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Topogenic Properties of Transmembrane Segments of *Arabidopsis thaliana* NHX1 Reveal a Common Topology Model of the Na^+/H^+ Exchanger Family

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The membrane topology of the *Arabidopsis thaliana* Na^+/H^+ exchanger isoform 1 (AtNHX1) was investigated by examining the topogenic function of transmembrane (TM) segments using a cell-free system. Even though the signal peptide found in the human Na^+/H^+ exchanger (NHE) family is missing, the N-terminal hydrophobic segment was efficiently inserted into the membrane and had an N-terminus lumen topology depending on the next TM segment. The two N-terminal TM segments had the same topology as those of TM2 and TM3 of human NHE1. In contrast, TM2 and TM3 of human NHE1 did not acquire the correct topology when the signal peptide (denoted as TM1) was deleted. Furthermore, there were three hydrophobic segments with the same topogenic properties as the TM9-H10-TM10 segments of human NHE1, which has one lumenal loop (H10) and two flanking TM segments (TM9 and TM10). These data indicate that the plant NHX isoforms can form the common membrane topology proposed for the human NHE family, even though it does not have a signal peptide.

Key words: endoplasmic reticulum, membrane protein, membrane topology, signal peptide, signal sequence.

Abbreviations: ER, endoplasmic reticulum; hNHE1, human Na^+/H^+ exchanger isoform 1; ProK, proteinase K; EndoH, endoglycosidase H; RM, rough microsomal membrane; PL, prolactin; g-loop, glycosylation-loop; AtNHX1, Na^+/H^+ exchanger isoform 1 of *Arabidopsis thaliana*; H-segment, hydrophobic segment.

The majority of membrane proteins on organelles of the secretory pathway are synthesized on membrane-bound ribosomes and cotranslationally integrated into the endoplasmic reticulum (ER) membrane, where they acquire their unique topology (*1*). The hydrophobic region of a signal sequence emerging from the free ribosome is recognized by a signal recognition particle, and then the ribosome-nascent chain complex is targeted to the ER. The signal recognition particle receptor on the ER releases the signal sequence from the signal recognition particle (*2*). Membrane proteins are integrated into the membrane, mediated by a so-called translocon (*3, 4*). The topogenic process is determined mainly by hydrophobic segments (H-segments), the so-called topogenic sequences. The signal peptide and type II signal anchor sequence translocate the following C-terminal side, while the type I signal anchor sequence translocates the preceding N-terminal side (*5, 6*). The translocation that is initiated by the signal peptide or type II signal-anchor sequence is stopped by the following hydrophobic segment, the so-called stop-transfer sequence. Thus, the topogenic properties of the TM segment should directly correlate with the final topology of membrane proteins (*7, 8*).

The topology of the human Na^+/H^+ exchanger isoform 1 (hNHE1) was extensively studied using cysteine scanning and reporter fusion experiments. It possesses 12 TM segments and a hydrophobic lumenal loop segment (termed H10) between TM9 and TM10 (*9, 10*). The first N-terminal H-segment of hNHE1, denoted as TM1, is a signal peptide. TM1 of NHE1, NHE3, and NHE6 is cleaved off (*11, 12*), and that of other isoforms is an orthologous segment. Because the C-terminal side of the TM1 segment should be located in the lumenal side and processed by the signal peptide, TM2 has an N_{lum}/C_{cyt} topology in which the N-terminus is on the lumenal side and the C-terminus is on the cytoplasmic side. On the other hand, a different topology model was proposed for the Na^+/H^+ exchanger isoform 1 of *Arabidopsis thaliana* (AtNHX1), in which various H-segments have a different membrane topology from the human isoforms (*13*). According to the similar hydropathy profiles and amino acid sequences, AtNHX1 and hNHE1, however, share the same topology, except that the region corresponding to the N-terminal TM1 of hNHE1 is missing in AtNHX1 (Fig. 1).

