Dimeric Interaction between the Cytoplasmic Domains of the Na^+/H^+ Exchanger NHE1 Revealed by Symmetrical Intermolecular Cross-Linking and Selective Co-immunoprecipitation†

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ABSTRACT: To investigate the oligomeric structure of Na^+/H^+ exchanger 1 (NHE1), permeabilized cells and membranes from cells expressing NHE1 variants were treated with the oxidizing agent Cu^{2+}/*o*-phenanthroline or the bifunctional sulfhydryl reagent methanethiosulfonate. These treatments resulted in symmetrical intermolecular cross-linking at intrinsic (Cys^{794} and Cys^{561}) or 15 exogenous cysteine residues introduced into the distal carboxyl- (C-) terminal cytoplasmic domain (after aa 600) but not at intrinsic Cys^{538} because of masking by its tight association with calcineurin B-homologous protein. Cross-linking was abolished in membranes solubilized with sodium dodecyl sulfate, which dissociates oligomeric NHE1, while it was preserved in those treated with Triton X-100. In addition, treatment with cross-linkers did not produce the tetrameric forms of NHE1 mutants with two cysteine residues. Thus, cross-linking presumably occurs between adjacent C-termini of the NHE1 dimer but not by a stochastic process via random collision of NHE1 molecules. The observations suggest that at least the distal C-termini of the NHE1 dimer are flexible or mobile and are thereby capable of easily making contact with each other over the large cytoplasmic portion of the molecule. Furthermore, co-immunoprecipitation experiments showed that the proximal C-termini (aa 503−580) have a strong propensity to interact directly with each other in parallel. Deletion of aa 562−579 resulted in disruption of disulfide cross-linking between the C-termini and markedly reduced the intracellular pH sensitivity of Na^+/H^+ exchange, suggesting that the dimeric interaction in this region may control the pH-dependent regulation of NHE1.

The Na^+/H^+ exchangers (NHEs,[1] SLC9 family) are electroneutral transporters that catalyze the exchange of Na^+ and H^+ in plasma membranes or other intracellular organellar membranes in various animal species (*1*−*5*). To date, nine NHE isoforms encoded by different genes have been identified in mammalian tissues, and these isoforms are thought to play different roles in various tissues or in various organellar compartments. The first isoform to be cloned, NHE1 (*6*), is expressed ubiquitously in the plasma membranes of essentially all tissues and plays a major role in maintaining pH_i and cell volume homoeostasis. NHE1 consists of two large functional domains, an amino- (N-) terminal membrane domain (∼500 amino acids) containing 12 putative membrane-spanning domains and a long carboxyl- (C-) terminal cytoplasmic domain (∼300 amino acids). The N-terminal membrane domain catalyzes substrate transport, while the C-terminal domain is involved in regulation (*7*).

The activity of NHE1 is controlled by various extrinsic factors, including growth factors, hormones, and mechanical stimuli, presumably through the H^+-regulatory site (*1*−*6*, *8*). Such pH-dependent regulation is thought to occur through the interaction of the C-terminal domain with various signaling molecules (*1*−*5*). For example, calcineurin B-homologous protein (CHP) was identified as such an accessory protein that associates tightly with NHE1 (*9*). Recently, we reported that CHP serves as an essential cofactor for the physiological Na^+/H^+ exchange activity through interaction with the juxtamembrane region of the C-terminal domain (*10*). Other factors in addition to CHP, including Ca^{2+}/calmodulin (*11*, *12*), an adaptor protein 14-3-3 (*13*), Nck-interacting kinase NIK (*14*), phosphatidyli-

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[1] Abbreviations: NHE, Na^+/H^+ exchanger; CHP, calcineurin B homologous protein; pH_i, intracellular pH; CuP, Cu^{2+}/*o*-phenanthroline; MTS-2, 1,2-ethanediylbis(methanethiosulfonate); MTS-6, 1,6-hexanediylbis(methanethiosulfonate); MTS-17, 3,6,9,12,15-pentaoxaheptadecane-1,17-diylbis(methanethiosulfonate); MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; DMEM, Dulbecco's modified Eagle's medium; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; LDS, lithium dodecyl sulfate; aa, amino acid.

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nositol 4,5-bisphosphate (*15*), and carbonic anhydrase II (*16*), have been reported to associate with NHE1. However, as structural information is extremely limited even for the hydrophilic C-terminal domain, it is still difficult to determine the precise regulatory mechanism.

In addition to its regulatory aspect, NHE is known to exist as an oligomer in the plasma membrane. A previous study (*17*) showed that NHE1 and NHE3 form the homooligomers by interacting via the transmembrane regions in intact cells although the functional unit may still be monomeric. Consistent with this, NHE1 in the placental brush border membranes was detected as a larger form (~205 kDa) cross-linked by disulfide bonds (*18*). In addition, pre-steady-state kinetics revealed that Na^+/H^+ exchange in kidney brush border membrane vesicles exhibits cooperative extracellular Na^+ dependence despite electroneutral exchange, suggesting that NHE may function as an oligomer (*19*−*21*). Electron cryomicroscopy using two-dimensional crystals indicated that the bacterial Na^+/H^+ antiporter NhaA also appears to form dimers (*22*). However, at present, the physiological significance of this oligomerization is not well understood.

In the present study, we analyzed dimeric interactions between the cytoplasmic domains of NHE1. One aim of this study was to elucidate the structural features of the cytoplasmic domains of the NHE1 dimer. The second aim was to determine whether adjacent cytoplasmic domains of the NHE1 dimer interact directly with each other. We found that symmetrical intermolecular cross-linking at native or introduced cysteine residues occurs between the cytoplasmic domains of the NHE1 dimer and that the expressed cytoplasmic domains interact strongly with each other in cells. This is the first report indicating dimeric interaction between adjacent cytoplasmic domains of two NHE1 molecules.

EXPERIMENTAL PROCEDURES

Cell Culture and cDNA Transfection. The exchanger-deficient cell line PS120 and corresponding transfectants were maintained in Dulbecco's modified Eagle's medium containing 25 mM $NaHCO_3$ and supplemented with 7.5% (v/v) fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL). Cells were maintained at 37 °C in the presence of 5% CO_2. All cDNA constructs were transfected into PS120 cells by the calcium phosphate−DNA coprecipitation technique, and stable clones were selected by repetitive H^+-killing selection procedures as described previously (*7*).

