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# NHE6 Protein Possesses a Signal Peptide Destined for Endoplasmic Reticulum Membrane and Localizes in Secretory Organelles of the Cell*

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**The NHE6 protein is a unique $Na^+/H^+$ exchanger isoform believed to localize in mitochondria. It possesses a hydrophilic N-terminal portion that is rich in positively charged residues and many hydrophobic segments. In the present study, signal sequences in the NHE6 molecule were examined for organelle localization and membrane topogenesis. When the full-length protein was expressed in COS7 cells, it localized in the endoplasmic reticulum and on the cell surface. Furthermore, the protein was fully *N*-glycosylated. When green fluorescent protein was fused after the second (H2) or third (H3) hydrophobic segment, the fusion proteins were targeted to the endoplasmic reticulum (ER) membrane. The localization pattern was the same as that of fusion proteins in which green fluorescent protein was fused after H2 of NHE1. In an *in vitro* system, H1 behaved as a signal peptide that directs the translocation of the following polypeptide chain and is then processed off. The next hydrophobic segment (H2) halted translocation and eventually became a transmembrane segment. The N-terminal hydrophobic segment (H1) of NHE1 also behaved as a signal peptide. Cell fractionation studies using antibodies against the 15 C-terminal residues indicated that NHE6 protein localized in the microsomal membranes of rat liver cells. All of the NHE6 molecules in liver tissue possess an endoglycosidase H-resistant sugar chain. These findings indicate that NHE6 protein is targeted to the ER membrane via the N-terminal signal peptide and is sorted to organelle membranes derived from the ER membrane.**

$Na^+/H^+$ exchangers (NHE)[1] have a crucial role protecting cells against internal acidification and maintaining intracellular pH (1, 2). They comprise a gene family; at present seven isoforms have been identified in mammalian cells (NHE1 to NHE7). The first five isoforms are in the secretory pathway, mainly in the plasma membrane (1). In contrast, NHE6 is suggested to localize in mitochondria (3), and NHE7 is suggested to be targeted to the *trans*-Golgi network (4). NHE6 is of particular interest to us, because it has a similar topology and hydrophobicity to plasma membrane counterparts but might localize in the mitochondria. The N-terminal hydrophilic portion of NHE6, which is rich in positively charged residues, is thought to be a mitochondrial-targeting sequence (see Fig. 1). The following membrane domain is highly hydrophobic, suggesting that it is targeted to ER via a signal-recognition particle-mediated mechanism. It is critical to establish the localization signal of the unique isoform of NHE proteins to clarify the intracellular sorting process of various membrane proteins.

The majority of membrane proteins in the endocytotic and exocytotic pathways are integrated into the ER membrane and then sorted to their final destinations via vesicle transport (5). The initial targeting process to the ER is mediated by the signal-recognition particle and signal-recognition particle receptor (6) and then the nascent polypeptides are integrated into the lipid bilayer via a translocon consisting of the Sec61 complex (7). The ER-targeting signal is defined by a hydrophobic segment of the nascent polypeptide chain. We have studied the topogenesis of the type I signal-anchor membrane proteins in the ER membrane and the mitochondrial outer membrane, whose hydrophobic segments have the amino-terminus on exoplasmic side and the carboxy-terminus on cytoplasmic side ($N_{exo}/C_{cyto}$ topology). Synaptotagmin II, a type I signal-anchor protein in the secretory pathway, is targeted to and integrated into the ER membrane just after the hydrophobic segment emerges from the ribosome, indicating that targeting to the ER membrane is a co-translational process (8). In contrast, Tom20 protein, which is a type I signal-anchor protein on the mitochondrial outer membrane and a primary receptor of precursor proteins for mitochondria (9), is post-translationally targeted to mitochondria. Mitochondria targeting of Tom20 protein is mediated by a characteristic signal in which there is a segment relatively less hydrophobic and a following motif with net positive charges (10). An increase in the hydrophobicity of the Tom20 membrane-anchor segment results in a switch of the targeting function from mitochondrial targeting to ER targeting, indicating that the high hydrophobicity of the segment is a dominant determinant for the co-translational ER-targeting pathway (10). These findings suggest that the NHE6 protein is targeted to the ER membrane rather than to the mitochondria.

The present study examined the intracellular localization of the NHE6 protein and the signal sequences for the membrane

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*The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EMBL Data Bank with accession number(s) AB074255 for NHE6.1.*

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[1] The abbreviations used are: NHE, $Na^+/H^+$ exchanger; EGFP, enhanced green fluorescent protein; Endo-H, endoglycosidase H; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; PNGaseF, peptide *N*-glycosidase F; ProK, proteinase K; RM, rough microsomal membranes; PBS, phosphate-buffered saline; H, hydrophobic segment.

topogenesis. The data of the present study indicated that NHE6 protein localizes exclusively on the membranes in the secretory pathway and that the N-terminal hydrophobic segment is a signal peptide that directs the molecule to the ER membrane and is then cleaved off.

## EXPERIMENTAL PROCEDURES

*Materials*—Enzymes for DNA manipulation and *in vitro* transcription (Toyobo and New England Biolabs), ProK (Merck), endoglycosidase H (Endo-H; New England Biolabs), and PNGaseF (New England Biolabs) were obtained from sources indicated. Rabbit reticulocyte lysate (11) and RM from dog pancreas (12) were prepared as described previously. RM were washed with 25 mM EDTA and treated with Staphylococcal nuclease (Roche Molecular Biochemicals) to remove endogenous mRNA as described previously (12). Prestained molecular weight markers (New England Biolabs) were used to estimate the approximate size of proteins using SDS-PAGE. The original clone of NHE6 missing several of the N-terminal residues was provided by Kazusa DNA Research Institute.

