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Chimeric Na^+/H^+ Antiporters Constructed from NhaA of *Helicobacter pylori* and *Escherichia coli:* Implications for Domains of NhaA for pH Sensing[1]

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In order to delineate regions which play a role in the regulation of Na^+/H^+ antiporter NhaA activity by pH, we analyzed the antiporter activities of various chimeric mutants constructed from specific portions of NhaA from *Escherichia coli* and *Helicobacter pylori* (EC and HP NhaA). HP NhaA contains 10 residues at the amino-terminus, and 38 residues in a loop region between the eighth and ninth transmembrane spans (loop 8), which are absent in EC NhaA. Deletion from HP NhaA or insertion into EC NhaA of the sequences caused almost no change in pH-dependent antiport activities relative to in the case of the wild-type parent molecules. Chimeras consisting of various combinations of the amino-terminal (amino terminus to sixth or eighth transmembrane span) and carboxy-terminal (seventh or ninth transmembrane span to the carboxy-terminus) regions of EC and HP NhaA showed antiporter activity profiles intermediate between those of the parent molecules. These results show that the two HP-specific sequences are not directly involved in the mechanism of pH sensing by HP NhaA and that the pH sensitivity of NhaA activity is not determined by the amino- or carboxy-terminal regions of NhaA alone, but may be due to interaction between unconserved residues in the two domains. In addition, it was suggested that loop 8 functions primarily as a hinge in both NhaA molecules.

Key words: Na^+/H^+ antiporter, NhaA, *Helicobacter pylori*, pH sensor.

Na^+/H^+ antiporters are ubiquitous membrane proteins found in the cytoplasmic and organelle membranes of cells of from microorganisms to higher plants and animals. Their primary function is to maintain the intracellular pH and sodium concentration by exchanging Na^+ for H^+. Regulation of the intracellular concentrations of these ions by antiporters is important in cell growth and resistance to salt (*1–4*).

In *Escherichia coli*, three Na^+/H^+ antiporters (NhaA, NhaB, and ChaA) are known and their functional characteristics have been well described (*5–9*). The activities of these antiporters are dependent on pH (*7–9*), and pH sensitivity is also characteristic of other transporters involved in general intracellular pH homeostasis (*4*). Defining the mechanism of regulation of the transporters by pH is important for understanding the mechanisms of intracellular pH regulation and salt homeostasis. Of the *E. coli* transporters that have been analyzed in terms of pH dependence of their activities, NhaA exhibits the greatest sensitivity to pH, with a change in maximum velocity of ion-transport by more than 3 orders of magnitude in response to a shift from pH 7 to 8.5 (*8*). NhaA therefore provides a useful model with which to examine the mechanism of pH regulation of membrane transport proteins.

We recently cloned the NhaA antiporter of *Helicobacter pylori* (HP NhaA) and expressed it as a functional protein in *E. coli* (*10*). *H. pylori* is a pathogenic bacterium involved in gastric inflammation, and lives in very acidic conditions (*11*). The activity of *E. coli* NhaA (EC NhaA) is negligible at acidic to neutral pH and increases significantly at alkaline pH, whereas HP NhaA is active in a pH-independent manner from pH 6.0 to 8.5 (*10*). HP and EC NhaA exhibit 49% identity and 82% similarity at the primary amino acid sequence level, and show very similar hydropathy profiles, the exceptions being two sequences which are found only in HP NhaA (Fig. 1, a and b). One of these is a 38 amino acid sequence which is inserted in a loop region between the eighth and ninth transmembrane spans (loop 8). Loop 8 contains many charged residues and is the most hydrophilic region in both NhaA molecules, although fewer charged residues are present in loop 8 of HP NhaA than in that of EC NhaA. The other additional HP-specific sequence consists of 10 residues at the amino terminus of HP NhaA. To date, three residues (Leu-73, His-225, and Gly-338) have been shown to be related to pH sensitivity in EC NhaA (*12–14*). However, since these residues are conserved in HP NhaA, the pH sensitivity of NhaA appears to be determined by some additional residues or regions.

We are attempting to identify regions and residues responsible for the different responses to pH shown by HP and EC NhaA, in order to further investigate the mechanisms of functional regulation of NhaA activity by pH. In

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a

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                                    TM 1
ECNhaA    1'          MKHLHRFFSSDASGGIILIIAAILAMIMANSGATSGWYHDFLETPVQLRV
HPNhaA    1" MNLKKTENALSLTLKNFIKSESFGGIFLFLNAVLAMVVANSFLKES-YFALWHTPFGFQI
                      *..*..*.. ***.*...*.***..***  ... * .. .** ...
                 TM 2                                  TM 3
ECNhaA   51' GSLEINKNMLLWINDALMAVFFLLVGLEVKRELMQGSLASLRQAAFPVIAAIGGMIVPAL
HPNhaA   60" GDFFIGFSLHNWIDDVLMALFFLMIGLEIKRELLFGELSSFKKASFPVIAAIGGMIAPGL
             *.. *. ..  **.*.***.***..***.****. *.*.*...*.**********.*.*
                 TM 4                                  TM 5
ECNhaA  111' LYLAFNYADPITREGWAIPAATDIAFALGVLALLGSRVPLALKIFLMALAIIDDLGAIII
HPNhaA  120" IYF-FLNANTPSQHGFGIPMATDIAFALGVIMLLGKRVPTALKVFLITLAVADDLGAIVV
             .*. *  *.. ...*..** **********. ***.*** ***.**..**. ******..
                      TM 6                  TM 7
ECNhaA  171' IALFYTNDLSMASLGVAAVAIAVLAVLNLCGARRTGVYILVGVVLWTAVLKSGVHATLAG
HPNhaA  179" IALFYTTNLKFAWLLGALGVVLVLAVLNRLNMRSLIPYLLLGVLLWFCVHQSGIHATIAA
             ******..*..* *  *  .. ******  . *.  *.*.**.**  * .**.***.*.
               TM 8                  Loop 8
ECNhaA  231' VIVGFFIPLK----------------------------------------EKHGR--SPAKR
HPNhaA  239" VILAFMIPVKIPKDSKNVELLELGKRYAETSSGALLSKEQQEILHSIEEKASALQSPLER
             **..*.**.*                                        **  .  ** .*
                      TM 9                          TM 10
ECNhaA  251' LEHVLHPWVAYLILPLFAFANAGVSLQGVTLDGLTSILPLGIIAGLLIGKPLGISLFCWL
HPNhaA  299" LEHFLAPISGYFIMPLFAFANAGVSVDSSINLEVDKVL-LGVILGLCLGKPLGIFLITFI
             *** * *  .*.*.***********... .  .....* **.* ** .****** *. ..
                           TM 11                  TM 12
ECNhaA  311' ALRLKLAHLPEGTTYQQIMVVGILCGIGFTMSIFIASLAFGSVDPELINWAKLGILVGSI
HPNhaA  358" SEKLKITARPKGISWWHILGAGLLAGIGFTMSMFISNLAFTSEHKDAMEVAKIAILLGSL
             . .**..  *.*... .*. .*.* *******.**..***.* . . .. **..**.**.