Construction of the NHE1 Mutant Plasmid. The plasmid carrying a cDNA encoding NHE1 containing unique restriction sites cloned into the mammalian expression vector pECE was described previously (*7*). The cDNA construct for NHE1 in which all endogenous cysteine residues were replaced by alanine, designated as Cys-less NHE1, was also described previously (*23*). Construction of plasmids for NHE1 containing point mutations was carried out by a PCR-based strategy using two template plasmids encoding the wild-type or Cys-less NHE1 as described previously (*23*). Similarly, plasmids containing nucleotide sequences corresponding to the hemagglutinin (HA) epitope YPYDVPDYAS or the c-Myc epitope EQKLISEEDL were constructed by inserting PCR fragments produced using antisense primers containing either epitope sequence and a stop codon just after the C-terminus of NHE1 into the appropriate restriction sites of the plasmid containing NHE1 cDNA. Expression vectors for the C-terminal cytoplasmic domain were constructed by a similar PCR-based strategy using HA- or Myc-tagged NHE1 plasmid as a template. The PCR products were inserted into pEGFP-N1 (Clontech, Palo Alto, CA), in which expression of green fluorescent protein (GFP) was blocked by introduction of a stop codon. Constructs were confirmed by sequencing plasmids with an ABI-PRISM DNA sequencer model 3100 (Applied Biosystems, Foster City, CA). In this study, we used the prefix "cl-" for point mutants produced from Cys-less NHE1 as a background.

Cross-Linking between Cysteine Residues of NHE1. For crude membrane preparation, PS120 cells stably expressing the NHE1 variants were grown to confluence on 100 mm culture dishes and collected with a cell scraper into 1.5 mL microtubes. The cells were then washed once with PBS containing 137 mM NaCl, 2.7 mM KCl, 9.6 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 at pH 7.4 and homogenized with a Physcotron for 30 s at 2800 rpm on ice. The homogenates were centrifuged for 5 min at 4000 rpm, and the supernatants were centrifuged again for 15 min at 50000 rpm. The pellets (crude membranes) were resuspended in PBS, and protein content was adjusted to 2−3 mg/mL.

The membranes (1−1.5 mg/mL) were treated with 100 μM CuP for 15 min at 4 °C. To stop the reaction, EDTA (final concentration, 5 mM) was added to the reaction mixture, and then the membranes were dissolved with LDS sample buffer (Invitrogen, San Diego, CA) and subjected to PAGE. For cross-linking experiments on cells, confluent cells were washed twice with 5 mL of PBSCM (PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4) and permeabilized for 15 min on ice with 30 μM β-escin (Sigma, St. Louis, MO) dissolved in medium (120 mM KCl, 2.5 mM $MgCl_2$, and 25 mM HEPES, adjusted to pH 7.2 with KOH) as described previously (*24*). The permeabilized cells were washed to remove β-escin and incubated with 100 μM CuP for 15 min at 4 °C, and the reaction was stopped by treating cells with 10 mM *N*-ethylmaleimide (NEM). In some experiments, cells were treated directly with cysteine cross-linkers MTS-2, MTS-6, or MTS-17 in PBSCM. The resultant cells were solubilized with PBS containing 2% SDS, 20 mM NEM, and 5 mM EDTA, mixed with concentrated LDS sample buffer, and subjected to PAGE.

Immunoprecipitation and Immunoblotting. Cells were transiently transfected with the HA- or Myc-tagged C-terminal cytoplasmic domain of NHE1, and 48 h later cells were solubilized for 20 min on ice with lysis buffer (1% Triton X-100, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 1 mM benzamidine in PBS) and then centrifuged at 15000 rpm for 5 min. The supernatant was incubated for 2 h at 4 °C with anti-tag antibody plus 30 μL of protein A−Sepharose beads (Amersham Biosciences Inc., Piscataway, NJ). The beads were washed five times with ice-cold lysis buffer, and proteins were eluted with LDS sample buffer containing 50 mM DTT. After PAGE on 3−8% gradient gels (Invitrogen) or handmade 7.5% gels (Figure 3), proteins were transferred electrophoretically onto poly(vinylidene difluoride) membranes and subjected to immunoblotting with anti-NHE1 (*11*), anti-HA (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or anti-Myc polyclonal antibody (Santa Cruz) as described previously (*11*). Proteins were visualized

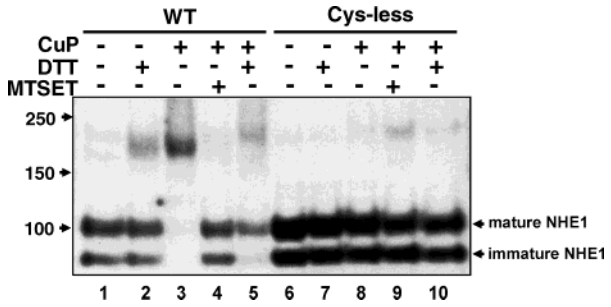

FIGURE 1: Cross-linking of NHE1 proteins with CuP. Crude membranes were prepared from cells expressing wild-type or Cys-less NHE1 and suspended in PBS at a protein concentration of 0.5−1 mg/mL. Membranes (60−90 μg) were treated with CuP (final concentration, 100 μM) for 15 min on ice, and then the reaction was stopped by adding EDTA (5 mM). In some experiments, membranes were briefly (5 min) treated with MTSET (1 mM) before CuP treatment. Samples of 12−18 μg of protein per lane were subjected to PAGE followed by immunoblotting with NHE1-specific antibody under reducing (+DTT) or nonreducing conditions.

by enhanced chemiluminescence detection (Amersham). Data were usually represented as a typical example of three independent experiments.

