NBCE1-A Transmembrane Segment 1 Lines the Ion Translocation Pathway

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The electrogenic Na\(^+\)/HCO\(_3\)\(^-\) cotransporter (NBCE1-A) transports sodium and bicarbonate across the basolateral membrane of the renal proximal tubule. In this study the structural requirement of transmembrane segment 1 (TM1) residues in mediating NBCE1-A transport was investigated. Twenty-five introduced cysteine mutants at positions Gln-424 to Gly-448 were tested for their sensitivity to the methanethiosulfonate reagents (2-sulfonatoethyl) methanethiosulfonate (MTSES), [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET), and (2-aminoethyl) methanethiosulfonate (MTSEA). Two mutants, T442C and A435C, showed 100 and 70% sensitivity, respectively, to inhibition by all the three methanethiosulfonate reagents, I441C had >50% sensitivity to MTSET and MTSEA, and A428C had 50% sensitivity to MTSEA inhibition.

A helical wheel plot showed that mutants T442C, A435C, and A428C are clustered on one face of TM1 within a 100° arc. Topology analysis of TM1 with biotin maleimide and 2-((5(6)-tetramethylrhodamine)carboxylymio)ethyl methanethiosulfonate (MTS-TAMRA) revealed Thr-442 marks the C-terminal end of TM1 and that extracellular FGGLLG stretch is in a small aqueous-accessible cavity. Functional studies indicated that Thr-442 residues in a narrow region of the ion translocation pore with strong δ-helical dipole influence. Analysis of the corresponding residue of NBCE1-A-Thr-442 in AE1 (Thr-422) shows it is functionally insensitive to MTSES and unlabeled with MTS-TAMRA, indicating that AE1-TM1 is oriented differently from NBCE1-A. In summary, we have identified residues Thr-442, Ala-435, and Ala-428 in TM1 lining the ion translocation pore of NBCE1-A. Our findings are suggestive of a δ-helical dipole at the C-terminal end of TM1 involving Thr-442 that plays a critical role in the function of the cotransporter.

Bicarbonate transport processes play an essential role in systemic acid-base balance and intracellular pH regulation (1). NBCE1-A belongs to the SLC4 bicarbonate transporter family containing three functionally distinct groups of transporters: Na\(^+\)-independent Cl\(^-\)/HCO\(_3\)\(^-\) exchangers, Na\(^+\)-HCO\(_3\) cotransporters, and Na\(^+\)-driven Cl\(^-\)/HCO\(_3\)\(^-\) exchangers, of which only NBCE1 and NBCE2 are electrogenic. Three NBCE1 variants have been reported; they are NBCE1-A, which is found in the kidney and eye, NBCE1-B, which is expressed in pancreas, duodenum, colon, and several other tissues, and NBCE1-C, which is predominantly expressed in the brain. NBCE1-A differs from NBCE1-B/NBCE1-C in the N-terminal initial 85 amino acids, and NBCE1-C differs from NBCE1-A/NBCE1-B in the last 61 amino acids in the C-terminal tail (1).

NBCE1 mediates the electrogenic cotransport of Na\(^+\) and HCO\(_3\)\(^-\) across plasma membranes in specific cell type. In kidney, NBCE1-A in proximal tubule cells mediates the basolateral efflux of HCO\(_3\)\(^-\) from cell to the blood, thereby reabsorbing ~80% of the filtered HCO\(_3\)\(^-\) load. In pancreas, NBCE1-B expressed in duct epithelial cells contributes to the basolateral influx of HCO\(_3\)\(^-\) from blood to cell, during the process of secretin-evoked pancreatic fluid secretion. The ion transport stoichiometry of NBCE1-A is 1 Na\(^+\)/3 HCO\(_3\)\(^-\) but can be altered to 1 Na\(^+\)/2 HCO\(_3\)\(^-\) upon phosphorylation of a serine residue near the C terminus (2). In addition, the cellular environment appears to play a role in modulating the transport strechiometry of NBCE1 proteins (3). Ten mutations in the SLC4 gene have been reported that cause autosomal recessive proximal renal tubular acidosis with ocular and intracerebral abnormalities (1).

NBCE1-A is a 140-kDa glycoprotein containing 1035 amino acids. Glycosylation studies indicate that it transverses the lipid bilayer a minimum of 10 times (4), and hydropathy analyses predict up to 14 transmembrane regions. Based on anion exchanger 1 (AE1) topology (5), NBCE1-A is predicted to transverse the lipid bilayer 13 times with 2 reentrant loops (6).

Both N and C termini of NBCE1-A are located in cytoplasm, with a large extracellular loop proposed between transmembrane segment (TM)2 and 6 containing two glycosylation sites (Fig. 1A). NBCE1-A is predominantly a homodimer that is composed of functionally active monomers and whose oligomeric state is dependent on S-S bond formation (7). We previously performed a large scale mutagenesis study on NBCE1-A protein-targeted acidic and basic amino acids near or outside putative transmembrane segments and had identified additional

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2 The abbreviations used are: TM, transmembrane; BM, biotin maleimide, 3-(N-maleimidylpropionylo) biocytin; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl) methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; MTS-TAMRA, 2-((5(6)-tetramethylrhodamine)carboxylymio)ethyl methanethiosulfonate; BCECF-AM, 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein tetrakis(acetoxyethyl) ester; LeuT, leucine transporter; AE1, anion exchanger 1; PBS, phosphate-buffered saline.
inactivating mutations affecting the cotransporter function (8). In addition, proposed TM8 was recently analyzed by cysteine-scanning mutagenesis, and several residues were found to be involved in forming the ion translocation pore (9). Protein domain swapping studies have suggested the electrogenicity of NBCe1-A is determined by the interaction of TMs in the lipid bilayer (10).