ECNhaA  371' SSAVIGYSWLRVRLRPSV
HPNhaA  418" ISGIIGALYLFALDKRAALKK
             *..**  .* .  . ..
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b

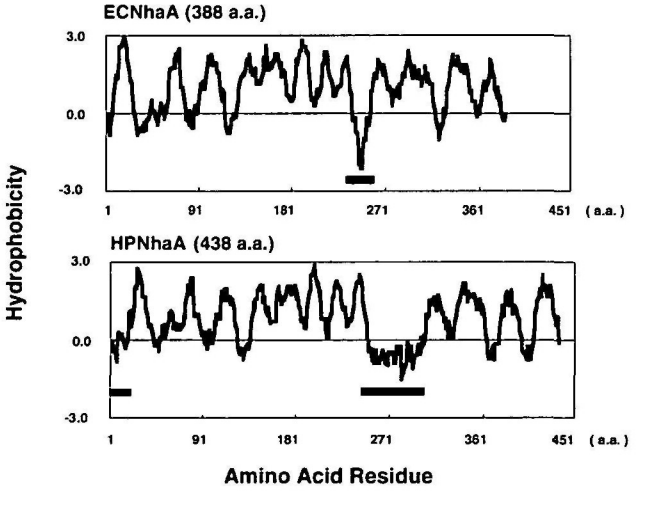

Fig. 1. **(a) Alignment of the deduced amino acid sequences of *E. coli* (EC) and *H. pylori* (HP) NhaA.** Identical and similar residues are indicated by asterisks and dots, respectively. Transmembrane segments (TM) of the NhaAs predicted by hydropathy analysis and results described by Rothman *et al.* (*27*) are indicated by TM 1–12. The loop 8 region between TM 8 and 9 is indicated by an arrow. The 10 residues at the amino-terminus and the 38 residues in loop 8 which are specific to HP NhaA are shadowed. **(b) Comparison of the hydropathy profiles of EC and HP NhaA.** The average hydrophobicity value at each residue, calculated by the method of Kyte and Doolittle (*28*), is plotted. The vertical and horizontal axes show the hydrophobicity values and amino acid residue numbers, respectively. Loop 8 in EC and HP NhaA, and the 10 additional residues of HP NhaA are indicated by bold bars.

this study, we constructed deletion, insertion and chimeric mutants from HP NhaA and EC NhaA. As a result, it was suggested that putative pH sensors are not localized in one consecutive region, but probably consist of a limited number of unconserved residues distributed throughout the amino- and carboxy-terminal regions.

EXPERIMENTAL PROCEDURES

E. coli Strains, Culture Conditions, and Plasmids—*E. coli* strains KNabc (*ΔnhaA*::Km^r, *ΔnhaB*::Em^r, *ΔchaA*::Cm^r, *sup*E, *hsd*D5, *thi*, *Δ*(*lac-pro*AB)/F′ (*traΔ*36, *pro*AB$^+$, *lac*I^q, *lacΔ*M15) (*15*) and JM109 (*16*) were used for expression of chimeric NhaA molecules and construction of various plasmids. Cells were cultured in L broth (LB) (*17*) containing 87 mM KCl instead of NaCl (LBK). For growth on solid plates, agar (1.5%) was added to the medium. Transformants were selected using an appropriate antibiotic. For analysis of the salt tolerance of KNabc transformed with various plasmids, different concentrations of NaCl or LiCl were added to LB plates containing 87 mM NaCl. The plates and broth cultures were incubated at 37°C.

The construction of EC and HP NhaA expression plasmids derived from pBR322 is described elsewhere (*10*). These plasmids express EC or HP NhaA with a FLAG epitope tag at the 3′ end of the coding region and under the control of the *tet* promoter. NhaAs expressed from these vectors contain an additional sequence, GMQ, at the junction with the FLAG tag sequence (DYKDDDDK). A CTG codon was substituted for the GTG initiation codon of *nhaA* of *E. coli* in order to avoid retardation of host cell growth, as described previously (*18*).

