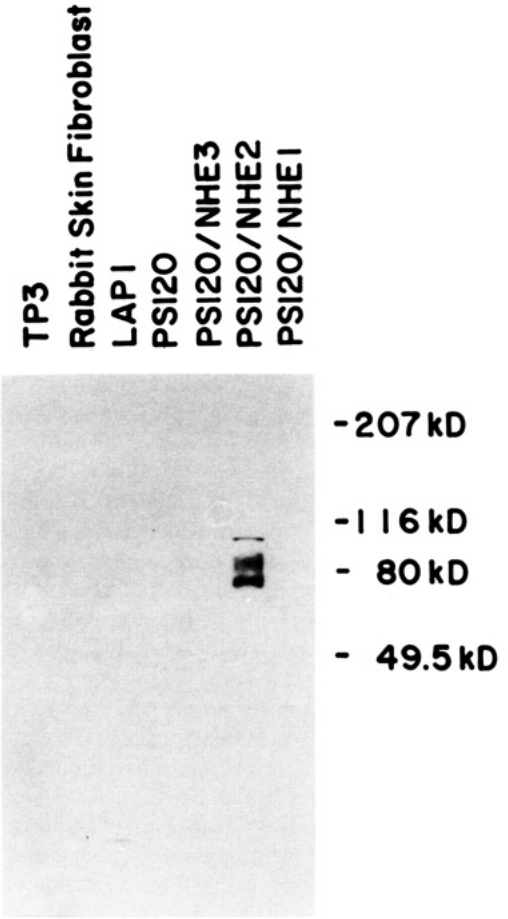

FIGURE 2: Western blotting of crude membrane proteins. Crude cell membranes were prepared as described in the Experimental Procedures from the cells shown on the top of the figure. They were separated by 9% SDS–PAGE, electrotransferred onto nitrocellulose membrane, and immunoblotted with Ab597 (1:1000 dilution). Each lane contained 10 μg of crude membrane protein. Prestained molecular weight markers (Bio-Rad) are shown on the right side.

RESULTS

Polyclonal Antibody against the GST/E2-C87 Fusion Protein. We have recently reported the cloning and functional characterization of a novel Na^+/H^+ exchanger isoform, NHE2 (Tse et al., 1993b). To further characterize NHE2 biochemically, we expressed, in bacteria, the last 87 C-terminal amino acids of NHE2 as a fusion protein of *Schistosoma japonicum* glutathione-*S*-transferase and raised a polyclonal antibody in rabbit against the affinity-purified fusion protein. Figure 1A shows the Coomassie Blue staining of SDS–PAGE of bacterial lysates isolated from bacteria transformed with either the pGEX vector (lanes 1 and 2) or the construct pGEX/E2-C87 (lanes 3 and 4), without (lanes 1 and 3) or with induction by IPTG (lanes 2 and 4). As shown in lane 4, upon induction with 0.1 mM IPTG, a 36 kDa GST/E2-C87 fusion protein was produced in bacteria transformed with pGEX/E2-C87, and this 36 kDa protein was not detected by Coomassie Blue staining under basal or uninduced conditions (lane 3). Similarly, as a positive control of fusion protein production in bacteria as induced by IPTG, a 26 kDa glutathione-*S*-transferase was produced in bacteria transformed with the pGEX vector when induced by 0.1 mM IPTG (lane 2) (Smith & Johnson, 1988). The 36 kDa GST/E2-C87 fusion protein was then affinity-purified from the bacterial lysates and used to immunize rabbits to raise polyclonal antibody. Two rabbits were immunized with the GST/E2-C87 fusion protein, and one rabbit produced an antiserum, called Ab597, that as shown by Western blotting in Figure 1B reacted with the GST/E2-C87 fusion protein. Ab597 recognized a 36 kDa protein in cell lysates from bacteria transformed with the pGEX/E2-C87 construct (lanes 2 and 3). When induced with 0.1 mM IPTG, the bacteria expressed quantitatively more 36 kDa protein than the uninduced bacteria (compare lanes 2 and 3). This 36 kDa protein was absent from the untransformed bacteria (lane 1). Apparently, the amount of several other proteins that were smaller than 36 kDa and reacted with Ab597 were also quantitatively higher in the IPTG-induced bacteria than in the uninduced bacteria. These proteins were likely degraded products of the 36 kDa fusion protein overexpressed in bacteria. These results

