The Yeast Na+/H+ Exchanger Nhx1 Is an N-Linked Glycoprotein

TOPOLOGICAL IMPLICATIONS*

Received for publication, March 1, 2000, and in revised form, August 30, 2000

Karen M. Wells and Rajini Rao‡

From the Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Nhx1, the endosomal Na+/H+ exchanger of Saccharomyces cerevisiae represents the founding member of a newly emerging subfamily of intracellular Na+/H+ exchangers. These proteins share significantly greater sequence homology to one another than to members of the mammalian Na+/H+ exchanger (NHE) family encoding plasma membrane Na+/H+ exchangers. Members of both subtypes are predicted to share a common organization, with an N-terminal transporter domain of transmembrane helices followed by a C-terminal hydrophilic tail. In the present study, we show that Nhx1 is an asparagine-linked glycoprotein and that the sites of glycosylation map to two residues within the C-terminal stretch of the polypeptide. This is the first evidence, to date, for glycosylation of the C-terminal region of any known NHE isoform. Importantly, the mapping of N-linked glycosylation to the C-terminal domain of Nhx1 is indicative of an unexpected membrane topology, particularly with regard to the orientation of the tail region. Although one recent study demonstrated that certain epitopes in the C-terminal domain of NHE3 were accessible from the exoplasmic side of the plasma membrane (Biemesderfer, D., DeGray, B., and Aronson, P. S. (1998) J. Biol. Chem. 273, 12391–12396), numerous other studies implicate a cytosolic disposition for the hydrophilic C-terminal tail of plasma membrane NHE isoforms. Our analysis of the glycosylation of Nhx1 is strongly indicative of residence of at least some portion of the hydrophilic tail domain within the endosomal lumen. These findings imply that the organization of the tail domain may be more complex than previously assumed.

Mammalian Na+/H+ exchangers (NHE)† are a ubiquitous family of transmembrane proteins that catalyze the antiport of Na+ and H+ at the plasma membrane. Under physiological conditions, NHE drive H+ out of the cell by coupling to the Na+ gradient, which is generated and maintained by the activity of the Na+/K+-ATPase. NHE are implicated in a variety of important physiological functions, including intracellular pH regulation, cell volume control, and Na+ homeostasis. Hydropathy analysis of the NHE1 isoform predicts that 10 to 12 transmembrane helices comprise the N terminus and that the C terminus constitutes a large cytoplasmic domain (1). This structure delineates function; the N-terminal domain, which is highly conserved among the various NHE isoforms, confers transport activity of the exchanger, whereas the considerably divergent C-terminal tail corresponds to a regulatory domain (2, 3). Numerous studies have shown that NHE isoforms can be post-translationally modified in a variety of ways. The cytosolic C-terminal tail domain modulates exchanger activity by becoming phosphorylated in response to extracellular signals like growth hormones (4) and through association with a variety of regulatory molecules, such as Ca2+/calmodulin (5). Some NHE isoforms are glycosylated in extracellular loops between transmembrane segments, although the functional significance of such modifications is unclear (6, 7).

Over the last several years, phenomenological evidence of intracellular Na+/H+ exchange has been documented in a variety of mammalian tissues (6–10), and in each case this activity colocalized with V-type H+-ATPase activity, suggesting an endosomal residence for Na+/H+ exchange. Our laboratory recently identified and cloned a Saccharomyces cerevisiae homologue of mammalian NHE, called Nhx1 (11), which represents the founding member of a unique and growing subfamily of intracellular Na+/H+ exchangers, including proteins identified in Schizosaccharomyces pombes, Caenorhabditis elegans, Arabidopsis thaliana, and humans. Nhx1 localizes to a late endosomal/prevacuolar compartment where it mediates intracellular sequestration of Na+ in a pH-dependent manner (11, 12), coupling Na+ movement to the proton gradient established by the vacuolar H+-ATPase (13, 34). Hydropathy analysis of Nhx1 reveals a domain structure similar to NHE isoforms, suggesting that the structure/function relationship for intracellular exchangers may be homologous to the plasma membrane-type antipoters. Here, we take a first step toward understanding how Nhx1 is regulated, specifically by investigating post-translational modification of the protein. We have determined that Nhx1 is an N-linked glycosylated protein and have shown that these glycosylations uniquely map to residues in the C-terminal hydrophilic tail portion of the exchanger. The presence of glycosylated asparagines in the C terminus predicts that at least some portion of the Nhx1 tail is exposed to the lumen of the late endosomal compartment. An earlier report demonstrated that epitopes within the NHE3 C-terminal tail were exposed to the exoplasmic side of the membrane (31), although there is no evidence for glycosylation of the tail region of NHE3 or any other NHE isoform. Taken together with the results of our study, these findings support an unpredicted topological arrangement for the C-terminal region of Na+/H+ exchangers that warrants further study.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Assays—R100, the Nhx1 null strain (Δnhx1), is isogenic to W303 and has been described previously

*This work was supported by an American Health Association Maryland Affiliate post-doctoral fellowship award (to K. W.) and by Grants GM52414 and DK54214 from the National Institutes of Health (to R. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Physiology, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-955-4732; Fax: 410-955-0461; E-mail: rrao@jhmi.edu.