Construction of Chimeric NhaAs—Most plasmids expressing NhaA chimeras were constructed by combining

TABLE I. **Oligo DNAs used in this study.**

Oligo DNA	Sequence (5'→3')	Position*
For Chimera HP-N		
HPN10	C T G A A T T C G A A A G A G A A A T A A A A - A A T G A G T T T G A C G C T T A A G A A C	HP(31–48)
For Chimera HP-38		
HP38-F	A C C G G T G A A G G A A A A A G C G A G C G C C T T A C A	HP(735–744)-HP(859–878)
HP38-R	T C G C T T T T T C C T T C A C C G G T A T C A T A A A A G	HP(868–859)-HP(744–725)
For Chimera EC38		
LoopN-F	T C C T T T G A A A A T C C C T A A A G A T T C T A A A A A	EC(711–720)-HP(745–764)
LoopN-R	C T T T A G G G A T T T T C A A A G G A A T A A A G A A G C	HP(754–45)-EC(720–701)
LoopC-F	T T C T A T T G A A G A G A A G C A T G G G C G T T C T C C	HP(849–858)-EC(721–740)
LoopC-R	C A T G C T T C T C T T C A A T A G A A T G C A A G A T T T	EC(730–721)-HP(858–839)
For Chimera NEC		
N10-F	A A A C G C G C T C A T G A A A C A T C T G C A T C G A T T	HP(21–30)-EC(1–20)
N10-R	T G T T T C A T G A G C G C G T T T T C T G T T T T T T T G	EC(8–1)-HP(30–9)
For Chimera E8H4		
E8H4-F	C C C G T G G G T G G G G T A T T T C A T C A T G C C C T T	EC(768–777)-HP(922–941)
E8H4-R	T G A A A T A C C C C A C C C A C G G G T G C A A C A C A T	HP(931–922)-EC(777–758)
For Chimera E6H6		
E6H6-F	G A A T C T G T G T A A T A T G C G C T C G C T C A T C C C	EC(591–600)-HP(625–644)
E6H6-R	A G C G C A T A T T A C A C A G A T T C A A T A C C G C G A	HP(634–625)-EC(600–581)
For Chimera H6E6		
H6E6-F	A A A C C G C C T G G G T G C A C G C C G C A C G G G C G T	HP(615–624)-EC(601–620)
H6E6-R	G G C G T G C A C C C A G G C G G T T T A A T A C G G C T A	EC(610–601)-HP(624–605)
For Chimera H8E4		
H8E4-F	C C C C A T T A G C G C G T A T C T G A T T T T G C C G C T	HP(615–624)-EC(601–620)
H8E4-R	T C A G A T A C G C G C T A A T G G G G G C T A G A A A A T	EC(787–778)-HP(921–902)

*This is the position when the first letter of the initiation codon is #1. For example, "EC(711–720)-HP(745–764)" means that this oligo DNA corresponds to the sequence from #711 to #720 of *E. coli nha*A and from #745 to #764 of *H. pylori nha*A.

TABLE II. **Mutant NhaAs constructed in this study.**

Mutant	Sequence contained in mutant*
HPΔN	HP11–438
HPΔ38	HP1–248, HP287–438
HPΔN38	HP11–248, HP287–438
NEC	HP1–10, EC1–388
EC38	EC1–240, HP249–286, EC241–388
NEC38	HP1–10, EC1–240, HP249–286, EC241–388
E8H4	EC1–259, HP308–438
E6H6	EC1–200, HP209–438
H6E6	HP1–208, EC201–388
H8E6	HP1–307, EC260–388

*The initiation methionine has been designated as #1. For example, "HP1–10, EC1–388" means that this chimera consists of amino acid residues #1 to #10 of HP NhaA and #1 to #388 of EC NhaA.

DNAs amplified by polymerase chain reaction (PCR) (*19*). pBluescriptII KS+ containing a cDNA encoding FLAG-tagged EC or HP NhaA was used as a DNA template, and the primers used are listed in Table I. Chimeras HPΔN38 and NEC38 were prepared by restriction endonuclease digestion and recombination (re-ligation) between chimeras HPΔN and HPΔ38, and between NEC and EC38, respectively. The regions of EC and HP NhaA contained in each chimera are shown in Table II. A CTG codon was substituted for the ATG initiation codon of chimeras NEC and NEC38 in order to avoid retardation of host cell growth, as described previously (*18*).

Preparation of Membrane Vesicles and Na^+/H^+ Antiport Assay—E. coli cells transformed with various expression plasmids were cultured in 300 ml of LBK at 37°C with vigorous shaking. After harvesting and washing, the cells were disrupted with a French Press and membrane vesicles were prepared by centrifugation as described previously (*10, 17*). The membrane vesicles (200 μg) were resuspended in 2 ml of assay buffer (10 mM Tricine and 140 mM KCl, adjusted to the desired pH with KOH), as described previously (*20*). Proton flow was measured by monitoring ACMA (9-amino-6-chloro-2-methoxyacridine) fluorescence quenching after the addition of potassium lactate (5 mM, pH 7.0) as a substrate of the electron transport respiratory chain (*15*). Fluorescence dequenching after the addition of NaCl or LiCl was monitored as a measure of antiporter activity. Fluorescence was measured with a Hitachi F-4500 fluorescence photometer.

Immunodetection of FLAG-Tagged Antiporters—Aliquots (5 μg) of the membrane vesicles prepared from various NhaA transformants were subjected to SDS polyacrylamide gel electrophoresis as described previously (*17*). The separated proteins were blotted onto a GVHP filter (Millipore) (*17, 18*) and the probed with anti–FLAG M2 monoclonal antibodies (Sigma). Immunoreactive bands were visualized with an ABC Vecta-Stain kit as described previously (*19*).

Other—Preparation of plasmids, and digestion by restriction endonucleases and ligation by T4 DNA ligase of the DNA fragments and other techniques related to handling of DNA were performed according to published procedures (*21*). The nucleotide sequences of DNA fragments cloned into the various expression plasmids in this study were determined with an automatic sequencer (PE Biosystems).

Restriction endonucleases, T4 DNA ligase, Taq, and KOD DNA polymerases were purchased from Toyobo and Takara. Oligonucleotides were synthesized by Pharmacia Biotech, Espec Oligo Service, and Hokkaido System Science. Other reagents and materials were of the highest grade commercially available.