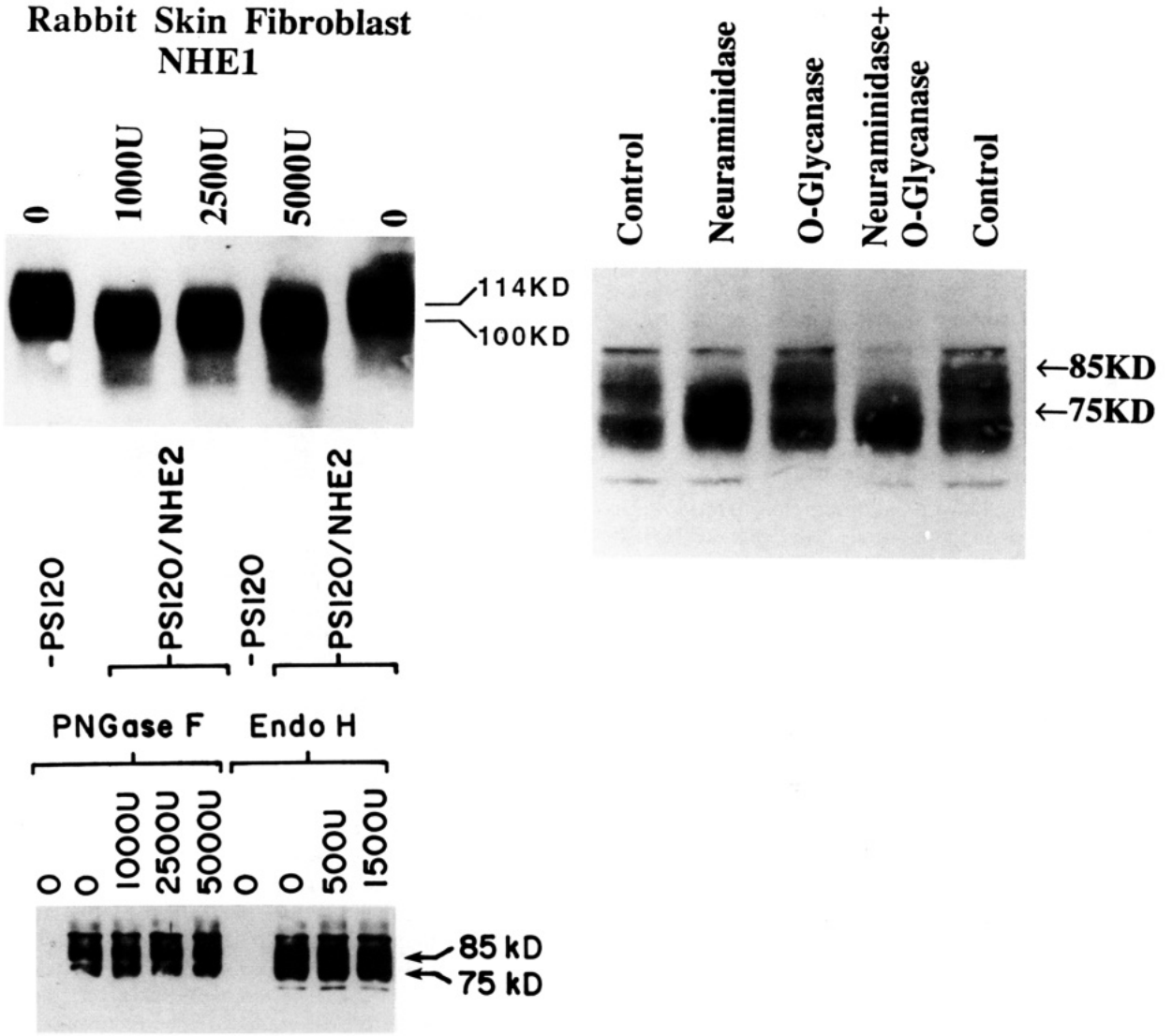

FIGURE 3: Endoglycosidase treatment of NHE1 and NHE2. (A, top left) effect of PNGase F on endogenous NHE1 in rabbit skin fibroblasts. Crude membranes (30 μg per lane) isolated from rabbit skin fibroblasts were treated with PNGase F [0–5000 New England BioLabs (NEB) units: 1 IUB munits = 500 NEB units], as described in the Experimental Procedures. The PNGase F-treated membranes were separated by 9% SDS–PAGE and analyzed by Western blotting with anti-NHE1 antibody, RP1-c28 (1:500 dilution). PNGase F shifted the mobility of NHE1 from 114 to 100 kDa. (B, bottom left) Effect of PNGase F and Endo H on NHE2. Crude membranes (20 μg per lane) prepared from PS120 and PS120/NHE2 cells were incubated with PNGase F (0–5000 NEB units) and Endo H (0–1500 NEB units, 1 IUB munit – 10 NEB units), as described in the Experimental Procedures and as shown in the figure. After endoglycosidase treatment, the membranes were separated by 9% SDS–PAGE and analyzed by Western blotting using Ab597 (1:1000 dilution). Neither enzyme had any effect on the 85 and 75 kDa NHE2 proteins. (C, right) Effect of neuraminidase and *O*-glycanase on NHE2. Crude cell membranes (30 μg) prepared from PS120/NHE2 cells were incubated with 20 munits of neuraminidase, 4 munits of *O*-glycanase, and the combination of 20 munits of neuraminidase and 4 munits of *O*-glycanase, as shown on the top of the figure. Endoglycosidase-treated membranes were analyzed by Western blotting using Ab597 (1:1000 dilution). Neuraminidase shifted the mobility of the 85 kDa protein to that of the 81 kDa protein, and neuraminidase plus *O*-glycanase shifted the 85 kDa protein to 75 kDa. *O*-Glycanase itself had no effect.

suggested that Ab597 cross-reacted with the 36 kDa GST/E2-C87 fusion protein and that even in the uninduced conditions there was a small amount of fusion protein expressed that was not detected by Coomassie Blue staining (Figure 1A), but was detected by the more sensitive Western blotting. The basal expression of fusion protein likely resulted from the presence of small amounts of inducer molecules in the culture medium.