Measurement of ^{22}Na Uptake. $^{22}Na^+$ uptake activity was measured by the K^+/nigericin pH_i clamp method (*25*). Briefly, serum-depleted cells in 24-well plates were preincubated for 30 min at 37 °C in Na^+-free choline chloride/KCl medium containing 20 mM HEPES/Tris (pH 7.4), 1.2−140 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 μM nigericin (Molecular Probes, Inc., Eugene, OR). $^{22}Na^+$ uptake was started by adding the same choline chloride/KCl solution containing ^{22}NaCl (37 kBq/mL; final concentration, 1 mM), 1 mM ouabain, and 0.1 mM bumetanide. In some wells, the uptake solution contained 0.1 mM EIPA. One minute later, cells were rapidly washed four times with ice-cold PBS to terminate $^{22}Na^+$ uptake. pH_i was calculated from $[K^+]_i/[K^+]_o = [H^+]_i/[H^+]_o$ by assuming an intracellular $[K^+]$ of 120 mM. The data were normalized by the protein concentration, which was measured using a bicinchoninic assay system (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard.

RESULTS

Detection of the NHE1 Dimer by Symmetrical Intermolecular Cross-Linking at Cysteine Residues. We first prepared crude membranes from cells expressing NHE1 or its cysteine-free derivative, Cys-less NHE1. Two protein bands were visible on immunoblotting with NHE1-specific antibody (Figure 1, lane 1). The upper band (~100−110 kDa) was the N- and O-linked glycosylated mature form, while the lower band (~80 kDa) was the immature form containing only high-mannose oligosaccharide (*25*). Following treatment of the membranes with the oxidizing agent CuP (100 μM), the NHE1 monomer band disappeared and a new protein band became detectable within the higher molecular mass range of 200−230 kDa, which was twice that of the monomer (Figure 1, lane 3). We also performed similar experiments using membranes pretreated with endoglycosidase F, which is known to cleave N-linked glycans in NHE1 (*26*). Although endoglycosidase F treatment reduced the apparent molecular mass of NHE1 to 92−96 kDa, further

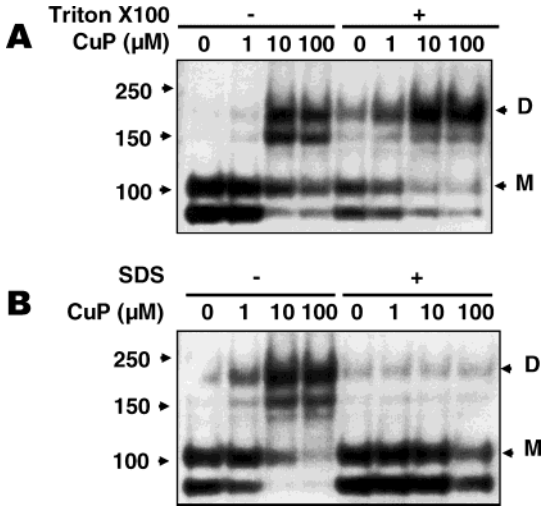

FIGURE 2: Effects of detergents on cross-linking of NHE1 with CuP. Crude membranes from PS120 cells expressing NHE1 were solubilized with or without 1% Triton X-100 (A) or 1% SDS (B). The solubilized membranes were centrifuged at 15000 rpm for 5 min to remove cell debris. Detergent-treated or untreated proteins were incubated with CuP (0−100 μM) for 15 min at 4 °C. After the reaction was stopped with EDTA, proteins were separated on PAGE under nonreducing conditions and subjected to immunoblotting with anti-NHE1 antibody. D and M indicate the positions of the NHE1 dimer and monomer, respectively.

CuP treatment resulted in a similar mobility shift to a higher molecular mass range (175−200 kDa), which was again twice that of the monomer (data not shown). The mobility shift was abolished almost completely by treatment with 10 mM DTT after oxidation or pretreatment with 1 mM cysteine-directed modifier reagent MTSET (Figure 1). In addition, this shift was not observed in Cys-less NHE1 (Figure 1). We also verified that the mature NHE1 expressed in the plasma membrane was cross-linked after surface labeling with NHS-biotin (data not shown). These results suggested that CuP treatment resulted in intermolecular disulfide bond formation at cysteine residues derived presumably from two NHE1 molecules. It should be noted that cross-linking between the immature forms of NHE1 was not detected in this experiment (Figure 1). However, it was often detected when we used membrane preparations including immature forms in relatively high quantities (Figures 2 and 5).

Cross-linking experiments suggested that two NHE1 monomers make contact with each other in the membrane. It is of interest to determine whether their interaction is preserved in the presence of detergents. Membranes were solubilized with 1% Triton X-100 or 1% SDS. After centrifugation the supernatant was treated with CuP and analyzed on immunoblot. As shown in Figure 2, the CuP-induced mobility shift of NHE1 on PAGE occurred in the presence of the relatively mild detergent Triton X-100 but not the more harsh detergent SDS. These findings suggested that two NHE1 monomers are still capable of interacting with each other upon treatment with Triton X-100 but not with SDS. Thus, it is unlikely that disulfide cross-linking is simply dependent on stochastic random collision.

Intermolecular Disulfide Cross-Linking Occurs between Cysteine Residues in Two Adjacent C-Terminal Cytoplasmic Domains of NHE1 Dimers. To identify the cysteine residue(s) that participate in cross-linking, we constructed NHE1