RESULTS

Deleted and Chimeric Antiporters of the Amino Terminal Region and Loop 8 of EC and HP NhaA—To determine whether or not 10 residues at the amino terminus and 38 residues from loop 8 of HP NhaA are involved in pH sensing, we constructed two types of mutant antiporter from EC and HP NhaA: (i) HP NhaA mutants with the HP-specific sequences deleted, and (ii) chimeric mutants in which the HP-specific sequences were inserted into EC NhaA.

The mutant HP NhaAs carried deletions of either 10 residues of the amino terminus (HPΔN, Fig. 2a and Table II), 38 residues of loop 8 (HPΔ38, Fig. 2a and Table II), or both regions (HPΔN38, Fig. 2a and Table II), and were expressed with a 3′-FLAG tag under the control of a *tet* promoter, as was wild-type HP NhaA. Na^+/H^+ antiporter–deficient *E. coli* strain KNabc (*15*) was transformed with appropriate expression vectors and then everted membrane vesicles were prepared from the transformants. Integration of the mutant NhaAs into the cytoplasmic membranes of host cells was analyzed by immunoblotting using the everted membrane vesicles and anti-FLAG antibodies. The transformants expressed comparable amounts of mutant and wild-type antiporters (Fig. 5). The Na^+/H^+ and Li^+/H^+ antiport activities of the mutants were measured by a fluorescence quenching method using the everted membrane vesicles. The profiles of the pH-dependent Na^+/H^+ antiport activities of all mutants were essentially the same as that of the wild-type HP NhaA (Fig. 2b). Since deletion of the two HP-specific sequences basically did not affect the pH-profiles of antiport activity, it appears that these regions are not required for pH-independent Na^+/H^+ antiporter activity in *H. pylori*. In contrast, the Li^+/H^+ antiport activities of the mutants lacking the 38 residues of loop 8 (HPΔ38 and HPΔN38) decreased at alkaline pH (Fig. 2c). These results suggest that the 38 loop 8 residues may be involved in the Li^+/H^+ antiport activity of HP NhaA at alkaline pH.

Next, three chimeras were constructed consisting of the amino terminal or loop 8 HP-specific region, or both regions, inserted into EC NhaA (NEC, EC38, and NEC38; Fig. 3a and Table II). KNabc transformed with plasmids expressing NEC or NEC38 did not grow even on media of normal salinity (LB and LBK). Similar growth retardation was observed for mutant EC NhaA with ATG as the initiation codon (wild-type EC NhaA has a GTG initiation codon) (*14*). Since the initiation codons of NEC and NEC38 are ATG derived from HP NhaA, we supposed that overproduction of the chimeric NhaAs due to elevation of the translation efficiency might have caused the observed growth retardation. A CTG codon was therefore substituted for ATG as the initiation codon of NEC and NEC38 (*20*). NEC and NEC38 with CTG as the initiation codon did not cause retardation of host cell growth, and normal expression of these chimeric NhaAs was observed (Fig. 5). The expression level of EC38 was also comparable to those of wild-type EC and HP NhaAs (Fig. 5). The Na^+/H^+ and Li^+/H^+ antiport activities of the chimeras exhibited almost the same pH-dependency as that of the wild-type EC NhaA (Fig. 3, b and c). These results show that neither addition of 10 residues of HP NhaA to the amino terminus of EC NhaA nor insertion of 38 residues from HP NhaA into loop 8 of EC NhaA

