The Na^+/H^+ Exchanger NHE-1 Possesses N- and O-Linked Glycosylation Restricted to the First N-Terminal Extracellular Domain†

Laurent Counillon,*,‡ Jacques Pouysségur,‡ and Reinhart A. F. Reithmeier§

Centre de Biochimie—CNRS, Université de Nice, Parc Valrose, 06108 Nice, Cedex 2, France

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ABSTRACT: The ubiquitously-expressed human Na^+/H^+ exchanger (NHE-1) contains three consensus sites (Asn-X-Ser/Thr) for N-linked glycosylation at asparagines 75, 370, and 410. The first extracellular loop is rich in serine and threonine residues which may contain O-linked carbohydrate. In order to determine unambiguously the sites of glycosylation and their role in biosynthesis and cation transport, site-directed mutagenesis at the individual potential N-glycosylation sites (Asn to Asp) was performed and all possible double and triple mutants were constructed. The mutated DNAs were expressed in PS120 hamster fibroblasts lacking endogenous exchanger, and the transfected cells were selected by their ability to survive acute intracellular acidification. All constructs produced functional exchangers that had transport rates and pharmacological profiles that were similar to that of wild-type. Immunoblot analysis of the expressed proteins with and without *N*-glycosidase F treatment showed that only the first N-glycosylation site (Asn 75) is utilized. In addition, treatment of NHE-1 with neuraminidase and *O*-glycosidase demonstrated that NHE-1 also contains O-linked oligosaccharide. Two forms of NHE-1 were consistently observed, a mature form with a molecular mass of 110 000 Da which contains N-linked and O-linked oligosaccharide and is expressed at the cell surface, and a lower molecular mass form (85 000 Da) present in the endoplasmic reticulum which only contains N-linked high-mannose oligosaccharide. NHE-3, an apically-expressed epithelial isoform which does not possess the N75 N-linked putative glycosylation site and any extracellular loops enriched in serine and threonine residues, does not exhibit any detectable glycosylation.

Eukaryotic Na^+/H^+ exchangers are members of a gene family (NHE, for Na^+/H^+ exchanger) of plasma membrane proteins that catalyze the electroneutral exchange of extracellular sodium for intracellular protons. The ubiquitous NHE-1 plays an essential role in the regulation of intracellular pH and volume [for review, see Grinstein (1988)]. This exchanger is also activated by a variety of external stimuli, suggesting an important role in cellular activation [for review, see Grinstein et al. (1989)]. In epithelial cells, other Na^+/H^+ exchanger isoforms are involved in transcellular sodium and bicarbonate reabsorption and proton secretion [for review, see Counillon and Pouysségur (1993b) and Tse et al. (1993b)].

All the cloned vertebrate Na^+/H^+ isoforms are polypeptides of about 800 amino acids that consist of two distinct domains: an amino-terminal membrane domain which carries out ion transport and a cytosolic carboxy-terminal domain which carries out the hormonal regulation of these proteins (Wakabayashi et al., 1992). The overall degree of amino acid identity among the cloned isoforms is on the order of 30–60% (Tse et al., 1993b). The topology of the membrane domain is still poorly defined, but on the basis of hydropathy analysis, the polypeptide is predicted to span the membrane 12 times (Figure 1A). The human NHE-1 isoform is an 815 amino acid protein containing three consensus N-glycosylation sites (Asn-X-Ser/Thr) at Asn 75, 370, and 410, all within the membrane domain (Sardet et al., 1989). Current topological models place the first two sites to the cell exterior and the third site in the cytosol (Figure 1A). All three sites are conserved across NHE-1 molecules from various species (Sardet et al., 1989; Tse et al., 1991; Reilly et al., 1991; Orlowski et al., 1992; Counillon & Pouyssegur, 1992) including the trout cAMP-activated isoform (Borgese et al., 1992). In contrast, only the second site (Asn 370) is conserved in all isoforms (Orlowski et al., 1992; Tse et al., 1992, 1993a). There is convincing evidence that the human NHE-1 isoform is a glycoprotein. Treatment of the protein expressed in fibroblasts with *N*-glycosidase F reduces the molecular mass from 110 to 90 kDa (Sardet et al., 1990). A similar shift (105 to 94 kDa) was observed for the protein in human placental brush border membranes (Haworth et al., 1993). When expressed in insect Sf9 cells, NHE-1 migrates with an apparent molecular mass of about 85 kDa (Fafournoux et al., 1991). This suggests that the protein is not glycosylated in these cells or that it contains a small core high-mannose structure. The NHE-1 cDNA cell-free translation product which is not glycosylated also has a molecular mass of about 85 kDa (Fafournoux et al., 1991). Haworth et al. (1993) have presented results which suggest that NHE-1 is heterogeneously glycosylated at 2–3 sites. These results are based on enzymatic deglycosylation which show the presence of additional bands intermediate between the fully glycosylated protein and the enzymatically deglycosylated form. However, which of the three potential N-linked oligosaccharide sites is used remains unknown. Also noteworthy in the first predicted extracellular domain of NHE-1 is the presence of 15 serine and threonine residues,

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* To whom all correspondence should be addressed. Tel: (33) 93 52 99 24; Fax: (33) 93 52 99 17.