Ab597 Recognized NHE2 as 75 and 85 kDa Proteins. In order to determine whether Ab597 cross-reacted with NHE2, Western blotting was used to screen a panel of crude membranes isolated from cells that are known to have no endogeneous Na^+/H^+ exchangers (PS120 cells and LAP1 cells) (Pouysségur et al., 1984; Franchi et al., 1986) and to contain NHE1 [rabbit skin fibroblasts, TP-3, a rabbit lymphoblast cell line, and PS120/NHE1 cells (Tse et al., 1991, 1992, 1993b)], NHE2 [PS120/NHE2 cells (Tse et al., 1993b; Levine et al., 1993)], and NHE3 [PS120/NHE3 cells (Levine et al., 1993; Tse et al., 1993c)] (Figure 2). It was found that Ab597 recognized proteins of 75 and 85 kDa only in PS120/NHE2 cells, but not in the other cell lines tested, suggesting that Ab597 is specific to NHE2 and does not cross-react with NHE1 or NHE3. Control preimmune sera from the same rabbit that gave the anti-serum, Ab597, did not give any reactivity in the cell lines tested (results not shown).

Ab597 also cross-reacted weakly with proteins of sizes 97 (Figure 2) and 70 kDa (not shown). These two proteins presumably were not the Na^+/H^+ exchanger, as they were also found in untransfected PS120 cells. Although the presence of these two reactive proteins in untransfected PS120 cell membranes is not demonstrated clearly in Figure 2, it

was detected in untransfected PS120 cell membranes by longer exposure of Western blots to enhanced chemiluminescence (data not shown). Further, these 97 and 70 kDa proteins could be immunoprecipitated by Ab597 in the untransfected PS120 cells (data not shown).

In some experiments, Ab597 was preabsorbed with glutathione-*S*-transferase. The preabsorbed antiserum was then incubated with a nitrocellulose membrane coated with fusion protein. The antibodies bound were then eluted with glycine/HCl (pH 2.2) and immediately neutralized with 1 M Tris to pH 7.5. This affinity-purified Ab597 gave results identical to those of the nonpurified antiserum, in studies identical to those shown in Figure 2 (results not shown). Therefore, all subsequent experiments were performed with the non-affinity-purified Ab597.

Endoglycosidase Treatment of NHE1 and NHE2. Rabbit skin fibroblasts endogenously express NHE1 message.[2] It has been reported that NHE1 is an N-linked glycoprotein of 110 kDa (Haworth et al., 1993, Fafournoux et al., 1994). In order to confirm this on the endogeneously expressed NHE1s in rabbit skin fibroblasts, crude rabbit skin fibroblast membranes were treated with PNGase F and analyzed by Western blotting with an anti-NHE1 antibody, RP1-c28 (Sardet et al., 1990). As shown in Figure 3A, RP1-c8 recognized NHE1 in rabbit skin fibroblasts as a protein of 114 kDa. Deglycosylation of NHE1 with PNGase F shifted its size from 114 to 100 kDa, confirming that it is N-glycosylated.