Currently, the structural basis underlying NBCe1-A transport function remains unknown. A recent crystal structure of a Na+-dependent leucine transporter (LeuT) revealed that LeuT-TM1 forms part of the sodium binding site and substrate translocation pathway (11). Interestingly, TM1 is also important for proper folding of the membrane domain of the AE1, a member of the SLC4 bicarbonate transport family that includes NBCe1-A. Studies of AE1 have shown that deletion of 9 amino acids (Ala-400—Ala-408) at the putative boundary of cytosolic domain and TM1 misfolds AE1 protein (12), resulting in inactive anion transport (13). The importance of TM1 in NBCe1-A is also suggested by two additional considerations. First, sequence alignment reveals all bicarbonate transporting proteins belonging to SLC4 family share a highly conserved ITFGGLLG amino acid stretch at the putative extracellular end of proposed NBCe1-A TM1 (Fig. 1B); second, a single amino acid mutation in TM1 of NBCe1-A (S427L) causes proximal renal tubular acidosis and dramatically decreases the transport activity of the cotransporter (14). Taken together, we hypothesize that the TM1 region of NBCe1-A may be potentially structurally and functionally important and may be involved in forming the substrate translocation pathway.

In this study we demonstrate for the first time that TM1 of NBCe1-A participates in forming the ion translocation pore. Furthermore, we have identified the residues in TM1 lining the ion translocation pore by cysteine-scanning mutagenesis coupled with methanethiosulfonate (MTS) reagents. Our findings show that NBCe1-A TM1 has one side facing the substrate translocation pathway, and the highly conserved FGGLLG amino acid stretch resides in an extracellular medium accessible small cavity. A preliminary version of this work has been published as an abstract (15).

**EXPERIMENTAL PROCEDURES**

**Materials**—Site-directed mutagenesis kits were from Stratagene. Biotin maleimide, BCECF-AM, Dulbecco’s modified Eagle’s medium, and all cell culture reagents were from Invitrogen. MTSEA, MTSET, MTSES, and MTS-TAMRA were from Toronto Research Chemicals Inc. Protein A-Sepharose, streptavidin/biotinylated-horseradish peroxidase complex and goat anti-sheep IgG-conjugated horseradish peroxidase were from GE Healthcare. IGEPAL, polyvinylsine, and nigericin were from Sigma. Polyvinylidene difluoride membrane was from Millipore.

**Site-directed Mutagenesis**—The human kidney variant of NBCe1-A cDNA and human AE1 cDNA cloned in pcDNA3.1 (+) expression vector (from Invitrogen) were used as the template for site-directed mutagenesis. Twenty-five cysteine codons were individually introduced into NBCe1-A cDNA at the position of amino acid Gln-424 to Gly-448, and one cysteine codon was introduced into human AE1 cDNA at the position of amino acid Thr-422. Mutagenesis was performed using Stratagene site-directed mutagenesis kit following the manufacturer’s instruction. The complete cDNA sequence of each mutant was verified by DNA sequencing.

**Protein Expression**—Mutant NBCe1-A proteins were expressed by transient transfection of human embryonic kidney 293 cells (HEK 293) using Lipofectamine 2000. Briefly, HEK 293 cells were plated onto 60-mm dishes in 4 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 200 mg/liter l-glutamine and penicillin-streptomycin. 16 h post-seeding, cells were transfected with mutant plasmids following the manufacturer’s instruction with a modification in that the transfection mixture was removed after 2 h of exposure. Cells were grown at 37 °C in a 5% CO₂ atmosphere and harvested 24–48 h post-transfection.

**Immunocytochemistry**—24 h post-transfection cells were rinsed with PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) and incubated with a rabbit anti-human NBCe1-A antibody (Ab-162, 1:100 dilutions in PBS) that specifically recognize the extracellular loop 3 of NBCe1-A protein. After 15 min of incubation at room temperature (RT), cells were rinsed with PBS and further incubated with goat anti-rabbit IgG conjugated with Cy3 (1:500 dilution in PBS, from Jackson ImmunoResearch) for 30 min at RT. Cells were then rinsed 3 times with PBS and mounted in Crystal/Mount (from Biomedica, Foster City, CA). In some experiments transfected cells were first permeabilized with 1 ml of ice-cold methanol for 2 min, and then the experiment proceeded as described above. Fluorescence images were acquired by a PXL charge-coupled device camera (model CH1; Photometrics) coupled to a Nikon Microphot-FXA epifluorescence microscope.