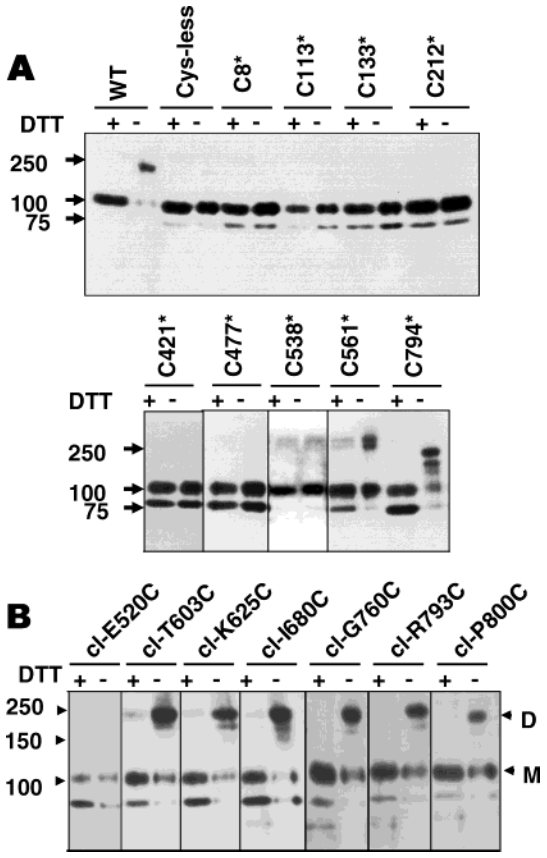

FIGURE 3: Cross-linking of NHE1 proteins containing a single cysteine residue. Crude membranes were prepared from cells stably expressing wild-type, Cys-less, mutants with a single native cysteine residue (A) or mutants with a single cysteine residue introduced into Cys-less NHE1 (B) and treated with 100 μM CuP for 15 min at 4 °C. The resulting membranes were analyzed by immunoblotting with the anti-NHE1 antibody under reducing or nonreducing conditions. Mutants with the native cysteine residue are indicated by an asterisk.

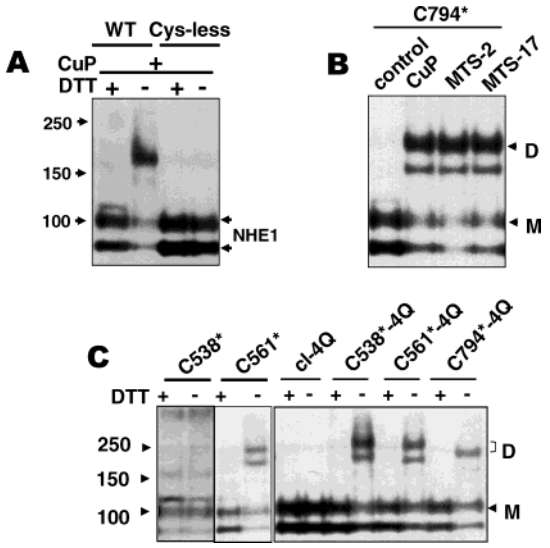

FIGURE 4: Cross-linking of NHE1 and its derivatives in permeabilized cells. (A) Cells stably expressing NHE1 or its Cys-less form were permeabilized with β-escin and then treated with 100 μM CuP for 15 min on ice and analyzed by immunoblotting with anti-NHE1 antibody under reducing and nonreducing conditions. (B) Permeabilized cells stably expressing C794* were treated with CuP, MTS-2, or MTS-17 (100 μM each) for 15 min on ice and analyzed by immunoblotting. (C) Cells stably expressing NHE1 mutants with a native cysteine residue or various derivatives of CHP-binding-defective mutant 4Q were permeabilized with β-escin, then treated with 100 μM CuP for 15 min on ice, and analyzed by immunoblotting with anti-NHE1 antibody. In 4Q, four hydrophobic residues of NHE1, Phe^{526}, Leu^{527}, Leu^{530}, and Leu^{531}, were replaced by four glutamine residues (*10*).

mutants with a single intrinsic cysteine residue using Cys-less NHE1 as the background template plasmid. Disulfide cross-linking with CuP did not occur at Cys^{8} in the cytoplasmic N-tail or at Cys^{113}, Cys^{133}, Cys^{212}, Cys^{421}, or Cys^{477} in the membrane-spanning domains (Figure 3A), consistent with our previous observation that the latter membranous cysteine residues are not accessible to the sulfhydryl reagent biotin maleimide (*23*). In contrast, cross-linking was observed at position 794 in the C-terminal cytoplasmic domain of NHE1 (Figure 3A). Significant but somewhat weaker cross-linking was also observed at position 561 but not at position 538. These results indicated that cross-linking of NHE1 occurs mainly at Cys^{794}. It should be noted that the apparent molecular mass (250 kDa or more) of cross-linked C561* was unexpectedly higher than the calculated mass of the NHE1 dimer. We considered it likely that cross-linking between juxtamembrane cysteine residues of NHE1 leads to a structural change resulting in aberrantly slow migration on PAGE.

We introduced a single exogenous cysteine mutation into several positions of the cytoplasmic domain of NHE1. Surprisingly, in addition to native cysteine residues Cys^{794} and Cys^{561}, CuP induced intermolecular cross-linking at various cytoplasmic amino acid positions, i.e., 603, 625, 680, 760, 793, 800 (Figure 3B), 615, 638, 650, 661, 701, 720, 740, 760, and 780 (data not shown), but not at position 520 (Figure 3B) (see also Figure 10 for amino acid positions in NHE1). We also tested cross-linking with CuP using β-escin-permeabilized cells, which are thought to maintain more nativelike conditions. As shown in Figure 4A, CuP-induced, DTT-dependent cross-linking was observed in permeabilized cells expressing wild-type NHE1 but not Cys-less NHE1. We also detected similar cross-linking in permeabilized cells expressing the NHE1 mutants with exogenous cysteine residues described above (not shown). Using permeabilized cells expressing C794*, we also tested other bifunctional cysteine cross-linkers, MTS-2 and MTS-17, which have spacer lengths of 5 and 25 Å, respectively. These agents were as effective as CuP for cross-linking of C794* (Figure 4B). Thus, the intermolecular cross-linking at cysteine residues from two NHE monomers appears to occur independently of cross-linker spacer length over the large cytoplasmic portion.

We postulated that lack of cross-linking of cysteine residues (positions 520 and 538) in the juxtamembrane domain may be due to the presence of CHP, which was shown to interact strongly with this region (*10*). We constructed several cysteine mutant NHE1s using the Cys-less version of 4Q that was reported previously to abolish CHP binding (*10*). As expected, in contrast to C538* (see Figure 4C), CuP treatment induced cross-linking in cells expressing C538*-4Q as well as other mutants, C561*-4Q and C794*-4Q (Figure 4C), suggesting that cross-linking at position C538 is masked by the presence of bound CHP. It should be noted that the apparent molecular masses for cross-linked products of C538*-4Q and C561*-4Q were higher than that for C794*-4Q (Figure 4C; see also Figure 3).