The abbreviations used are: NHE, Na+/H+ exchangers; HA, hemagglutinin; PCR, polymerase chain reaction; Eno H, endoglycosidase H; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethane-sulfonic acid.

This paper is available on line at http://www.jbc.org
Nhx1 Is an N-Linked Glycoprotein

Nhx1 is an N-linked glycoprotein. Western blots of HA-tagged Nhxl are shown. A, Endo H treatment. Membranes (50 μg) pooled from sucrose gradients, as described under “Experimental Procedures,” were from left to right, untreated, incubated in reaction buffer alone (Mock), or incubated in reaction buffer plus 1 unit/mg of Endo H (Endo H). The positions of molecular weight markers are shown at the extreme left; the arrow to the right indicates disappearance of the upper set of bands. B, tunicamycin treatment. 10 μg/ml tunicamycin (Tu) was added to yeast cultures (W303/pRin73), and aliquots were removed at the indicated times. The cleared lysates were prepared, and 150 μg of protein was loaded per lane. The arrow to the right indicates the upper set of bands.

RESULTS

Nhxl Is an N-Linked Glycosylated Protein—Nhxl exhibits a consistently multi-handed appearance upon gel electrophoresis, strongly suggestive of post-translational modification of the N-linked region (12). To determine whether Nhxl was N-glycosylated, a combination of in vivo and in vitro biochemical and genetic approaches were employed. Membrane fractions enriched for Nhxl (see “Experimental Procedures”) were treated with endoglycosidase H, an enzyme that cleaves high-mannose complex-type N-linked glycosylations (21, 22). As shown in Fig. 1A,

FIG. 1. Nhxl is an N-linked glycosylated protein. Western blots of HA-tagged Nhxl are shown. A, Endo H treatment. Membranes (50 μg) pooled from sucrose gradients, as described under “Experimental Procedures,” were from left to right, untreated, incubated in reaction buffer alone (Mock), or incubated in reaction buffer plus 1 unit/mg of Endo H (Endo H). The positions of molecular weight markers are shown at the extreme left; the arrow to the right indicates disappearance of the upper set of bands. B, tunicamycin treatment. 10 μg/ml tunicamycin (Tu) was added to yeast cultures (W303/pRin73), and aliquots were removed at the indicated times. The cleared lysates were prepared, and 150 μg of protein was loaded per lane. The arrow to the right indicates the upper set of bands.

FIG. 2. Expression of Nhxl in temperature-sensitive sec mutant strains. Western blots of HA-tagged Nhxl are shown; arrows on the left indicate the position of Nhxl. NHX1::HA (CN, heat shock promoter) was transformed into sec18 (A) and sec7 (B) mutant strains. Cultures were grown to an A600 value of ~1, then divided, and allowed to grow for an additional 2 h at either permissive (25 °C) or nonpermissive (37 °C) temperatures. Total membranes were prepared, and 50 μg of protein was loaded in each lane. C, NHX1::HA (pRin73) was transformed into a sec53 mutant strain. The procedure was as above, except 70 μg of total protein was loaded in each lane.

spheroplasts using yeast lytic enzyme (10 mg/600 OD units; ICN) for 45 min at 37 °C. Spheroplasts were suspended in 3–6 ml of lysis buffer (0.3 m sorbitol, 20 mM triethanolamine, pH 7.2, 1 mM EDTA, and protease inhibitors) and homogenized with ~60 strokes of a Wheaton A Dounce homogenizer. The cleared lysate (~3 ml) was layered on top of centrifuged through (2 h, 27,000 rpm, Beckman SW28 rotor, 4 °C) a 10-step sucrose gradient (3 ml each of 18–54% (w/w) increments). Fractions were collected from the top, pooled, and centrifuged for 50 μl of buffer (0.5 M sucrose, 10 mM MES/ROH, pH 6, 150 mM KCl) plus protease inhibitors. 30–50 μg of total protein was denatured for 10 min at 60 °C in 0.5% sodium dodecyl sulfate and 1% β-mercaptoethanol, then diluted into 50 mM sodium citrate buffer, pH 5.5, and incubated either with or without Endo H at 37 °C for 18 h, as specified in legends to figures. Following treatments, sample preparations were incubated in 10% (v/v) trichloroacetic acid and centrifuged for 30 min at 4 °C, and pellets were resuspended in sample buffer prior to loading on gels.

