Detection of Oligomerization and Conformational Changes in the Na\(^+\)/H\(^+\) Antiporter from Helicobacter pylori by Fluorescence Resonance Energy Transfer* 

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Oligomerization and conformational changes in the Na\(^+\)/H\(^+\) antiporter from Helicobacter pylori (HPNhaA) were studied by means of fluorescence resonance energy transfer (FRET) analysis. Na\(^+\)/H\(^+\) antiporter-deficient Escherichia coli cells expressing C-terminal fusions of HPNhaA to green fluorescent protein (GFP) variants exhibited wild-type levels of antiporter activity in their everted membrane vesicles. Vesicles containing both HPNhaA-CFP and HPNhaA-YFP or HPNhaA-Venus exhibited FRET from CFP (donor) to YFP or Venus (acceptor), suggesting that HPNhaA forms an oligomer. Co-purification of HPNhaA tagged by Venus and FLAG sequences confirmed oligomerization. FRET decreased extensively after treatment of the vesicles with proteinase K; FRET was not observed by merely mixing vesicles expressing the donor or acceptor fusion alone. Fluorescence of Venus is less sensitive to anions and stronger than that of anion-sensitive YFP. Using HPNhaA-Venus as the acceptor, Li\(^+\) was found to cause a significant decrease in FRET regardless of the presence or absence of \(\Delta pH\) across the membranes, whereas Na\(^+\) caused a much weaker effect. This Li\(^+\) effect was minimal in vesicles prepared from cells expressing HPNhaA containing an Asp\(^{141}\) to Asn mutation, which results in defective Li\(^+\)/H\(^+\) antiporter activity, possibly Li\(^+\) binding. These results demonstrate that monomer interactions within the HPNhaA oligomer are weakened possibly by Li\(^+\) binding. Dynamic interactions between HPNhaA monomers were detectable in membranes by FRET analysis, thus providing a new approach to study dynamic conformational changes in NhaA during antiporter activity.

Na\(^+\)/H\(^+\) antiporters are ubiquitous membrane proteins found in cytoplasmic and organelle membranes of most organisms, from bacteria to human. The gradient of H\(^+\) or Na\(^+\) across biomembranes for bacteria and yeast or for mammals, respectively, drives the secondary transport of the counterions by Na\(^+\)/H\(^+\) antiporters. The antiporters play a major role in pH and Na\(^+\) homeostasis of cells (1–5). NhaA\(^2\) (ECNhaA), one of three Na\(^+\)/H\(^+\) antiporters in Escherichia coli, plays a major role in the regulation of intracellular pH and cellular Na\(^+\) concentrations in bacteria (6–9). NhaA has a highly conserved primary structure among various bacteria including Helicobacter pylori and is estimated to have 12 transmembrane domains (4). The intramembrane domains have also been determined by phoA fusion analyses (10). Three Asp residues essential for ion transport, as well as several other functionally important residues, have been identified for ECNhaA (11) and H. pylori NhaA (HPNhaA) (12). These residues are clustered in transmembrane domains (TM) 4, 5, 10, and 11 in both HPNhaA (12) and ECNhaA (11). Several primary missense mutations defective in antiporter activity have been identified in these TMs, and suppressor mutations were mapped to these same TMs and flanking regions, suggesting mutual association of these TMs (12). This was directly shown by cross-linking of ECNhaA TM4 and TM11 (13).

An apparent pH-dependent antiporter activity has been characterized for ECNhaA, in which the antiport activity is 2,000-fold higher at pH 8.5 than at that at pH 7.0 (8). In sharp contrast, HPNhaA exhibits consistently high activity from pH 6.5 to 8.5 (14). We have shown for HPNhaA that the enhancement of activity at alkaline pH is associated with Loop 7 and TM8, whereas the high activity at acidic pH is caused by a structure formed by TM4 and TM10 (12). A chimeric NhaA composed of the N- and C-terminal halves of ECNhaA and HPNhaA, respectively, showed maximum antiporter activity in the intermediate pH range, providing additional evidence for functional interactions between the N- and C-terminal halves of the protein (15). Recently, the topology of HPNhaA TM4 was analyzed by cysteine-scanning mutagenesis and N-ethyl maleimide probing of Cys residue accessibility (16). Part of TM4 was shown to face a water-filled channel-like structure. Asp\(^{141}\) and Thr\(^{140}\) in TM4 could be the binding site for transporting ions (16).

Recent studies on the crystallographic structures of the lac permease (17) and glycerol 3-phosphate transporter (18) have predicted that a dynamic conformational change coincides with the operation of these transporters. A crystallographic structure of ECNhaA at 7.0 Å has been reported and shows clustering of \(\alpha\)-helical transmembrane domains (19). It may be too early to predict a dynamic conformational change for NhaA; however, pH-dependent conformational changes in ECNhaA have been detected by means of trypsin susceptibility (20). This implies that a pH-dependent conformational change during antiport is quite possible. It is important to establish a procedure to detect this putative conformational change during operation of the Na\(^+\)/H\(^+\) antiport in real time to understand the energy coupling mechanisms. Therefore, in this study we developed methods to use fluorescence resonance energy transfer (FRET) between two green fluorescent protein (GFP) variants fused to HPNhaA.