Since Ab597 recognized NHE2 as more than one single protein, i.e., as 75 and 85 kDa proteins, it is likely that there was posttranslational modification of NHE2, e.g., glycosylation. Furthermore, NHE2 has three putative N-linked glycosylation sequences found at N^{350}, N^{623}, and N^{685}, and N^{350}, which is located on the putative extracellular loop between membrane-spanning domains 7 and 8 (Sardet et al., 1989; Tse et al., 1992), is conserved in the other NHE isoforms. Thus, it was suggested that NHE2 was likely to be N-glycosylated (Tse et al., 1993b; Wang et al., 1993). To determine whether NHE2 was N-glycosylated, NHE2 was exposed to PNGase F and Endo H. As shown in Figure 3B, both the 85 and 75 kDa proteins were resistant to PNGase F and Endo H digestion, even after 18 h of incubation with excess endoglycosidases. This suggested that NHE2 was not N-glycosylated, and the possibility that NHE2 was O-glycosylated was then studied. Neuraminidase is an exoglycosidase. It removes sialic acids from both N- and O-glycosylated proteins. Incubation of NHE2 with 20 munits of neuraminidase for 6 h shifted the mobility of the 85 kDa protein to that of 81 kDa (Figure 3C). *O*-Glycanase alone had no effect on the mobility of the 85 and 75 kDa proteins. It is known that *O*-glycanase activity is inhibited by the presence of sialic acids on the O-linked core disaccharide, and the sialic acids must be removed to see the effect of *O*-glycanase, for instance, by neuraminidase exposure during treatment with *O*-glycanase. Therefore, NHE2 was first incubated with 20 munits of neuraminidase for 1 h. Four milliunits of *O*-glycanase was then added, and NHE2 was incubated further with these two glycosidases for an additional 6 h. It was found that the combination of neuraminidase and *O*-glycanase further shifted the mobility of the 85 kDa protein to that of 75 kDa. The mobility of the 75 kDa protein was not affected by the glycosidases. These results suggest that the 85 kDa protein is the O-glycosylated form of NHE2 and the 75 kDa protein is unglycosylated.

[2] C. M. Tse and M. Donowitz, unpublished results.

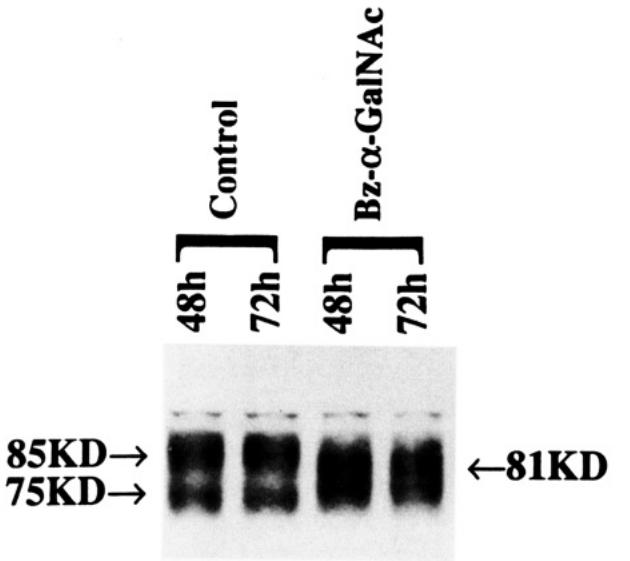

FIGURE 4: Western blot analysis of NHE2 in BzαGalNAc-treated PS120/NHE2 cells. PS120/NHE2 cells were incubated with and without 3 mM BzαGalNAc in the culture medium for 48 and 72 h. Crude cell membranes were then prepared and analyzed by Western blotting with Ab597 as described in the Experimental Procedures. BzαGalNAc decreased the mobility of the 85 kDa protein to 81 kDa and had no effect on the 75 kDa protein. This effect on NHE2 was the same after a 48 or 72 h incubation of the PS120/NHE2 cells with BzαGalNAc.