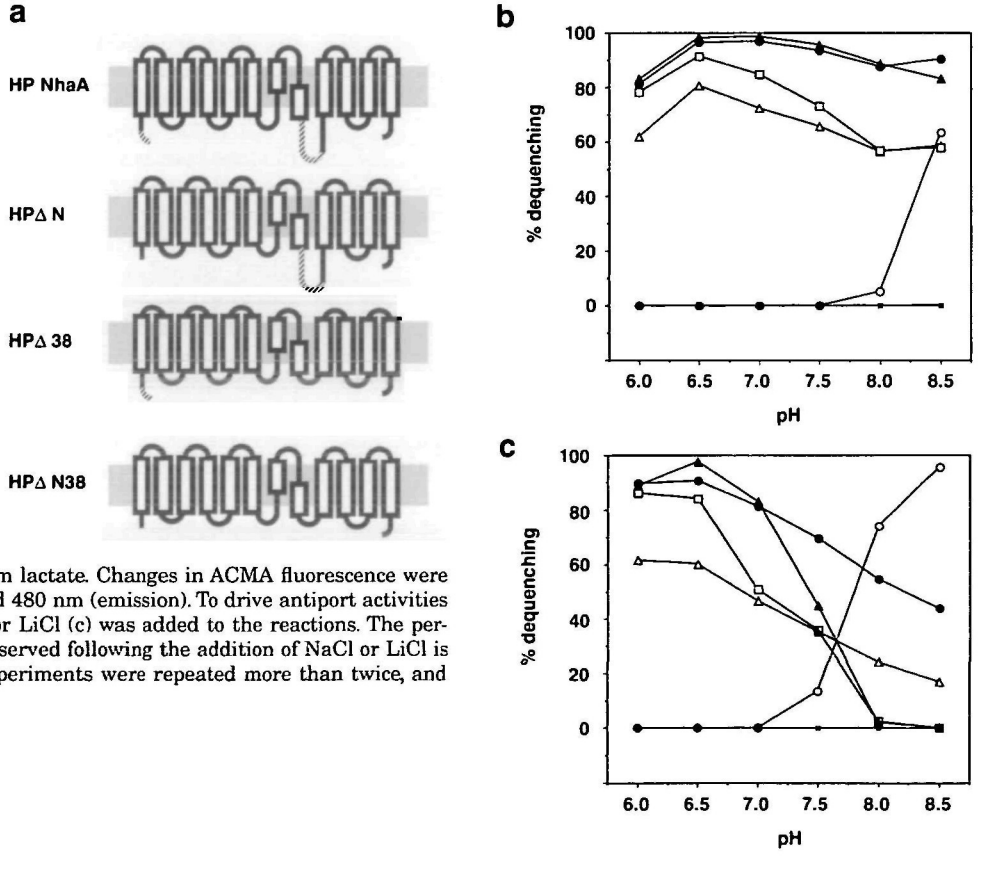

Fig. 2. **(a) Secondary structure models of wild-type HP NhaA and deletion mutants derived from HP NhaA (HPΔN, HPΔ38, and HPΔN38).** Gray regions indicate the 10 residues at the amino terminus and the 38 residues in loop 8 which are specific to HP NhaA. **(b and c) pH profiles of the Na^+/H^+ (b) and Li^+/H^+ (c) antiport activities of wild-type EC and HP NhaA, and deletion mutants.** Everted membrane vesicles were prepared from KNabc transformed with plasmids expressing wild-type EC (open circles) or HP (filled circles) NhaA, HPΔN (open triangles), HPΔ38 (filled triangles), HPΔN38 (open squares), or pBR322 (crosses). The membrane vesicles (200 μg) were suspended in 2 ml of assay buffer containing 1 μM ACMA and then respiration was initiated by the addition of potassium lactate. Changes in ACMA fluorescence were monitored at 410 nm (excitation) and 480 nm (emission). To drive antiport activities in the membranes, 5 mM NaCl (b) or LiCl (c) was added to the reactions. The percentage fluorescence dequenching observed following the addition of NaCl or LiCl is plotted against the assay pH. All experiments were repeated more than twice, and typical results are shown.

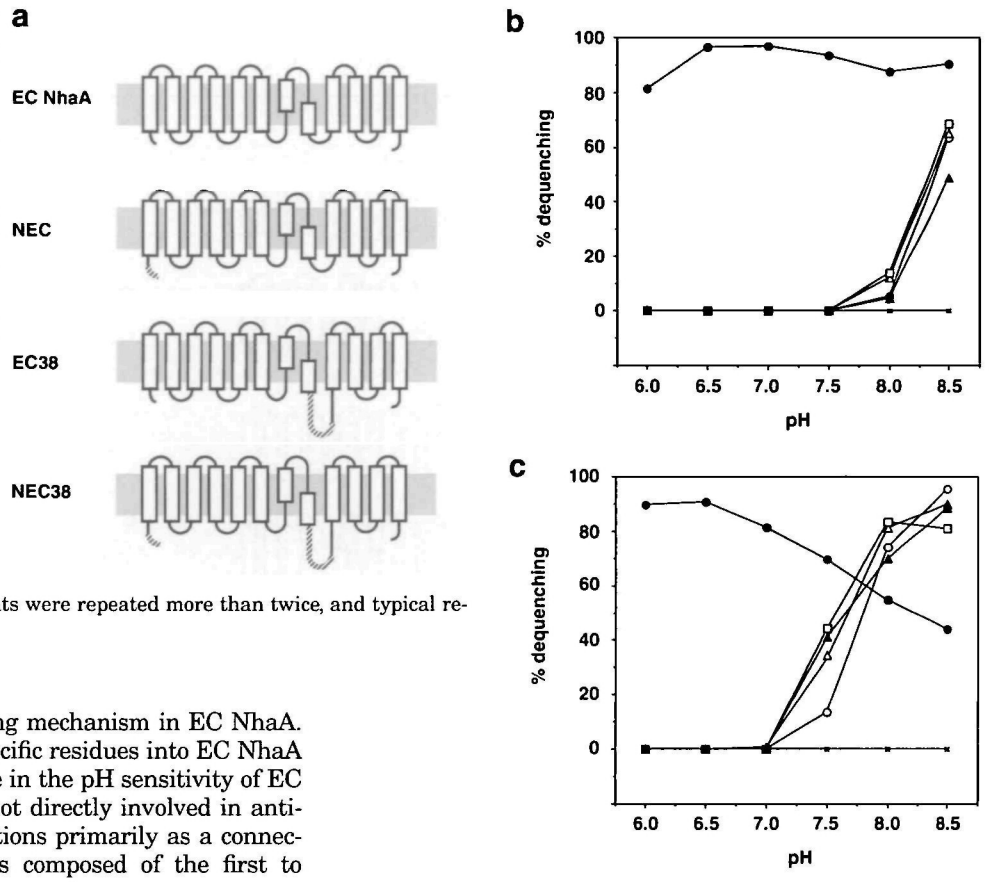

Fig. 3. **(a) Secondary structure models of wild-type EC NhaA and chimeric mutants of EC NhaA (NEC, EC38, and NEC38).** Gray regions indicate the 10 residues at the amino terminus and the 38 residues in loop 8 derived from HP NhaA. **(b and c) pH profiles of Na^+/H^+ (b) and Li^+/H^+ (c) antiport activities of wild-type EC and HP NhaA, and chimeric mutants of EC NhaA.** Everted membrane vesicles were prepared from KNabc transformants: wild-type EC (open circles) and HP (filled circles) NhaA, NEC (open triangles), EC38 (filled triangles), NEC38 (open squares), or pBR322 (crosses), and Na^+/H^+ and Li^+/H^+ antiport activities were determined as described in the legend to Fig. 2. The percentage fluorescence dequenching observed following the addition of NaCl or LiCl is plotted against the assay pH. All experiments were repeated more than twice, and typical results are shown.

has any effect on the pH-sensing mechanism in EC NhaA. That insertion of the 38 HP-specific residues into EC NhaA loop 8 did not cause any change in the pH sensitivity of EC NhaA suggests that loop 8 is not directly involved in antiporter activity, but that it functions primarily as a connection between the two domains composed of the first to eighth and ninth to twelfth transmembrane spans.