FRET can occur between donor and acceptor chromophores when they are located within 100 Å of each other and arranged properly in terms of their transition dipole moments (21). The ECNhaA dimer is reported to occupy 48 × 90.5 Å based on two-dimensional crystal data (25). Therefore, it is reasonable to expect that FRET could be used to...
detect oligomerization of HPNhaA containing suitable chromophores. Here, we used HPNhaA and GFP fusion proteins, in which GFP variants (CFP, YFP, or Venus) fused to the N or C terminus or inserted into Loop 8 at residue 286 are shown schematically. Procedures for construction of the fusion constructs are described under “Materials and Methods.” Symbols shown in parentheses correspond to those in B and C. B, pH-dependent Na\(^+\)/H\(^+\) antiporter activity in the everted membrane containing various fusions shown in A. Open square indicates the vector alone. C, pH-dependent Li\(^+\)/H\(^+\) antiporter activity in the everted membrane containing various fusions shown in A. Membrane vesicles were prepared from HPNhaA-GFP variant fusion protein transformants of E. coli (HIT\(\Delta\)LAB\(^{+}\)). Membrane vesicles (100 \(\mug\)) were incubated in 2 ml of assay buffer, and pH-dependent antiporter activity was measured as the fluorescence change of ACMA in the presence of a \(\Delta\)pH across the membrane driven by 5 mM lactate, 5 mM NaCl (B), or 5 mM LiCl (C). The percentage of fluorescence dequenching observed before and after addition of NaCl or LiCl is plotted against assay pH.

FIGURE 1. Schematic illustration of HPNhaA-GFP variant fusions and antiporter activities. A, schematic illustration of HPNhaA-GFP variant fusions: HPNhaA (438 amino acids) and GFP variants (CFP, YFP, or Venus) fused to the N or C terminus or inserted into Loop 8 at residue 286 are shown schematically. Procedures for construction of the fusion constructs are described under “Materials and Methods.” Symbols shown in parentheses correspond to those in B and C. B, pH-dependent Na\(^+\)/H\(^+\) antiporter activity in the everted membrane containing various fusions shown in A. Open square indicates the vector alone. C, pH-dependent Li\(^+\)/H\(^+\) antiporter activity in the everted membrane containing various fusions shown in A. Membrane vesicles were prepared from HPNhaA-GFP variant fusion protein transformants of E. coli (HIT\(\Delta\)LAB\(^{+}\)). Membrane vesicles (100 \(\mug\)) were incubated in 2 ml of assay buffer, and pH-dependent antiporter activity was measured as the fluorescence change of ACMA in the presence of a \(\Delta\)pH across the membrane driven by 5 mM lactate, 5 mM NaCl (B), or 5 mM LiCl (C). The percentage of fluorescence dequenching observed before and after addition of NaCl or LiCl is plotted against assay pH.
structure (24, 25). However, dimerization has neither been shown to occur under native conditions in membranes, nor in intact cells. Here, we detected oligomerization between two HPNhaAs fused to different GFP variants (CFP, YFP, or Venus) by FRET in the same E. coli cell. We established a FRET system for HPNhaA and detected oligomerization in intact membranes and cells. Further, through FRET, we detected conformational changes in HPNhaA during changes in pH and during Li\(^+\)/H\(^+\) antipit, possibly as a result of Li\(^+\) binding.

**MATERIALS AND METHODS**

**Bacteria Strains and Culture Conditions—** E. coli strains HIT\(\Delta\)AB\(^{-}\) (\(\Delta lacY, \Delta nhaA, nhaB^{+}\)) (26) and JM109 (27) were used for the expression of HPNhaA fused to GFP variants and for the construction of various plasmids, respectively. Cells were cultured in L broth (LB) (28) containing 87 mM KCl instead of NaCl (LBK). For growth on solid plates, agar (1.5%, w/v) was added to the medium. Transformants were selected using an appropriate antibiotic. For analysis of salt resistance of HIT\(\Delta\)AB\(^{-}\) transformed with various plasmids, additional amounts of NaCl or LiCl were added to LB plates. The plates and liquid cultures were incubated at 37°C.

**Construction of Expression Plasmids Encoding Fusion Protein Genes—** Construction of HPNhaA expression plasmids derived from pBR322 (pBR-HP) has been described previously (14). The HindIII/Sall fragment including the entire coding region of HPNhaA was excised from pBR-HP and inserted into the pACYC184 plasmid (24), and named pACYC-HP. CFP and YFP were joined to the C or N terminus of HPNhaA in pBR-HP or in pACYC-HP. For the C terminus fusion, CFP and YFP were amplified by PCR from the eCFP or eYFP vector (Clontech, Palo Alto, CA) using the primer sequences: ACATGCATGCGACTTGTGAGCAAGGGCGAGG and CCGTGACATCTCTTGAAGACTGCTGC, which include Sall or Spal restriction sites. The PCR products were ligated into the Sall/Spal fragment of pBR-HP or pACYC-HP, creating the HPNhaA-CFP and HPNhaA-YFP plasmids, respectively. For the N terminus fusion, a two-step PCR was used to create a linker sequence (Gly-Gly-Ser-Gly-Gly) to link the C terminus of CFP or YFP and the N terminus of HPNhaA with the primer sequences: CCGGAATTCGAAAGAGAAATAAAAAATGTGAGCA-AGGGCGAGG and ATTTTCTCCTAATCCTGTTAAGACTGCTG- TCCAT, for CFP and YFP, and GAGGAGATGGAGGAAATCCTCAA-AAAAAGAAGAAAAAGCGGCTCAGT and GGAGACCGATGACCAT, for HPNhaA. Then the primer sequences AATTAAACCTCTACTA- AAGGG and CCGGAATTCGAAAGAAATATTTTTTGTGAGCA- AGGGCGAGG (29), were used to amplify the fused sequences and create the restriction sequences to ligate the fragments into the appropriate plasmids to create CFP-HPNhaA and YFP-HPNhaA or CFP-HPNhaA- YFP and YFP-HPNhaA-CFP. The plasmid expressing an HPNhaA-Venus fusion was constructed by essentially the same method as described above using the Venus/pCS2 plasmid (RIKEN, Wako City, Japan) instead of the vector plasmid for eYFP.

