Topological Location and Structural Importance of the NBCe1-A Residues Mutated in Proximal Renal Tubular Acidosis*

Quansheng Zhu, Liyo Kao, Rustam Azimov, Debra Newman, Weixin Liu, Alexander Pushkin, Natalia Abuladze, and Ira Kurtz

From the Division of Nephrology, David Geffen School of Medicine, UCLA, Los Angeles, California 90095

NBCe1-A electrogenically cotransports Na\(^+\) and HCO\(_3^-\) across the basolateral membrane of renal proximal tubule cells. Eight missense mutations and 3 nonsense mutations in NBCe1-A cause severe proximal renal tubular acidosis (pRTA). In this study, the topologic properties and structural importance of the 8 endogenous residues mutated in pRTA and the in situ topology of NBCe1-A were examined by the substituted cysteine accessibility method. Of the 55 analyzed individually introduced cysteines, 8 were labeled with both membrane permeant (biotin maleimide (BM)) and impermeant (2-((5(6)-tetramethylrhodamine)carboxylamino)ethyl methanethiosulfonate (MTS-TAMRA)) sulfhydryl reagents, 4 with only BM, and 3 with only MTS-TAMRA. The location of the labeled and unlabeled introduced cysteines clearly indicates that the transmembrane region of NBCe1-A contains 14 transmembrane segments (TMs). In this in situ based NBCe1-A topology, residues mutated in pRTA (pRTA residues) are assigned as: Ser427, TM1; Thr442 and Gly486, TM3; Arg510 and Leu522, TM4; Ala799, TM10; and Arg881, TM12. Substitution of pRTA residues with cysteines impaired the membrane trafficking of R510C and R881C, the remaining membrane-processed constructs had various impaired transport function. Surprisingly, none of the membrane-processed constructs was accessible to labeling with BM and MTS-TAMRA, nor were they functionally sensitive to the inhibition by (2-aminoethyl)methanethiosulfonate. Functional analysis of Thr442 and with different amino acid substitutions indicated it resides in a unique region important for NBCe1-A function. Our findings demonstrate that the pRTA residues in NBCe1-A are buried in the protein complex/lipid bilayer where they perform important structural roles.

The electrogenic Na\(^+\)-HCO\(_3^-\) cotransporter 1 (NBCe1-A)\(^2\) is responsible for reabsorbing 60–80% of the filtered HCO\(_3^-\) load in the mammalian kidney. NBCe1-A belongs to the SLC4 HCO\(_3^-\) cotransporter family that contains several HCO\(_3^-\)-coupled transporters including Na\(^+\)-independent Cl\(^-\)/HCO\(_3^-\) exchangers, Na\(^+-\)HCO\(_3^-\) cotransporters (electrogenic and electroneutral), and a Na\(^+\) -driven Cl\(^-\)/HCO\(_3^-\) exchanger (1–3). Additionally, the SLC4 family contains a transporter, SLC4A4.1 (NaBC1), which has minimal sequence homology to the other SLC4 proteins and is purported to transport Na\(^+\) and borate electrogenically. NBCe1 is encoded by the SLC4A4 gene and has 3 variants. NBCe1-A is expressed in the kidney and eye, NBCe1-B is expressed in pancreas, duodenum, colon, and several other tissues, and NBCe1-C 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 C-terminal amino acids (1–3).

In the kidney, NBCe1-A is expressed on the basolateral membrane of the proximal tubule cells where it mediates the Na\(^+\)-coupled efflux of HCO\(_3^-\) from cells to blood. NBCe1-A exists as a membrane-embedded 140-kDa homodimer, of which each monomer is fully functional (4). The proposed transmembrane segments (TMs) 1 and 8 were recently analyzed by cysteine scanning mutagenesis and several residues were found to be involved in forming the ion translocation pore (5, 6). In particular, residue Thr442 in TM 1 was proposed to form an external gate for the transported substrates (5). Interestingly, substitution of Asp555 to glutamic acid in the proposed TM 5 of NBCe1-A induced an outward rectifying Cl\(^-\) current and altered the transport substrate selectivity, indicating it plays an important role in HCO\(_3^-\) selectivity (7). Notably, this residue is in close proximity of the proposed DIDS (a functional inhibitor) binding site of NBCe1-A (Lys\(^{559}\)) (8).

Eight missense mutations (R298S, S427L, T485S, G486R, R510H, L522P, A799V, and R881C), and 3 nonsense mutations (Q29X, W516X, and a frameshift deletion at nucleotide 2311A) in NBCe1-A have thus far been identified that cause autosomal recessive proximal renal tubular acidosis (pRTA) with ocular and intracerebral abnormalities (9–13). The disease causing mechanism of the nonsense mutations is due to absence of the full-length NBCe1 protein, whereas the missense mutations result from either reduced transport function or impaired plasma membrane processing (R510H, L522P, A799V, and R881C) (11, 12, 14). In general, mutant membrane transporter proteins are thought to have reduced function as a result of three possible mechanisms that are not mutually exclusive: 1) misfolding of the transporter protein; 2) alteration of the substrate translocation pathway; and 3) impairment of the transporter conformation (static/dynamic) required for substrate translocation. Interestingly, one of the pRTA causing
mutations, S427L, is located adjacent to Ala428 in TM 1; an identified pore lining residue in NBCe1-A (5), suggesting that this and other missense mutations may potentially alter the structure of the substrate translocation pore. Indeed, a recent study also reported that Arg298 may be involved in constructing a “HCO3⁻/Htunnel” in NBCe1-A and that mutation R298S disrupts the local structure of the ion permeation pathway thereby impairing the HCO3⁻ entrance (15).