In addition to analysis of the HP-specific regions described above, we conducted analysis of another sequence which may be involved in pH sensing. The entire loop 8 region, including the 38 HP-specific residues, is the region of greatest difference between EC and HP NhaA in terms of hydrophobicity (Fig. 1, a and b). We constructed two chimeras in which the entire loop 8 region was exchanged between EC and HP NhaA in order to examine the contribution of sequences other than the 38 HP-specific residues to the pH-dependent antiport activities. The activities of the chimeras were almost the same as those of the relevant wild-type parent molecules (data not shown), suggesting that the loop region is not involved in the regulation of NhaA activity by pH.

Chimeras Composed of Two Domains of EC and HP NhaA—The results described above clearly show that the two regions showing the greatest variation between EC and HP NhaA are not involved in pH sensing. This further suggests that the comparatively slight sequence differences in conserved regions might be important in the regulation of NhaA activity by pH. Since the results described above also suggested that NhaA might be divided into two domains at loop 8, we examined whether one or both of these two domains are involved in pH sensing. For this purpose, we constructed chimeras carrying combinations of the amino- and carboxy-terminal domains of EC and HP NhaA separated by loop 8 (E8H4 and H8E4; Fig. 4a and Table II). In addition, chimeras consisting of combinations of the domains separated by the central loop (loop 6) were also constructed (E6H6 and H6E6; Fig. 4a and Table II).

Following transformation with the various expression plasmids, all of these chimeras except H6E6 showed integration into the cytoplasmic membranes of host cells at levels comparable to in the case of the wild-type NhaA (Fig. 5). Since expression of chimera H6E6 was observed in whole cell extracts (data not shown), this chimeric NhaA appears not to be efficiently integrated into the membranes. Of the chimeras, H8E4 showed higher activity than other chimeras. The Na^+/H^+ antiport activity of H8E4 was negligible at pH 6.0 and 6.5, but was enhanced from neutral to alkaline pH (Fig. 4b). This profile of pH sensitivity was not identical to either that of HP NhaA (which is active from pH 6.0 to 8.5) or that of EC NhaA (which is dramatically activated at alkaline pH). H8E4 exhibited moderate Li^+/H^+ antiport activity at pH 6.0 (60% dequenching) and very high activity from pH 6.5 to 8.5 (90–100% dequenching) (Fig. 4c). This was again different from the pH responses of wild-type NhaAs from *H. pylori* and *E. coli*. In summary, although H8E4 was fully active in both Na^+/H^+ and Li^+/H^+ antiport, the pH profile of this chimera differed from those of both EC and HP NhaA.

Chimeras E8H4 and E6H6 showed Na^+/H^+ antiport activities which were only slightly activated by a shift from neutral to alkaline pH; these profiles were somewhat similar to that of EC NhaA. Although the Li^+/H^+ antiport activities of these chimeras increased with increasing pH, no activation was observed at pH values greater than 8.0. Thus the Li^+/H^+ activities of these chimeras also did not resemble that of either of the wild-type parent molecules.

Taken together, these results show that the pH sensitivity of NhaA is not determined solely by either the amino-