In this study, we examined whether the absence of the signal peptide induced a different membrane topology of the AtNHX1 and whether the topogenic properties of the TM segments indicate a different membrane topology model. We evaluated topogenic functions using model fusion proteins with AtNHX1 sequences. Even though it does not possess the signal peptide, the N-terminus of the first H-segment was positioned in the ER lumen when assessed in the presence of the following second H-segment. The C-terminal portion of the second H-segment was positioned in the lumenal side. The H-segments corresponding to the TM9-H10-TM10 region of NHE have the

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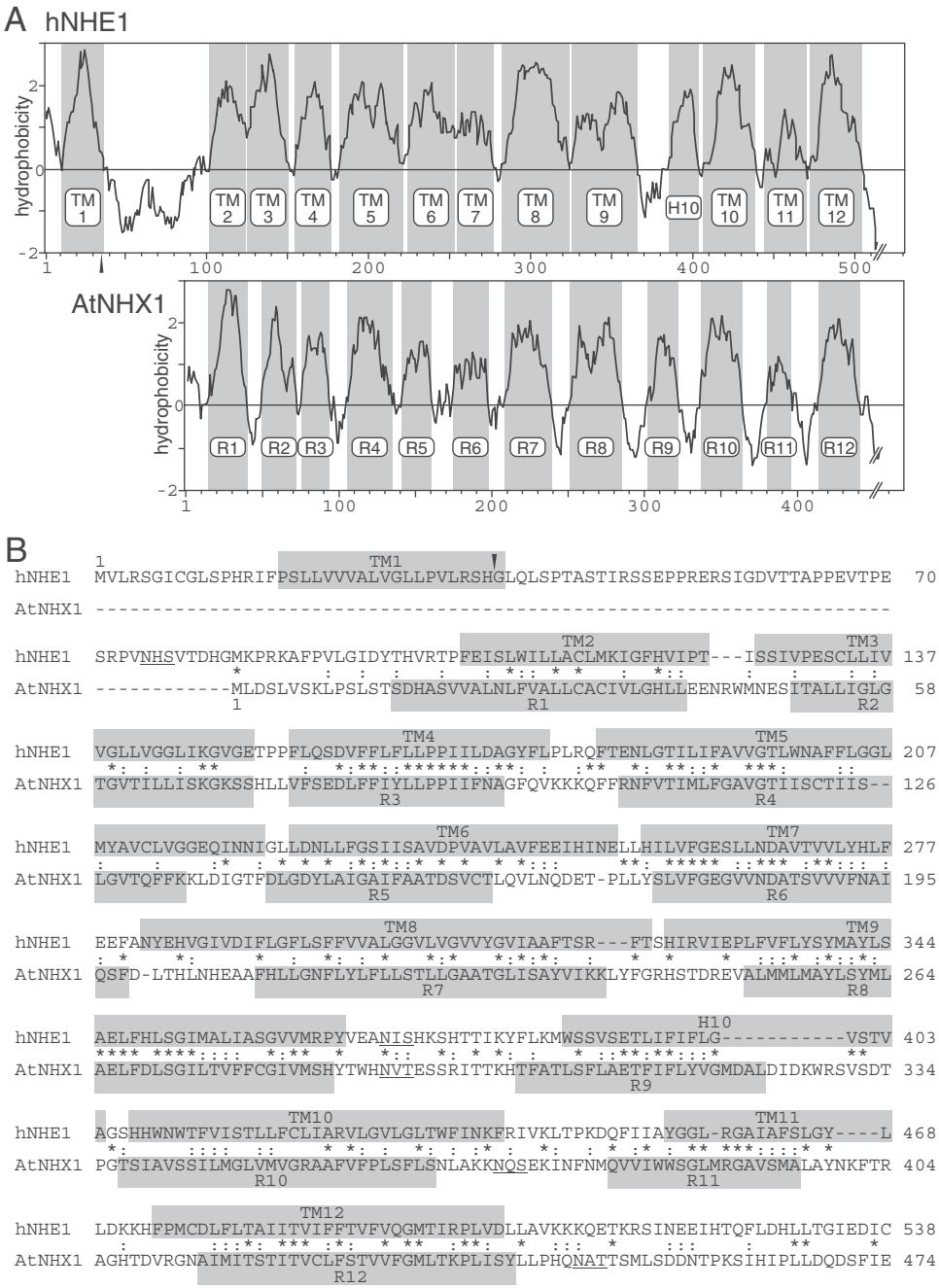