‡ Université de Nice.

§ Department of Medicine and Biochemistry, Room 7344, Medical Sciences Building, 1 Kings College Circle, University of Toronto, Toronto, Ontario, Canada.

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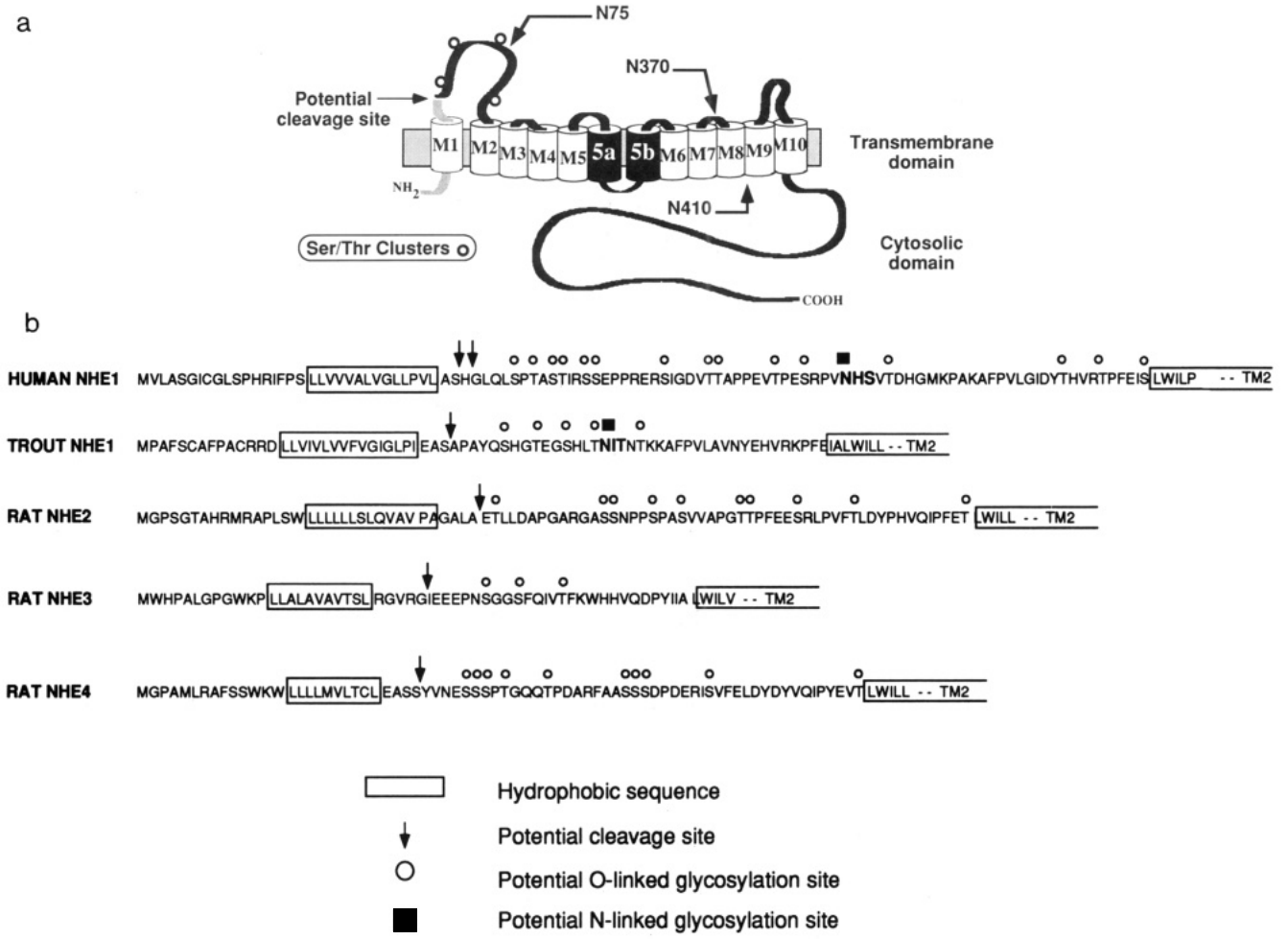

FIGURE 1: (Panel A) Folding model showing the location of consensus glycosylation sites and potential signal sequence cleavage sites in NHE-1 and other isoforms. (Panel B) Sequence conservation of the N-terminal region of the NHE-1, β NHE-1, NHE-2, NHE-3, and NHE-4 Na^+/H^+ exchanger isoforms.

many of which are clustered (Figure 1B). Clusters of serine and threonine residues in external segments are a common feature of O-glycosylation sites in membrane proteins (Jentoft, 1990). Such an arrangement of serine and threonine residues is conserved in the first extracellular domain of all NHE isoforms except trout NHE-1 and rat and rabbit NHE-3, which retain only 5, 3 (Figure 1B), and 1 potential O-glycosylation sites, respectively. In addition, there are only a few isolated serine and threonine residues in other predicted extracellular loops.