To create Loop 8 fusions, CFP or Venus were inserted into the Loop 8 of HPNhaA as follows. A BanIII restriction site between amino acid residues 286 and 287 of HPNhaA was created by two-step PCR (29) with the primer sequences: CCGCATCGAGGGAGAGGAGGAAATGTGGAGG- CAGGGAGGAGG and CCGCATCGATCTCTCCTCCTCTCCT- CTGTACAGCTGCTGCAT, which include the BanIII site and the linker sequence Gly-Gly-Gly-Gly. The PCR products were ligated into the BanIII fragment of pBR-HP-BanIII, creating HPNhaA(Loop8-CFP)-Venus Loop 8/C terminus and HPNhaA(Loop8-Venus)-FLAG. Venus was fused to the C terminus of HPNhaA in the resultant plasmids by ligating the fragment into the Sall/Spal restriction site, as described above, creating HPNhaA(Loop8-Venus)-FLAG. The EcoRI/Spal fragments of these plasmids were inserted into the pACYC vector encoding HPNhaA fused to CFP or Venus. A fusion gene with mutant HPNhaA and GFP variants was created as described above, using a plasmid encoding a mutant HP- NhaA (12) as the template.

**TABLE ONE**

| Fusion protein | Location of GFP variant | Cell growth 
|----------------|------------------------|----------------|
|----------------|------------------------|----------------|
| HPNhaA-FLAG    | -                      | ++ + + +       |
| Vector         | -                      | - - - -        |
| CFP-HPNhaA-FLAG| N terminus             | ++ +           |
| YFP-HPNhaA-FLAG| N terminus             | ++ +           |
| HPNhaA-CFP     | C terminus             | ++ + + +       |
| HPNhaA-VFP     | C terminus             | ++ + + +       |
| HPNhaA-Venus   | C terminus             | ++ + + +       |
| CFP-HPNhaA-YFP | N/C terminus           | + -            |
| YFP-HPNhaA-CFP | N/C terminus           | - +            |
| HPNhaA(Loop8 CFP)-FLAG | Loop 8              | ++ + +         |
| HPNhaA(Loop8 Venus)-FLAG | Loop 8            | ++ + + +       |
| HPNhaA(Loop8 CFP)-Venus Loop 8/C terminus | ++ + + + | |

**FRET Analysis of Conformational Changes in HPNhaA**

Plasmids expressing various NhaA-GFP variant fusions were introduced to E. coli HIT\(\Delta\)AB\(^{-}\). Transformed cells were replicated on LB plates with 0.65 μM (pH 8.0) NaCl or 0.15 μM (pH 7.5) LiCl and incubated for 36 h. Cell growth yield on the plate was classified to three categories (+, ++, ++++) depending on the density of cells on the plate.

Preparation of Membrane Vesicles and Measurement of Na\(^+\)/H\(^+\) Antipporter Activities—Everted membrane vesicles from E. coli cells transformed with various plasmids were prepared as described previously (15). After centrifugation of E. coli cells disrupted with a French press, the collected membrane vesicles (100 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 (11). Proton flow was measured by monitoring 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching after addition of potassium lactate (5 mM, pH 7.0) as a substrate of the electron transport respiratory chain (30). Fluorescence quenching, as a measure of the antipporter activity,
was monitored with a fluorospectrophotometer (JASCO FP-750, Jasco Ltd., Tokyo, Japan) after adding 5 mM NaCl or LiCl.

Detection of Dimerization by Coprecipitation—Membrane vesicles from *E. coli* HIT/AB- cells transformed with various expression plasmids were prepared as described previously (15). Membrane vesicles (0.25 mg of protein) were suspended in 300 μl of 10 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl and 1% Triton X-100. The suspension was centrifuged at 178,000 g for 30 min with an Airfuge (Beckman Coulter, Fullerton, CA) to remove insoluble materials. The resultant supernatant was subjected to immunoprecipitation with an anti-FLAG M2-agarose affinity gel (Sigma-Aldrich). Immunoprecipitates were solubilized in 0.125 M Tris-HCl buffer (pH 6.8) containing 4% SDS, 20% glycerol, and 0.004% bromphenol blue. The proteins were detected by Western blot analysis after separation on SDS-PAGE, as described previously (15). The antibodies used were anti-FLAG M2 monoclonal antibodies (Sigma-Aldrich) and anti-GFP antibodies (Molecular Probes, Eugene, OR). Horseradish peroxidase-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA) and Vector Labs (Burlingame, CA), respectively.