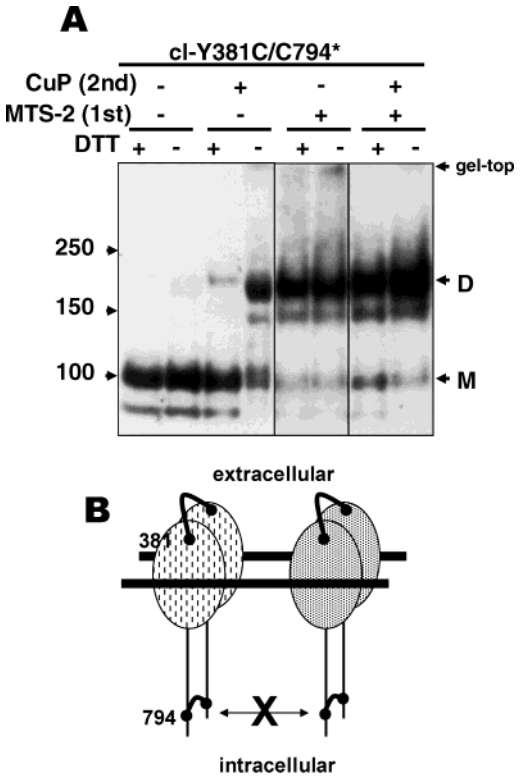

FIGURE 5: Cross-linking of the double-cysteine mutant of NHE1. (A) Cells expressing the NHE1 mutant (cl-Y381C/C794*) with two cysteine residues at positions 381 and 794 were first treated with 100 μM MTS-2 for 15 min on ice, and then crude membranes were prepared. Membranes were further treated with 100 μM CuP for 15 min at 4 °C and analyzed by PAGE under reducing and nonreducing conditions followed by immunoblotting with anti-NHE1 antibody. (B) Schematic drawing showing the possible cross-linking between cysteine residues of two NHE1 monomers.

We further analyzed the oligomerization of NHE1 using cells expressing a mutant with two cysteine residues at amino acid positions 381 and 794; the former (Tyr in wild-type NHE1) is located in the extracellular loop 5 (*23*), while the latter is located in the cytosol. In a preliminary search, we found that cysteines at 381 (and also at 153 and 283) can form intermolecular cross-links on treatment with the uncleavable cross-linker MTS-2. This reagent was not accessible to Cys^{794} from the outside of living cells at least under our experimental conditions (not shown). Treatment of cells expressing this double cysteine mutant with MTS-2 produced the DTT-insensitive protein band at the dimer position on PAGE (Figure 5A, lane 6), suggesting that the intermolecular cross-linking occurred at cysteines introduced into position 381, as shown schematically in Figure 5B. After treatment with MTS-2, crude membranes were prepared. Further treatment of membranes with CuP increased the intensity of the protein band at the dimer position but did not produce the protein band with higher molecular mass corresponding to the tetrameric form (i.e., dimer of dimers) (Figure 5A, lane 8), suggesting that cytoplasmic cross-linking does not occur between dimers cross-linked externally (Figure 5B).

Direct Interaction between the Cytoplasmic Domains of NHE1. Cross-linking experiments suggested that the cytoplasmic domains of NHE1 may be located in close proximity. To test whether the cytoplasmic domains of NHE1 make contact with each other homotypically, we transiently coexpressed two cytoplasmic domains (aa 503−815) with

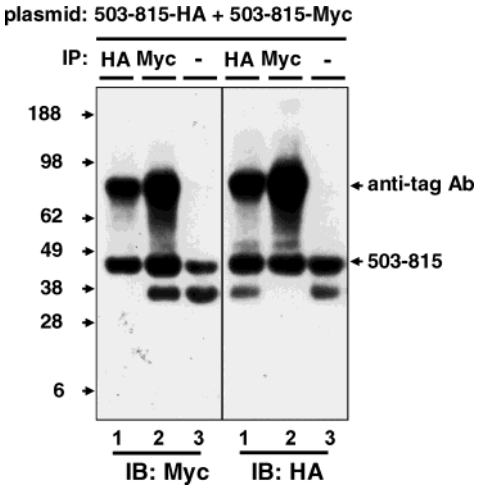

FIGURE 6: Co-immunoprecipitation of the C-terminal cytoplasmic domains of NHE1 labeled with different tags. Two plasmids, 503−815-HA and 503−815-Myc, were transiently cotransfected into PS120 cells. Forty-eight hours after transfection, Triton X-100-solubilized proteins were subjected to immunoprecipitation (IP) with anti-HA or anti-Myc antibodies followed by immunoblotting (IB). Proteins were also analyzed without immunoprecipitation (lanes 3 in both panels).

different tags, HA and Myc, in PS120 cells (see Figure 6). These proteins were expressed well as shown by immunoblotting with each specific antibody, although shorter bands (~35 kDa) were also visible (Figure 6, lane 3 in left and right panels). We observed that the upper bands bind CHP, while the lower bands do not (data not shown). As CHP binds to the juxtamembrane domain (aa 510−540), the former is probably the full-length cytoplasmic domain although its apparent molecular mass (~48 kDa) is higher than the calculated value (~37 kDa), while the latter may be the N-terminally degraded protein. The HA- and Myc-tagged cytoplasmic domain proteins were recovered in the immunoprecipitates with Myc- and HA-specific antibodies (Figure 6, lane 1 in left and lane 2 in right), respectively, suggesting that the two expressed cytoplasmic domains interacted with each other in cells.