Little is known about the biological roles of oligosaccharide chains in membrane transport proteins. Glycosylation may play an important role in the biosynthesis of the protein, promoting the proper folding of the polypeptide chain [for review, see Rasmunsen (1992)]. It may also be responsible for maintaining the conformation of a protein domain required for function. Oligosaccharide chains are also known to protect proteins from proteolytic attack, to act as cell surface antigens and adhesion molecules, and to mediate cell–cell contact (Feizi, 1993). In addition, glycosylation may also be involved in targeting the protein to the cell surface or to the proper subcellular compartment. These latter roles imply that the function of the oligosaccharide may be independent of the transport function of the protein. The roles of the oligosaccharide on Na^+/H^+ exchangers are not well established. Enzymatic deglycosylation of human placental BBM vesicles containing NHE-1 has no effect on the protein transport capabilities (Haworth et al., 1993). In contrast, a similar treatment of rat renal BBM vesicles containing an apical NHE isoform, probably NHE-3, resulted in a 50% decrease in transport activity without effect on the apparent affinity of the exchanger for sodium (Yusufi et al., 1988). These experiments do not however adequately define the role of oligosaccharide chains on the antiporter since the enzymatic treatment results in global deglycosylation of multiple membrane components that could indirectly affect transport.

In order to determine the positions of the N-linked oligosaccharide and the role of glycosylation in antiporter synthesis and function, we have used here an approach combining both enzymatic deglycosylation and biochemical characterization of NHE-1 molecules mutated on their N-glycosylation consensus sites.

MATERIALS AND METHODS

Site-Directed Mutagenesis. A human NHE-1 cDNA deleted of its 5′ noncoding region and cloned into pECE mammalian expression vector (Δ5pEAP) was used for the site-directed mutagenesis studies (Wakabayashi et al., 1992). A 1.1-kb *Hin*dIII–*Sac*I restriction fragment of Δ5pEAP containing the first glycosylation site (Asn 75) of the exchanger and a 0.6-kb *Acc*I–*Acc*I fragment containing the codons (Asn 370 and 410) for last two glycosylation sites were subcloned into the PTZ 18 vector (Bio-Rad). Site-directed mutagenesis was performed on single-stranded plasmids according to Kunkel (1985). The following oligonucleotides containing the point mutations (in **bold**) in codons (underlined) were

purchased from Eurogentec (Liège, Belgium): N75D (coding): 5′-CGCCCTGTTGATCATTCCGTC-3′; N370D (anticoding): 5′-GTGGGAGATGTCGGCCTCCAC-3′; N410D (anticoding): 5′-CGAAGGTCCAGTCCCAGTGGTGG-3′.

Mutagenized cDNA fragments in PTZ18 were confirmed by sequencing (Sanger et al., 1977). The Δ5pEAP vector was modified (pEAPK) to remove the bulk of the 3′ noncoding region and multiple restriction enzyme sites (e.g., *Acc*I) in the polylinker region. A synthetic oligonucleotide linker (5′-CTAGAGGTACCT-3′) containing an internal *Kpn*I site was used to produce a deletion from the *Kpn*I site within the 3′ noncoding cDNA to the end of the polylinker. The mutated *Hin*dIII–*Sac*I and *Acc*I–*Acc*I fragments were excised from PTZ18 and introduced into pEAPK by restriction enzyme cutting and religation. The presence of the mutations in the plasmid DNA to be used for transfection was confirmed by sequencing.

Transfection and Selection of Cells. Mutant Chinese hamster lung fibroblasts (PS120 cell line) devoid of Na^+/H^+ exchange activity (Pouysségur et al., 1984) were grown in 100-mm Petri dishes and transfected with 20 μg of DNA using the calcium phosphate precipitation method (Wigler et al., 1979). Positive transfectants were selected by the ability of the cells to survive a 1-h intracellular acidification induced by NH_4^+ cell loading. The intracellular acidification kills all nontransfected cells but allows cells expressing a functional Na^+/H^+ exchanger to survive. This proton suicide assay was repeated twice a week until a cell population of stable transfectants resistant to intracellular acidification was produced.

As an alternative method of selection, PS120 cells expressing a functional exchanger were obtained by cotransfection of antiporter DNA with a plasmid encoding a gene for neomycin resistance. In this case 18 μg of antiporter DNA and 2 μg of pcDNAneo (Invitrogen) plasmid were transfected into PS120 cells which were then grown in the presence of 400 μg/mL neomycin (G418). The ability of neomycin-resistant cells to withstand proton suicide was tested by plating 10^4 cells in 60-mm dishes and performing 3 proton suicide selections beginning 2 days after plating, followed by counting colonies.

Inhibition of Initial Rates of $^{22}Na^+$ Uptake by Amiloride. Transfected PS 120 cells (10^5) were plated into 24-well dishes and grown to confluence. Cells were incubated for 1 h in 15 mM Hepes, pH 7.5, 50 mM NH_4Cl, and 70 mM choline chloride. Cells were washed twice with choline chloride buffer. Transport was initiated by incubating cells at 37 °C in uptake media: 120 mM choline chloride, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4, 1 mM ouabain, and 1 μCi/mL carrier-free ^{22}Na. In order to determine the pharmacological profiles of Na^+ transport by the mutated NHE-1 molecules, increasing amounts of amiloride (a competitive inhibitor) were added to the uptake media, and the initial rates of $^{22}Na^+$ uptake were followed. Influx of sodium was stopped by rinsing the cell monolayers four times with ice-cold phosphate-buffered saline. Cells were solubilized in 1 mL of 0.1 N NaOH, and radioactivity was determined by γ counting.