For treatments with tunicamycin, 20–200-ml cultures were grown overnight at 30 °C to an A600 value of ~1, divided into two, and grown for two more hours following addition of tunicamycin (10 μg/ml final concentration) or an equal volume of carrier (5 μl NaOH). Aliquots (10–40 ml) were removed at indicated times, and total membranes were prepared by a glass bead method (18). Briefly, cells from each aliquot were harvested and resuspended in ~200 μl of bead buffer (10 mM Tris/HCl, pH 7.4, 0.3 M sorbitol, 0.1 mM NaCl, 5 mM MgCl2) plus protease inhibitors. Acid-washed glass beads were added to the minicoccus, and samples were vortexed 3 times for 1 min. Lysates were washed 3 times with 1 ml of bead buffer and, using a drawn-out Pasteur pipette, removed to new tubes. Cleared lysates were centrifuged for 1 h at 45,000 rpm (Beckman Ti50 rotor, 4 °C), and final pellets were resuspended in ~30 μl of 20 mM K-Hepes, pH 7.4, plus protease inhibitors. Samples (30–100 μg) were diluted in sample buffer prior to loading on gels.

Protein concentrations were determined by the method of Lowry et al. (19) for total membrane preparations and by a modified Lowry method (20) for fractionation and pooled preparations. SDS-PAGE and Western blotting were as described previously (12). Antibodies used were mouse anti-HA monoclonal antibody, 12CA5 (Roche Molecular Biochemicals) at 1:5,000, and horseradish peroxidase-coupled sheep anti-mouse antibody (Amersham Pharmacia Biotech) at 1:10,000.

RESULTS

Nhxl Is an N-Linked Glycosylated Protein—Nhxl exhibits a consistently multi-handed appearance upon gel electrophoresis, strongly suggestive of post-translational modification of the N-linked region (12). To determine whether Nhxl was N-glycosylated, a combination of in vivo and in vitro biochemical and genetic approaches were employed. Membrane fractions enriched for Nhxl (see “Experimental Procedures”) were treated with endoglycosidase H, an enzyme that cleaves high-mannose complex-type N-linked glycosylations (21, 22). As shown in Fig. 1A,

FIG. 2. Expression of Nhxl in temperature-sensitive sec mutant strains. Western blots of HA-tagged Nhxl are shown; arrows on the left indicate the position of Nhxl. NHX1::HA (CN, heat shock promoter) was transformed into sec18 (A) and sec7 (B) mutant strains. Cultures were grown to an A600 value of ~1, then divided, and allowed to grow for an additional 2 h at either permissive (25 °C) or nonpermissive (37 °C) temperatures. Total membranes were prepared, and 50 μg of protein was loaded in each lane. C, NHX1::HA (pRin73) was transformed into a sec53 mutant strain. The procedure was as above, except 70 μg of total protein was loaded in each lane.

spheroplasts using yeast lytic enzyme (10 mg/600 OD units; ICN) for 45 min at 37 °C. Spheroplasts were suspended in 3–6 ml of lysis buffer (0.3 M sorbitol, 20 mM triethanolamine, pH 7.2, 1 mM EDTA, and protease inhibitors) and homogenized with ~60 strokes of a Wheaton A Dounce homogenizer. The cleared lysate (~3 ml) was layered on top of centrifuged through (2 h, 27,000 rpm, Beckman SW28 rotor, 4 °C) a 10-step sucrose gradient (3 ml each of 18–54% (w/w) increments). Fractions were collected from the top, pooled, and centrifuged for 30 min at 4 °C, and pellets were resuspended in sample buffer prior to loading on gels. For treatments with tunicamycin, 20–200-ml cultures were grown overnight at 30 °C to an A600 value of ~1, divided into two, and grown for two more hours following addition of tunicamycin (10 μg/ml final concentration) or an equal volume of carrier (5 μl NaOH). Aliquots (10–40 ml) were removed at indicated times, and total membranes were prepared by a glass bead method (18). Briefly, cells from each aliquot were harvested and resuspended in ~200 μl of bead buffer (10 mM Tris/HCl, pH 7.4, 0.3 M sorbitol, 0.1 mM NaCl, 5 mM MgCl2) plus protease inhibitors. Acid-washed glass beads were added to the minicoccus, and samples were vortexed 3 times for 1 min. Lysates were washed 3 times with 1 ml of bead buffer and, using a drawn-out Pasteur pipette, removed to new tubes. Cleared lysates were centrifuged for 1 h at 45,000 rpm (Beckman Ti50 rotor, 4 °C), and final pellets were resuspended in ~30 μl of 20 mM K-Hepes, pH 7.4, plus protease inhibitors. Samples (30–100 μg) were diluted in sample buffer prior to loading on gels.