*Antibodies and Organelle Probe*—A synthetic peptide (GDHELVIRGTRLVLPMDDSE) corresponding to the C terminus of human NHE6 was coupled to keyhole limpet hemocyanin and used to raise antibodies in rabbit. Specific IgG was affinity-purified using the synthetic peptide coupled to Thiol-link resin (Pierce) according to the standard protocol supplied by the company. Anti-EGFP antiserum was raised in rabbit by immunizing with recombinant EGFP purified from *Escherichia coli*. Antibodies to H-450 (13), Tom40 (14), and cytochrome P450(2C11) (15) were used as markers for the cell fractions containing cytosol, mitochondria, and the microsomal membranes, respectively. Mouse monoclonal antibody against human CD4 (NU-TH/I; Nichirei) and rabbit antibodies against calnexin (StressGen) were obtained from the indicated sources.

*Isolation of cDNA-encoding NHE6 Protein and Construction of Expression Plasmids*—Total cDNAs from human liver total RNA were kindly supplied by Dr. Ukaji (Kyushu University). For isolation of NHE6 cDNA, the 5′-half and 3′-half of the NHE6 open reading frame were separately obtained by PCR amplification using human liver cDNA and the following primer pairs: AGGCGGGGGGAGACATG (sense for 5′-end), GAGGAAGGTACTCCAGGACATCAA (antisense for the former portion), CGGGAGTTCCAGTTGTTGGAGACA (sense for the latter half portion), and GTATTAGTAAGCTTAGGCTGGACCATG (antisense for 3′-end).

Each obtained DNA was denatured at 90 °C for 2 min, quickly cooled on ice, and phosphorylated with T4-polynucleotide kinase. After the phosphorylation reaction, the denatured strands were annealed and ligated with pGem5fZ(+) (Promega), which had been digested with *Eco*RV. The two cDNA fragments were connected at the endogenous *Stu*I site, which appears in the middle portion of the NHE6 coding sequence. The full-length cDNA was subcloned between the *Hin*dIII and *Xba*I sites of the pRcCMV vector (InvitroGen). The obtained NHE6 cDNA was sequenced. Unexpectedly, there were 96 bases inserted between $Leu^{143}$ and $Val^{144}$ of the originally reported sequence (3) (see Fig. 1*A*). The full-size clone without the insert was also constructed by connecting the N-terminal portion of our cDNA with the original open reading frame cDNA missing the N-terminal portion (KIAA0267) using the overlap extension method (16). To distinguish the newly obtained cDNA from the original, we termed them NHE6.1 and NHE6.0, respectively.

*DNA Construction*—For N-terminal and GFP fusion proteins, each DNA fragment coding for $Met^1$–$Gly^{46}$, $Met^1$–$Phe^{98}$, and $Met^1$–$Pro^{123}$ was obtained by PCR amplification from human liver cDNA and inserted between *Hin*dIII and *Xba*I of the GFP fusion plasmid, pIJGFP (10). For NHE1-GFP fusion constructs, the N-terminal portion of NHE1 ($Met^1$–$Arg^{100}$, $Met^1$–$Cys^{133}$) was isolated with PCR amplification from human cDNA using the primers ATGCAAGCTTCCACCATGGTTCTGCGGTCT (sense at initiation methionine, *Hin*dIII site, the Kozak sequence, and ATG are underlined), TACGTCTAGAGCGCACGTGTGTGTAGTC (antisense at $Arg^{100}$, with *Xba*I site), and TACGTCTAGAGCAGCTCTCCGGGACGAT (antisense at $Cys^{133}$, with *Xba*I site). The obtained fragments were ligated with pIJGFP digested with *Hin*dIII and *Xba*I.

For CD4 derivatives, cDNA encoding human CD4 kindly supplied by Dr. Nakauchi (RIKEN) was subcloned between *Eco*RI and *Hin*dIII of pKCRH2PL (17) to obtain pCD4 (15). The thirteen C-terminal residues were deleted by site-directed mutagenesis. Mutagenesis exposed the di-lysine motif on the C terminus, so that the resulting molecule, CD4D, localized only in the ER membrane in the cells (15, 18).

*Cell Cultures and Immunofluorescent Microscopy*—COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum under 10% $CO_2$ atmosphere. Transfection was performed according to standard procedures using FuGene reagent (Roche Molecular Biochemicals). The cells were grown on glass coverslips in culture dishes for 24 h after the transfection. The cells on the coverslips were fixed with 50% methanol, 50% acetone for 2 min at room temperature. After washing two times with phosphate-buffered saline (PBS), the coverslips were incubated with PBS containing 2% bovine serum albumin for 15 min and then incubated with the primary antibodies: affinity-purified anti-NHE6 IgG, anti-EGFP serum, anti-CD4 monoclonal antibody, or anti-calnexin antibodies. After incubating for 1 h, the coverslips were washed three times with PBS containing 0.1% Tween 20 and then incubated for 30 min with the FITC or Texas Red-labeled secondary antibodies. The coverslips were washed and mounted on glass slides with VECTASHIELD (Vector Laboratories, Burlingame, CA). The sample was examined using confocal laser scanning microscopy. Where indicated, mitochondria were stained with MitoTracker (Molecular Probes).

*Immunoblotting Analysis*—After transfection, COS7 cells (on 10-cm culture dishes) were cultured for 24 h and harvested with a cell lifter (Costar) into 2 ml of PBS and precipitated by centrifugation at 5000 × *g* for 2 min. Cells were resuspended with PBS containing Complete, mini, and EDTA-free protease inhibitor mixture tablets (Roche) and sonicated in a microtube with Bioruptor (Cosmobio) at maximum intensity for 1 min. After brief centrifugation at 3000 × *g* for 5 min, membranes were precipitated by ultracentrifugation (163000 × *g* for 10 min). Where indicated, aliquots were treated with either Endo-H or PNGaseF for 2 h at 37 °C under the buffer conditions recommended by the supplier. The aliquots, containing the same equivalents, were analyzed by SDS-PAGE (10% gel) and subsequent immunoblotting using the C-terminal antibodies. Protein bands were visualized with horseradish peroxidase-labeled anti-rabbit IgG (BIOSOURCE) and ECL reagent (Amersham Biosciences, Inc.). Chemiluminescence was detected with a LAS1000plus (Fuji, Tokyo, Japan).