Membrane Preparation and Immunodetection of NHE-1. All steps were carried out at 0–4 °C unless stated otherwise. Confluent cells were washed three times with phosphate-buffered saline and scraped from the plates using 1 mL of lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and the following protease inhibitors: 0.1 mM PMSF, 1 mM *o*-phenanthroline, and 1 mM iodoacetamide). Cells were lysed in the same buffer by passage through a 27-gauge needle. Unbroken cells and nuclei were removed by centrifugation at 500*g*, 10 min. The membrane fraction was then collected by centrifugation of the supernatant at 100000*g* for 30 min. The pellet was resuspended in 100 μL of lysis buffer, and the protein concentration was determined using the Pierce bicinchoninic acid (BCA) assay with bovine serum albumin as the standard.

Protease Treatment. To test for cell surface expression of the antiporter, confluent cells were treated with various proteases [trypsin (1.5 mg/mL), chymotrypsin (1 mg/mL), and proteinase K (1 mg/mL)] at 37 °C for 15 min. After inhibition of the proteases with 0.1 mM PMSF, the cells were scraped from the dishes and recovered by centrifugation (1000*g*, 10 min) followed by washing in ice-cold phosphate-buffered saline. Cells were suspended in lysis buffer, and membranes were prepared as described above.

Enzymatic Deglycosylation. Membranes samples (20–50 μg of protein) diluted in lysis buffer to 1–2 mg of protein/mL were boiled in 0.5% sodium dodecyl sulfate and 1% 2-mercaptoethanol for 3 min followed by addition of NP40 to a final concentration of 1% and sodium phosphate, pH 7.5, to 50 mM. *N*-Glycosidase F (New England Biolabs) was added at 0.2 IUB mU/μg of protein, and the sample was incubated at 37 °C for 1 h. For Endo H digestion, samples were denaturated as described above, and sodium citrate (pH 5.5) was then added to a final concentration of 50 mM. Endo H (New England Biolabs) was added at 2.5 IUB mU/μg of protein, and the samples were incubated overnight at 37 °C. For *O*-glycanase treatment, samples were treated with 0.5 mU of neuraminidase (Boehringer Mannheim)/μg of protein and with 0.05 mU of *O*-glycanase (Boehringer Mannheim)/μg of protein at 37 °C for 1 h or overnight.

Western Blotting of Protein Samples. Samples were dissolved in Laemmli sample buffer, and the proteins (20–50 μg) were resolved by SDS minigel electrophoresis (Bio-Rad) and transferred to nitrocellulose (Hybond C, Amersham) in 20% methanol, 25 mM Tris, and 0.19 M glycine. The blot was probed in 150 mM NaCl and 50 mM Tris-HCl, pH 8.0, containing 5% nonfat milk powder with a 1/500 dilution of a polyclonal rabbit antibody (RP-C28) raised against a fusion protein encoding residues 658 to the carboxyl terminus of the protein (Sardet et al., 1990). After washing with 0.1% Triton X-100 in 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0, the blot was incubated with peroxidase-coupled goat anti-rabbit IgG antibody at a 1/1000 dilution. Blots were again washed and developed using the enhanced chemiluminescence system (ECL, Amersham). Prestained molecular mass markers were purchased from Bio-Rad (110 kDa: phosphorylase B; 80 kDa: bovine serum albumin).

RESULTS

A topological profile of human NHE-1 and the location and conservation of the three consensus N-glycosylation sites (Asn-X-Ser/Thr) is illustrated in Figure 1A. Single or in combination site-directed mutants (Asn to Asp) of the glycosylation sites were constructed to prevent glycosylation at particular acceptor sites. All mutant cDNAs were expressed in antiporter-devoid fibroblasts (PS120 cells) which were subjected to intracellular acidification. The cells that were resistant to this selection procedure expressed functional Na^+/H^+ exchange activity. Under these conditions, 100% of the mock transfected cells (cells transfected with 20 μg of salmon sperm DNA) were killed. All mutant cDNAs, when expressed in PS120 cells, produced functional transporters, despite the elimination of one or all glycosylation sites.

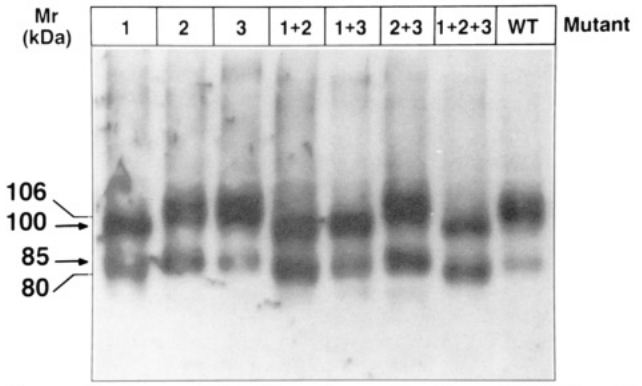

FIGURE 2: Immunoblot of membrane fractions of PS120 cells transfected with wild-type or mutant NHE-1 cDNAs. Membrane protein samples were separated on 10% SDS–PAGE gels, transfered to nitrocellulose (Amersham), and revealed with the NHE-1-specific RPc28 antibody as described in Materials and Methods. In the figure, mutation 1 represents the N75D substitution, mutation 2 the N370D substitution, and mutation 3 the N410D substitution.