Protein concentrations were determined by the method of Lowry et al. (19) for total membrane preparations and by a modified Lowry method (20) for fractionation and pooled preparations. SDS-PAGE and Western blotting were as described previously (12). Antibodies used were mouse anti-HA monoclonal antibody, 12CA5 (Roche Molecular Biochemicals) at 1:5,000, and horseradish peroxidase-coupled sheep anti-mouse antibody (Amersham Pharmacia Biotech) at 1:10,000.

RESULTS

Nhxl Is an N-Linked Glycosylated Protein—Nhxl exhibits a consistently multi-handed appearance upon gel electrophoresis, strongly suggestive of post-translational modification of the N-linked region (12). To determine whether Nhxl was N-glycosylated, a combination of in vivo and in vitro biochemical and genetic approaches were employed. Membrane fractions enriched for Nhxl (see “Experimental Procedures”) were treated with endoglycosidase H, an enzyme that cleaves high-mannose complex-type N-linked glycosylations (21, 22). As shown in Fig. 1A,
and tunicamycin sensitivities, shifting Nhx1 from permissive (25 °C) to nonpermissive (37 °C) temperatures in sec53 resulted in loss of the upper bands on SDS-PAGE (Fig. 2C).

Nhx1 Contains Six Consensus Sites for N-Linked Glycosylation—Hydropathy analysis of Nhx1 (Fig. 3A) by the Kyte-Doolittle method (26) predicts multiple hydrophobic regions capable of spanning the lipid bilayer 10 to 13 times. Although less well defined, the hydrophilic peaks numbered 6 and 7 share a high degree of identity with other NHE isoforms and are predicted to be involved in transport (27). Based on hydropathy analysis, transmembrane predictions, and models of plasma membrane NHE isoforms (3), we developed a working model for the membrane topology of Nhx1 (Fig. 3B). As drawn, the six consensus sites for N-linked glycosylation, -NX(S/T)- (28), are polarized into two groups; Asn-121, Asn-334, and Asn-420 are in loop regions of the N-terminal transmembrane domain and are predicted to face the lumen of the prevacuolar compartment, and Asn-515, Asn-560, and Asn-563 are in the C-terminal tail domain and are predicted to reside in the cytosol.

Nhx1 Is Glycosylated in the C-terminal Tail Domain—Based on the preliminary topological model and the known sites of glycosylation in other NHE isoforms, we hypothesized that the likely site(s) of N-linked glycosylation were on the loops between transmembrane segments, namely at one or more of the residues Asn-121, Asn-334, and Asn-420. Each of these asparagines was individually replaced with aspartate, as described under “Experimental Procedures.” Mutants N334D and N420D were as abundant as wild-type Nhx1; however mutant N121D was expressed at significantly lower levels. Regardless of expression levels, the aspartate point mutants conferred salt-tolerant growth that was indistinguishable from wild-type (Fig. 4A) and was clearly above the Δnhx1 null strain (11). An alternate substitution, N121A, was generated; this mutant exhibited normal abundance levels, although salt tolerance was intermediate to wild-type and null mutant (not shown). The
individual point mutants (N121A, N334D, and N420D) each exhibited a sensitivity to tunicamycin similar to that of wild-type (Fig. 4B), indicating a retention of N-linked glycosylation in vivo.

It was possible that replacement of a glycosylated asparagine residue could lead to improper glycosylation at another, normally unmodified, consensus site for N-linked glycosylation. To address this concern, we created the triple loop mutant, N121A/N334D/N420D, and determined its glycosylation state. This triple mutant exhibited normal abundance and subcellular distribution but had decreased activity, as inferred from a reduction of salt tolerance to levels similar to the null strain (not shown). Most importantly, treatment with tunicamycin (Fig. 4B) and Endo H (Fig. 4C) resulted in a collapse of the upper collection of bands to a lower molecular weight, as in the single point mutants. Taken together, these results prove that mutation of all N-linked glycosylation consensus sites in the N-terminal domain does not prevent the normal glycosylation of Nhx1.