Inhibition of O-Linked Glycosylation by BzαGalNAc. To further elucidate the role of *O*-linked glycosylation on Na^+/H^+ exchange by PS120/NHE2 cells, the O-linked glycosylation of NHE2 was inhibited by incubating PS120/NHE2 cells with BzαGalNAc. BzαGalNAc is a competitive inhibitor of mucin glycosylation and prevents the extension of O-glycylation by competing with the substrate of UDP-GalNAc: GalNAc-β-1,3-galactosyltransferase (Kuan et al., 1989; Kojima et al., 1992). Therefore, PS120/NHE2 cells were incubated in the presence and absence (control) of 3 mM BzαGalNAc for 48 and 72 h. Treatment of PS120/NHE2 cells with 3 mM BzαGalNAc for up to 72 h had no effect on the morphology and the growth of PS120/NHE2 cells (data not shown). Membranes were then purified from the control and BzαGalNAc-treated cells and analyzed by Western blotting with Ab597. As shown in Figure 4, incubation of the PS120/NHE2 cells for 48 or 72 h with BzαGalNAc decreased the size of the 85 kDa protein to 81 kDa. BzαGalNAc had no effect on the 75 kDa protein. Since BzαGalNAc inhibited the O-linked glycosylation of NHE2, we tested whether this inhibition causes any change in the initial rate of Na^+/H^+ exchange by PS120/NHE2 cells. As shown in Figure 5, incubation of PS120/NHE2 cells with BzαGalNAc for 72 h had no effect on the initial rate of Na^+/H^+ exchange by PS120/NHE2 cells.

Topology of NHE2. Like other members of the Na^+/H^+ exchanger gene family, NHE2 is predicted to have 10–12 membrane-spanning domains and a long hydrophilic C-terminus putatively located on the cytoplasmic surface (Tse et al., 1994b; Wang, et al., 1993). The C-terminus of NHE1 has been shown to be cytoplasmic (Sardet et al., 1990). To confirm this putative membrane topology of NHE2, immunolocalization of NHE2 expressed in PS120/NHE2 cells was performed. Ab597 did not react with nonpermeabilized PS120 on nonpermeabilized PS120/NHE2 cells (results not shown). Further, it did not recognize the permeabilized PS120 cells studied as a negative control (Figure 6B). However, Ab597 immunolabeled the PS120/NHE2 cells when they were

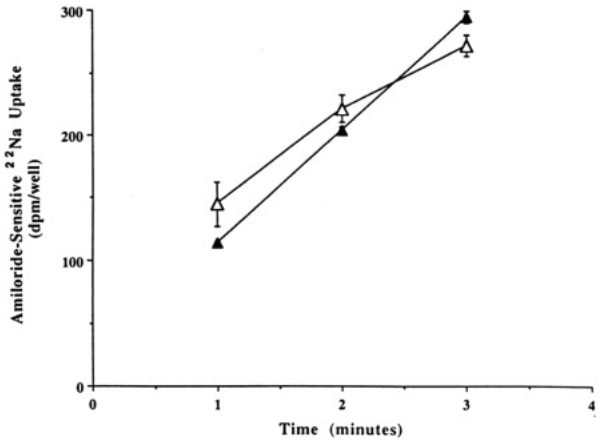

FIGURE 5: Effect of BzαGalNAc on Na^+/H^+ exchange in PS120/NHE2 cells. PS120/NHE2 cells were incubated with (△) and without (▲) 3 mM BzαGalNAc in the culture medium for 72 h, and this incubation resulted in the inhibition of O-linked glycosylation in NHE2 (Figure 4). A time course of carrier-free amiloride-sensitive ^{22}Na uptake (1 mM amiloride) in PS120/NHE2 cells acidified with NH_4Cl was performed as described previously (Tse et al., 1993b) over a period of 3 min, during which time it was previously shown that the uptake was linear. Each data point represents a mean of triplicates ± SEM.

permeabilized with 0.06% digitonin (Figure 6D). These results suggested that Ab597 reacted with NHE2 from the cytoplasmic surface and that the C-terminus of NHE2 was cytoplasmic. Furthermore, the fluorescence signal given by the bound antibody was diffuse, and its intensity was increased at the cell periphery and, perhaps, at adhesion plaques (Figure 6D) (Grinstein et al., 1993). This suggested that at least some of the expressed NHE2 was targeted properly to the plasma membrane. This is supported further by the functional Na^+/H^+ exchange activity expressed in PS120/NHE2 cells (Tse et al., 1993b; Wang et al., 1993; Levine et al., 1993; Yu et al., 1993).