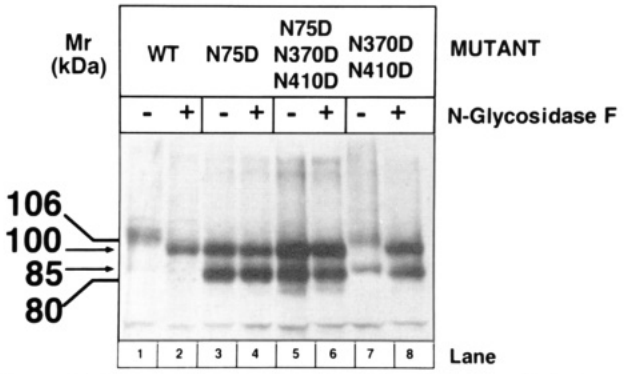

FIGURE 3: Immunoblot showing the effect of enzymatic deglycosylation (*N*-glycosidase F) on the mobility of wild-type and mutant NHE-1 proteins. Membrane preparations obtained from PS120 cells transfected with cDNAs encoding the wild-type NHE-1 and the N75D, N370D-N410D, and N75D-N370D-N410D mutants were submitted to *N*-glycosidase F digestion prior to SDS–PAGE electrophoresis and Western blotting, as described in Materials and Methods.

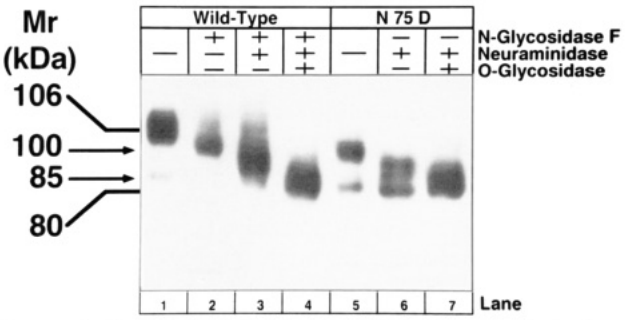

FIGURE 4: Immunoblot showing the effect of enzymatic deglycosylation (*N*-glycosidase F, neuraminidase, *O*-glycosidase) on wild-type and mutant NHE-1 proteins. Membrane preparations obtained from PS120 cells transfected with cDNAs encoding the wild-type NHE-1 and the N75D mutant were submitted to *N*-glycosidase F, neuraminidase, and *O*-glycosidase digestion, as described in Materials and Methods. Proteins were separated on SDS–PAGE, transferred to nitrocellulose membrane, and revealed as described in Materials and Methods.

The calculated molecular mass of NHE-1 based on its amino acid sequence is approximately 91 kDa. In order to examine the mobility of wild-type NHE-1 and of the different mutants on SDS–PAGE, we performed immunoblots of crude membrane fractions prepared from PS120 fibroblasts transfected with wild-type or mutant cDNAs. The Na^+/H^+ exchanger migrates as a diffuse band with an apparent molecular mass of 110 000 Da (Figure 2, lane WT). Mutation of Asn 75 (N75D) resulted in the production of a protein band with a lower molecular mass (100 000 Da) than the wild-type protein (Figure 2, lane 1). Also present in most membrane preparations is a lower molecular mass band (85 000 Da). The intensity of this band varied from preparation to preparation. The nature of this band is discussed later in the article. Mutation of either Asn 370 (N370D) or 410 (N410D) produced a 110 000-Da band as for the wild-type protein (Figure 2, lanes 2 and 3). The shift in the mobility observed only in the presence of the D75N mutation (compare lane 1 with lane WT) strongly suggests that the Na^+/H^+ exchanger is glycosylated solely at this position. An alternative explanation would be that the introduction of such a negative charge (Asp for Asn) could drastically change the mobility of the mutated protein on SDS gels, but this seems unlikely since similar mutations introduced at the other glycosylation consensus sites do not produce the same effect. The lack of mobility shift with the N370D and N410D mutants suggests that the protein either is not glycosylated at these positions or contains a small oligosaccharide chain whose absence is not detected by changes in mobility upon SDS gel electrophoresis.

Further evidence to suggest that Asn 75 is the only site of glycosylation comes from the double and triple mutants. The N75D mutant in combination with mutation at either Asn 370 or 410 or with both Asn 370 and 410 in the triple mutant gave a 100-kDa band identical to that of the N75D mutant (Figure 2). In contrast, the double N370D + N410D mutant gave a 110-kDa band similar to that of wild type (Figure 2).