**Fluorescence Measurements and Microscopic Observation—** All fluorescence measurements were performed using an FP-750 fluorometer. *E. coli* cells (HITΔAB−) expressing fusion protein were grown at 37 °C in LBK medium overnight, washed, resuspended in phosphate-buffered saline, and diluted to an *A*<sub>600</sub> of 1.0. This suspension was used for fluorescence measurements. FRET measurements using everted membrane vesicles from cells expressing fusion proteins were performed in Tricine-KOH buffer (pH 8.5, Tricine, 10 mM; KCl, 140 mM). Proteinase K (50 μM), and NaCl, LiCl, or choline Cl (in various concentrations) were added to the reaction mixture. Cell suspensions (2 ml) from the early logarithmic growth phase or 100 μg of membranes in 2 ml were irradiated at 433 nm to excite CFP and fluorescence emission was recorded at 450–600 nm. For excitation of YFP or Venus, cells or membranes were illuminated at 473 nm, and then fluorescence emission was
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recorded at 500–600 nm. FRET was determined by subtracting the control emission spectra from the FRET emission spectrum. The control emission was obtained by excitation of two types of control cells or membranes, one expressing only the CFP-tagged protein and the other expressing only the YFP or Venus-tagged protein (31). The apparent efficiency of FRET was calculated by dividing the maximum value of fluorescence intensity of the FRET spectrum by the maximum value of fluorescence intensity obtained upon direct excitation of YFP or Venus.

Fluorescence images of cells coexpressing CFP- and Venus-tagged fusion proteins were captured using a CCD camera (ORCA, Hamamatsu Photonics, Shizuka, Japan) mounted on an Olympus BX51 microscope equipped with a FRET filter set (U-MF2, Olympus, Tokyo, Japan).

**Immunological Detection**—Aliquots of the membrane vesicles prepared from various HPNhaA transformants were subjected to SDS-polyacrylamide gel electrophoresis, as described previously (15). The separated proteins were blotted onto GVHP filters (Millipore, Billerica, MA), and probed with anti-GFP antibodies (Molecular Probes). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences), as described previously (15).

**Gene Manipulation and DNA Sequencing**—Preparation of plasmids, digestion of DNA with restriction endonucleases, ligation with T4 DNA ligase, and other techniques for handling DNA were performed according to published procedures (34). The nucleotide sequences of DNA fragments cloned into various expression plasmids in this study were verified using an automated sequencer (PE Biosystems, Foster City, CA).

**Materials**—Restriction endonucleases, T4 DNA ligase, Taq, and KOD DNA polymerases were purchased from Toyobo Co. (Osaka, Japan). Oligonucleotides were synthesized by Invitrogen. Other reagents and materials were of the highest grade commercially available.

**RESULTS**

**Construction of Active HPNhaA-GFP Fusion Proteins**—To detect interactions between HPNhaA monomers by FRET, we constructed N- or C-terminal fusions of HPNhaA with CFP or YFP (Fig. 1A). We have shown previously that the N- and C-terminal halves of HPNhaA interact functionally with each other based on the analysis of a chimeric NhaA construct of the *E. coli* and *H. pylori* proteins (15). HPNhaA fused at both its N- and C-terminal ends to GFP variants (CPF-HPNhaA-YFP, YFP-HPNhaA-CFP) was also constructed to detect interactions between its N- and C-terminal halves (Fig. 1A). The antipporter activity of the fusion proteins in *E. coli* HITΔAB− cells transformed with the fusion genes was analyzed by measuring their ability to grow on medium containing high concentrations of NaCl or LiCl. All of the transformed cells were able to grow on high salt agar plates, suggesting that the fusion proteins exhibited antipporter activity. Whereas cell growth was vigorous for cells transformed with the C-terminal GFP fusion (TABLE ONE), the cell growth in high salt medium was very low for fusions with GFP variants at both their N and C termini (TABLE ONE).

The Na+/H+ antipporter activities were directly measured in everted membrane vesicles prepared from transformed cells. Consistent with the results of salt-resistant cell growth, HPNhaA fused at its C terminus to GFP variants showed Na+/H+ and Li+/H+ antipporter activity comparable to that of the wild type (Fig. 1, B and C). However, the other fusions showed much lower activity (data not shown). The expression of the fusion proteins in the membranes was confirmed by Western blot analyses with anti-GFP antibodies (Fig. 2, A and B). The expression levels of HPNhaA-CFP and HPNhaA-YFP were about 20-fold greater than the other fusions (data not shown). Thus, we concluded that the lower antipporter activity of the N-terminal end or both N- and C-terminal end fusion proteins was because of lower levels of expression or integration of the proteins into the membrane.

In summary, HPNhaA-CFP and HPNhaA-YFP fusions were active Na+/H+ and Li+/H+ antipporters, and exhibited profiles of pH-dependent antipporter activity similar to those of the wild type. These results indicate that the fusion proteins were suitable for FRET analyses.

**Detection of FRET in Everted Membrane Vesicles Containing Fusion Proteins**—To detect interactions between HPNhaA monomers in the membranes by FRET, we introduced both HPNhaA-CFP and HPNhaA-YFP into *E. coli* HITΔAB−. If HPNhaA-CFP and HPNhaA-YFP interact closely within the membrane vesicles, FRET from CFP to YFP should be observed. The everted membrane vesicles were prepared from the transformants, and then fluorescence was measured.
Fluorescence emission profiles for the membranes of *E. coli* cells expressing HPNhaA-CFP or HPNhaA-YFP alone (controls) or expressing both HPNhaA-CFP (donor) and HPNhaA-YFP (acceptor) were compared using an excitation wavelength of 433 nm. The fluorescence emission spectra differed among the transformants tested. The fluorescence peak shifted to 528 nm (emission peak of YFP) from the emission peak for CFP (480 nm) in membranes containing both HPNhaA-YFP and HPNhaA-CFP (Fig. 3A). This shift in the fluorescence peak also occurred when the expression vectors were exchanged (pBR HPNhaA-CFP and pACYC HPNhaA-YFP versus pBR HPNhaA-YFP and pACYC HPNhaA-CFP) (Fig. 3A), implying that the expression levels of the fusion proteins do not appreciably affect FRET. However, the emission profiles sometimes differed between experiments. This was because of two factors. One was the slight differences in the expression levels of the fusion proteins between different batches of culture. The other was the slightly higher expression of the NhaA-GFP variants from the pBR vector than from the pACYC (Fig. 2, A and B, lanes 2 and 3). Therefore, the apparent differences in the fluorescence profiles probably reflect differences in promoter efficiencies between the different vectors.