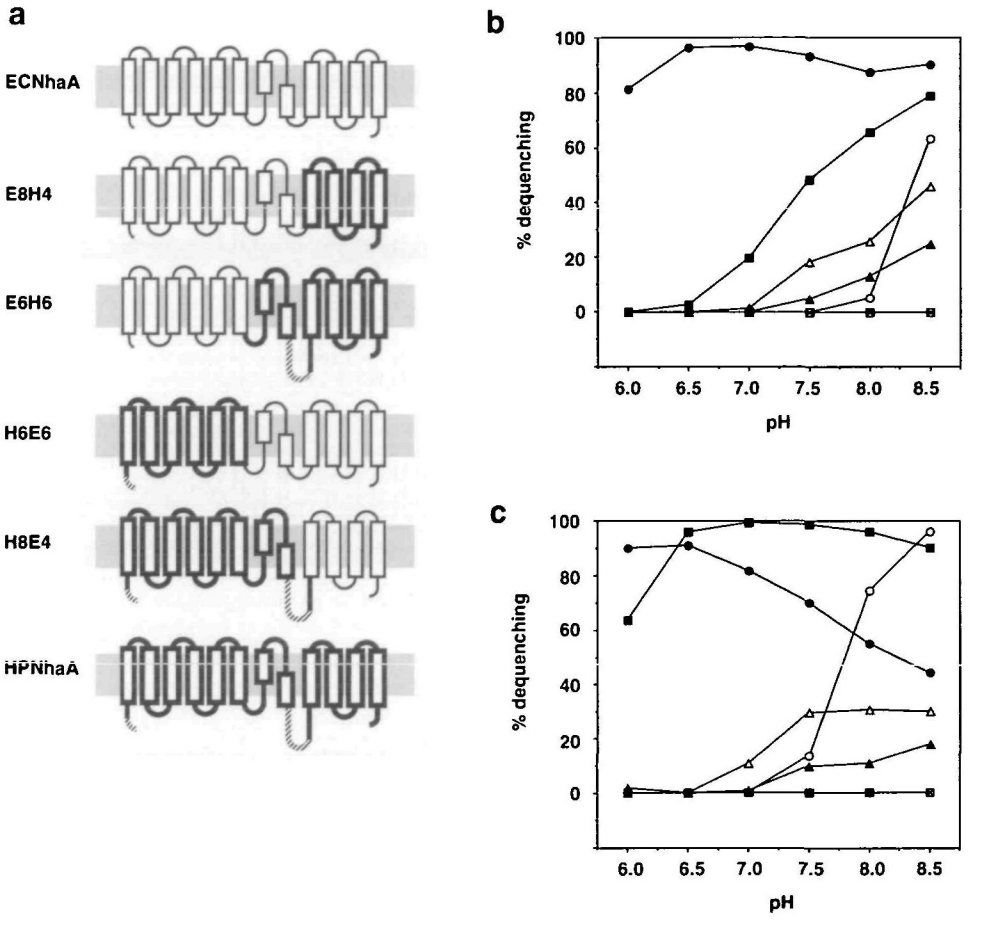

Fig. 4. **(a) Secondary structure models of wild-type EC NhaA, and chimeric mutants of EC and HP NhaA (E8H4, E6H6, H6E6, and E8H4).** Regions derived from EC NhaA are indicated by thin lines, while regions derived from HP NhaA are shown by thick lines. Gray regions indicate the 10 residues at the amino terminus and the 38 residues in loop 8 derived from HP NhaA. **(b and c) pH profiles of Na^+/H^+ (b) and Li^+/H^+ (c) antiport activities of wild-type EC and HP NhaA, and chimeric mutants of EC and HP NhaA.** Everted membrane vesicles were prepared from KNabc transformants: wild-type EC (open circles) and HP (filled circles) NhaA, E8H4 (open triangles), E6H6 (filled triangles), H6E6 (open squares), H8E4 (filled squares), or pBR322 (crosses), and Na^+/H^+ and Li^+/H^+ antiport activities were determined as described in the legend to Fig. 2. The percentage dequenching observed following the addition of NaCl or LiCl is plotted against the assay pH. All experiments were repeated more than twice, and typical results are shown.

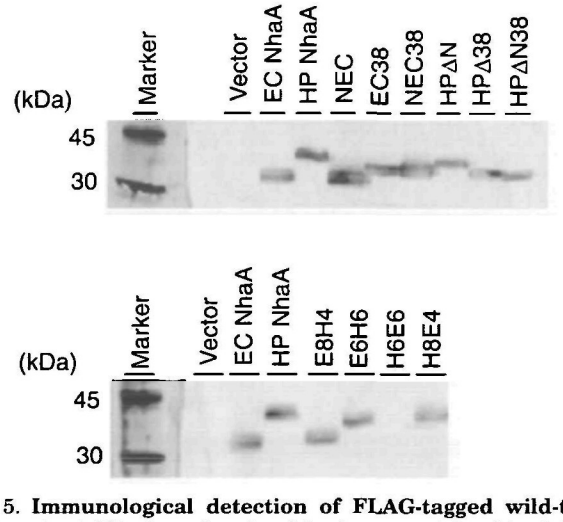

Fig. 5. **Immunological detection of FLAG-tagged wild-type and mutant NhaA molecules.** Membrane proteins (5 μg) from KNabc transformed with plasmids expressing the FLAG epitope-tagged wild-type and mutant NhaAs were subjected to SDS polyacrylamide gel electrophoresis (12.5% acrylamide). As a negative control, membrane proteins of pBR322 transformants were also analyzed. After electrophoresis, the proteins were transferred to a GVHP filter (*18*) and probed with anti-FLAG monoclonal antibody M2 (Sigma), and then immunoreactive bands were visualized (*18*). Molecular size markers (M) were stained with Coomassie Brilliant Blue.

TABLE III. **Growth of the transformants on medium containing a high concentration of NaCl or LiCl.**

Expressed NhaA	Growth of transformant	
	0.65 M NaCl	0.15 M LiCl
Empty Vector	–	–
EC NhaA	+++	++
HP NhaA	+++	++
HPΔN	+++	++
HPΔ38	+++	++
HPΔN38	+++	+
NEC	+++	++
EC38	+++	++
NEC38	+++	++
E8H4	+++	++
E6H6	+++	++
H6E6	–	–
H8E4	++	++

E. coli KNabc (ΔnhaA, ΔnhaB, ΔchaA) transformed with various expression vectors expressing chimeric NhaAs was incubated on an agar plate containing LBK and varying concentrations of NaCl (pH 8.0) or LiCl (pH 7.5) for 24 h at 37°C. The diameters of the colonies were divided into four categories, +++ (large), ++ (middle), + (small), and – (very small or none).

terminal or carboxyl-terminal domain, but rather that both regions of NhaA appear to be involved in pH sensing.

Function of the Chimeras In Vivo—In order to determine whether or not the chimeras constructed in this study were functional *in vivo*, the growth of transformants was analyzed on media containing high concentrations of NaCl or

LiCl (Table III). All transformants as well as strains expressing wild-type EC or HP NhaA grew on media containing 0.65 M NaCl at pH 8.0 or 0.15 M LiCl at pH 7.5, with the exception of cells expressing H6E6. However, relatively slower growth was observed in two cases, H8E4 at pH 8.0 with NaCl and HPΔN38 at pH 7.5 with LiCl. In these cases, in spite of slower growth, higher antiport activities were found in *in vitro*.

DISCUSSION

We searched for structures responsible for the different responses of EC and HP NhaAs to a pH change, in order to investigate the structural basis of pH sensing by NhaA molecules. The results of the present study show that the different pH profiles of the Na^+/H^+ and Li^+/H^+ antiport activities are essentially independent of the 38 loop 8 and 10 amino-terminal residues found in HP but not in EC NhaA, the only exception being the effect of the 38 loop 8 residues on Li^+/H^+ antiport in HP NhaA. This conclusion further suggests that the difference in pH sensing depends on unconserved residues within highly conserved sequences distributed over the length of the molecule. Deletion of the 38 HP-specific residues from loop 8 of HP NhaA caused a decrease in Li^+/H^+ antiport at alkaline pH, but insertion of this sequence into EC NhaA did not have a similar effect. Moreover, the loop 8-deleted HP NhaA mutant was able to grow on medium containing 0.15 M LiCl. These results suggest that the HP-specific loop 8 region has essentially no functional importance; the tertiary structure of HP NhaA lacking these 38 residues might indirectly destabilize the ion transport pathway, leading to the altered response of Li^+/H^+ antiport to alkaline pH.