To confirm the glycosylation status of the various mutants, the isolated membrane fractions from PS120 cells expressing the various NHE-1 constructs were solubilized in SDS and subjected to digestion with *N*-glycosidase F. This enzyme cleaves the entire oligosaccharide chain from N-linked glycoproteins, leaving an aspartate in place of the original asparagine. The enzymatically deglycosylated protein will therefore have the same amino acid at the glycosylation site as the mutants. *N*-Glycosidase F digestion increased the mobility of the wild-type protein to 100 000 Da, the same molecular mass as for the N75D mutants (Figure 3, lane 2). As expected, *N*-glycosidase F treatment had no effect on the mobility of the triple mutant (Figure 3, lane 6). Importantly, enzymatic deglycosylation had no effect on the mobility of the two bands of the N75D mutant (Figure 3, lane 4). In contrast, *N*-glycosidase F treatment resulted in a shift in the mobility of the N370D + N410D mutant which retains the Asn 75 site (Figure 3, lane 8). For the wild-type and all the mutants possessing an intact N75 glycosylation site, *N*-glycosidase F treatment also shifted the mobility of the lower 85 000-Da band to the same molecular mass (82 000 Da) as that of the N75D mutant (Figure 3), showing that this form also contains N-linked oligosaccharide.

Treatment of NHE-1 with *N*-glycanase followed by neuraminidase and *O*-glycanase results in a further shift in the mobilty of the protein to the position of the lowest molecular mass form (82 000 Da) (Figure 4, lanes 1–4). This shows that in addition to N-linked oligosaccharide the NHE-1 contains O-linked sugars. Neuraminidase and *O*-glycanase treatment of the N75D mutant lacking the N-linked sugar also resulted in a shift to the same lower molecular mass (Figure 4, lanes 5–7). This indicates that N-glycosylation is not a prerequisite for O-glycosylation.

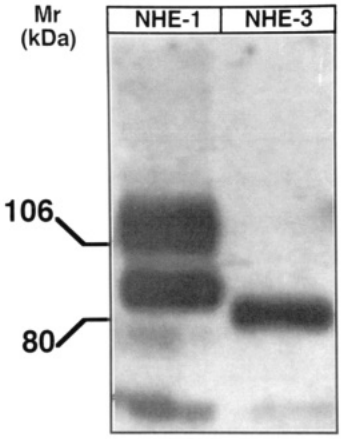

FIGURE 5: Immunoblot showing the difference in electrophoretic mobility between NHE-1 and NHE-3. An epitope-tagged rat NHE-3 cDNA (VSVG tag) was expressed in PS120 fibroblasts. Membrane proteins from NHE-1 and NHE-3/VSVG-transfected cells were separated on SDS–PAGE and blotted onto nitrocellulose. The blot was hybridized first with the NHE-1-specific RPc28 antibody. After revelation, the blot was washed extensively and rehybridized with the anti-VSVG serum in order to specifically reveal the epitope-tagged NHE-3 protein.

NHE-3 contains only the second highly conserved consensus glycosylation site (Asn 370 in NHE-1) and, in light of the above-mentioned results, was therefore expected not to be N-glycosylated. Interestingly, rat NHE-3 runs as a sharp band with an apparent molecular mass of 82 000 Da on SDS gels (Figure 5). The mobility of NHE-3 on SDS gels is similar to the mobility of the fully deglycosylated NHE-1. The nucleotide sequence of human NHE-1 cDNA predicts a protein of 815 amino acids with a molecular mass of 90 704 Da while rat NHE-3 is 831 amino acids in length with a predicted molecular mass of 92 997 Da. Treatment of rat NHE-3 with *N*-glycosidase F, neuraminidase, and *O*-glycanase failed to change the rate of migration of the protein on SDS gels (data not shown). This suggests that NHE-3 contains little if any N- or O-linked oligosaccharide. Furthermore, preliminary experiments suggest that trout βNHE-1 contains primarily N-linked oligosaccharide, but no O-linked oligosaccharide (M. Malapert, F. Borgese, J. Pouysségur, and R. Motais, unpublished results). It is important to note that NHE-3 and the trout βNHE-1 isoform lack the highly enriched clusters of serine and threonine residues found in the first loop of other NHE-1 isoforms (Figure 1B). Moreover, NHE-2, which possesses clusters of serines and threonine but no N-linked glycosylation site in its first extracellular loop, appears to be O-glycosylated but not N-glycosylated (M. Tse, and M. Donowitz, unpublished results).

In order to investigate the nature and the cellular localization of the 110- and 85-kDa forms of antiporters detected in cells expressing NHE-1, we decided to perform cell surface expression assays by the use of externally-applied proteases. Treatment of PS120 cells expressing NHE-1 with trypsin, chymotrypsin, or proteinase K resulted in digestion of essentially all of the 110-kDa band while the lower molecular mass band remained intact (data not shown). This confirms that the 110-kDa form of NHE-1 is present at the cell surface and probably represents the mature form of the polypeptide. The lower molecular mass band, resistant to digestion, is not exposed to the cell surface and may reside within the cell. This is consistent with observations obtained from enzymatic deglycosylation of NHE-1: The 110-kDa band is resistant to endoglycosidase H, while the molecular weight of the 85-kDa band is reduced upon endoglycosidase H digestion (Figure 6).