*In Vitro Translation and Topology Assay*—*In vitro* transcription and translation were performed as described previously (19). The plasmid (1 μg) was linearized with *Eco*RI and then transcribed with T7 RNA polymerase at 37 °C for 60 min. The mRNAs were translated in the presence or absence of RM using the cell-free system containing 20% reticulocyte lysate. Salt conditions were optimized as 100 mM potassium acetate and 1.0 mM magnesium acetate. Aliquots were treated with ProK (333 μg/ml) on ice for 30 min in the presence or absence of 0.5% Triton X-100. To improve the resolution of SDS-PAGE, RM was isolated from the translation mixture through a high salt cushion with ultracentrifugation where indicated, as previously described, and was treated with Endo-H (20, 21). After the enzyme reactions were terminated with 10% trichloroacetic acid, the precipitated protein was dissolved in SDS sample buffer and analyzed using SDS-PAGE. The gel image was visualized using a phosphoimager (FLA2000; Fuji, Tokyo, Japan).

*Cell Fractionation of Rat Hepatocytes*—Cell fractionation was performed as described previously (22). Rat liver (7.8 g) was homogenized with the homogenization buffer using a Potter homogenizer (10 strokes at 4 °C). The homogenized mixture was centrifuged at 3000 × *g* for 10 min, and the post-nuclear supernatant was centrifuged at 8000 × *g* for 10 min to obtain precipitates of the mitochondrial fraction. The post-mitochondrial supernatant was further centrifuged at 200000 × *g* (55000 rpm) for 1 h to separate the cytosol fraction and microsomal membranes (Ms).

## RESULTS

*NHE6 Is Localized in the ER in Cultured Cells*—NHE6 protein contains two characteristic sequences that seem to possess an organelle-targeting function; the N-terminal sequence rich in positively charged residues, which is predicted to be a mitochondrial-targeting signal, and the highly hydrophobic sequences that seem to be a signal destined for the ER membrane (Fig. 1*A*). To assess the localization of human NHE6, we constructed expression plasmids for NHE6 proteins in which the expression was regulated by the cytomegalovirus promoter.

Both cDNAs isolated from human fetal brain and adult liver contained the same 96-base inserts, which encode 32 amino acid residues between $Leu^{143}$ and $Val^{144}$ of the reported NHE6 molecule (Fig. 1*A*, underline). We termed the new version of the