Because FRET between CFP and YFP fusion proteins was clearly evident, we decided to use a GFP variant Venus for further analyses. Fluorescence of the HPNhaA-Venus fusion was minimally affected by pH or monovalent anions, including Cl⁻ (data not shown). The pH-dependent antiporter activity in everted membrane vesicles from *E. coli* cells expressing HPNhaA-Venus, or both HPNhaA-CFP and HPNhaA-Venus, were similar to the wild type (Fig. 1, B and C). We tested FRET between HPNhaA-CFP and HPNhaA-Venus (Fig. 3B). The calculated difference in emission intensities between HPNhaA-Venus and HPNhaA-CFP alone plus NhaA-Venus alone showed a clear peak at 528 nm (emission peak of Venus) (Fig. 3B, dashed line), indicating that FRET occurred.

The fluorescence spectral profiles of FRET were slightly different between experiments as described above. Therefore, to compare the FRET profiles from different experiments more precisely, FRET efficiency was used as an index. We calculated the apparent FRET efficiency by dividing the fluorescence intensity of FRET by the intensity obtained by excitation at 473 nm, which excites Venus alone (31). Thus, differences in Venus expression could be cancelled in this way.
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FRET efficiency was 13.0% for the HPNhaA-CFP and HPNhaA-Venus pair (Fig. 3C). When the membrane vesicles containing HPNhaA-CFP or HPNhaA-Venus alone were mixed and then excited by 433-nm light, the fluorescence profile was almost the same as the sum of fluorescence intensities of CFP alone and Venus alone (Fig. 3D), and very different from the FRET fluorescence profile (Fig. 4B). The apparent efficiency of FRET in this case was 0.3% (Fig. 3E), being much lower than with both fusion proteins in the same membranes.

To confirm that the FRET observed really depended on interactions between HPNhaA monomers fused to CFP or Venus, and not on interactions mediated by the fluorescent tags, we analyzed the effect of proteinase K on FRET. At low concentration, proteinase K does not degrade GFP variants but cleaves at the fusion site (32). Following a 600-s incubation with proteinase K, the FRET fluorescence peak at 528 nm decreased, and the peak at 480 nm increased concomitantly (Fig. 4A). HPNhaA-CFP and HPNhaA-Venus were analyzed by Western blot analysis with the anti-GFP antibodies before and after the addition of proteinase K. Both fusion proteins disappeared after proteinase K treatment for 600 s (Fig. 4B), and the bands corresponding to CFP and Venus increased. When proteinase K was added to membranes containing either HPNhaA-CFP or HPNhaA-Venus, or to a mixture of membranes containing each of these proteins, the fluorescence did not change (data not shown). These results exclude the possibility that FRET resulted from interactions mediated by the GFP variants alone or between HPNhaA GFP variant fusions integrated into different membrane vesicles.

We have shown that the FRET between HPNhaA monomers is caused by their tight interaction within membranes, and not by intermembrane vesicle interactions, or random collision of free GFP variants. However, we could not rule out the possibility that FRET resulted from random collisions between HPNhaA and other proteins present within the same membrane. To assess this possibility, we tested whether or not HPNhaA-CFP in the presence of Fob-Venus, LacY-Venus, or the tetracycline carrier TetAB-Venus, which are proteins that should not interact with HPNhaA, could generate a FRET signal when present together within the same membrane. A significantly lower or no FRET signal was generated with these proteins, although a strong FRET signal was detected for the HPNhaA-HPNhaA interaction under the same conditions (Fig. 4C). These results confirmed that the FRET signal generated by HPNhaA-HPNhaA in everted membranes is caused by specific interactions reflecting dimerization or oligomerization. Based on these observations together with the results of other control experiments described in the previous section, we concluded that HPNhaA undergoes oligomerization in membranes.

**HPNhaA Monomer Interactions Change Depending on the Antiporter Activity**—Next, we analyzed the effect of different antiport substrate ions on FRET in the presence of ΔpH across the membrane to observe the conformational changes that are expected to occur between HPNhaA monomers when the antiport is active. The fluorescence ratio at 528 nm versus 480 nm was used as an index of the change in FRET. Na⁺, the normal substrate for the HPNhaA, did not cause extensive changes in FRET, similar to the control ion, choline (Fig. 5A). However, Li⁺ significantly affected the FRET at various substrate concentrations (Fig. 5A), but did not cause significant changes in Venus fluorescence up to 100 mM at pH 8.5 (data not shown). FRET decreased with increasing Li⁺ concentrations, suggesting that the interactions between HPNhaA monomers were reduced by Li⁺/H⁺ antiport activity. The addition of lactate alone caused a decrease in FRET (Fig. 5B), and further addition of Li⁺ caused an additional decrease. NH₄Cl is known to acidify the intravesicular pH, leading to dissipation of the ΔpH across the membrane and inhibition of the antiporter (33). Therefore, we tested whether NH₄Cl could restore the decrease in FRET or not (Fig. 5B). The addition of NH₄Cl in the presence or absence of ΔpH induced by lactate caused a slight decrease, instead of an increase (Fig. 5B, panels a and b). These results suggested that the Li⁺-dependent decrease in FRET is primarily because of a conformational change in HPNhaA induced by Li⁺ binding.