Next, we investigated whether the C-terminal tail harbored site(s) of N-linked glycosylation. There are three strong consensus sites for N-linked glycosylation at Asn-515, Asn-550, and Asn-563, as well as several weak sites. To test the hypothesis that some or all of these sites are normally glycosylated, we engineered two mutant Nhxl constructs (see Fig. 5B), a C-terminal truncation at asparagine 512 (Δ512), which deleted the three strong consensus sites for N-linked glycosylation in the tail region, and a triple mutant (N515D/N550D/N563D) in which aspartates replaced each of the three consensus asparagines. Fig. 5A clearly shows that unlike the wild-type control, the Δ512 mutant was insensitive to Endo H treatment. As indicated in Fig. 5B, both Δ512 and the triple mutant also appeared insensitive to tunicamycin treatment, strongly suggesting a loss of the site(s) of in vivo glycosylation. In contrast, the N334D mutant, used as a positive control because of its strong upper banding pattern (see Fig. 4B), showed a clear sensitivity to tunicamycin. Additionally, expression of either Δ512 or the triple mutant in sec7 and sec18 mutant strains resulted in no change in banding pattern upon shift to the nonpermissive temperature of 37 °C (Fig. 5C), an observation in stark contrast...
to that of the wild-type protein (see Fig. 2). It was possible that deletion of the C-terminal tail or mutation of the three asparagine residues resulted in polypeptides that were misfolded or abnormally localized and therefore not properly glycosylated. In earlier work, we have described the subcellular fractionation of yeast lysates on sucrose density gradients and shown colocalization of Nhx1 with endosomal markers (12). Here we show that the triple mutant was identical to wild-type Nhx1 in its distribution on sucrose density gradients (Fig. 6A), and in its tolerance to high salt (Fig. 6B), indicating that this protein was properly localized and fully functional. Distribution of the ΔN12 mutant showed some overlap with Golgi-containing fractions (12), suggesting a delay in trafficking out of the Golgi (Fig. 6A). Interestingly, ΔN12 had a sodium-sensitive phenotype intermediate to that of wild-type Nhx1 and Δnhx1 (Fig. 6B), suggesting that the N-terminal transmembrane domain retains some transport function.

Asn-515 and Asn-550 Are the Sites of N-Linked Glycosylation of Nhx1—To map the precise site(s) of N-linked glycosylation, we created the single point mutants N515D, N550D, and N563D. Each mutant appeared fully functional, as evidenced by salt-tolerant growth (Fig. 7A). The abundance of each mutant was approximately equal to that of wild-type, but the banding pattern was upwardly shifted for the N515D and N550D mutants (Fig. 7B). Based on this observation, we constructed the double mutant N515D/N550D. Fig. 7C shows that although each of the three single point mutants appeared to retain tunicamycin sensitivity, the double mutant N515D/N550D, like the C-terminal triple mutant, was insensitive to tunicamycin treatment. The double aspartate mutant also showed an upward shift compared with wild-type, perhaps because of the substitution with charged residues. To test this hypothesis, we created a double mutant substituted with alanines rather than aspartates. As illustrated in Fig. 7D, mutant N515A/N550A does not show the mobility shift of the aspartate mutant and also exhibits an insensitivity to tunicamycin. Thus, the data indicate that Nhx1 is normally glycosylated at residues Asn-515 and Asn-550. We also verified a normal subcellular localization of the double mutant on sucrose density gradients (data not shown). These results confirmed that (i) both Asn-515 and Asn-550 were the sites of N-linked glycosylation; and (ii) none of the remaining consensus sites were glycosylated, as evidenced by the insensitivity of the double mutant to tunicamycin.

**DISCUSSION**

*N-Linked Glycosylation of the Na⁺/H⁺ Exchanger Nhx1—* The present study shows that Nhx1, the yeast endosomal Na⁺/H⁺ exchanger, is post-translationally modified with N-linked glycosylations. Two independent experimental approaches, employing tunicamycin (*in vivo*) and Endo H (*in vitro*), resulted in similar effects; the uppermost collection of bands on Western blots of Nhx1 disappeared following treatment. The sensitivity of Nhx1 to Endo H indicates that the N-linked glycosylations are of the high mannose complex-type, created first by attachment of a core glycosylation in the endoplasmic reticulum and followed by maturation of that modification by enzymes in the Golgi. Temperature-sensitive blocks in vesicular traffic out of the endoplasmic reticulum (sec18) or Golgi (sec7) resulted in the expected loss or accrual of the higher molecular weight bands of Nhx1, respectively. These observations are consistent with those documented for other N-linked glycosylated membrane proteins in yeast (24, 29).