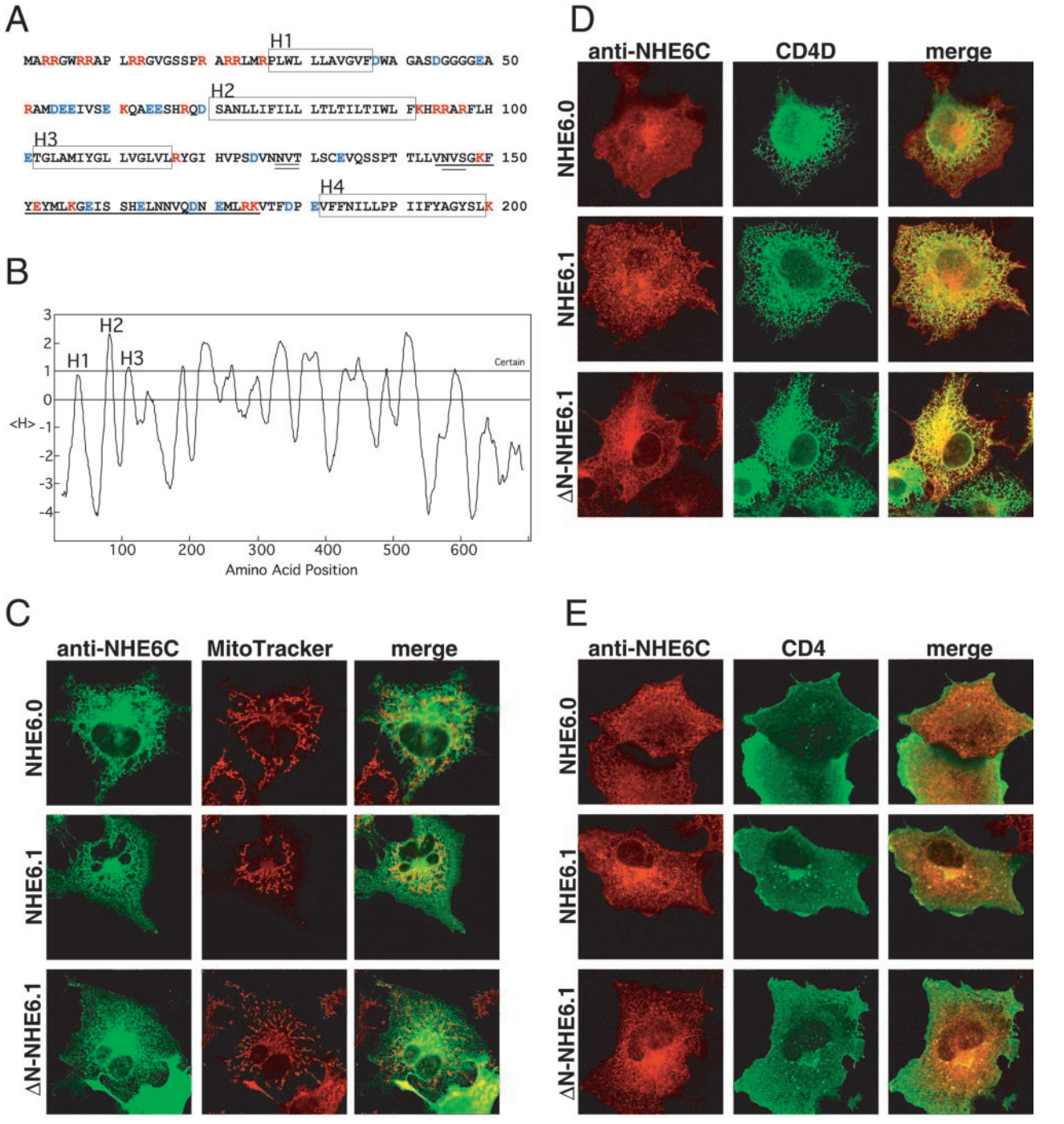


FIG. 1. **NHE6.0 and NHE6.1 locate in the ER and secretory organelle membranes but not in the mitochondria of COS7 cells.** *A*, N-terminal sequence of human NHE6.1. The hydrophobic segments (*H1–H4*) are indicated by *boxes*. Potential *N*-glycosylation sites between H3 and H4 are indicated by *double underlines*. Positively and negatively charged residues are indicated by *red* and *blue characters*, respectively. The 32-amino acid insert in NHE6.1 ($Val^{144}$–$Lys^{175}$) is *underlined*. *B*, hydrophobicity profile, <*H*>, was calculated according to TopPredII using the default parameters (38). The *line* at <H> = 1 indicates the standard cutoff value for predicting "certain" transmembrane helices. The N-terminal three hydrophobic segments are indicated in the *panel*. *C–E*, intracellular location of the NHE6 variants, NHE6.0 and NHE6.1. The expression plasmids for NHE6.0, NHE6.1, and the mutant of NHE6.1 in which 39 N-terminal residues were deleted (Δ*N-NHE6.1*) were transfected into COS7 cells. The NHE6 molecules were detected by using fluorescent microscopy with antibodies against the C-terminal synthetic peptide. In *panel C*, cells were also stained with MitoTracker (*red*) as a mitochondrial marker. NHE6 proteins were detected with FITC-conjugated anti-rabbit IgG. *D* and *E*, NHE6 proteins were coexpressed with CD4D and CD4, which locate in the ER membrane and plasma membrane, respectively, as the control. The NHE6 molecules were detected with Texas Red-conjugated anti-rabbit antibodies, and the CD4 molecules were detected with FITC-labeled anti-mouse IgG.

NHE6 sequence NHE6.1 to distinguish it from the original sequence (NHE6.0). With the 96-base insertion, the loop between H3 and H4 became more hydrophilic and gained an additional *N*-glycosylation site (double underlines). The expression plasmid for the NHE6.0 cDNA was also constructed from the previously reported cDNA clone.

The constructs for NHE6.0 and NHE6.1 were transiently expressed in COS7 cells, and the location was examined using indirect immunofluorescent microscopy with polyclonal antibodies against the synthetic peptide corresponding to the 15 C-terminal residues of human NHE6. Both constructs localized in the reticular structure and on the cell surface, which is

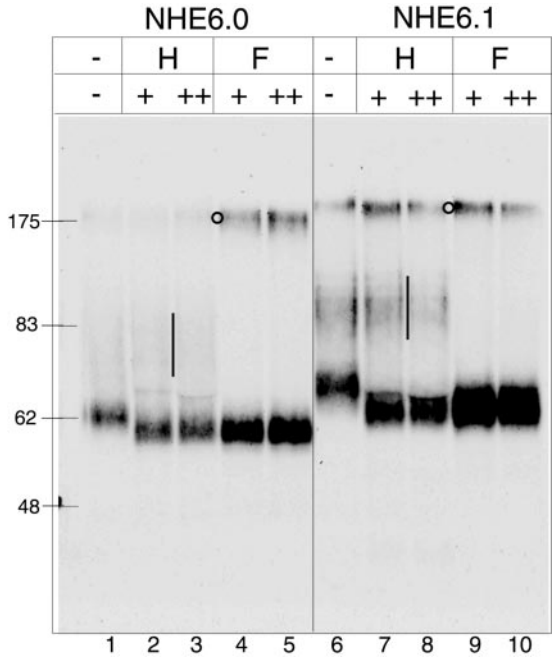


FIG. 2. **NHE6.0 and NHE6.1 were *N*-glycosylated in COS7 cells.** Expression plasmids for NHE6.0 and NHE6.1 were transfected into COS7 cells. After incubation for 24 h, cells were disrupted by sonication, and membranes were recovered by ultracentrifugation. Aliquots of the membranes were treated with Endo-H (*H lanes*; +, 1000 units; ++, 2000 units) or PNGaseF (*F lanes*; +, 500 units; ++, 1000 units). The samples were subjected to SDS-PAGE (10% gel) and subsequent immunoblotting analysis using the C-terminal antibodies. The diffuse higher molecular weight signals, which disappeared only after PNGaseF treatment, are indicated by *vertical lines*. The *open circles* indicate multimeric or aggregated NHE6 protein.

completely different from the mitochondrial marker (MitoTracker; see Fig. 1*C*). The NHE6 protein signals were not observed in the nontransfected cells (data not shown), indicating that they were specific for the transfected NHE6 gene. To address whether the NHE6 protein signals were located on membrane organelles in the secretory pathway, the expression plasmid for either the marker protein for ER (CD4D) or the cell surface (CD4) was co-transfected (*D* and *E*). CD4 is a well known cell surface marker of T-cells, and CD4D is a mutant in which 13 C-terminal residues were deleted to expose the dilysine motif at the C-terminal and to localize in the ER membrane (18). The marker proteins located to the ER and cell surface as expected (*D* and *E*). The expressed NHE6.1 and NHE6.0 proteins partially co-localized with the ER resident protein, as well as with the cell surface protein, strongly suggesting that both of the proteins existed in the secretory organelles between the ER and plasma membranes. Even when the N-terminal 39-residue-long positive charge-rich sequence was deleted (ΔN-NHE6.1), the localization of the protein was essentially the same as the original (*C*, *D*, and *E*). Essentially the same results were obtained using other cultured cells, PS120 cells, which had been stably transfected with the expression plasmids for NHE6.0 and NHE6.1 (data not shown).