Interestingly, tag-specific antibodies did not co-immunoprecipitate the shorter, possibly degraded fragments (Figure 6), suggesting that the N-terminus is important for interaction between the cytoplasmic domains. To identify the site of interaction, we constructed epitope-tagged cytoplasmic domains of different sizes and performed co-immunoprecipitation experiments. Deletion of the N-terminus up to aa 580 abolished co-immunoprecipitation, while deletion up to aa 562 preserved it (Figure 7B). Therefore, at least aa 562−580 is important for the interaction between cytoplasmic domains, but aa 580−815 is not involved. Similar experiments were repeated with combinations of cytoplasmic domains of different sizes. While 562−815-Myc interacted with 503−815-HA, the same protein did not interact with 503−815(Δ562−579)-HA (Figure 8B, parts a and b), indicating that aa 562−579 binds to the same region of the partner protein. Thus, the region 562−579 appears to be crucial for interaction. Furthermore, the cytoplasmic domains deleted of aa 540−579 were also capable of interacting with each other (Figure 8B, part d). In addition, 503−815-Myc interacted with 503−815(Δ562−579)-HA (Figure 8B, part e) and 503−815(Δ540−579)-HA (data not shown). Thus,

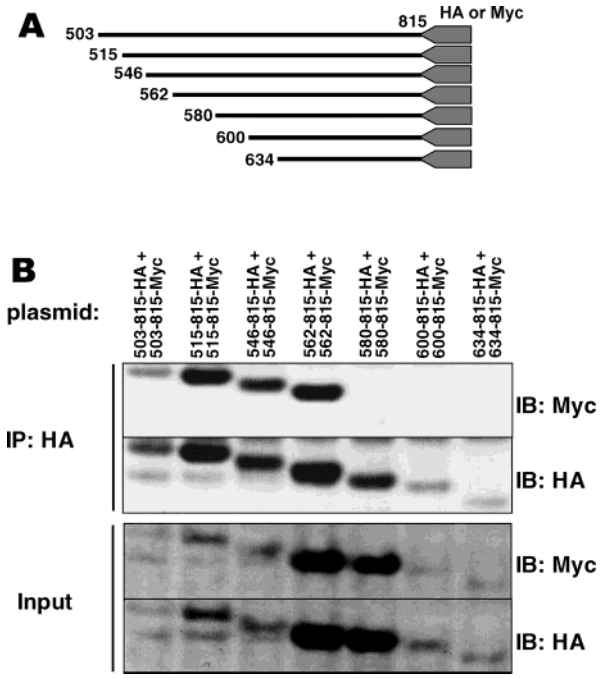

FIGURE 7: Co-immunoprecipitation of the various cytoplasmic domains of NHE1 labeled with different tags. (A) Schematic drawing of cytoplasmic domain constructs. (B) The two plasmids indicated in the figure were transiently transfected into PS120 cells. Triton X-100-solubilized proteins were subjected to immunoprecipitation (IP) with anti-HA antibody followed by immunoblotting (IB) with anti-Myc or anti-HA antibody. Solubilized lysates without immunoprecipitation (input).

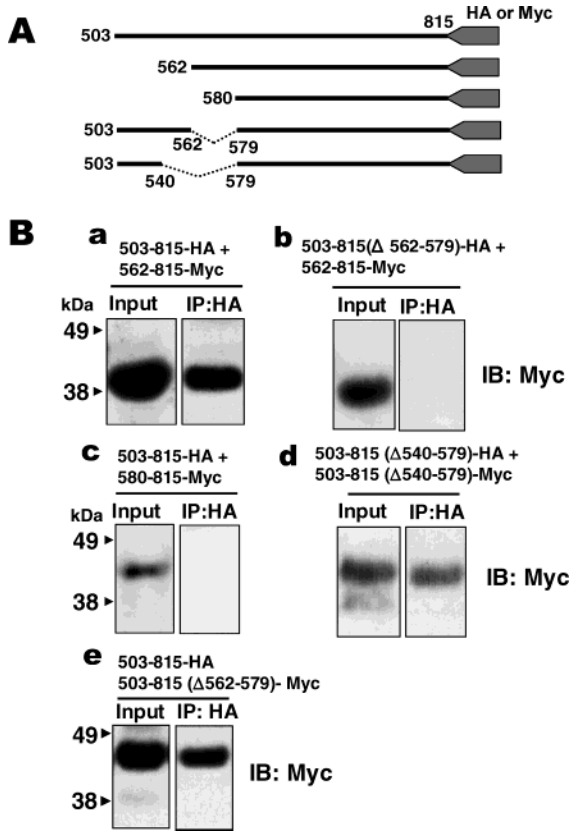

FIGURE 8: Co-immunoprecipitation performed on different combinations of the various cytoplasmic domains of NHE1. (A) Schematic drawing of cytoplasmic domain constructs. (B) The two plasmids indicated in the figure were transiently transfected into PS120 cells. Detergent-solubilized proteins were subjected to immunoprecipitation (IP) with anti-HA antibody followed by immunoblotting (IB) with anti-Myc antibody. Solubilized lysates without immunoprecipitation (input).

in addition to aa 562−579, the more proximal region also has the ability to interact.

We expressed an NHE1 mutant with deletion of part of the cytoplasmic interaction domain (aa 562−579) in PS120 cells and carried out the cross-linking experiment using the crude membrane fraction. In contrast to the appearance of a discrete dimer band for wild-type NHE1, CuP treatment resulted in smears on the gel for Δ562−579 (Figure 9A), suggesting that this mutant protein may form complexes with other cellular proteins through Cys^{794}. Thus, deletion of aa 562−579 markedly inhibited the intermolecular cross-linking between the NHE1 monomers. It should be noted that CuP treatment also produced relatively discrete, cross-linked products with lower molecular mass (~150 kDa), which were presumably derived from the immature form of Δ562−579.

We next measured the EIPA-sensitive $^{22}Na^+$ uptake in transfectants stably expressing wild-type or Δ562−579. Deletion of aa 562−579 reduced the maximal activity at acidic pH_i by about 10-fold (not shown). Furthermore, the same deletion greatly shifted the p*K* value for intracellular pH to the acidic side as compared to wild-type NHE1 (Figure 9B) and abolished NHE1 activation in response to various stimuli, such as growth factors and hyperosmotic stress (data not shown). These observations suggested that this region is critical for preserving the pH-dependent regulation of NHE1.

DISCUSSION

This study was initiated on the basis of our finding that NHE1 in the membranes can be cross-linked with the zero-length cross-linker CuP. Treatment of permeabilized cells or membranes expressing various NHE1 variants with CuP or other MTS reagents resulted in a mobility shift of NHE1 from the monomer to the higher molecular mass range corresponding to the dimer. We also showed that treatment of a double-cysteine mutant NHE1 (positions 381 and 794) with two different cross-linkers did not produce the higher molecular mass products corresponding to the tetrameric form (see Figure 5). The latter observation suggests that two NHE1 dimers are not further cross-linked with each other at the cytoplasmic end. These observations were consistent with the previous findings that treatment of cells with the amino-directed cross-linker disuccinimidyl suberate resulted in production of homodimeric forms of NHE1 or NHE3 (*17*) and that intermolecular disulfide cross-linking of NHE1 occurred in the placental brush border membranes (*18*). A previous study (*17*) indicated that homodimers would be formed somewhere between the N-terminal transmembrane domains of NHE1 (aa 1−500). Unexpectedly, we found that intermolecular cross-linking occurred predominantly at Cys^{794} in the C-terminal cytoplasmic domain, suggesting that, in addition to the transmembrane domain, the two C-termini of the NHE1 dimer might be located in close proximity.