Using site-directed mutagenesis of the consensus sites for N-linked glycosylation, we have mapped Nhx1 glycosylation to the C-terminal hydrophilic tail domain, specifically to residues Asn-515 and Asn-550. Importantly, loss of N-linked glycosylation had no apparent effect on *in vivo* exchanger function, as evidenced by normal profiles of sodium-tolerant growth. Polypeptide expression levels and subcellular localization of nonglycosylated Nhx1 appeared similar to that of wild-type. Thus, the specific role of N-linked glycosylation of Nhx1 remains to be determined. Although the triple loop mutant was glycosylated normally, its sodium-sensitive growth was identical to the null strain, suggesting that this combination of point mutations (N121A/N334D/N420D) is deleterious for exchanger function. This observation may not be surprising, as each residue lies within the N-terminal transporter domain of the exchanger, and the single point mutant N121A exhibited a salt tolerance intermediate to wild-type and the null strain (data not shown).

Expression levels were drastically reduced only for the N121D mutant, which was not unexpected, given the predicted location of Asn-121 one to four amino acid residues inside the α-helical M4 transmembrane segment. Introduction of the negatively charged aspartate within the membrane may have led to destabilization and possibly increased degradation of the mutant polypeptide. It was noteworthy that the relative intensities and mobilities of the Nhx1 bands varied with the position(s) of substitution, as well as with the host strain and growth medium. For example, the mobilities of the bands in N334D was markedly changed, whereas N420D appeared very similar to wild-type. One explanation is that introduction of a negative charge at certain sites in the polypeptide affects mi-
Nhx1 Is an N-Linked Glycoprotein

Thus far, only NHE1 has been shown to be novel for the following reasons: (i) to date, there is no evidence -linked glycosylation in the C-terminal tail region of NhX1, particularly as a model protein for the new subfamily of intracellular Na+/H+ exchangers. The demonstration of N-linked glycosylation in the C-terminal tail region of NhX1 is novel for the following reasons: (i) to date, there is no evidence for glycosylation of any NHE isoform on its C-terminal tail domain; and (ii) the location of glycosylations implies an unusual topological disposition of the C-terminal tail of NhX1. Thus far, only NHE1 has been shown to be N-linked glycosylated, and this site has been mapped to an extracellular loop between transmembrane domains M1 and M2 (6). Both NHE1 and NHE2 are O-linked glycosylated, each at sites between M1 and M2 (6, 7). There is no evidence, for any of the known NHE isoforms, that N-linked glycosylations exist on the C-terminal tail domain. Numerous functional studies suggest that the large hydrophilic C-terminal tail of NHE assumes a cytosolic orientation and constitutes a regulatory region of the exchanger. It is in fact this cytosolic domain that is implicated in pH "set point," calmodulin binding, protein kinase C-dependent phosphorylation, and volume sensitivity (27, 30). However, and of particular significance with regard to the present study, recent results indicate that some regions of the C-terminal domain of NHE3 assume an exoplasmic orientation. Two monoclonal antibodies directed against the C-terminal tail of NHE3 reacted with epitopes exposed at the extracellular surface (one epitope lies between residues 702 and 756, and the other epitope lies C-terminal to residue 756) (31). In contrast, during the preparation of this manuscript, a substituted cysteine-based topological analysis of the NHE1 isoform demonstrated that three residues (558, 561, and 794) in the C-terminal tail assumed the predicted cytosolic orientation (33). Our results, combined with these recent topological studies of NHE1 and NHE3, suggest either that there is considerable topological divergence in the C-terminal tail topology of various exchanger isoforms or that the organization of the tail domain is considerably complex.

The mapping of glycosylation sites in NhX1 to the C-terminal domain strongly suggests that the hydrophilic tail, or some portion thereof, resides within the lumen of the prevacuolar compartment, rather than in the cytosol. Although this observation was unexpected in terms of homology to NHE isoforms, the physiological function(s) of intracellular Na+/H+ exchangers remains to be elucidated and thus may be consistent with some structural divergence from plasma membrane-type antiporters. A hallmark of the NHE family is the strong sequence conservation within the N-terminal transmembrane domain, which mediates transport function, whereas the hydrophilic C-terminal tail has diverged considerably, allowing for tissue and cell-specific regulation (2, 3). A luminal disposition of at least some portion of the putative regulatory domain of NhX1 may be relevant for its proposed roles in endosomal volume control, Na+ sequestration, and pH homeostasis (12, 32).