To investigate further the change in FRET induced by Li⁺ binding, we tested the effect of single amino acid substitutions in HPNhaA, D141N,
D171N, and D172N, that decrease antiporter activity to zero (12). As shown in Fig. 6A, the point mutations slightly reduced FRET in the absence of Li$^+$. The decrease in FRET was most extensive for D141N. In the presence of Li$^+$, the decrease in the FRET efficiency of the mutants was no longer apparent for D141N, whereas a slight but a significant decrease was observed for D171N and D172N (Fig. 6B). It was suggested that Asp141 is involved in Li$^+$ binding, whereas Asp171 and Asp172 are involved in H$^+$ binding (16). Therefore these results support a model in which the change in monomer interactions detected by FRET reflects a conformational change induced by Li$^+$ binding.

Intramolecular Domain Interaction Detected by FRET with Insertion of GFP Variants into Loop 8—We have previously found that the insertion of 38 amino acid residues into Loop 8 of ECNhaA, which corresponds to the hydrophilic region between transmembrane region 8 and 9, does not affect antiporter activity (15). Here we tested whether or not insertion of the much larger GFP variants into Loop 8 (Fig. 1A) affects antiporter activity with the aim of using such a construct for further FRET analyses. Although the insertion of CFP caused a slight decrease in antiporter activity, the fusion protein still retained significant Na$^+$/H$^+$ and Li$^+$/H$^+$ antiporter activity across the pH range tested (TABLE ONE and Fig. 1B). The additional fusion of Venus to the C terminus of this construct, creating the HPNhaA(Loop8 CFP)-Venus construct (Fig. 1A), decreased Na$^+$/H$^+$ antiporter activity by 50% (Fig. 1B). ECNhaA contains a trypsin-sensitive site in Loop 8 whose cleavage is pH-dependent. ECNhaA activity is also known to be highly dependent on pH (8), and HPNhaA contains a pH-dependent antiporter activity similar to ECNhaA (12). These observations suggested that the conformation of Loop 8 is pH-dependent. Therefore, we tested NhaA constructs containing GFP variants in Loop 8 that would enable us to detect interactions between Loop 8 and the C terminus of NhaA by FRET.

A significant FRET, with an apparent FRET efficiency of 18.3%, was observed between CFP in Loop 8 and Venus at the C terminus of NhaA (Fig. 7A). Association of the GFP variants may have occurred within the same HPNhaA molecule, or between variants fused to different HPN-
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![Figure 7](image)

haA molecules. We examined the apparent FRET efficiency for co-expressed HPNhaA(Loop8 CFP)-FLAG and HPNhaA-Venus to differentiate between these two possibilities. The apparent FRET efficiency was 4.7% (Fig. 7A), which was significantly lower than the FRET efficiency of HPNhaA(Loop8 CFP)-Venus. Therefore, the contribution of FRET between Venus and CFP attached to different molecules is low. The apparent FRET efficiency between HPNhaA(Loop8 CFP) and HPNhaA(Loop8 Venus) was 24.7% (Fig. 7A), which was much higher than that of co-expressed HPNhaA-CFP and HPNhaA-Venus (Fig. 3C). This result suggests that Venus and CFP in Loop 8 of different HPNhaA monomers are located in the closest distance among the fusions tested.

Using HPNhaA(Loop8 CFP)-Venus, the effect of pH on the conformational changes that occur within an NhaA monomer was analyzed by FRET. The apparent FRET efficiency increased with increasing pH (Fig. 7B). In comparison, the pH did not affect the apparent FRET efficiency between HPNhaA-CFP and HPNhaA-Venus (Fig. 7B). These results suggest that the conformation of the C-terminal region does not change with pH, as a change in conformation of this region would be expected to alter the FRET efficiency between HPNhaA-CFP and HPNhaA-Venus. Thus, the pH-dependent change in FRET efficiency detected with the HPNhaA(Loop8 CFP)-Venus construct suggests that the Loop 8 region may move toward the C-terminal region upon alkalinization. We also tested the effect of substrate ions at pH 6.5, because the Li⁺/H⁺ antiporter activity was null at alkaline pH (Fig. 1C). A decrease in the ratio of fluorescence intensity was observed (Fig. 7C), suggesting that Loop 8 and the C terminus move away from each other in the presence of Li⁺, but not in the presence of Na⁺ or choline.

**Observation of Oligomerization in Living Cells**—Once detection of FRET in the everted membrane vesicles was established, we tried to detect FRET in intact cells. The fluorescence of the expressed fusion constructs was observed primarily in the cell periphery, possibly the cytoplasmic membrane (Fig. 8A). This observation is consistent with the detection of the constructs in the membrane fraction using antibodies against GFP (Fig. 4B). As shown in Fig. 4B, although some fusion proteins were cleaved and free GFP variants were generated as a result, most of the fusion proteins remained intact. Fluorescence spectrum analysis revealed FRET in intact *E. coli* cells expressing HPNhaA-CFP and HPNhaA-Venus in the same cells (Fig. 8B). As shown in Fig. 3E, FRET between free CFP and Venus was negligible. Therefore, we concluded that the FRET observed in the intact cells reflects the presence of oligomeric HPNhaA in intact cell membranes.