One important observation is that CuP-induced cross-linking of NHE1 occurred even when membranes were solubilized with Triton X-100 (Figure 2A), suggesting that the NHE1 dimer did not dissociate on solubilization with this detergent. Such a strong interaction between monomers was also reported in the bacterial Na^+/H^+ antiporter NhaA,

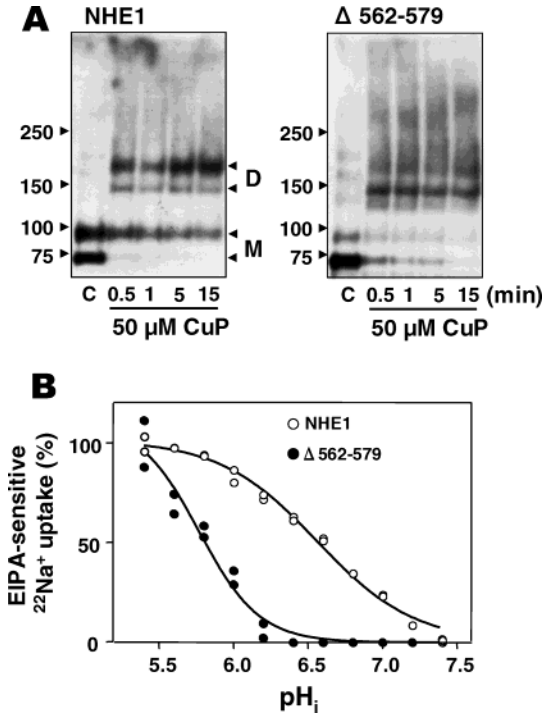

FIGURE 9: Properties of deletion mutant Δ562−579 of NHE1. (A) Membranes were prepared from cells expressing wild-type NHE1 or deletion mutant Δ562−579, then treated with 50 μM CuP for 0.5−15 min on ice, and analyzed by immunoblotting with anti-NHE1 antibody. A typical example of three independent experiments was shown. (B) The pH_i dependence of $^{22}Na^+$ uptake in cells expressing wild-type NHE1 or deletion mutant Δ562−579 was measured as described in Experimental Procedures.

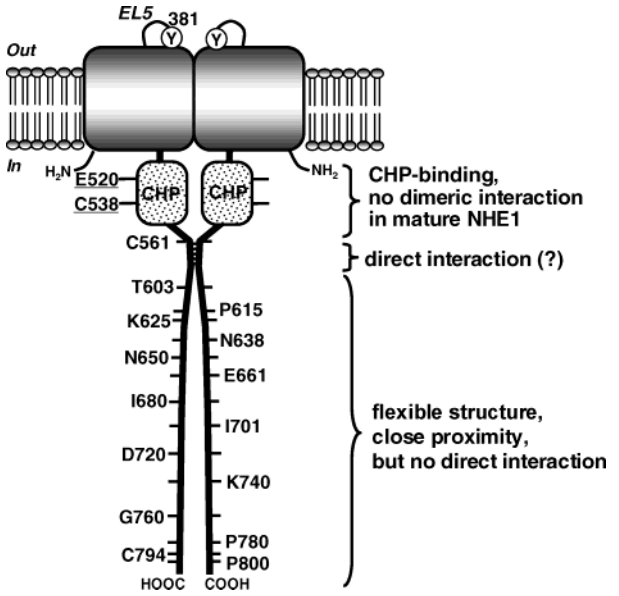

FIGURE 10: Possible dimer model of NHE1. Positions of amino acid residues mutated in this study were indicated. The region aa 562−579 would confer dimerization of the cytoplasmic regulatory domain. Although the region aa 503−540 has also the ability to interact with each other, it would not make contact in the mature NHE1 expressed in the plasma membrane because CHP tighly associates with this region. The distal region (aa 600−815) would have a flexible structure.

which did not dissociate on solubilization with dodecyl maltoside (*27*). However, CuP-induced cross-linking was abolished when membranes were solubilized with the harsher detergent SDS, which is expected to dissociate the NHE1 dimer (Figure 2B). These observations suggest that dimerization is not a stochastic process resulting from random collisions between monomers.

We found that CuP-induced cross-linking of NHE1 occurs predominantly at Cys^{794} and weakly at Cys^{561}. Surprisingly, such symmetrical cross-linking was also observed between almost all cysteine residues introduced into the distal C-terminal region (amino acid positions 615, 625, 638, 650, 661, 680, 701, 720, 740, 760, 780, and 800), suggesting that two C-termini are able to make contact at various cytoplasmic sites, despite the interaction of various signaling molecules with this region of NHE1. This unusual phenomenon, however, does not appear to be due to artifacts resulting from protein denaturation or aggregation because cross-linking at multiple sites also occurred in permeabilized cells which are expected to preserve the relatively intact membrane integrity and because biotin-labeled surface NHE1 proteins were also cross-linked in a similar way (see Results). We observed that CuP-induced cross-linking between Cys^{538} residues does not occur normally but becomes detectable when CHP binding is disrupted by mutation (4Q) (Figure 4D), suggesting that tightly bound CHP sterically blocks the homotypic interaction between CHP-binding domains (aa 510−540) of wild-type NHE1. Although Ca^{2+}/calmodulin (*11*, *12*), 14-3-3 (*13*), tescalcin (*28*, *29*), carbonic anhydrase II (*16*), and heat shock protein (30) were reported to bind to several regions within the distal C-terminal domain, the results of the present study suggest that, unlike CHP, these proteins may interact with NHE1 only in a spatially or temporally restricted fashion.