*NHE6.0 and NHE6.1 Molecules Are N-Glycosylated in the COS7 Cells*—To examine the glycosylation states of the expressed proteins, microsomal membranes were analyzed using SDS-PAGE and immunoblotting with the C-terminal antibodies. Each construct had a single major band and diffused signals (vertical bars) of higher molecular weight (Fig. 2, *lanes 1* and *6*). These bands were not observed in the COS7 cells transfected with only a vector plasmid (data not shown), indicating that the antibodies reacted only with the NHE6 molecule expressed by the plasmid. Following Endo-H treatment, the major bands of NHE6.0 and NHE6.1 were shifted down, but the diffused signals were not affected (*lanes 2*, *3*, *7*, and *8*). PNGaseF treatment, which cleaves off all of the *N*-linked sugar chains including the complex type, made the diffuse larger signals and the major band the smaller form (*lanes 4*, *5*, *9*, and *10*). The larger bands of ~170 kDa, indicated by open circles, are likely to be multimers or aggregates of the NHE proteins, as reported previously (23). The enzyme treatments produced essentially the same patterns, even when twice the amount of enzyme was used (compare lanes + and ++), indicating that the enzyme treatment proceeded completely. Take together it is indicated that both NHE6.1 and NHE6.0 were fully *N*-glycosylated and were sorted beyond the Golgi compartment to some degree in the cultured cells. The NHE6.0 and NHE6.1 molecules expressed in COS7 cells were primarily targeted to the ER membrane and *N*-glycosylated.

*N-terminal Sequence Contains a Signal for ER but Not for Mitochondria*—To examine the organelle-targeting function, various N-terminal GFP fusion proteins were constructed (Fig. 3, *A* and *B*). When the fusion proteins were expressed in COS7 cells, H1-H2-GFP and H1-H2-H3-GFP produced a reticular pattern that was identical with that of the ER marker protein, calnexin; the signals overlapped, producing the yellow color shown in *panel A*. These results indicated that the N-terminal portion, including H1 and H2, contains sufficient information to target the ER membrane. As a control, we used the NHE isoform, NHE1, which is targeted to the ER (24, 25). H1-H2-GFP, in which GFP was fused after the second hydrophobic segment of NHE1, produced a reticular pattern that overlapped with that of calnexin in COS7 cells (Fig. 3*C*).

The mitochondrial-targeting function of the N-terminal-positive-rich sequence was also examined using N30-GFP fusion protein (Fig. 3*B*). This construct did not produce a significant fluorescent GFP signal. Only trace amounts of signal were detected, even when using the anti-GFP antibodies. To address the possibility of rapid degradation via the proteasome system, the effect of lactacystin was examined. After incubation for 8 h with lactacystin, the antibodies produced a significant GFP signal throughout the cell. This signal is diffuse cytoplasmic and not ER, as well as not mitochondrial, suggesting that the N-terminal sequence does not possess the mitochondrial-targeting function. It is likely that the 30 N-terminal residues prevent correct folding of the GFP polypeptide chain and lead to degradation via a proteasome.

*N-terminal Sequence of 46 Residues Contains a Signal Peptide*—The data described above indicated that the 98 N-terminal-residue sequence of NHE6, including the two hydrophobic segments, has an ER-targeting function. To further examine the topogenic functions of the N-terminal portion, the topologies of the N-terminal fusion constructs were assessed using a cell-free expression system. GFP fusion after the H1 segment (H1-GFP) was also constructed (Fig. 4*A*). These constructs were expressed in a reticulocyte lysate system in the presence or absence of RM. Major bands were observed with the expected molecular weights (Fig. 4*A*, *lanes 1*, *5*, and *9*, *downward arrowhead*). When synthesized in the presence of RM, all of the constructs were efficiently processed (*lanes 2*, *6*, and *10*, *upward arrowhead*). The processed form of H1-GFP was not degraded by ProK treatment (*lane 3*), whereas it was accessible to the protease in the presence of non-ionic mild detergent (Triton X-100; *lane 4*). In contrast, the processed form of the H1-H2-GFP construct was degraded by ProK, even in the absence of detergent (*lane 7*), indicating that the reporter domain was on the cytoplasmic side of RM. The band in *lanes 4* and *7* is highly likely to be a ProK-resistant core of the folded GFP molecule. The processed form of the H1-H2-H3-construct was ProK-re-