**Functional Transport Assay of NBCe1-A**—HEK 293 cells grown on coated coverslips were transfected with various NBCe1-A mutant cDNAs using Lipofectamine 2000. 24 h post transfection cells were loaded with fluorescent pH probe BCECF-AM and assayed using a microfluorometer (16). Cells were initially bathed in HEPES-buffered Na⁺-free solution (140 mM tetramethyl ammonium chloride, 2.5 mM K₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 5 mM HEPES, pH 7.4). After 25 min of equilibration, intracellular pH values were acutely acidified by exposing cells to HCO₃⁻-buffered Na⁺-free solution (115 mM NaCl, 2.5 mM K₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 25 mM tetramethyl ammonium-HCO₃, pH 7.4). Cells were then exposed to a HCO₃⁻-buffered Na⁺-containing solution (115 mM NaCl, 2.5 mM K₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 25 mM NaHCO₃, pH 7.4). 5′-(N-ethyl-N-isopropyl) amiloride (5 μM) was included in all assay solutions to block endogenous Na⁺-H⁺ exchange activity. At the end of each experiment, pHᵢ was calibrated by monitoring the 500/440-nm fluorescence excitation ratio using high-K⁺ nigericin. The rate of pHᵢ recovery, dpHᵢ/dt, was calculated in the initial 15 s after exposure to Na⁺. In HCO₃⁻-containing solutions, the total cell buffer capacity (βᵢ) was equal to β plus the HCO₃⁻ buffer capacity calculated as 2.3 × [HCO₃⁻]. Equivalent base flux was calculated as dpHᵢ/dt × βᵢ, where dpHᵢ/dt represents the initial rate of change of pHᵢ after exposure to the Na⁺-containing HCO₃⁻-buffered solution. For transport assays with methanethiosulfol
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... (MTS) reagents, cells were exposed to the reagents (1 mM MTSEA for 1 min, 4 mM MTSES/MTSET for 5 min) in the HEPES-buffered Na\(^{+}\)-free solution before the Na\(^{+}\)-induced flux measurements. In some studies transfected cells were preincubated under various ion/buffer conditions before the addition of MTSES. Transport function of mutant proteins were depicted as a percent of wild-type NBCe1-A flux for comparison, and sensitivity of mutants to MTS reagent treatment were depicted as a percentage of activity in the absence of MTS reagents. At least of seven different experiments were performed in each protocol.

Functional Transport Assay of AE1—HEK 293 cells were grown on coated coverslips and transfected with human AE1 wild-type or mutant cDNA as described above. 24 h post-transfection, AE1-mediated transport was measured using BCECF. After BCECF loading in a HEPES-buffered Na\(^{+}\)-containing solution, the cells were initially bathed in a HCO\(_3\)-buffered Na\(^{+}\)- and Cl\(^{-}\)-containing solution (115 mM NaCl, 2.5 mM K\(_2\)HPO\(_4\), 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM glucose, 25 mM tetramethyl ammonium–HCO\(_3\), pH 7.4). After a base-line pH was measured, Cl\(^{-}\) was acutely removed by bathing the cells in a Cl\(^{-}\)-free solution (115 mM sodium gluconate, 2.5 mM K\(_2\)HPO\(_4\), 7 mM calcium gluconate, 2 mM magnesium gluconate, 5 mM glucose, 25 mM NaHCO\(_3\), pH 7.4). 5\(^{-}\)(N-Ethyl-N-isopropyl) amiloride (5 mM) was included in all assay solutions to block endogenous Na\(^{+}\)-H\(^{+}\) exchange activity. Intracellular pH was calibrated as described above. In experiments involving MTSES exposure, the cells were treated with MTSES (4 mM; 5-min exposure) in a HEPES-buffered Na\(^{+}\)- and Cl\(^{-}\)-containing solution before switching to the HCO\(_3\)-buffered solutions. The rate of pH\(_i\) increase, dpH\(_i\)/dt, was calculated in the initial 15 s after removal of extracellular Cl\(^{-}\). Transport function and sensitivity to MTSES were depicted as a percent of wild-type AE1 function. At least of seven different experiments were performed in each protocol.

Biotin Maleimide Labeling Assay and Immunoprecipitation—Whole cell labeling with biotin maleimide proceeded as described previously (5, 17). Briefly, transfected HEK 293 cells were incubated with 0.2 mM biotin maleimide in 1 ml of PBS containing 0.1 mM CaCl\(_2\) and 1 mM MgCl\(_2\), pH 7.0, for 10 min at room temperature. Subsequently, the reaction was stopped by adding a 5-fold molar excess of dithiothreitol, and cells were lysed with 500 μl of IPB buffer (5 mM EDTA, 150 mM NaCl, 1% (v/v) IGEPAL, 0.5% (w/v) sodium deoxycholate, 10 mM Tris-HCl, pH 7.5) containing 0.2% (w/v) bovine serum albumin and protease inhibitors (from Roche Applied Science) on ice for 10 min. Insoluble material was removed by centrifugation at 20,000 × g for 15 min at 4 °C. The supernatant was precleared with preimmune rabbit serum and protein A-Sepharose resin, and then NBCe1-A protein was immunoprecipitated by rabbit anti-human NBCe1-A N terminus polyclonal antibody (18). For detailed protocols, see Zhu et al. (5).

SDS-PAGE and Immunoblotting—Protein samples were resolved on 7.5% polyacrylamide gels and transfected to polyvinylidene difluoride membranes. Biotinylated proteins were detected by incubation of blots with 1:10000 diluted streptavidin–biotinylated horseradish peroxidase (GE Healthcare) in TBST buffer (0.1% (v/v) Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.5, containing 0.5% (w/v) bovine serum albumin. Protein expression level was assessed by probing the blot with anti-NBCe1 C-terminus polyclonal antibody (4) at a 1:3000 dilution in TBST buffer (TBST buffer containing 5% (w/v) nonfat milk). Preliminary experiments for assessing mutant protein expression level were performed as described above.