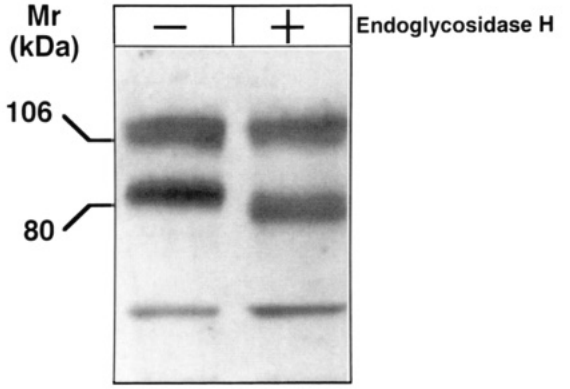

FIGURE 6: Immunoblot showing the effect of endoglycosidase H digestion on the wild-type NHE-1. Membrane preparations obtained from PS120 cells expressing NHE-1 were submitted to endoglycosidase H digestion as described in Materials and Methods. Proteins were separated on SDS–PAGE, transferred to nitrocellulose membrane, and revealed as described.

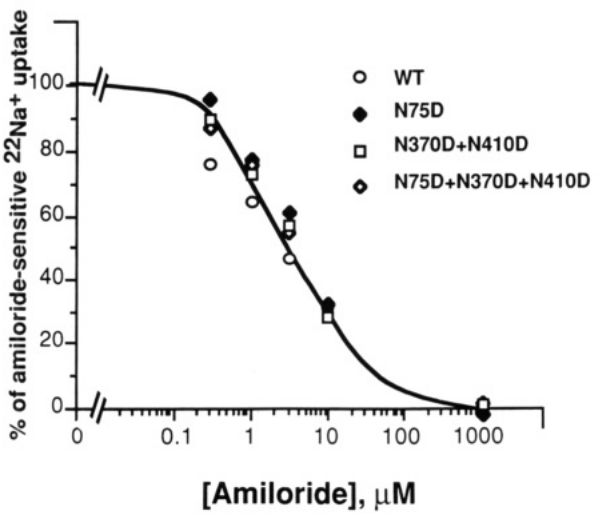

FIGURE 7: Amiloride sensitivities of wild-type and NHE-1 mutants expressed in PS120 cells. Inhibition of amiloride-sensitive $^{22}Na^+$ influx initial rates was determined in PS120 cells expressing the NHE-1 mutants in the presence of increasing concentrations of amiloride, as described in Materials and Methods.

This result indicates that the 85-kDa band is an internal form of the antiporter which contains only a high-mannose structure and, therefore, resides in the endoplasmic reticulum.

In order to determine if the removal of the glycosylation sites may have affected the function or the biosynthesis of NHE-1, the activity of the mutated antiporters was assayed by performing both pharmacological experiments and Na^+/H^+ exchange activity measurements. The sensitivities of the various mutants to inhibition by amiloride or *N*5-(methylpropyl)amiloride, as measured by dose-dependent inhibition of amiloride-sensitive initial rates of $^{22}Na^+$ uptake (Figure 7), were similar to those of the wild-type protein. These results show that removal of one or all of the consensus sites for N-linked glycosylation does not affect the sensitivity of the exchanger to inhibition by amiloride or an amiloride analogue. Initial rates of $^{22}Na^+$ uptake measurements were also performed on the transfected cells, and these results showed that both wild-type NHE-1 and all mutants have a similar rate of sodium uptake. In order to use an alternative method of selection which would not impose any threshold to the Na^+/H^+ exchange activities of the NHE-1 mutants prior to the functional assays, cells cotransfected with NHE-1 mutants and a neomycin resistance marker were selected in the presence

of G418. To determine the Na^+/H^+ exchange activity of the various mutants, the positive transfectants obtained using this protocol were then assayed for their ability to survive an acute intracellular acidification. Under these acid-loading conditions, a functional Na^+/H^+ exchanger is necessary and sufficient to protect the cells against the otherwise lethal acidification (Pouysségur, 1985), and therefore, the determination of the number of cells surviving this selection procedure provides a very sensitive test for monitoring the Na^+/H^+ exchange activity. Any neomycin-resistant cells that express a functional antiporter will survive this proton suicide selection. The triple glycosylation mutant gave comparable yields of proton suicide-resistant colonies as the wild-type. A comparable yield of H^+ suicide-surviving colonies in PS120 cells expressing wild-type and mutated antiporters suggests that the mutations do not significantly impair the cell surface expression and function of NHE-1.

DISCUSSION

In addition to the investigation of the possible functional importance of protein glycosylation, the identification of glycosylation sites within membrane proteins can be used to investigate the topology of the protein in the membrane. The Na^+/H^+ exchanger contains a major site of glycosylation at Asn 75 which is present within a 70 amino acid hydrophilic sequence. We can therefore unambiguously establish that this loop is extracellular. In addition, inspection of the amino acid sequence of this extracellular loop reveals the presence of clusters of serine and threonine residues which probably serve as acceptor sites for O-linked carbohydrate (Figure 1B). It is well established that the carboxy-terminal domain of the antiporter is cytosolic (Sardet et al., 1990); therefore, the polypeptide deduced from the cDNA sequence is predicted to span the membrane an even number of times. In addition, the amino-terminal region of the antiporter (Figure 1) has features of a signal sequence based on the the von Heijne rules (von Heijne, 1983, 1986). If such a signal peptide cleavage occurs, the new N-terminal end of the mature protein will then be extracellular, and the mature polypeptide will therefore span the membrane an odd number of times. Hence, the exact topology of NHE-1 remains to be further investigated.