Fig. 1. **Orthologous H-segments of hNHE1 and AtNHX1.** (A) Hydrophobic profiles of hNHE1 and AtNHX1. The plot was generated by the method of Kyte and Doolittle (*24*). A predicted processing site is indicated by the arrowhead. (B) Sequence comparison of hNHE1 and AtNHX1. Identical (*) and similar (:) residues are indicated. Hydrophobic regions are shaded. *N*-glycosylation motifs are underlined.

same topogenic properties as the orthologous region of AtNHX1. All of the topogenic properties of AtNHX1 are consistent with the common topology model proposed for the human NHE family. We concluded that plant NHE isoforms are integrated into the membrane in the same way as the human isoforms, even though the plant NHE isoforms do not have an N-terminal signal peptide.

MATERIALS AND METHODS

Materials—Enzymes for DNA manipulation and *in vitro* transcription (Takara), proteinase K (ProK; Merck), and endoglycosidase H (EndoH; New England Biolabs) were obtained from the sources indicated. Rough microsomal membrane (RM) (*14*) and rabbit reticulocyte lysate (*15*) were prepared as previously described. To remove endogenous mRNA, RM was washed with 25 mM EDTA and treated with *Staphylococcus aureus* nuclease as described previously (*14*).

DNA Construction—The hNHE1 cDNA was previously described (*9*). The AtNHX1 open reading frame was obtained by polymerase chain reaction amplification using an *A. thaliana* cDNA library, which was a kind gift form Drs. K. Iba and O. Matsuda (Kyushu University), and the following primers: ATGTTGGATTCTCTAGTGTC (sense) and TCAAGCCTTACTAAGATCAGGAGG

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(antisense). For fusion protein construction, each DNA fragment (*Nde*I/*Xho*I) encoding various regions was amplified and ligated with the DNA fragment (*Xho*I/*Xba*I) coding for the reporter domain of prolactin (PL) on pCITE-2a (Novagen) (*Nde*I/*Xba*I) (*16*). Where indicated, a glycosylation site was included in the PL domain by point mutation (T60N) (*7*). For the glycosylation-loop (g-loop) insertion, the DNA fragment containing *N*-glycosylation sites derived from human band 3 (Gln^{629}–Trp^{662}, *Eco*RI/*Eco*RI) (*7*) was inserted after the Val^{291} or Trp^{328} of AtNHX1. The oligonucleotide sequences and details of construction are available upon request from the author.

In Vitro *Analysis*—Plasmids were linearized using *Sca*I and then transcribed using T7 RNA polymerase. The obtained mRNAs were translated in a reticulocyte lysate cell-free system as previously described (*5*). The translation mixture contained 100 mM KCl, 1.5 mM $Mg(OAc)_2$, 32% reticulocyte lysate, and 15.5 kBq/μl EXPRESS protein-labeling mix (Perkin Elmer). Aliquots (5 μl) of translation mixture were treated with EndoH at 37°C for 60 min under denaturing conditions. Other aliquots were treated with 400 μg/ml ProK on ice for 40 min (*17*). After ProK treatment, the reaction was terminated with 10% trichloroacetic acid, and the ^{35}S-labeled proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and image analysis.

RESULTS

Integration of the N-Terminal Region of AtNHX1—In this study, the H-segments of hNHE1 were labeled numerically as 12 TM segments (TM1–TM12) and one lumenal membrane loop segment (H10) between TM9 and TM10 as described previously (*9*). H-segments of AtNHX1 were serially numbered according to the previous model (*13*). To distinguish the molecular species, the H-segments of AtNHX1 were labeled numerically starting with R. Hydropathy analysis of hNHE1 and AtNHX1 indicated a clear difference in the N-terminus; the H-segment corresponding to TM1 of hNHE1 is missing and other segments had a very similar pattern (Fig. 1A). The sequence alignment also identified corresponding segments between human and plant isoforms except for TM1 (Fig. 1B).