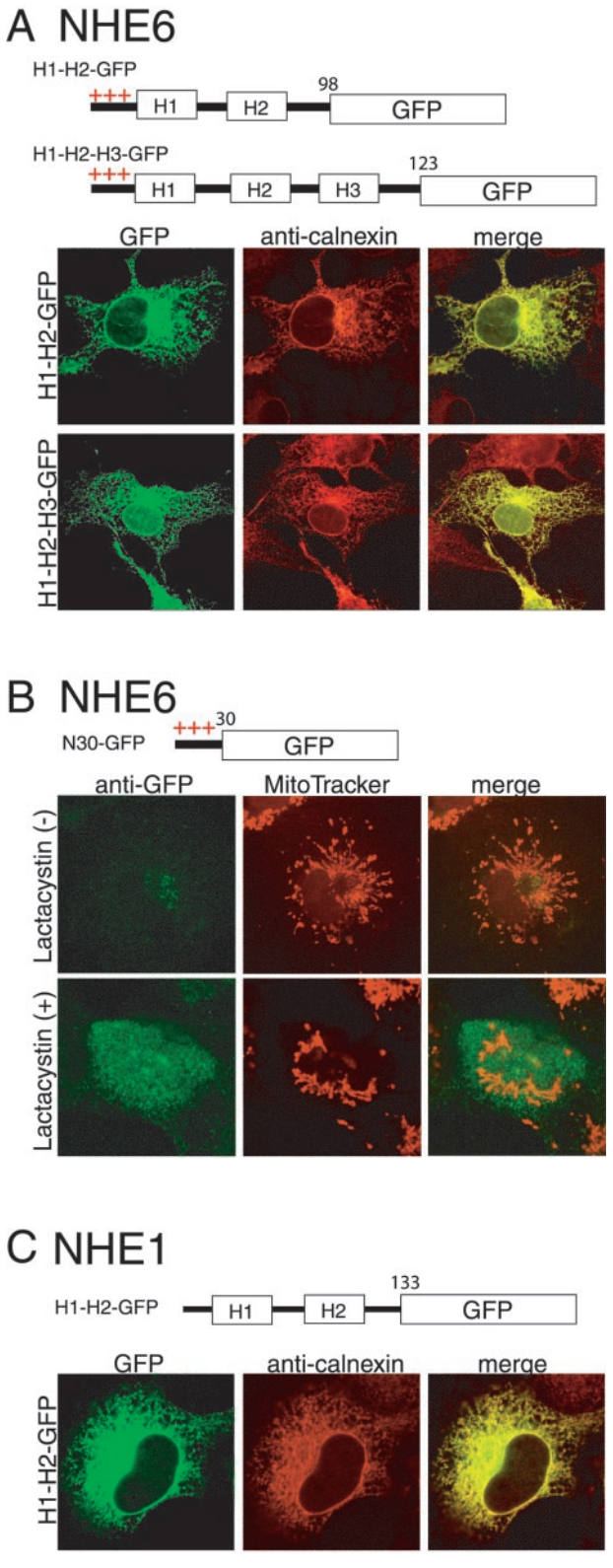


FIG. 3. **N-terminal sequences of NHE6 and NHE1 possess an ER-targeting function.** *A*, N-terminal sequences indicated in the figure were fused to GFP and expressed in COS7 cells. The ER marker protein, calnexin, was stained with Texas Red-conjugated anti-rabbit IgG. *B*, N-terminal sequence including only the N-terminal-positive charge-rich segment of NHE6 was fused to GFP. 12 h after transfection, cells were further incubated for 8 h in the presence (+) or absence (−) of 20 $\mu$M lactacystin. Because GFP fluorescence was not detected at any time, cells were stained with the anti-GFP antibodies and FITC-conjugated anti-rabbit IgG. Cells were also stained with MitoTracker (*red*). *C*, the fusion protein of the N-terminal sequence of NHE1 and GFP was expressed in COS7 cells.

sistant (*lane 11*), indicating that its GFP domain was on the lumenal side, suggesting that the H3 segment was inserted into the membrane and mediated translocation of the following reporter domain (see Fig. 4*C*). These results indicated that the N-terminal portion, including the H1 segment, is the signal peptide that mediates translocation of the following portion, the H2 segment interrupts translocation, and the H3 segment again mediates translocation of the GFP domain to the lumenal side (*C*). These NHE6 fusion proteins had no *N*-glycosylation sites.

A similar experiment was performed using the NHE1 N-terminal portion as a control of the ER-directed NHE isoform (Fig. 4*B*). The H1-GFP construct shifted little in mobility, even in the presence of RM (*lane 2*), whereas the full-size band was resistant to ProK (*lane 3*). Similarly, H1-H2-GFP did not shift down (*lane 6*) but was degraded by ProK even in the absence of detergent (*lane 7*). When RM vesicles were isolated by ultracentrifugation and treated with Endo-H, the band clearly shifted down by 2.5 kDa (*lanes 9–12*). Thus, the H1 of NHE1 behaved as a signal peptide and was then processed off, and the endogenous glycosylation site in the C-terminal flanking region of the processing site was efficiently glycosylated. This is the reason that the mobility of the bands obtained in the absence and presence of RM appeared to be the same. It also indicates that H2 is the stop-transfer sequence, which leaves the following portion on the cytoplasmic side. Taken together, topogenic functions of the N-terminal portions of NHE1 and NHE6 are highly similar; H1 is a signal peptide, and H2 is a stop-transfer sequence, as shown in *panel C*.

*NHE6 Localizes on the Membranes in the Secretory Pathway*—To elucidate intracellular localization of NHE6 protein in liver cells, a cell fractionation study was performed. We found a sequence similar to human NHE6 in the rat expression sequence tag data base (GenBank™ accession number AI501017). It is 91% homologous to the region from $Thr^{558}$ to $Ala^{669}$ of human NHE6. The peptide sequence used for making antibodies is 100% identical in human and rat. Each cell fraction from rat liver was probed using the affinity-purified antibodies (Fig. 5*A*). A major single band was detected in the microsomal fraction (*lane 3*) but not in the cytosol or mitochondrial fractions. There was no shift in mobility of this band following Endo-H treatment (*lane 5*), whereas major and minor larger bands shifted down following treatment with PNGaseF (*lane 6*). The endogenous polypeptide of NHE6 was fully *N*-glycosylated and modified into the complex form. To confirm separation of these fractions, they were examined by immunoblotting with the antibodies against H450 (cytosol), cytochrome P450(2C11) (microsomes), and Tom40 (mitochondria) (*B*). The majority of each marker protein was detected in the expected fractions, indicating that microsomal membranes and mitochondria were clearly separated. The obtained microsomal fraction contained secretory organelles, Golgi, plasma, and ER membranes. These data suggest that the NHE6 molecule locates in the membrane of the secretory pathway and becomes modified in the Golgi compartments.