Our results indicate that the consensus glycosylation site at Asn 370 which is absolutely conserved among the NHE isoforms is not used or if used contains only a very small oligosaccharide chain. This suggests that utilized sites of N-linked glycosylation are restricted to a single extracellular loop within NHE-1. This restriction may be a general feature of multispan membrane proteins (Landolt-Marticorena & Reithmeier, 1994). The isoforms of the protein (NHE-2,3,4; Tse et al., 1992, 1993a; Orlowski et al., 1992) which contain only this single consensus glycosylation site are therefore predicted not to be N-glycosylated. The lack of change in the mobility of NHE-3 upon treament with *N*-glycosidase F is consistent with this conclusion. It is possible that the glycosylation pattern of the Na^+/H^+ exchanger varies with cell type and that this site is used in epithelial cells but not in fibroblasts like PS120. However, NHE-1 expressed in epithelial OK or MDCK cells comigrates with NHE-1 in PS120 cells on SDS gels, suggesting that the protein has a similar glycosylation pattern in these epithelial cells (Helmle-Kolb et al., 1993; Noël, Roux, and Pouysségur, unpublished results). Haworth et al. (1993) have reported the presence of multiple bands upon enzymatic deglycosylation of NHE-1, suggesting that NHE-1, when expressed in placenta, contains multiple glycosylation sites. These reported multiple bands may be explained by the presence of O-linked carbohydrate, since *N*-glycosidase treatment of NHE-1 produces a protein which still contains O-linked oligosaccharide. In our studies, neuraminidase treatment of protein lacking N-linked oligosaccharide resulted in a further shift in molecular mass that was completed by *O*-glycanase treatment. These observations clearly indicate that the NHE-1 form is indeed O-glycosylated.

Membrane preparations from NHE-1-transfected PS120 cells often contained a lower molecular mass form (85 kDa) of the antiporter which is endoglycosidase H-sensitive and resistant to external proteolytic treatment of intact cells. By contrast, the higher molecular mass form of the protein is sensitive to proteolytic attack, indicating that it is expressed at the cell surface, and its mobility can be increased by *O*-glycanase treatment. As O-glycosylation occurs in the trans-Golgi, the lack of cell surface expression of the lower molecular mass form is consistent with its presence in a pre-trans-Golgi compartment. In addition, these results suggest that O-glycosylation of NHE-1 occurs prior to the cell surface expression of the antiporter. By the use of *N*-glycosidase and endoglycosidase H, it was possible to show that the the lower molecular mass form, which lacks O-linked sugars, contains a small N-linked oligosaccharide which has a high-mannose structure. We therefore conclude that the lower molecular mass form represents an internal precursor of the mature protein, containing N-linked oligosaccharide and deficient in O-linked carbohydrate. The endoglycosidase H sensitivity of this precursor localizes it in the endoplasmic reticulum.

The topological arrangement of the Na^+/H^+ exchanger deduced from its hydropathy plot is remarkably similar to the proposed topology of the Na^+/Ca^{2+} exchanger (Nicoll et al., 1990; Hryshko et al., 1993). This protein is produced with a cleavable signal sequence, and the first extracellular domain contains the only site of N-linked glycosylation in the protein. In neither of these proteins are the oligosaccharide chains required for transport function. Similarly, neither the single site of glycosylation of the Na^+-dependent glucose carrier (Hirayama & Wright, 1992) nor that of Band 3, the erythrocyte anion exchanger (Casey et al., 1992), is required for transport activity. In agreement with our mutagenesis studies, Haworth et al. have shown that enzymatic deglycosylation of NHE-1 has no effect on Na^+/H^+ exchange activity (Haworth et al., 1993). This is in contrast to a study of rat renal BBM vesicles where enzymatic deglycosylation of N-linked oligosaccharide was shown to reduce the V_{max} of transport by 50% (Yusufi et al., 1988). These authors also report a decrease in exchanger activity in BBM vesicles isolated from rats treated with swainsonine, an inhibitor of α-mannosidase II. These results may be due to indirect effects since all glycoproteins present in the BBM, not only the antiporter, would be affected by deglycosylation and the drug treatment. The observation that NHE-3, which is the most abundant isoform expressed in rat kidney cortex (Orlowski et al., 1992), is not glycosylated supports our view that the inhibition of transport is indirect.

In conclusion, we have shown that the NHE-1 isoform of the Na^+/H^+ exchanger contains both N-linked and O-linked oligosaccharide. Although N-linked oligosaccharides appear to be a common feature of transmembrane transporters, the discovery of O-linked oligosaccharides was more unexpected. The investigation of O-linked glycosylation among transporters constitutes an area worthy of further investigation. Mutagenesis studies have shown that the elimination of the N-glycosylation site does not significantly impair the function

and the biosynthesis of this transporter. In addition, lack of a direct role for glycosylation in the transport process is highlighted by the observation that the NHE-3 isoform is most likely devoid of N- and O- linked carbohydrates. Taken together with several results obtained from studies on other transmembrane transporters, the data suggest that we must look beyond substrate translocation in our search for the functional importance of glycosylation in transport proteins.

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