A revised topological model for NhX1 is essential, based on our new evidence that this protein is N-linked glycosylated on its hydrophilic C-terminal tail. Because we know very little about the physiological function or the orientation of the C terminus for this emerging subfamily of intracellular Na+/H+ exchangers, a variety of topology models are possible; one commonality must be that at least some portion of the C terminus (including residues Asn-515 and Asn-550) is lumenally accessible. The simplest model would assume a luminal disposition for the entire C-terminal tail; the loss or gain of one transmembrane segment in NhX1 would result in a luminaly oriented tail. Wakabayashi et al. (33) discovered in their recent study on NHE1 topology that a hydrophobic region, located between M11 and M12 of the original model, in fact constitutes an additional transmembrane segment. The homologous hydrophobic peak in NhX1, indicated by an asterisk in Fig. 3A, could provide an unpredicted membrane-spanning segment that would dispose the C terminus to the lumen. An alternative

Fig. 7. NhX1 is glycosylated at residues Asn-515 and Asn-550. A, NaCl sensitivity of point mutants, as described in the legend to Fig. 4. Western blots of HA-tagged NhX1 are shown in B-D. B, abundance of point mutants. Total membranes were prepared, and 50 µg of protein was loaded in each lane; duplicate lanes represent separate yeast transformants. C, tunicamycin treatment. Lanes correspond to 2-h treatments with (+) or without (-110 µg/ml tunicamycin; protein loaded was 50 µg (−Tu) and 100 µg (+Tu). Double is the double point mutant N515D/N550D. D, tunicamycin treatment, as in C; protein loaded was 100 µg (−Tu) and 150 µg (+Tu). DblA is the N515A/N550A mutant, and DblID is the N515D/N550D mutant.

The transmembrane regions (A–D) of the NhX1 topology model illustrate the possible membrane-spanning segments for NhX1, including the probable second transmembrane segment. The possible NH2-terminal segments and cytosolic domains are marked by B. The corresponding region of the original model for NhX1 is shown in bold letters. Transmembrane segments are indicated by boxes with the corresponding numbers. An alternate orientation of the NH2-terminal segment is shown in (C). The orientation and location of the cytosolic domain (II) is indicated by the asterisk and the orientation of the lumenal domain (I) by the triangle. The region of the hydrophilic C-terminal tail is shown in gray, and highlighted in blue, indicating the direction of the orientational change. A revised topological model for NhX1 is essential, based on our new evidence that this protein is N-linked glycosylated on its hydrophilic C-terminal tail. Because we know very little about the physiological function or the orientation of the C terminus for this emerging subfamily of intracellular Na+/H+ exchangers, a variety of topology models are possible; one commonality must be that at least some portion of the C terminus (including residues Asn-515 and Asn-550) is lumenally accessible. The simplest model would assume a luminal disposition for the entire C-terminal tail; the loss or gain of one transmembrane segment in NhX1 would result in a luminaly oriented tail. Wakabayashi et al. (33) discovered in their recent study on NHE1 topology that a hydrophobic region, located between M11 and M12 of the original model, in fact constitutes an additional transmembrane segment. The homologous hydrophobic peak in NhX1, indicated by an asterisk in Fig. 3A, could provide an unpredicted membrane-spanning segment that would dispose the C terminus to the lumen. An alternative
model is that only certain portions of the Nhx1 C-terminal tail are accessible to the lumen; this would imply that part of the tail would have to traverse the lipid bilayer. Hydropathy analysis of Nhx1 and NHE isoforms indicates no putative membrane-spanning α-helices in the C-tail regions; hence, for NHE3, Biemesderfer et al. (31) suggest that region(s) of the C terminus may span the membrane as a β-sheet. Such a scenario represents another plausible model that could explain our observations about Nhx1. To address these and other possibilities, we are currently pursuing a rigorous experimental dissection of the membrane topology of Nhx1.

The present study supports the idea that the modular domain structure of Nhx1 parallels exchanger function in a way similar to that of NHE isoforms. In particular, the large N-terminal transmembrane domain appears sufficient for sodium/proton exchange activity; the truncation mutant Δ512, in which 120 amino acids were deleted from the C-terminal end, can still confer salt tolerance, albeit at levels somewhat lower than wild-type. However, the C-terminal deletion also resulted in significantly increased abundance of the polypeptide, as well as a partial shift in subcellular localization into Golgi-containing fractions of the sucrose density gradient. It is possible that slower trafficking of the truncated protein out of the Golgi also delays its proteolytic turnover, accounting for increased abundance.

It is interesting that even in the absence of N-linked glycosylation, Nhx1 retains some multi-banded appearance upon gel electrophoresis. Expression of Nhx1 in the sec53 mutant, which blocks all O-linked and N-linked glycosylations, retains some measure of multi-banded character, as well. These data suggest the existence of additional post-translational modifications of Nhx1. In particular, the sequence of the first predicted transmembrane helix reveals features reflective of a signal peptide, and we have preliminary evidence for an N-terminal cleavage event. Additionally, there are numerous consensus sites for protein kinase C- and casein kinase II-dependent phosphorylation distributed throughout the polypeptide.