MTS-TAMRA Labeling Assay—HEK 293 cells expressing mutant NBCe1-A proteins were washed 3 times with PBS and incubated with 100 μM MTS-TAMRA (1:1000 dilution in PBS, pH 7.4) on ice. After 3 min of incubation, cells were washed twice with PBS, and NBCe1-A labeling was analyzed under a microscope (Olympus BH2) at excitation wavelength 545 nm and emission wavelength 590 nm.

Image and Data Analysis—Films from immunoblots and biotinylation blots were scanned with a Hewlett-Packard Scanjet 5590. Scanned images were quantified with UNSCAN-IT gel¹⁰M Version 6.1 software. Biotinylation levels were calculated according to Zhu et al. (5).

Statistical Analysis—Means ± S.E. were calculated with SigmaPlot 10 software. Statistical analysis was performed using SigmaPlot 10 software. Dunnett’s t test was used to assess statistical significance with p < 0.05 considered significant.

RESULTS

Characterization of NBCe1-A-TM1-introduced Cysteine Mutants

Wild-type NBCe1-A Is Functionally Insensitive to Methane-thiosulfonate Reagents—MTSES, MTSET, and MTSEA are small MTS reagents that specifically react with the free sulphydryl group of cysteine, which have been used widely to probe residues lining the substrate translocation pore of membrane channel and transporters (19, 20). If an endogenous or introduced cysteine residue is in the ion translocation pore, covalent modification with any of these reagents will potentially block the substrate pathway and, thus, sterically inactivate the transport activity (21). Wild-type (wt) human NBCe1-A contains 15 endogenous cysteine residues (Fig. 1A). Before utilizing MTS reagents in experiments involving mutant NBCe1-A constructs, we performed control experiments to determine whether these agents alter the function of the wild-type transporter. Three cysteine reactive MTS reagents were utilized in these experiments: MTSEA, MTSES, and MTSET. At the extracellular pH of 7.4 in the transport assay, a significant fraction of MTSEA is unprotonated and membrane-permeable (22), whereas MTSES carries a fixed negative charge, and MTSET carries a fixed positive charge. Both of the latter reagents are membrane-impermeant. After 1 min of incubation with 1 mM MTSEA, we observed no change in wt-NBCe1-A transport activity (Fig. 2B). Similar results were observed with MTSES/MTSET (4 mM; 5 min) treatment (see Fig. 5, A and B). None of the MTS reagents had an effect in mock-transfected cells (data not shown). Therefore, our results show that all three MTS reagents have no effect on wt-NBCe1-A transport activity. These results complement our recent finding using MTSES (7) and experiments performed in the Xenopus oocyte expression system (9).
Construction and Cellular Localization of NBCe1-A TM1-introduced Cysteine Mutants—Given that the three MTS reagents had no effect on transport function of wt-NBCe1-A, 25 cysteine codons were individually introduced into the wt-NBCe1-A cDNA at each position corresponding to amino acid Gln-424 to Gly-448, which covers the putative TM1 and the highly conserved ITFGGLLG stretch (Fig. 1B). Because mutation of structurally important residues in a transmembrane protein may affect protein folding (23), we initially determined whether each of the constructs was processed normally and trafficked to the plasma membrane. To clearly determine the membrane presence of mutant proteins, we developed an anti-NBCe1-A polyclonal antibody against residues Asp-598—His-616 in the extracellular loop 3 of NBCe1-A (Ab-162; Fig. 3A). This antibody recognizes all NBCe1 variants. Ab-162 directly binds to NBCe1-A protein on the plasma membrane without cell permeation and, therefore, only detects the native protein if is localized on the plasma membrane. Fig. 3B, a and b, shows that mock-transfected HEK 293 cells have no Ab-162 membrane staining, whereas cells transfected with wt-NBCe1-A have very strong membrane staining (Fig. 3Bc). Table 1 shows most of the mutant proteins expressed strongly on the plasma membrane, except mutants Q424C, Y433C, N439C, G444C, and L447C. To further determine whether the membrane-unprocessed mutants are expressed, we permeabilized the transfected cells with methanol followed by Ab-162 staining. Results showed the membrane-unprocessed mutants are well expressed but fully arrested intracellularly (Fig. 3B, d–f). Notably, all these residues are conserved among the bicarbonate transporter family except residue Asn-439. Before the immunocytochemistry analysis, the protein expression level of each construct was analyzed by immunoblot with anti-NBCe1-A C-terminal antibody, and no significant difference was observed (data not shown).