**Detection of Oligomerization of HPNhaA by Coprecipitation of Differently Tagged NhaA**—Oligomer formation of HPNhaA was confirmed by coprecipitating differently tagged monomers. C-terminal fusions of HPNhaA to a FLAG sequence or to GFP variant Venus were co-expressed in *E. coli* cells (Fig. 9, A and B). When extracts from these cells were immunoprecipitated with an anti-FLAG monoclonal antibody, both HPNhaA-FLAG and the HPNhaA-Venus fusion were detected in

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**Figure 7.** Apparent FRET efficiency in everted membrane vesicles containing HPNhaA with the Loop 8 insertion and C-terminal fusions of GFP variants or HPNhaA with the Loop 8 insertion alone. A, fluorescence was measured for membrane vesicles derived from *H. influenzae* cells expressing HPNhaA with the Loop 8 insertion fused on its C-terminal side to GFP variants. The apparent FRET efficiency (E) was calculated as described under "Materials and Methods." Data are averages of three independent experiments. B, effect of pH on FRET efficiency for HPNhaA(Loop8-CFP)-Venus. Membrane vesicles containing HPNhaA(Loop8 CFP)-Venus (closed squares) or HPNhaA-CFP/HPNhaA-Venus (closed diamonds) were suspended in assay buffer adjusted to different pH values. Fluorescence was measured by excitation at 433 nm or 473 nm, and then FRET efficiency (E) was calculated as described under "Materials and Methods." Data are the average of three independent experiments. C, membrane vesicles containing HPNhaA(Loop8 CFP)-Venus were suspended in Tricine-KOH buffer (pH 6.5). Respiration was induced by adding 5 mM lactate, and then the fluorescence ratio of 528 nm/480 nm was measured in the presence of various concentrations of NaCl, LiCl, or choline Cl as described in the legend to Fig. 5. NaCl, closed diamonds; LiCl, closed squares; choline Cl, closed triangles.
the immunoprecipitates by Western blot analysis using the anti-FLAG and anti-GFP antibody, respectively (Fig. 9, A and B). The \( \beta \) subunit of the \( F_{1}\)Fo ATPase (Fob) did not co-precipitate with HPNhaA-FLAG (Fig. 9B). These results confirmed that HPNhaA forms an oligomer.

**Functional Interaction of HPNhaA Monomers in Vivo**—To address whether HPNhaA monomer interactions play a functional role in vivo, we tested for a dominant negative effect of the functionally defective mutant HPNhaA. This type of experiment requires a functionally defective protein whose expression is much higher than that of the normal protein. Because overexpression of active or defective NhaA causes severe growth retardation, as we reported previously (29), significantly higher expression of the defective NhaA over that of the wild type could not be achieved. Accordingly, clear results were not obtained for these experiments. Next, the possibility of functional suppression through the concomitant expression of two functionally defective HPNhaA proteins in the same cell was tested. Here, we analyzed combinations of H233R with D141N, D171N, or D172N. The H233R mutation caused lower antiporter activity within the alkaline pH range compared with the wild type (Fig. 10, B and C). Expression of H233R/D141N or the H233R/D171N combination resulted in the recovery of LiCl-resistant cell growth (Fig. 10A) as well as Li\(^{+}/H^{+}\) antiporter activity at pH 7 (Fig. 10B), but Na\(^{+}/H^{+}\) antiporter activities were not recovered. Other combinations, D141N/D171N, D141N/D172N, D171N/D172N, and H233R/H233R did not result in the recovery of antiporter activity (Fig. 10A). These results suggested that a functional interaction takes place between HPNhaA monomers, although this could only be detected under certain restrictive conditions.

**DISCUSSION**

The present results by means of FRET analyses suggest that HPNhaA forms an oligomer, possibly a dimer, in the intact membrane. Coprecipitation of differently tagged HPNhaA proteins and/or \( F_{\beta} \) b were solubilized by 1% Triton X-100 treatment. The proteins in the solubilized samples were separated by SDS-PAGE and then analyzed by Western blot with anti-GFP antibodies (Molecular Probes) and anti-FLAG M2 monoclonal antibodies. Lane 1, Vector; lane 2, HPNhaA-Venus; lane 3, HPNhaA-FLAG; lane 4, HPNhaA-Venus/HPNhaA-FLAG; lane 5, F\(_{\beta}\)-b-Venus/HPNhaA-FLAG. B, Triton X-100-solubilized samples were immunoprecipitated using anti-FLAG M2-agarose affinity gel. Immunoprecipitates were analyzed by Western blot analysis as described previously (15).

D172N, and H233R/H233R did not result in the recovery of antiporter activity (Fig. 10A). These results suggested that a functional interaction takes place between HPNhaA monomers, although this could only be detected under certain restrictive conditions.

**FRET Analysis of Conformational Changes in HPNhaA**

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**FIGURE 8.** FRET of HPNhaA-GFP variant fusions in the intact cells. *E. coli* HT115 cells co-expressing HPNhaA-CFP and HPNhaA-Venus were cultured to late logarithmic phase (\( A_{600} = 1.0 \)) in LB medium and harvested. Fluorescence in the cells suspended in phosphate-buffered saline was observed by fluorescence microscopy (Olympus BX51) using an excitation wavelength of 433 nm. A, typical optical field showing transformed cells. B, fluorescent spectra of the cells obtained by fluorometry. HPNhaA-CFP/HPNhaA-Venus, thin line; HPNhaA-CFP, dotted line; HPNhaA-Venus, dashed line; FRET, thick line.