In conclusion, our finding that Nhx1 is N-linked glycosylated at two residues in its hydrophilic C-terminal tail domain makes an experimental dissection of membrane topology imperative; this would contribute greatly to future studies on molecular structure and function. The observation that a C-terminally truncated mutant and the N515D/N550D double mutant retain heterogeneity on SDS-PAGE Western blots paves the way for further characterization of post-translational modification(s) and of how this intracellular exchanger might be regulated.

REFERENCES

1. Sardet, C., Franchi, A., and Pouyssegur, J. (1989) Cell 25, 271–280
2. Wakabayashi, S., Fafournoux, P., Sardet, C., and Pouyssegur, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2424–2428
3. Orlowksi, J., and Grinstein, S. (1997) J. Biol. Chem. 272, 22373–22376
4. Biemesderfer, D., DeGray, B., and Arnesen, P. S. (1998) J. Biol. Chem. 273, 723–726
5. Bertrand, B., Wakabayashi, S., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994) J. Biol. Chem. 269, 13733–13709
6. Counillon, L., Pouyssegur, J., and Reitmeier, R. A. F. (1994) Biochemistry 33, 10463–10469
7. Tse, C. M., Levine, S. A., Yun, C. H. C., Khurana, S., and Donowitz, M. (1994) J. Biol. Chem. 269, 12954–12961
8. Hilden, S. A., Ghoshroy, K. B., and Madias, N. E. (1999) Am. J. Physiol. 28, F1311–F1319
9. Van Dyke, R. W. (1995) Am. J. Physiol. 269, C943–C954
10. Thevenod, A. F. (1996) J. Membr. Biol. 152, 195–205
11. Nass, R., Cunningham, K. W., and Rao, R. (1997) J. Biol. Chem. 272, 26145–26152
12. Nass, R., and Rao, R. (1998) J. Biol. Chem. 273, 21054–21060
13. Gaxiola, R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L., and Fink, G. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1480–1485
14. Novick, P., and Schekman, R. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1858–1862
15. Rodriguez-Navaro, A., and Ramos, J. (1984) J. Bacteriol. 159, 940–945
16. Sardet, C., Franchi, A., and Pouyssegur, J. (1989) Biotechniques 8, 404–407
17. Fisher, C. L., and Pei, G. K. (1997) J. Biol. Chem. 272, 26145–26152
18. Counillon, L., Pouyssegur, J., and Reitmeier, R. A. F. (1994) Biochemistry 33, 265–275
19. Lowry, O. H., Rosebrrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
20. Bensadoun, A., and Weinstein, D. (1976) Anal. Biochem. 70, 241–250
21. Kobata, A. (1979) Anal. Biochem. 100, 1–14
22. Tse, C. M., Levine, S. A., Yun, C. H. C., Khurana, S., and Donowitz, M. (1994) J. Biol. Chem. 269, 10463–10469
23. Duskin, D., and Mahoney, W. C. (1982) J. Biol. Chem. 257, 3105–3109
24. Herscovics, A., and Orlean, P. (1993) FEBS Lett. 310, 76–81
25. Kepe, F., and Schekman, R. (1988) J. Biol. Chem. 263, 9155–9161
26. Kepe, F., and Schekman, R. (1988) J. Biol. Chem. 263, 9155–9161
27. Wakabayashi, S., Shigekawa, M., and Pouyssegur, J. (1997) Physiol. Rev. 77, 1–51
28. Marshall, R. D. (1972) Annu. Rev. Biochem. 41, 673–702
29. Orlean, P., Kuranda, M. J., and Albright, C. F. (1991) Methods Enzymol. 194, 682–697
30. Counillon, L., and Pouyssegur, J. (2000) J. Biol. Chem. 275, 1–4
31. Biemesderfer, D., DeGray, B., and Arnesen, P. S. (1998) J. Biol. Chem. 273, 12391–12396
32. Nass, R., and Rao, R. (1999) Microbiology 145, 3221–3226
33. Wakabayashi, S., Pang, T., Su, X., and Shigekawa, M. (2000) J. Biol. Chem. 275, 7942–7949
34. Quintero, F. J., Blatt, M. R., and Pardo, J. M. (2000) FEBS Lett. 471, 224–228
The Yeast Na\(^+\)/H\(^+\) Exchanger Nhx1 Is an N-Linked Glycoprotein: TOPOLOGICAL IMPLICATIONS
Karen M. Wells and Rajini Rao

J. Biol. Chem. 2001, 276:3401-3407.
doi: 10.1074/jbc.M001688200 originally published online October 17, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001688200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 16 of which can be accessed free at http://www.jbc.org/content/276/5/3401.full.html#ref-list-1