Transport Function of NBCe1-A-introduced Cysteine Mutants—Before determining the sensitivity of each mutant to MTS reagents, we examined the base-line transport activity of membrane-processed constructs and compared the functional activity to wt-NBCe1-A. Fig. 4 shows that of 20 assayed cysteine mutants, the majority had greater than 50% that of the wild-type trans-
**Pore-lining Residues of NBCe1-A**

![Image](https://example.com/figure3.png)

**FIGURE 3. Membrane-processing analysis of NBCe1-A-introduced cysteine mutants with Ab-162.** A, immunoblot analysis of Ab-162. HEK 293 cells expressing wt-NBCe1-A were lysed in SDS-PAGE sample buffer without reducing reagents. Samples were resolved on 7.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blot was probed with Ab-162 in TBST at 1:1000 dilution. a, lysate of wt-NBCe1-A-transfected cells shows two bands detected using Ab-162 representing two oligomeric states, dimer and monomer (lane 1), whereas no bands were detected in mock-transfected cell lysate (lane 2). b, peptide-blocking assay of Ab-162. Ab-162 was preincubated with peptide DTLAPEYLPTMSSTDMYH before probing the lysate of wt-NB Ce1-A-transfected cells. A, representative images of Ab-162 staining. HEK 293 cells expressing various NBCe1-A-introduced cysteine mutants were stained with Ab-162 for 15 min at room temperature, and fluorescence images were acquired by a PXL charge-coupled device camera coupled to a Nikon Microphot-FXA epifluorescence microscope. a, mock-transfected cells; b, Nomarski image of the cells in a; c, cells expressing wt-NB Ce1-A; d, cells expressing mutant Q424C; e, Nomarski image of the cells in d; f, cells expressing mutant Q424C stained with Ab-162 after methanol permeation.

**TABLE 1**

**Summary of membrane processing of NBCe1-A introduced cysteine mutants**

Transfected cells were stained with Ab-162 at 1:100 dilution in PBS. +, positive staining; −, negative staining.

| Mutants | Ab-162 staining | Mutants | Ab-162 staining |
|---------|-----------------|---------|-----------------|
| Q424C   | −               | V437C   | +               |
| A425C   | +               | T438C   | +               |
| L426C   | +               | N439C   | −               |
| S427C   | +               | A440C   | +               |
| A428C   | +               | I441C   | +               |
| T429C   | +               | T442C   | +               |
| L430C   | +               | F443C   | +               |
| L431C   | +               | G444C   | −               |
| I432C   | +               | G445C   | +               |
| Y433C   | +               | L446C   | +               |
| L434C   | +               | L447C   | −               |
| A435C   | +               | G448C   | +               |
| T436C   | +               |         |                 |

port activity; 5 had less than 40% that of the control function (A425C, I429C, I432C, T436C, L446C). Of note, mutation of Ala-425 and Ile-432 to cysteine impaired the transport function of wt-NB Ce1-A by ~75%, suggesting they are functionally or structurally important residues. Interestingly, helical wheel analysis shows the functional impaired mutants (A425C, I429C, I432C, T436C) all reside on one helical face (see Fig. 11B), suggesting this face of TM1 is functionally important, probably by interacting with other TMs. Our results showing

**FIGURE 4. Functional characterization of NBCe1-A-introduced cysteine mutants.** Transport function of various NBCe1-A-introduced cysteine mutants was assayed as described under “Experimental Procedures.” Transport data were expressed as a percentage of wt-NB Ce1-A function. The constructs labeled with a black circle were not functionally characterized due to lack of plasma membrane expression. Error bars represent the mean ± S.E. (n = 7–12). *, p < 0.05 Dunnett’s t test.

that mutant S427C retains ~50% transport function complements a previous report where the spontaneous mutation of Ser-427 to nonpolar amino acid (Leu) inactivated NBCe1-A transport function in oocytes, whereas mutation of Ser-427 to polar amino acid, Thr, has no functional effect (14).

**Sensitivity of NBCe1-A-TM1-introduced Cysteine Mutants to MTS Reagents**

To determine whether residues in TM1 of NBCe1-A line the ion translocation pore, we scanned all the 20 membrane-processed-introduced cysteine mutants for the sensitivity to three MTS reagents, MTSES, MTSET, and MTSEA. HEK 293 cells expressing introduced cysteine mutants were preincubated with MTS reagents followed with the transport activity assay. Functional sensitivity of every mutant protein to MTS reagents was calculated as the percentage change in function in comparison to the absence of MTS reagent treatment. Fig. 5, A–C, shows that the transport activity of mutant T442C and A435C are very sensitive to the inhibition of all the three MTS reagents (100% and ~70% inhibition respectively), mutant I441C is highly sensitive to MTSET and MTSEA, and mutant A428C is highly sensitive to MTSEA inhibition. Helical wheel analysis shows that highly functionally sensitive mutants are clustered on one face of TM1 within a 100° arc, except mutant I441C (Fig. 11B). In addition to the MTS reagent-inhibited mutants, some mutants showed increased transport activity after MTS reagent treatment. This effect has been reported in studies of introduced cysteine mutants of both transporters and ion channels (9, 24, 25).

**Topology Determination of the C Terminus of NBCe1-A-TM1**

**Labeling of NBCe1-A-introduced Cysteine Mutants with Biotin Maleimide**—Given that the transport function of mutant NBCe1-A-T442C is completely blocked by all three MTS reagents, our results suggest that Thr-442 resides in an aqueous-accessible environment. A recent N-glycosylation scan-
ning mutagenesis study of human AE1 proposed the C-terminal end of TM1 is at residue Phe-423 (26) and that the GGLLG stretch is part of the first extracellular loop. To define the location of Thr-442 and the stretch of ITFGGLLG in NBCe1-A protein, we assessed the accessibility of introduced cysteine mutants to biotin maleimide (BM), a membrane-permeant sulfhydryl reactive reagent that was used previously for AE1 topology analysis (5). wt-NBCe1-A contains 15 endogenous cysteines, of which 3 are in the N-terminal cytoplasmic domain, 10 are in the transmembrane region, and 2 are in the cytoplasmic C-terminal tail (Fig. 1A). Fig. 6A shows that wt-NBCe1-A is strongly labeled with BM, suggesting there are free aqueous-accessible cysteine residues in the protein. Mutation of the five proposed intracellular cysteines (Cys-120, -389, -399, -992, -1035) to serine (NBCe1-A-5C−) completely abolished BM labeling (Fig. 6A), indicating the remaining endogenous cysteines either reside in the lipid bilayer (non-aqueous environment) or in a folded inaccessible conformation, which will not interfere BM labeling of introduced cysteines. In transport experiments, the NBCe1-A-5C− protein was fully functional (Fig. 4).