We hypothesized that R1 of AtNHX1 is can be integrated into the membrane as a type I signal-anchor sequence. To address this possibility, a reporter domain of PL was fused after the segment and expressed in the cell-free system including RM (Fig. 2). Where indicated, a potential glycosylation site was included in the PL domain (gPL) and/or the N-terminus (g-R1). When synthesized in the absence of RM, a single major band was observed with each construct (Fig. 2, lanes 1 and 5). When translated in the presence of RM, both the C-terminal PL domain and the N-terminal region were significantly glycosylated (lanes 2 and 6). The larger bands were sensitive to EndoH treatment, indicating that the sites were glycosylated (lanes 3 and 7). After EndoH treatment, the no mobility shift was observed, indicating that the N-terminal portion was not processed. The mobility shifts induced by glycosylation were different depending on the positions; *e.g.*, the mobility shift by glycosylation of the gPL domain is smaller than that caused by glycosylation of the g-R1 domain. It is likely that the differences in the local conformation caused the differences in the mobility shifts. When the glycosylation sites were incorporated in both domains, two glycosylated bands were observed (lane 10). To examine the membrane topology, the membrane vesicles were treated with ProK. The glycosylated PL domain was shifted down only slightly (lanes 4 and 12), while the protein glycosylated at the N-terminus was degraded (lanes 8 and 12). The mobility shift of the PL domain-fragment was due to degradation of the short N-terminal region. The PL domain of the N-terminally glycosylated form was degraded almost completely (case a), while the R1-PL domain translocated through the membrane was resistant to ProK (case b). All the data indicated that the R1-PL fusion proteins had a mixed topology (cases a and b). Thus, the R1 segment alone cannot perform the type I signal-anchor function.

To examine the topology of the two N-terminal H-segments (R1 and R2), PL was fused after the R2 segment. Both the N-terminus and the C-terminal PL domain were more efficiently glycosylated than the fusion construct with R1 alone (Fig. 2, lanes 14, 18, and 22). In particular, the g-R1–2-gPL construct produced the dominant di-glycosylated form (lane 22). The glycosylated forms were resistant to ProK (lane 24). The short loop between R1 and R2 should be inaccessible to the protease. In contrast to the mixed topology with the R1 segment alone, the R1-R2 region was integrated into the membrane with a unique topology with both ends in the lumenal side, even though it did not have a signal peptide. The topology was identical to those of TM2 and TM3 in the hNHE1 molecule.

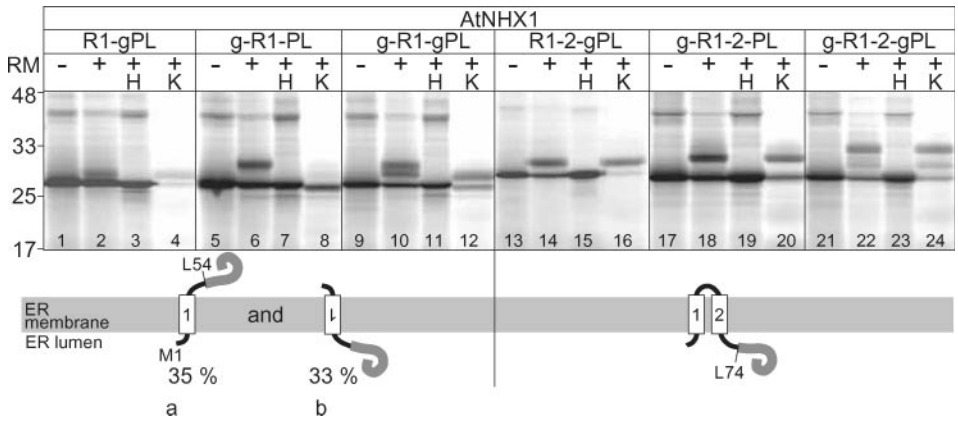

Fig. 2. **Integration of the N-terminal region of AtNHX1.** A PL or gPL reporter domain was fused at various sites. The fusion proteins were expressed *in vitro* in the absence (–) or presence (+) of RM. Aliquots were treated with EndoH (H) or ProK (K). The proteins were analyzed by SDS-PAGE (13%) and image analysis. gPL and g-R1 indicate the PL domain and N-terminal segment with a glycosylation site, respectively. Topologies deduced from the results are illustrated.