## DISCUSSION

All lines of evidence in the present study demonstrate that the NHE6 molecule localizes on the organelle membranes in the secretory pathway that are derived from ER membranes. The N-terminal hydrophobic segment (H1) of NHE6 is a signal peptide and is cleaved off. The positive charge-rich sequence of the N-terminal portion does not possess a mitochondrial-targeting function. Thus, the H1 portion primarily determines ER targeting as in the case of NHE1. The next hydrophobic segment (H2) of NHE6 and NHE1 interrupts translocation and becomes the transmembrane segment (the so-called stop-trans-

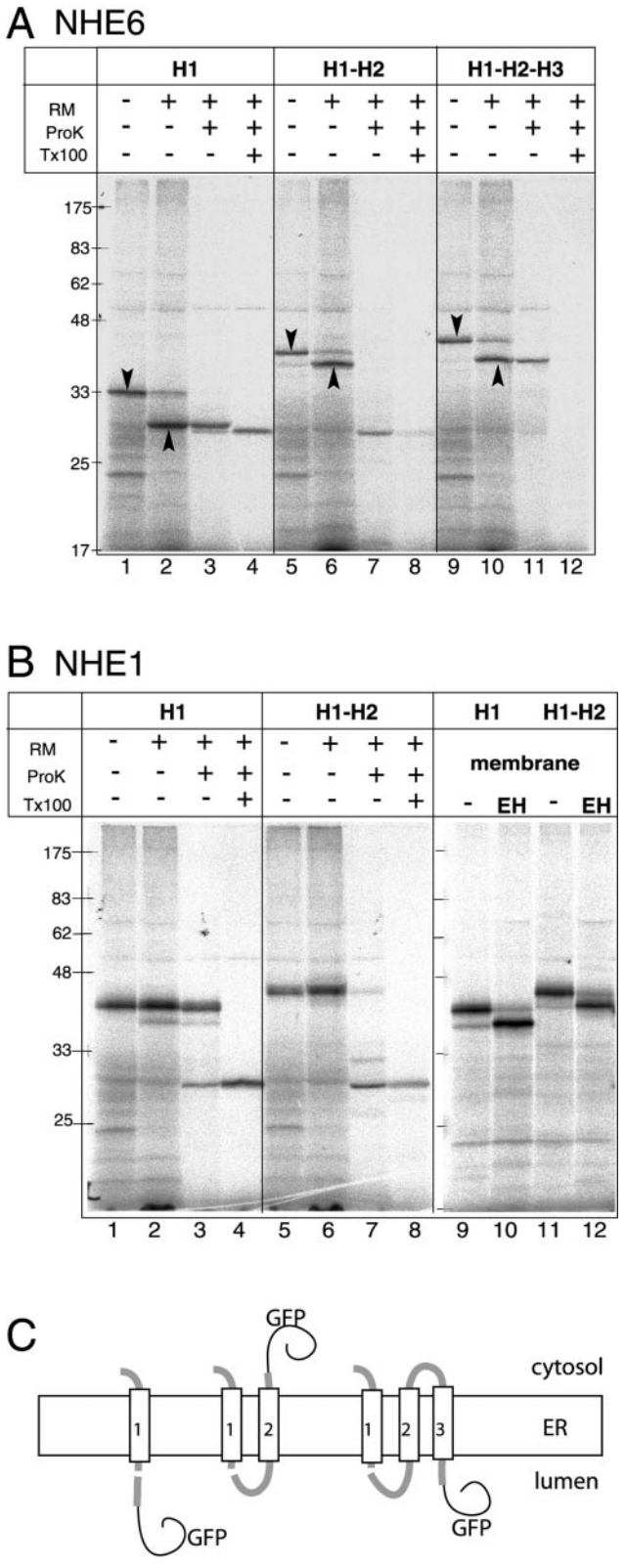


FIG. 4. **The N-terminal hydrophobic segment (*H1*) of NHE6 and NHE1 is a signal peptide.** *A*, GFP fused after the H1 ($Gly^{46}$), the H2 ($Phe^{98}$), and the H3 ($Pro^{123}$) of NHE6 were expressed in a reticulocyte lysate cell-free system in the presence or absence of RM. Aliquots were treated with ProK in the presence (+) or absence (−) of Triton X-100. The *downward* and *upward arrowheads* indicate precursor and processed forms, respectively. *B*, GFP fused after the H1 ($Arg^{100}$) or H2 ($Cys^{133}$) of NHE1 was expressed as shown in *panel A*. Where indicated (*membrane*), RM vesicles were isolated after the translation reaction through a 0.5 M sucrose cushion and subjected to Endo-H treatment (*EH lanes*). *C*, schematic presentation of topology of each construct as follows: H1 construct translocated the GFP domain through the membrane and processed off, H1-H2 constructs left the GFP domain on the cytoplasmic side because of the stop-transfer function of H2, and H1-H2-H3 constructs again translocated the GFP domain through the membrane because of the reinitiation function of H3. Note that the H1 segment of all the constructs was processed in the membrane as a signal peptide and that the processed GFP domain of NHE1 constructs contained sugar chains.