We individually introduced 14 cysteine codons into NBCe1-A-5C− cDNA at amino acid positions between Pro-439 and Asp-452. Whole cell labeling of intact HEK 293 cells expressing introduced cysteine mutant NBCe1-A protein was performed with BM at 48 h expression. A single labeled endogenous cysteine residue NBCe1-A (Cys-1035) and NBCe1-A-5C− construct were included in every labeling experiment as positive and negative labeling control. Fig. 6A shows NBCe1-A-Cys-1035 is strongly labeled with BM, whereas the negative control NBCe1-A-5C− construct is barely labeled.

The biotin signal of each introduced cysteine mutant was quantified by densitometry of BM blot and the corresponding immunoblot. NBCe1-A appears as two bands on the immunoblot that represent the glycosylated (higher molecular weight band) and unglycosylated (lower molecular weight band) form of NBCe1-A protein (Fig. 6A). Although the glycosylated form of NBCe1-A is the major biotinylated band on the BM blot, the unglycosylated form of wt-NBCe1-A and NBCe1-A-Cys-1035 is also significantly labeled. Because endoplasmic reticulum (ER)-retained membrane protein is reported to be minimally labeled by BM (5), our data suggest that the biotinylated unglycosylated form of NBCe1-A resides in a compartment other than ER. To simplify the calculation, we quantified the major biotinylated band of each mutant (glycosylated form), except mutant G444C, whose major BM-labeled band is the unglycosylated form. Data were normalized to mutant Cys-1035, which was served as an internal standard and positive control in each experiment. Fig. 6B shows mutants N439C to T442C were not labeled, whereas mutants F443C, G444C, L447C, G448C, A450C, and T451C were significantly labeled with BM. Four mutants (G445C, L446C, D449C, and D452C) were not biotinylated, yet reside in the region with residues that were otherwise labeled. The labeling pattern suggests this region has 3-fold periodicity (Fig. 6C).

Labeling of NBCe1-A-introduced Cysteines with MTS-TAMRA—Although six introduced cysteine mutants were observed significantly labeled with BM, the intensity of labeling is rather weak; that is, less than 50% that of the positive control. Therefore, we decided to further define the accessibility of these mutants to a membrane-impermeant sulfhydryl-specific chemical MTS-TAMRA. MTS-TAMRA is a membrane-impermeable chemical carrying tetramethylrhodamine group that can be localized optically (fluorescence excitation 545 nm/emission 590 nm). Fig. 7, C–E shows the mock-transfected and wt-NBCe1-A and L430C (negative control)-transfected cells had minimal surface labeling with MTS-TAMRA, whereas cells transfected with T442C, F443C, G444C, G448C, A450C, and T451C were significantly labeled with BM. Four mutants (G445C, L446C, D449C, and D452C) were not biotinylated, yet reside in the region with residues that were otherwise labeled. The labeling pattern suggests this region has 3-fold periodicity (Fig. 6C).
struct is accessible to MTS reagents but not available for BM labeling, we propose that residue Thr-442 resides at the C-terminal end of NBCe1-A-TM1, facing the ion translocation pore and that extracellular loop 1 starts from residue Phe-443.

**Functional and Structural Insight of Residue Thr-442 in NBCe1-A**

The full inhibition of mutant T442C transport activity by three MTS reagents implicated that it resides in a functional or structural important region. To determine whether Thr-442 is involved in the significant conformational changes during transport cycle, we probed mutant T442C with MTS-TAMRA at various conditions: (a) HCO₃⁻-buffered Na⁺-containing solution, (b) HCO₃⁻-buffered Na⁺-free solution, (c) HEPES-buffered Na⁺-containing solution, and (d) HEPES-buffered Na⁺-free solution. Fig. 8A, a–d, shows T442C is highly accessible to MTS-TAMRA irrespective to the absence or presence of Na⁺ in either HCO₃⁻-buffered or HEPES-buffered solution. We further probed mutant T442C with MTSES in functional assays under the same conditions. Fig. 8B shows MTSES can access T442C and produce an equal inhibitory effect under different substrate conditions. These observations suggest NBCe1-A-Thr-442 is not involved in the conformational changes that will shield its exposure to an aqueous environment.