In addition to CuP treatment, cross-linking at intrinsic or other introduced cysteine residues also occurred upon treatment with the thiol-directed cross-linkers MTS-2 and MTS-17, the spacer arms of which have lengths of 5 and 25 Å, respectively (*31*). These observations strongly suggest that the distal C-terminus of NHE1 has a flexible structure (Figure 10), thereby allowing the distance between the cytoplasmic domains of the NHE1 dimer to change easily. The distal regions thus do not directly interact, consistent with the recent result that the last C-terminal 183 amino acids of NHE1 have only a slight tendency to dimerize on their own (*29*). On the basis of circular dichroism measurements, Li et al. (*29*) reported that the distal region of NHE1 contains essentially a β-sheet, β-turn, and an unstructured part, but almost no α-helix, and that the secondary structure is significantly altered upon changes in pH or Ca^{2+} concentration. The high degree of flexibility of the NHE1 cytoplasmic domain may play an important role in its function. Previously, we proposed that Ca^{2+}-induced activation of NHE1 occurs through derepression of the autoinhibitory domain (aa 635−656) caused by calmodulin binding (*11*, *12*). The mobile cytoplasmic domain might provide structural benefits for such a Ca^{2+}-induced activation because the autoinhibitory domain is predicted to interact with an acceptor region(s) within the transmembrane domain of NHE1 in the resting state and to leave the putative acceptor in response to Ca^{2+} mobilization (*32*). In addition, NHE1 may control actin cytoskeletal reorganization through interaction with ERM family proteins (*33*), thereby regulating stress fiber formation and cell locomotion (*33*−*35*). Two different functions of NHE1, ion exchange and cytoskeletal rearrangement, appear to be regulated independently (*33*−*35*). Flexibility of the

cytoplasmic domain may help to alleviate constraints on the transmembrane ion transport domain, which may be produced by interaction with the cytoskeleton. Some degree of flexibility was also observed in the N-terminal cytoplasmic domain of the anion exchanger AE1 (band 3), which is known to bind the membrane-cytoskeleton anchoring protein ankyrin (*36*, *37*), although this domain of AE1 has a relatively compact dimeric structure (*38*). It is interesting to note that, in contrast to NHE1, intermolecular cross-linking occurs between two different endogenous cysteine residues, Cys^{201} and Cys^{317}, in the cytoplasmic domain of AE1 (*39*).

The structure of the proximal C-terminal region of NHE1 appears to be quite different from the distal end of the molecule. We found that the expressed C-terminal domains interact strongly in cells through several sites within aa 503−580. The requirement of symmetrical regions within aa 503−580 for the interaction (Figure 8) suggests that this homotypic association is specific and not due to nonspecific binding or aggregation. These observations indicate that this region (aa 503−580) of NHE1 has a strong propensity to interact with the same region in adjacent molecules in a parallel fashion. Thus, in addition to the transmembrane domains (*17*), this cytoplasmic region may be another candidate for the dimer interface. However, it was difficult to determine whether these regions of the NHE1 dimer interact when the whole NHE1 is expressed in the plasma membrane. The CuP-induced Cys^{538}-Cys^{538} cross-linking of NHE1 was blocked by tightly bound CHP (Figure 4D). In addition, purified CHP complexed with aa 503−545 of NHE1 was confirmed to be a monomer on gel filtration (*40*). Therefore, at least the N-terminal halves (aa 503−545) in aa 503−580 of NHE1 would not interact with each other in NHE1 expressed in the membrane (Figure 10). However, the C-terminal halves may associate because CuP-induced cross-linking occurred through Cys^{561} (Figure 3B). Thus, the region aa 562−579 may confer dimerization of the cytoplasmic regulatory domain, which in turn appears to bring the remaining distal flexible portion (aa 580−815) of the respective monomers into close proximity of each other. Previously, we reported that deletions of different regions in subdomain I (aa 515−595) of the NHE1 cytoplasmic domain markedly disrupted the NHE1 function, particularly the pH_i sensitivity (*25*). Here, we observed that deletion of aa 562−579 disrupted the intermolecular disulfide cross-linking of NHE1 and shifted the pH_i dependence of the exchange activity to the acidic side (Figure 9), suggesting that deletion-induced inhibition of NHE1 activity may be due to the structural distortion caused by disruption of dimeric interaction in this subdomain. These observations raise the interesting possibility that NHE1 may function as a cooperative dimer in which two subunits are functionally coupled in the regulation of pH_i sensitivity. This was reinforced by our recent finding that intermolecular cross-linking at cysteine residues introduced into the extracellular loop abolished pH_i regulation by NHE1 (T. Hisamitsu, unpublished observations).

It is also possible that the parallel association between the relatively long juxtamembrane regions (aa 503−580) has some other important physiological roles in the function of NHE1. These relatively hydrophobic stretches of two NHE1 monomers may be masked by interacting with each other during biosynthesis of NHE1, thereby passing the "quality control" checkpoint of the endoplasmic reticulum (ER). Control of retention/retrieval in the ER by oligomerization has been demonstrated previously for some membrane proteins, such as ATP-sensitive potassium channels (*41*), γ-aminobutyric acid receptor (*42*), and *N*-methyl-D-aspartate receptor (*43*). Recently, we obtained some evidence that CHP does not bind to the immature form of NHE1 which is retained in the ER (our unpublished results). Therefore, it is plausible that NHE1 and CHP are capable of interacting only after these proteins are separately transported to the plasma membrane. The finding that the immature form of Δ562−579 can be cross-linked (Figure 9) suggests that the more proximal CHP-binding regions may dimerize in the ER but not in the plasma membrane.

In summary, we obtained evidence that the distal C-terminal cytoplasmic regions of the NHE1 dimer are flexible and that they are able to make contact easily, while the proximal regions have the intrinsic ability to interact directly with each other. To date, attempts to crystallize the cytoplasmic domain of NHE1 have been hampered by the high degree of degradation and aggregation of proteins expressed in *Escherichia coli* and other organisms, which may result from the unusual structural properties of the C-terminus. Although future studies are required to elucidate the precise structure, the results of the present study provide basic information regarding the dimeric structure of the C-terminal cytoplasmic domains of NHE1 expressed in living cells.

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