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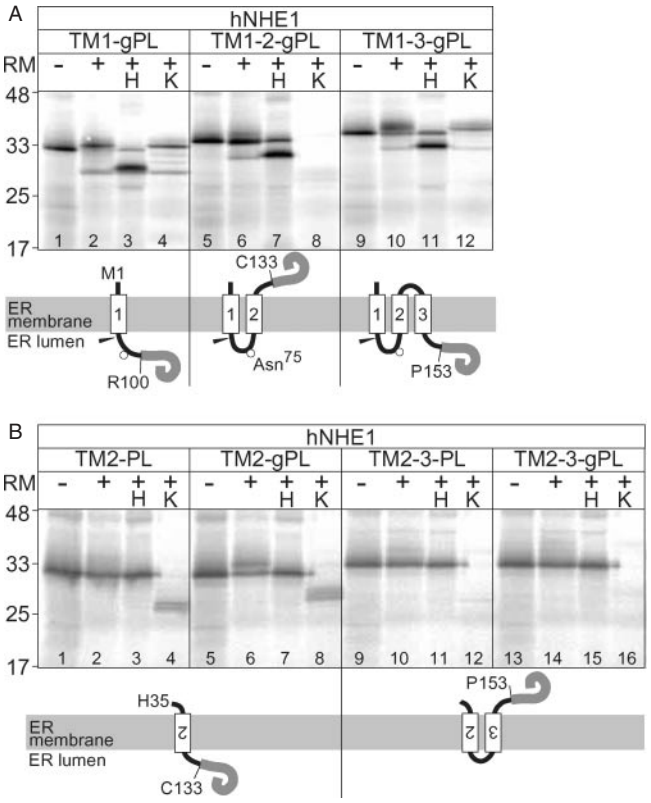

Fig. 3. **Signal peptide-dependent integration of the N-terminal region of hNHE1.** The indicated constructs were examined *in vitro*. Endogenous glycosylation and processing sites are shown as circles and arrowheads, respectively. (A) The PL or gPL reporter domain at various sites was fused after the indicated site. (B) PL or gPL reporter domain was fused after TM2 or TM3, which was initiated by His^{35}.

Signal Peptide Is Required for Proper Integration of TM2-TM3 of hNHE1—Next, we examined whether TM2-TM3 of hNHE1 were correctly integrated into the membrane in the absence of the signal peptide, as observed with R1-R2 of AtNHX1. When the gPL domain was fused after the TM1 segment, the TM1 initiated translocation of the reporter domain and produced a ProK-resistant and glycosylated form of the PL domain as previously described (*11*) (Fig. 3A, lanes 2, 3, and 4). The synthesized fusion proteins were translocated through the membrane, processed by signal peptidase, and glycosylated. When the gPL domain was fused after the TM2 segment, the TM2 segment stopped the translocation and the reporter domain was positioned on the cytoplasmic side of the membrane, where only the endogenous glycosylation site Asn^{75} of hNHE1 was glycosylated (lanes 6 and 7) and the following PL domain was sensitive to ProK (lane 8). When the PL domain was fused after the TM3, the PL domain was again translocated through the membrane to be glycosylated (lanes 10 and 11) and to become ProK resistant (lane 12). These data indicated that the three fusion proteins were integrated into the membrane with the expected topology (Fig. 3A). The mobilities of the products obtained in the absence of RM (lanes 1, 5, and 9) were similar to those obtained in the presence of RM (lanes 2, 6, and 10). This is because the decrease of molecular weight by the removal of the signal peptide (TM1) in the presence of RM is compensated by the attachment of a sugar chain. Anyway, in contrast to the R1 segment of AtNHX1, the TM1 segment was processed. When the TM1 segment was deleted, TM2 alone induced translocation of the following portion, resulting in a ProK-resistant PL domain (Fig. 3B, lanes 4 and 8) and glycosylation of the gPL domain (lane 6). Furthermore, when the PL domain was fused after TM3, there was neither a ProK-resistant form (lanes 12 and 16) nor a glycosylated form (lanes 10, 11, 14, and 15). Thus, TM2 and TM3 had inverted topology in the absence of the signal peptide of TM1. TM2 and TM3 were integrated into the membrane depending strictly on the signal peptide.