DISCUSSION

We and Shull *et al.* have recently cloned and functionally expressed NHE2 (Tse et al., 1993b; Wang et al., 1993; Levine et al., 1993). In the present study, we used an anti-NHE2 antibody, Ab597, to characterize the molecular properties of NHE2 expressed in PS120 cells, including its molecular size as revealed by Western blotting, the presence and the type (N and O) of glycosylation, and a test of its putative membrane topology. Our results show that NHE2 is made up of 85 and 75 kDa proteins. Although NHE2 has N-linked glycosylation consensus sequences (Tse et al., 1993b; Wang et al., 1993), NHE2 is not N-glycosylated as both the 85 and 75 kDa proteins of NHE2 are resistant to PNGase F and Endo H digestion. Neuraminidase treatment of NHE2 shifted the mobility of the protein from 85 to 81 kDa. This suggested that NHE2 might be O-glycosylated, as it is not N-glycosylated and sialic acids are usually found on O-linked disaccharides (Schachter, 1985). Although *O*-glycanase by itself had no effect on NHE2, *O*-glycanase was able to further shift the mobility of the neuraminidase-treated NHE2 from 81 to 75 kDa. These results strongly suggested that the 85 kDa protein of NHE2 contained O-linked glycosylation. This was further supported by incubating PS120/NHE2 cells with BzαGalNAc to inhibit O-linked glycosylation; this treatment reduced the size of the 85 kDa protein to 81 kDa. The 75 kDa NHE2 protein was not affected by either the glycosidase treatment of PS120/NHE2 membranes or the BzαGalNAc treatment of PS120/NHE2 cells. These results, therefore, indicate that the 75 kDa protein is an unglycosylated form of NHE2. When NHE1 is expressed in PS120 cells, NHE1 is made up of a 110 kDa N- and O-glycosylated protein and a 85 kDa protein that contains a small amount of N-linked glycosylation (Counillon et al., 1994). The latter is an internal precursor of the mature NHE1 protein. Thus, by analogy with NHE1, the 75 kDa protein is likely an internal precursor of NHE2, and this form might be present in a significant amount in an overexpression system, such as in PS120/NHE2 cells. We have observed that there is some variability of the relative amounts of the 85 and 75 kDa proteins in membranes prepared from PS120/NHE2 cells (compare Figures 2, 3B,C, and 4). The reason for such variability is not known and perhaps might be due to the saturation of the O-linked glycosylation machinery in PS120/NHE2 cells in which NHE2 is overexpressed.

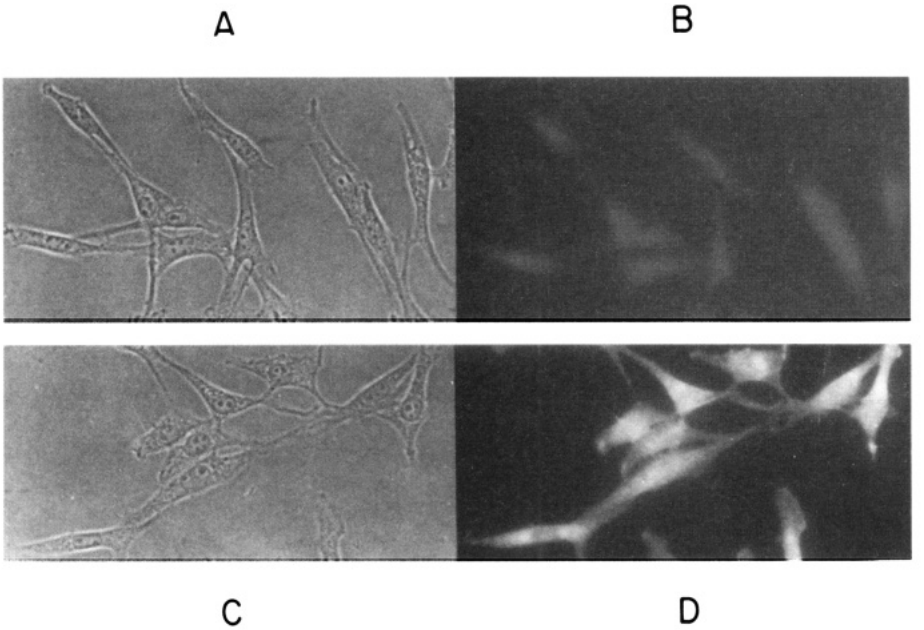

FIGURE 6: Immunocytochemistry of NHE2 in PS120/NHE2 cells. Panels A and C are phase-contrast images, and panels B and D are the fluorescence microscope images of PS120 cells (A, B) or PS120/NHE2 cells (C, D) permeabilized with 0.06% digitonin and stained with the Ab597 (1:100 dilution) and a FITC-conjugated anti-rabbit IgG secondary antibody.