We noted that insertion of the 38 HP-specific amino acid residues into loop 8 of EC NhaA did not affect the pH sensitivity or transport activity of EC NhaA. This implies that NhaA can be divided into two domains at loop 8. Such a two-domain structure has been reported for several other proteins, including lactose transporter and tetracycline/H^+ antiporter (*22, 23*). Chimeras consisting of combinations of the amino- and carboxy-terminal regions of EC and HP NhaA (H8E4, E8H6, and E6H6) showed pH sensitivity profiles intermediate between those of the wild-type parent molecules. This observation suggests that the structure required for pH sensing does not reside solely within either the amino- or carboxy-terminal domain, and raised the possibility that interaction between residues in the two domains is required for pH sensing. This conclusion is consistent with the observation that the change in EC NhaA pH sensitivity caused by substitution of Ser for Gly-338 in the 11th transmembrane region is suppressed by a second site mutation at residue 127, 129, or 130 in the 4th transmembrane region (*14*).

Single amino acid substitutions at residues Leu-73, His-225, and Gly-338 have been reported to alter the pH sensitivity of EC NhaA (*12–14*). These residues may be involved in the pH sensing mechanism, but the observation that all three are conserved in EC and HP NhaA suggests that other residues are also involved in pH sensing. For preliminary mutational analyses, among the unconserved residues in relatively well-conserved regions, we focused on Asp-73 of HP NhaA. Since Asp-73 is only one unconserved and negatively charged residue in the transmembrane regions, we replaced it with Asn (the corresponding residue in EC NhaA). The mutant HP NhaA exhibited almost the same activities as the wild-type parent molecule (data not shown), indicating that Asp/Asn-73 is not involved in the regulation of NhaA activity by pH. Since EC NhaA Na^+/H^+ antiport activity is enhanced by three orders of magnitude in response to a pH change from 7.0 to 8.5 (less than two orders of magnitude) (*8*), multiple residues which function in pH sensing by accepting and releasing protons are probably involved. Consistent with this observation, the separate membrane-integral portion and the cytoplasmic domain of Cl^-/HCO_3^- exchanger AE2 were shown to be involved in the pH sensor (*24*). This observation also suggests that the structure responsible for pH sensing may involve more than a single residue. Dynamic conformational changes have been detected in the amino terminus and loop 8 region of EC NhaA (*25, 26*) as susceptibility to trypsin or binding to specific antibodies, suggesting that the molecular mechanism of Na^+/H^+ antiport involves a dynamic conformational change during ion transport. The pH sensing mechanism might be related to these dynamic conformational changes. The present results, however, show that the amino terminal region and loop 8 do not play key roles in the response to a pH change and suggest that conformational changes in these domains are unlikely to have an essential role in pH sensing.

We also observed differences between the pH profiles of the Na^+/H^+ and Li^+/H^+ antiporter activities. The Li^+/H^+ antiport activity of EC NhaA increases in the pH range of 7.5 to 8.0, while the Na^+/H^+ antiport activity does so in the pH range of 8.0 to 8.5. The Li^+/H^+ antiporter activity of HP NhaA decreases from pH 7.0 to 8.5, while the pH profile of the Na^+/H^+ antiporter activity is flat from pH 6.0 to 8.5. The different responses are even more obvious for chimera H8E4. The Na^+/H^+ antiporter activity of this chimera, at least between pH 7.0 and 8.5, was intermediate between those of the parent molecules. In contrast, the pH profile of H8E4 Li^+/H^+ antiporter activity showed higher relative activity from pH 7.0 to 8.0 than in the case of either of the parent molecules (Fig. 4, b and c). The reason for the higher activity found for the Li^+/H^+ antiport in H8E4 is not clear at present. One possibility is that the mutation caused a conformational change in the binding site of Li^+ and/or Na^+ leading to enhanced Li^+/H^+ antiport activity. Deletion of the 38 HP-specific residues from HP NhaA caused a decrease in Li^+/H^+ but not Na^+/H^+ antiport activity at alkaline pH (Fig. 2, b and c). Since Asp-133, Asp-163, and Asp-164 have been reported to be essential for Na^+/H^+ and also Li^+/H^+ antiport activities (*20*), the two antiport mechanisms probably depend on a number of common residues. The results of the present study, however, show that the fine mechanisms of Na^+/H^+ and Li^+/H^+ antiport are supported, at least in part, by different structures.

The deletion, insertion, or chimeric mutants of NhaA constructed in this study were integrated into the membranes of transformed host cells at levels similar to those exhibited by the wild-type EC and HP NhaAs, with the sole exception of the H6E6 chimera. These results suggest that most of the mutant NhaA molecules tested were not dramatically structurally different from the wild-type parent molecules, leading to proper assembly into the membranes. An aberrant structure may therefore be responsible for the deficient integration of H6E6 into cell membranes.

Although H8E4 and HPΔN38 exhibited comparable activities *in vitro* to the wild-types, the growth of cells expressing H8E4 in the presence of NaCl at pH 8.0 and HPΔN38 in that of LiCl at pH 7.5 was slower than that of the wild-types (Figs. 2c and 4b, and Table III). Although the reasons for the differences observed in *in vitro* antiport activities and cell growth are not clear at present, they might be due to differences in delicate ionic conditions and driving forces for NhaA.

In the present paper, we present evidence that candidate residues involved in the pH sensing mechanism of NhaA lie within a limited number of residues in the amino- and carboxy-terminal domains divided by loop 8, which are not conserved in EC and HP NhaAs. A survey of these candidate residues in terms of their roles in pH sensing is in progress.

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