Topogenic Properties around the Membrane Loop Segment—The H10 of hNHE1 is a lumenal membrane loop between TM9 and TM10 (*9, 10*). The H-segments are proposed to have different topology in the model proposed for AtNHX1 (*13*). R8, R9, and R10 of AtNHX1 were, however, expected to be orthologous to the TM9, H10, and TM10 of hNHE1, respectively (Fig. 1). To evaluate the topogenic properties of this orthologous region, a reporter domain of PL was fused at various positions around the region (Fig. 4). The constructs were initiated from the R8 segment but not from the N-terminus, because the full-length protein possesses so many H-segments that the product had a very diffuse band on SDS-PAGE (data not shown). In the presence of RM, the Thr^{287}-gPL fusion was glycosylated in the gPL domain (Fig. 4B, lane 2). When the translation mixture was treated with ProK, the glycosylated form in lane 2 was ProK-resistant (lane 4), indicating that a gPL domain was translocated through the ER membrane and protected from ProK by the membrane. The R8 segment alone is sufficient to initiate translocation of the following portion with an N_{cyt}/C_{lum} topology, indicating that the orientation of the following H-segments should be the same as that in the full-length molecule. The di-glycosylated forms were observed with Ile^{297}- and Ala^{304}-fusions in addition to the mono-glycosylated form (lanes 6 and 10). These two glycosylated forms were ProK-resistant (lanes 8 and 12). In these model proteins, the potential glycosylation site at Asn^{290} was partially accessible to the enzyme. When the fusion site was shifted downstream, di-glycosylation was not observed (Fig. 4B, lane 14), indicating that the potential Asn^{290} site was sequestered from the enzyme by the R9 segment. The polypeptide chain was resistant to ProK treatment. Thus, we concluded that the R9 segment did not have a stop-transfer function and that the translocation initiated by the R8 segment was stopped only by the R10 segment. The glycosylation site was sequestered by the R9 segment, although the R9 segment does not have a stop-transfer function.

To confirm the orientation of the R9 segment, the loop sequence (g-loop) of human band 3 was inserted into the flanking sites of the R9 segment (Fig. 4C). This loop has little inhibitory effect on the polypeptide chain translocation and thus has been used for probing membrane topology of multispanning membrane proteins; e.g., band 3, K^+-channel, and various ion transporters (*18–21*). Both of the constructs were significantly glycosylated (Fig. 4C, lanes 6, 9, 12, and 15), whereas the original construct was not glycosylated (lane 2). After ProK treatment, there were no resistant bands (Fig. 4C, lanes 4, 7, 10, 13, and 16), indicating that the following reporter domain was

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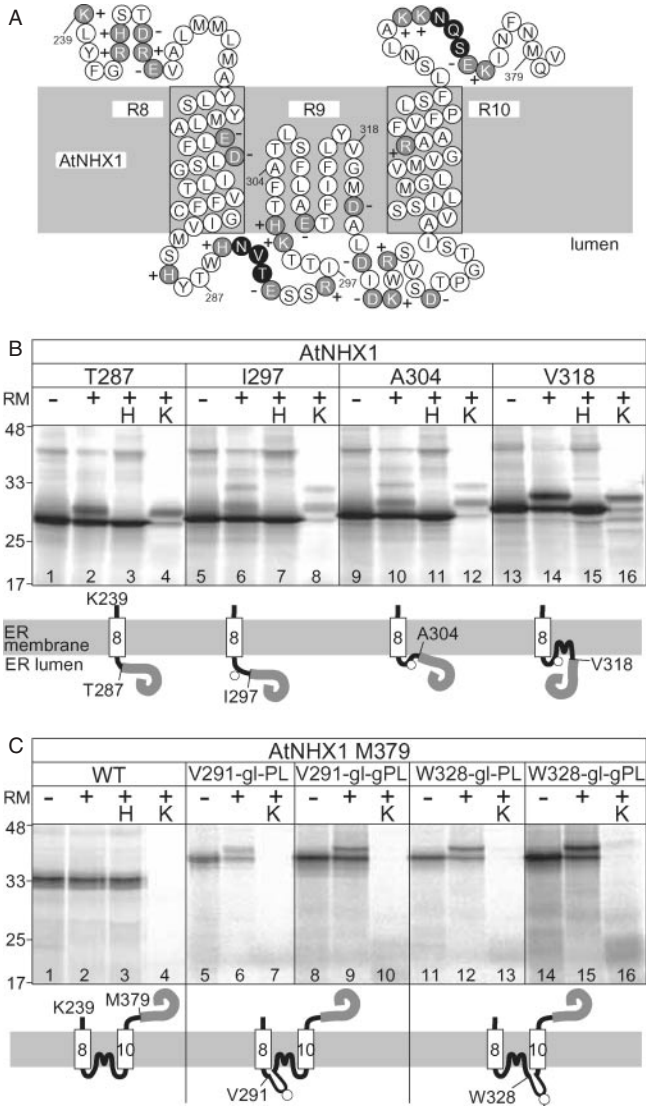