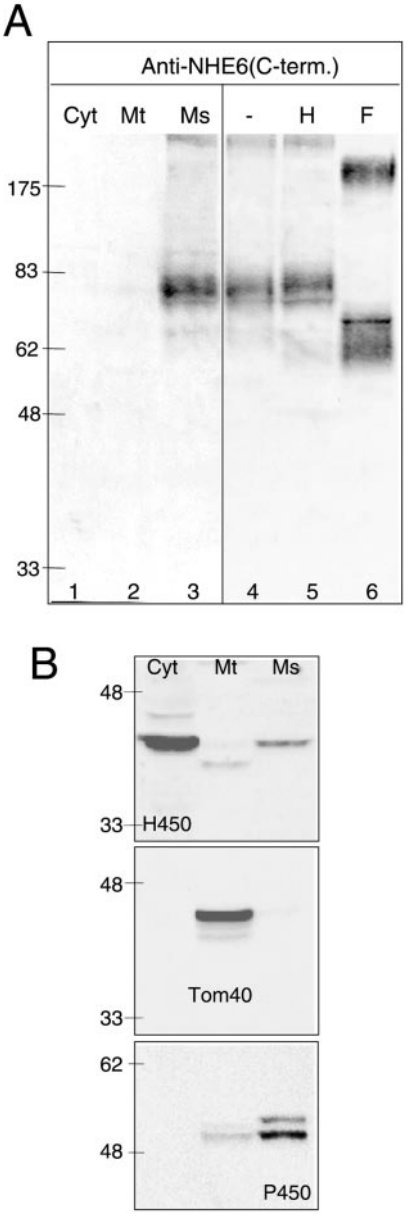


FIG. 5. **NHE6 molecules locate in the microsomal fraction and possess complex sugar chains in liver cells.** *A*, cell fractionation of NHE6 protein using rat liver. Rat liver was homogenized and fractionated using differential centrifugation. Mitochondrial precipitates (*Mt*), microsomal precipitates (*Ms*), and cytoplasmic supernatant (*Cyt*) were subjected for immunoblotting analysis. NHE6 was noticed only in the microsomal fraction. Aliquots of the microsomal membrane fraction were treated by Endo-H (*H*) or PNGaseF (*F*). *B*, H450, Tom40, and cytochrome P450(2C11) were used as cytosol, mitochondria, and microsomes markers, respectively.

fer sequence). Furthermore, the following H3 segment is able to start the integration of the following portion again (so-called translocation reinitiation sequence). Thus, membrane integration of the N-terminal three transmembrane segments is explained by the conventional co-translational integration model

(26–28). The two hydrophobic segments, H2 and H3, meet the requirements for the topogenic functions (28–30).

The NHE6 molecule is initially targeted to the ER membrane and is sorted to the organelle membranes toward the plasma membrane. Cell fractionation studies indicated that the molecule expressed in the rat liver cells is sorted toward the plasma membrane as are the other NHE proteins and then becomes resistant to Endo-H treatment. In the cultured cells, both the NHE6.0 and NHE6.1 molecules were located in membranes between the ER and plasma membrane. NHE6.0 and NHE6.1 were more dispersed than the ER marker (CD4D) and less dispersed than the plasma membrane marker (CD4). In contrast to liver cells, significant amounts expressed in COS7 cells were Endo-H-sensitive. It is probable that they were too highly expressed in the cell to be fully sorted through the Golgi compartment. The exit efficiency from the ER seems to be highly dependent on the molecular character; *e.g.* the H1-H2-GFP fusion protein almost completely merged with the ER marker protein, indicating that it was not sorted out from the ER. Generally, a variety of membrane proteins are retained in the ER by the ER quality control mechanisms (31, 32). Anyway, it is clearly demonstrated that in liver cells, as well as COS cells, the NHE6 proteins are completely *N*-glycosylated in the ER membrane.

The present results have implications for membrane topogenesis of the NHE family proteins. The N-terminal first hydrophobic segment (H1) of NHE1 and NHE6 was cleaved off *in vitro*. The algorithm for prediction of signal peptides by Nielsen *et al.* (33) suggests that both H1 segments are cleavable signal sequences, and the processing sites of NHE6 and NHE1 are $Ala^{56}$ and $Ala^{35}$, respectively. The H1 segment of NHE3 is a signal peptide (34). Other isoforms, NHE2, NHE4, NHE5, and NHE7, are also predicted to have signal peptides. Thus, it is reasonable to assume that the N-terminal hydrophobic segments of other isoforms are also signal peptides.

The positive charge-rich segment is not a mitochondrial-targeting sequence. The sequence is too rich in positive charges, and hydrophobic residues are rare, so it does not meet the proposed requirements for the presequence of the matrix-targeted sequence (35–37). The previous reports that the NHE6.0 protein located exclusively in mitochondria (3, 4) are inconsistent with the present results, and the reason for the discrepancy remains to be clarified. They provide one immunofluorescent experiment, where NHE6-GFP fusion was located in dot structures in HeLa cells (3). As mentioned by the authors, the GFP signal was not very correspondent with the mitochondrial marker, and the protein aggregates could have resulted in the apparent colocalization (3). It is also reported that NHE6-HA fusion expressed in the stably transfected Chinese hamster ovary cell line under the control of inducible promoter was fractionated in the mitochondrion-enriched fraction (P10 pellet), whereas the NHE7-HA fusion protein was associated with the microsomal enriched membrane fraction (P100 pellet) (4). They provided no data concerning glycosylation status. Although the difference among the cell-fractionation studies are unresolved at present, all the data in this study of the localization in the COS cells, the *N*-glycosylation status, the presence of the ER-targeting signal peptide, and the localization of NHE6 protein with Endo-H-resistant and PNGaseF-sensitive sugar chain in microsomal fraction from liver cells, demonstrated the localization of the NHE6 protein in the membrane organelles derived from ER.

In the present study, the isolated NHE6.1 cDNA was identical to the reported NHE6 sequence (3) except that it possesses a 96-base insert coding for 32 amino acid residues (Fig. 1*A*). The insertion is directly connected to previous sequences in the human genome data base (GenBank™ accession number AC025232), indicating that this 96-base sequence is transcribed as a single exon including the preceding sequence. We isolated the NHE6.1 version of cDNAs from three independent human mRNA sources, fetal brain, fetal liver, and adult liver (data not shown), suggesting that the NHE6.1 mRNA is expressed in various tissues. NHE6.0 cDNA, however, was not detected (data not shown). The mechanisms for expression regulation and multiplicity of NHE6 mRNA require further investigation.

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