Helical dipoles play a critical role in ion channel and transporter proteins, as suggested by results obtained from crystallized potassium and chloride channels (27, 28) as well as biochemical analysis of AE1 (20). Our observation that Thr-442 resides at the extracellular end of TM1 prompted us to investigate the functional importance of Thr-442, as mutant T442C is functional and fully inhibited by MTS reagents. According to helical dipole theory, the C terminus of a transmembrane helix carries a partial negative charge, and the N terminus carries a partial positive charge (27). Therefore, mutation of NBCe1-A-Thr-442 to cysteine is expected to retain normal transport function, as both amino acids have uncharged polar side chains. To determine whether a δ⁺ charge helical dipole at the C-terminal end of TM1 is critical for NBCe1-A transport function, we individually mutated Thr-442 to amino acids with polar small (Ser) or bulky side chains (Gln), nonpolar small (Ala) or bulky side chains (Trp), and polar with negative charge (Asp) or positive charge side chains (Arg). Transport function of mutant proteins was assessed. Fig. 9 shows that mutation of Thr-442 to the polar residue Ser had no functional effect, but substitution with Gln impaired transport function by...
Moreover, substitution of Thr-422 with nonpolar residue Ala minimally affected the transport function, but substitution with Trp significantly decreased the transport function to ~50% of wt-NBCe1-A. However, mutation either to negative (Asp) or positive (Arg) charged residues decreased transport function to 20–30% of wt-NBCe1-A. These results suggest the charge helical dipole at the C-terminal end of TM1 plays a critical role in maintaining the normal NBCe1-A transport function, and the local steric constraint restricts the size of a given residue.

Orientation of Residue Thr-422 in AE1-TM1

Our results suggested that the pathway for ion transport through NBCe1-A is in part composed of specific residues in TM1 that are in contact with the aqueous environment. To determine whether our findings can be generalized to other members of the SLC4 family, we examined the role of the NBCe1-A-Thr-442 corresponding residue in anion exchanger AE1 (Thr-422). We substituted AE1-T422 with cysteine and assessed its sensitivity to MTS reagents. Transport function was measured before and after treatment with MTSES. In contrast to NBCe1-A-T442C, there was no effect of MTSES reagents on the transport function of AE1-T422C, suggesting Thr-422 does not line a water-filled pathway in AE1 (Fig. 10A, a–f). In addition, MTS-TAMRA failed to label AE1-T422C, suggesting that the pathway for ion transport through NBCe1-A is in part composed of specific residues in TM1 that are in contact with the aqueous environment.
unlike NBCe1-A-T442C (Fig. 10, a and b). Given the high homology of TM1 residues in both transporters, the results suggest that the orientation of TM1 in relation to other transmembrane segments differs between the two transporters and that AE1-T422 is not exposed to the aqueous environment.

**DISCUSSION**

NBCe1 electrogenerically co-transport Na\(^+\) and HCO\(_3^-\) across plasma membranes; however, limited knowledge is available regarding the structural basis for NBCe1 transport. In the present study we examined amino acids Gln-424–Gly-448 in the TM1 region of human NBCe1-A to identify potential residues lining the ion translocation pore by analyzing the functional sensitivity of NBCe1-A-introduced cysteine mutants to MTS reagents. By using the well characterized cysteine accessibility method, we demonstrated that the TM1 region of NBCe1-A forms part of the ion translocation pathway, and furthermore, the highly conserved FGGLLG sequence in SLC4 proteins is located in a small aqueous-accessible cavity in NBCe1-A. Our results complement the recent crystallization of LeuT (11) and vibrio parahaemolyticus sodium/galactose symporter (29), which sheds light on how sodium coordinates with substrate in a sodium-dependent cotransporter protein and the possible transport mechanism involved.

We have identified two introduced cysteine mutants (A435C and T442C) in the TM1 region of human NBCe1-A that are highly inhibited by three MTS reagents, MTSES, MTSET, and MTSEA. Furthermore, I441C was significantly inhibited by MTSET and MTSEA and A428C by MTSEA alone. The locations of these residues have a helical periodicity, and all reside on one face of a predicted \(\alpha\)-helix except I441C (Fig. 11). The magnitude of inhibition of T442C and A435C by MTSES, MTSET, and MTSEA was 100 and \(\sim\)70%, respec-
tively, irrespective of the charges carried with the reagents. These findings suggest that Thr-442 and Ala-435 are in an environment that does not expel charged chemicals that differs from AE1, which has a filter exclusively selective for anions over cations (20). The basis for inhibition by these reagents is their ability to permeate aqueous regions, to form a covalent adduct, and to sterically block the confined space of the ion translocation pathway (21). The high sensitivity of T442C to MTS reagent blockage irrespective of their net charge properties is compatible with three possible locations for the Thr-442 residue in NBCe1-A TM1; that is, 1) one of the ion coordination sites in TM1, 2) a position in TM1 that is critical for conformational changes during transport cycle, and 3) a very narrow region of a pore structure involving TM1. The first possibility is unlikely as the inhibitory effect of MTS reagents on the T442C construct is not affected by the presence of Na\(^{+}\) or HCO\(_3\)\(^{-}\) ion. The lack of a detectable conformational change of T442C probed with either MTS-TAMRA or MTSES under different conditions also ruled out the second possibility. We speculate Thr-442 may reside at the entry of the ion translocation channel, a very narrow region similar to a “gate,” and thus, attachment of a small chemical moiety could completely block the ion movement. This would explain the observation that Ala-435 has less inhibition to MTS reagents, although it resides deeper into the lipid bilayer. This hypothesis is further supported by the observation that Thr-442 resides at the C-terminal end of TM1 and that alteration of either the side chain size or charge of the residue at this position dramatically affects the transport function of NBCe1-A, indicating the \(\delta^-\) charge helical dipole involving Thr-442 contributes to the formation of the gate. MTS reagents have a size of 6 Å in diameter, and 10 Å in length (21). The full blockage of NBCe1-A transport function by MTS reagents suggests the maximal gate size at open conformation is less than 10 Å. This hypothesis is supported by the observation that substitution of Thr-442 with either polar (Cys, Ser) or nonpolar (Ala) amino acids that have similar or smaller size side chains (<2.5 Å) had minimal functional effect. However, substitution with Gln or Trp, whose side chains are twice as large (>5 Å) as that of Thr, decreased transport function to 50% that of wt-NBCe1-A. Indeed, the crystal structure of the Na\(^{+}\)-dependent LeuT demonstrates that an arginine close to the extracellular end of TM1 forms a salt bridge with an aspartic acid, thereby defining the extracellular gate of the substrate channel (11).