Fig. 4. **Topogenic properties of the R8 to R10 region are similar to those of the TM9-H10-TM10 region of hNHE1.** (A) Topology model around R9. Numbers indicate amino acid residues at which the constructs start or are fused to the reporter domain. (B and C) PL or gPL reporter domain was fused at various sites. The g-loop insertion positions are indicated.

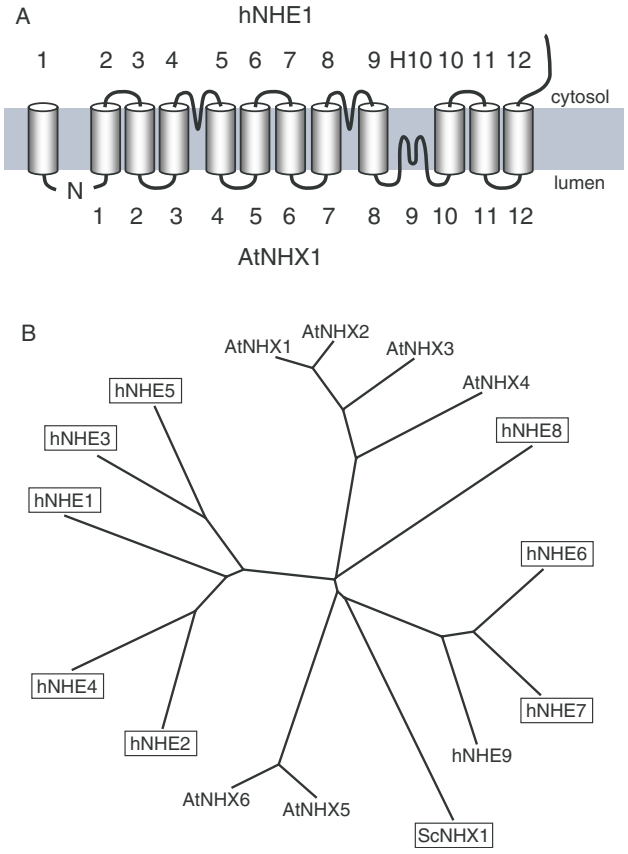

Fig. 5. **AtNHX1 share the same topology with the equivalent hydrophobic regions in hNHE1.** (A) The topology models of hNHE1 and AtNHX1. (B) Phylogenic diversity of Na^+/H^+ exchangers. Alignment analysis was performed using the CLUSTAL W method (*25*). Proteins with a signal peptide are boxed. The accession numbers and sources of the Na^+/H^+ antiporters are as follows: human isoforms, hNHE1 (P19634), hNHE2 (AAD41635), hNHE3 (P48764), hNHE4 (NP_001011552), hNHE5 (AAC98696), hNHE6 (NP_006350), hNHE7 (NP_115980), hNHE8 (Q9Y2E8), hNHE9 (Q8IVB4); *A. thaliana* isoforms, AtNHX1 (AAD16946), AtNHX2 (AAM08403), AtNHX3 (NP_187288), AtNHX4 (AAM08405), AtNHX5 (AAM08406), AtNHX6 (AAM08407); yeast, ScNHX1 (NP_010744).

on the cytoplasmic side. These results are a clear indication that both ends of the R9 segment were exposed to the lumenal side of the membrane. The R9 segment was integrated as a membrane loop structure, and R10 has a TM-topology. The topogenic properties around the R8–R10 segments were identical to those observed with the hNHE1 molecule (Fig. 5A). Even the presence of the potential glycosylation site and its sequestration behavior are quite similar.