Although N-linked glycosylation has been suggested to be important for the function of the renal epithelial brush border NHE (Yusufi et al., 1988), the fact that NHE2 lacks N-linked glycosylation calls into question the importance of N-linked glycosylation for Na^+/H^+ exchange. NHE3 is another intestinal and renal brush border isoform NHE. NHE3 has both the pharmacological and functional characteristics of the brush border NHE and has also been localized to the brush border of kidney proximal tubule cells and the brush border of intestinal villus cells (Biemesderfer et al., 1993; Bookstein et al., 1994; Hoogerwerf et al., 1994). However, this isoform lacks both N- and O-linked glycosylation (Counillon et al., 1994), further calling into question the requirement of N-linked glycosylation for Na^+/H^+ exchange activity. In fact, the only NHE isoform identified to have N-linked glycosylation is NHE1, and N-linked glycosylation is not required for Na^+/H^+ exchange catalyzed by NHE1. It was shown that deglycosylation of NHE1 with glycopeptidase F in placental brush border membrane vesicles had no effect on its Na^+/H^+ exchange activity (Haworth et al., 1993). It was also demonstrated by Counillon et al. (1994) that removal of the N-linked glycosylation sites in NHE1 by site-directed mutagenesis and expression of these N-linked glycosylation deficient mutants of NHE1 in PS120 cells did not affect the functional and pharmacological properties of NHE1. Inhibition of the extension of O-linked glycosylation with BzαGalNAc in PS120/NHE2 also did not affect the Na^+/H^+ exchange activity. Thus, no functional role for glycosylation in NHEs has been determined.

The other conclusion from the finding that NHE2 lacks N-linked glycosylation is that the N-linked glycosylation consensus sequence, which is conserved in all cloned NHEs (N^{350} in NHE2, N^{370} in NHE1, N^{325} in NHE3, and N^{342} in NHE4), is not linked to any oligosaccharides. NHE1 has two N-linked glycosylation sites located on its putative extracellular loops: N^{75} and N^{370} (Sardet et al., 1989; Tse et al., 1991). By site-directed mutagenesis, the N^{75}, but not the N^{370}, was shown to be linked to oligosaccharides. Although, it is not yet known whether NHE4 is N-glycosylated, NHE4 it unlikely to be N-glycosylated as it has only the conserved N-linked glycosylation site, N^{342}, which is not used for N-linked glycosylation of NHE1, NHE2, and NHE3.

Although there is known no consensus sequence for O-linked glycosylation, it has been suggested by Jentoft (1990) that O-linked oligosaccharides are clustered in heavily glycosylated 20-70 amino acid long domains in which serine and threonine residues constitute 25–40% of the amino acids. Upon examination of the deduced amino acid sequence of rabbit NHE2, it is found that the first putative extracellular loop of NHE2 fits this criterion. There are 12 serine and threonine residues in this putative loop of 54 amino acids (22%), and most of the serine and threonine residues are clustered in the region, T^{45}SSSPLSPASVVAPGTTAFEES^{66}, in which serine and theonine residues constitute 41% of the amino acids. NHE1 has both N- and O-linked glycosylation (Counillon et al., 1994). The first putative extracellular loop of rabbit NHE1 contains 72 amino acids, with 17 serine and theonine residues (23%). This domain of NHE1 also contains the used N-linked glycosylation consensus sequence (N^{75}RS). Interestingly, rabbit NHE3 has only two threonine residues in this putative first extracellular loop of 32 amino acids, and NHE3 is not O-glycosylated.

In conclusion, NHE2 is made up of a 85 kDa O-linked sialoglycoprotein and a 75 kDa unglycosylated protein. This study identifies that not all membrane transport proteins are N-glycosylated or closely linked to N-glycosylated proteins (such as the α-subunit of Na-K-ATPase). It is anticipated that Ab597 will be useful in elucidating the molecular and biochemical properties of NHE2, in particular, the mechanism by which growth factors regulate NHE2.

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