**FIGURE 9.** Detection of HPNhaA dimers by coimmunoprecipitation. A, membrane vesicles from *E. coli* HT115 cells (0.25 mg of protein) expressing HPNhaA-GFP variant fusion proteins and/or \( F_{\beta} \) b were solubilized by 1% Triton X-100 treatment. The proteins in the solubilized samples were separated by SDS-PAGE and then analyzed by Western blot analysis using anti-FLAG M2-agarose affinity gel. Immunoprecipitates were analyzed by Western blot analysis as described previously (15).
control experiments, including measuring FRET following proteinase K release of the GFP variants from NhaA and testing for nonspecific interactions between NhaA and the b subunit of the F1Fo ATPase, LacY, or TetAB. Although nonspecific interactions between NhaA and LacY or TetAB were higher than that between NhaA and the Fo-b subunit, FRET was significantly higher for the NhaA monomer interaction. Therefore, these experiments showed that FRET truly reflects close interactions between NhaA monomers. During the preparation of this report, the crystal structure of ECNhaA at 3.4-Å resolution was reported. Because the dimer structure was not presented, the precise distance between the two monomers still remains open to question (34).

The crystallographic structures of lac permease (LacY) (17) and glycerol-3-phosphate transporter (G3PT) (18) as well as ECNhaA (34) were solved and have revealed the precise atomic structures of these transporters, especially the topological arrangement of essential and important residues involved in the transport mechanisms. The results have revealed that a central pore, required for the LacY and G3PT transporters, is formed between the pseudo-symmetrical N- and C-terminal domains. The structural information predicts that dynamic conformational changes take place in the transporters to open the pore to the opposite sides of the membrane in an alternating fashion (17, 18, 34). However, only a crystal structure representing one conformation of the expected two or more states has been shown for each transporter (17, 18, 34). The prediction of dynamic conformational change during ion transport was partly supported by the observation that ECNhaA is susceptible to digestion by trypsin at alkaline pH, conditions under which ECNhaA is active, although it is not cleaved by trypsin at acidic pH, conditions under which the transporter is inactive (20). Thus, the present observations provide the first evidence of a conformational change in intact HPNhaA and further indicate that an extensive conformational change in both the HPNhaA monomer and oligomer occurs during Li\textsuperscript{+}/H\textsuperscript{+} antiport.

A significant decrease in apparent FRET efficiency was observed between monomers, and also between the Loop 8 and the C-terminal domain within a monomer, in the presence of Li\textsuperscript{+}, but not Na\textsuperscript{+}. This result suggests that the interaction between Loop 8 and the C terminus, and also interactions between monomers, is disrupted during Li\textsuperscript{+}/H\textsuperscript{+} antiport, but not during Na\textsuperscript{+}/H\textsuperscript{+} antiport. The apparent affinity of Li\textsuperscript{+} for NhaA is much higher than Na\textsuperscript{+}, as indicated by the lower $K_m$ value for the Li\textsuperscript{+} antiport. Therefore, it is reasonable to assume that release of Li\textsuperscript{+} from the putative binding site requires more energy and more extensive confor-
Conformational change in Li\textsuperscript{+}/H\textsuperscript{+} antipporter activity was supported further by the finding that the decrease in the apparent FRET change caused by the Li\textsuperscript{+}/H\textsuperscript{+} antipporter was not observed in HPNhaA constructs carrying point mutations that have been shown to cause an extensive decrease in antipporter activity. We have previously found that Asp\textsuperscript{141} and Thr\textsuperscript{140} may be involved in Na\textsuperscript{+} and Li\textsuperscript{+} binding, while Asp\textsuperscript{171} and Asp\textsuperscript{172} may be required for H\textsuperscript{+} binding (16).\textsuperscript{3} The effect of these changes on the conformational change during FRET induced by Li\textsuperscript{+}/H\textsuperscript{+} antipporter was more prominent for D141N than D171N and D172N, a result that is again consistent with conformational changes depending on Li\textsuperscript{+} binding. This result also suggested that the conformational change during Li\textsuperscript{+}/H\textsuperscript{+} antipporter may be caused by binding of Li\textsuperscript{+} rather than the entire process of antipporter. This hypothesis is consistent with the experiment showing that NH\textsubscript{4}Cl-induced dissipation of the \(\Delta\text{pH}\) did not significantly affect Li\textsuperscript{+} binding.

Recovery of Li\textsuperscript{+}/H\textsuperscript{+} antipporter activity in cells expressing the HPNhaA mutant H233R together with the D141N or D171N mutant was observed. Interactions between the two defective mutant NhaAs might change the conformation of one of the mutant NhaAs, leading to partial recovery of activity. As discussed above, Asp\textsuperscript{141} and Asp\textsuperscript{171} may be involved in ion binding. Therefore, these residues, which are intact in the HPNhaA of the H233R mutant, may enhance Li\textsuperscript{+}/H\textsuperscript{+} antipporter activity after a putative conformational change. For ECNhaA, the combination of H225R and D163C (H225R/D163C), corresponding to H233R/D171N in this report, has been reported to reestablish the wild-type pattern of pH-dependent Na\textsuperscript{+}/H\textsuperscript{+} antipporter activity (24). Thus, the precise structure required for antipporter activity may be different between HPNhaA and ECNhaA.

As shown in this study, the conformational change that NhaA undergoes upon ion transport and pH change is detectable by FRET using fusions of NhaA-GFP variants. This study demonstrates the feasibility of precisely identifying the structural elements required for conformational change. A detailed analysis such as this will require labeling residues with small fluorescent probes.

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