DISCUSSION

The topologies of the N-terminal H-segments (R1 and R2) are consistent with the common model proposed for human NHE isoforms (Fig. 5A), even though AtNHX1 does not possess the N-terminal signal peptide. The combined R1 and R2 segments have a sufficient topogenic property with the same membrane topology as TM2 and TM3 of hNHE1. The similar hydropathy profiles and amino acid sequences also support this conclusion.

The characteristic lumenal membrane loop region of TM9-H10-TM9 in hNHE1 is also assigned in the plant NHE isoform. The R9 segment corresponding to H10 has no stop-transfer function, is translocated through the ER membrane, and both ends are in the lumenal side of the membrane. The cryptic glycosylation site just after the R8 segment is sequestered from the lumenal enzyme depending on the R9 segment. All these behaviors are the same as those of the orthologous segments of hNHE1 (*9*).

All of the topogenic properties observed above are consistent with the topology model of hNHE1 and inconsistent with that proposed for AtNHX1 (*13*). In the latter model, the N-terminus of R1 is on the cytoplasmic side, R2 is a TM segment with an N_{lum}/C_{cyt} topology, R9 is a TM segment with an N_{lum}/C_{cyt} topology. The model was based on another sequence assignment different from that in this

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study; the R1 segment was assigned as a TM1 segment, even though the TM1 segment is a signal peptide and should not exist in the AtNHX1. This model is based on experiments in which systematically constructed AtNHX1 molecules were expressed in yeast cells and the topology was assigned based on the mobility of the product after protease treatment of vacuole membranes. Topology assignment might be affected by somewhat unexpected degradation of the products by yeast proteases and unexpected mobility of highly hydrophobic polypeptides on the SDS-PAGE. The topogenesis process on the ER membrane should be common to all eukaryotic cells. Similar systems are conserved in the topogenesis on the cytoplasmic membrane of bacteria cells. AtNHX1 topology is the same as the topology model proposed for the NHE family.

For the correct unique topology formation of R1, the membrane insertion must be supported by the following R2 segment. When the R1 of AtNHX1 was examined alone, the orientation was not determined uniquely, resulting in a mixed topology. Although the hydrophobicity seemed to be sufficient, the charge distribution was not sufficient for the SA-I topology; *e.g.*, positively-charged residues were present in the N-domain and the charge difference between the 15-residue N-domain and C-domain did not favor type I orientation. Despite the insufficiency, R1 was integrated into the membrane in a unique authentic topology depending on the following R2 segment. Interactions between H-segments should be involved in achieving the correct integration, as in previous observations in which the nearest flanking TM segments affected the insertion of the preceding H-segment (*22*).

AtNHX1 has the same topology as hNHE1, even though it possesses no signal peptide. Interestingly, integration of the hNHE1 strictly depends on the signal peptide. When the signal peptide was deleted, the TM2-TM3 region was integrated in an inverted topology. Other plant isoforms and human NHE9 are missing the orthologous regions of the signal peptide. Phylogenic relations do not correlate with the existence of the signal peptide (Fig. 5B). Whether there was a signal peptide in the ancestral molecule of NHE family is not known. There are two possibilities. First, during evolution of the NHE family isoforms, the signal peptide was inserted in front of the first H-segments and then the original topogenic properties of the following H-segments were lost. Second, initially the signal peptide was present in the N-terminal portion and lost after the following H-segments acquired the topogenic property, as noted in AtNHX1. The deletion of the pre-existing segment is more likely than insertion of the functional segment into the specific position. In the G-protein–coupled receptor family that possess seven TM segments, some populations in specific subfamilies possess signal peptides in addition to the N-terminus of the TM1 segment (*23*). In this case, the length of the N-terminal hydrophilic segment seems to correlate with the existence of a signal peptide; the signal peptide is essential for translocation of the long N-terminal domain of specific subfamilies.

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