The I441C construct was inhibited by MTSET and MTSEA by more than 50% but insensitive to MTSES. Ile-441 may have two possible locations, 1) lining the ion translocation pore (but a local environment is unfavorable for MTSES access) and 2) aqueous-accessible but not lining the ion translocation pore. On the helical wheel plot, Ile-441 resides at the C-terminal end of TM1 starting from residue Thr-442 and the sequence proceeds clockwise.
Pore-lining Residues of NBCe1-A

A428C transport function suggests that a substrate binding site similar to LeuT exists in NBCe1-A, which is selective for Na⁺ and HCO₃⁻. On the helical wheel plot, A428C is located within a 100° arc from T424C and A435C, suggesting it lines the ion translocation pore facing the intracellular milieu.

Our observation that the highly conserved FGGLLG sequence is not highly accessible to BM but to MTS-TAMRA labeling indicates this stretch of residues lies in a small aqueous-accessible cavity. Biotin maleimide is a bulky reagent with a size of ~22 Å; it covalently reacts with cysteine to introduce a biotin group. MTS-TAMRA is composed of a cysteine-reactive MTS group and rhodamine linked with biotin group. MTS-TAMRA is composed of a cysteine-reactive MTS group and rhodamine linked with biotin group. The differences in labeling can be explained by the local steric hindrance that limits the accessibility of bulky chemicals to these residues.

It is known that certain mutations in a multitransmembrane segment protein can cause protein misfolding and prevent processing to the plasma membrane. In this study we observed five mutations in TM1 causing NBCe1-A intracellular retention, of which two reside in the highly conserved ITPFGGLLG region. The 3-fold periodicity of biotinylation in this region suggests it may have an extended β-strand structure. In human AE1, TM2 and TM3 are initially expressed in the endoplasmic reticulum lumen and then fold back into the protein complex at a later stage of maturation (26). We speculate that this highly conserved region may act as a hinge for the proper folding of TMs during protein maturation.

Mutation of Ser-427 to Leu inactivates NBCe1-A transport function and causes proximal renal tubular acidosis (14). Here, we observed that mutation of Ser-427 to Cys retains 50% NBCe1-A transport activity. Our finding is consistent with the observation the mutation of Ser-427 to Thr does not affect NBCe1-A transport function (14). It is likely that the polarity of the residue at this position is important for cotransporter function. Interestingly, the corresponding residue of NBCe1-A Gln-424 in human AE1 (Gln-404) lies in the missing stretch of residues (Ala-400—Ala-408) causing Southeast Asian ovalocytosis (30). Our data suggest that missing residue Gln-404 in AE1 may contribute significantly to the cause of Southeast Asian ovalocytosis.

The structural differences among the SLC4 transporters that account for their varying ion transport are currently unknown. Our results are the first experimental evidence suggesting a structural difference between the two members of the SLC4 family, the sodium-dependent bicarbonate transporter NBCe1-A and sodium-independent chloride bicarbonate exchanger AE1. The insensitivity of T422C in human AE1 to MTSEA inhibition suggests that AE1 has a different folding structure compared with NBCe1-A. Although residues in NBCe1-A TM8 were previously reported to line the ion translocation pore as in AE1, the accessibility pattern of NBCe1-A TM8 cysteine mutants to MTSEA differed from AE1 (9, 31). Further studies are needed to determine the orientation and potential interaction between TM1 and TM8 in NBCe1-A.

The anion translocation pore of AE1 has been intensively studied and is currently viewed as an hourglass-shape structure with open pores on both side of the membrane leading to a narrow substrate translocation site in the middle of the lipid bilayer (20, 31). Our data show that the smallest of the three MTS reagents, MTSEA, could completely block the ion translocation in NBCe1-A, whereas the inhibitory effect on AE1 pore-lining mutants was maximally about 40% (20, 31). These differences complement our findings wherein NBCe1-A has an ion translocation pathway significantly different from AE1, potentially more closely resembling the LeuT Na⁺/Cl⁻-dependent leucine transporter.

In summary, we have identified three residues on one helical face of TM1 lining the ion translocation pathway of NBCe1-A. Furthermore, residue Thr-442 marks the C-terminal end of TM1 and is involved in forming a very narrow region for the ion entrance into the substrate translocation pore. The highly conserved FGGLLG sequence resides in a small aqueous-accessible cavity on the extracellular surface of NBCe1-A protein. Our results suggest that the structure of the NBCe1-A ion translocation pathway of NBCe1-A is significantly different from that of AE1.

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