The gross functional and targeting defects caused by NBCe1-A pRTA missense mutations have been previously analyzed in Xenopus oocyte (10–12, 14), ECV304 (9, 11, 12), and Madin-Darby canine kidney cells (14, 16). However, a systematic analysis of the potential molecular and structural and topological significance of the wild-type residues involved in causing pRTA (pRTA residues) is lacking, in part due to the presence of two NBCe1-A topology models that are distinctly different and have their respective limitations. One model is derived from in vitro glycosylation studies (Fig. 1) (17) and the other is based on topology of anion exchanger 1 (AE1) (3, 18). Importantly, the model based on in vitro data may not represent the actual folding of the protein in the cellular environment, and AE1 topology may not represent NBCe1-A folding as well. To understand the molecular mechanisms whereby NBCe1-A missense mutations cause pRTA, a deeper understanding of the topological properties of the transporter is essential.

In this study, we analyzed the potential structural mechanism(s) involved in the functional impairment of NBCe1-A in the context of known pRTA missense mutations by the substituted cysteine accessibility method. To address this question in detail, we determined the topology of NBCe1-A in the native cellular environment by analyzing the accessibility of 55 introduced cysteines to cysteine-specific reagents (in situ topology). On the basis of results, we propose a new NBCe1-A topology that differs from the models either based on in vitro glycosylation or on the sequence homology with AE1. Our results provide novel insights into the mechanistic abnormalities involving the NBCe1-A residues mutated in pRTA.

**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 and MTS-TAMRA were from Toronto Research Chemicals Inc. Sulfo-NHS-SS-biotin and streptavidin-agarose resins were from Pierce. Protein A-Sepharose, streptavidin/biotinylated-horseradish peroxidase complex, and goat anti-sheep IgG-conjugated horseradish peroxidase were from GE Healthcare. Igepal, polylysine, and nigericin were from Sigma. Polyvinylidene difluoride membrane was from Millipore.

**Site-directed Mutagenesis**—A wild-type and a modified human NBCe1-A construct with 5 endogenous cysteines substituted with serines (NBCe1-A-5C⁻) were used as the templates for site-directed mutagenesis. 63 amino acids throughout the transmembrane region of NBCe1-A were individually substituted with cysteine in the NBCe1-A-5C⁻ construct including the 8 residues involved in pRTA missense mutations. Mutagenesis was performed using the Stratagene site-directed mutagenesis kit following the manufacturer’s instructions. The
**pRTA Causing Mutations in NBCe1-A**

**Complete cDNA sequence of each mutant was verified by DNA sequencing.**

**Protein Expression**—Mutant NBCe1-A proteins were transiently expressed in human embryonic kidney 293 cells (HEK 293) using Lipofectamine 2000 transfection. 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 of l-glutamine and penicillin-streptomycin. 16 h post-seeding, cells were transfected with mutant plasmids following the manufacturer’s instruction with modification, that the transfection mixture was removed after a 2-h exposure. Cells were grown at 37°C in a 5% CO2 atmosphere and harvested 24 to 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 interacts with an epitope in putative extracellular loop 3 (5). After a 15-min incubation at room temperature, 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 room temperature. Cells were then rinsed three times with PBS and mounted in Crystal/Mount (from Biomeda, Foster City, CA). In some experiments, transfected cells were permeabilized with 1 ml of ice-cold methanol for 2 min prior to immunostaining. Fluorescence images were acquired by a PXL charge-coupled device camera (model CH1; Photometrics) coupled to a Nikon Microphot-FXA epifluorescence microscope.

**Sulfo-NHS-SS-biotin Surface Labeling**—Whole cell labeling with sulfo-NHS-SS-biotin was performed following the manufacturer’s instruction (Pierce). Briefly, transfected HEK 293 cells (60-mm plates) were washed 3 times with ice-cold PBS (pH 8.0) and incubated with 0.8 mM sulfo-NHS-SS-biotin in 2 ml of PBS (pH 8.0) for 30 min at 4°C. Reaction was stopped by adding 2 ml of 50 mM Tris buffer (containing 140 mM NaCl, pH 8.0) and further incubated for 10 min at 4°C. Cells were then collected, washed 3 times with PBS (pH 8.0), and lysed in 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 protease inhibitors (from Roche) on ice for 10 min. After a 10-min centrifugation at 20,000 × g for 15 min at 4°C, the supernatant was collected and incubated with 50 μl of streptavidin-agarose resins on a rotating shaker for 4 h at 4°C. Resins were then collected by brief centrifugation at 6,000 × g and washed 3 times with IPB buffer. The bond proteins were eluted with 2× SDS sample buffer containing 2% β-mercaptoethanol heated at 60°C for 5 min.

**Functional Transport Assay of NBCe1-A**—HEK 293 cells grown on coated coverslips were transfected with various NBCe1-A mutant constructs using Lipofectamine 2000. 24 h post-transfection, cells were loaded with fluorescent pH probe BCECF-AM and assayed using a microfluorometer (19). Cells were initially bathed in HEPES-buffered Na+-free solution (140 mM TMACl, 2.5 mM K2HPO4, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 5 mM HEPES, pH 7.4). After 25 min equilibration, intracellular pH were acutely acidified by exposing cells to HCO3−-buffered Na+-free solution (115 mM TMACl, 2.5 mM K2HPO4, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 25 mM 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)amilorida (30 μM) was included in all assay solutions to block endogenous Na+-H+ exchange activity. At the end of each experiment, pH was calibrated by monitoring the 500/440-nm fluorescence excitation ratio using high-K+ nigericin. The rate of pH recovery, dpH/dt, was calculated in the initial 15 s following exposure to Na+. For transport assays with the methanethiosulfonate reagent MTSEA, cells were exposed to MTSEA (1 mM for 1 min) in a HEPES-buffered Na+-free solution prior to the Na+-induced flux measurements. Transport function of mutant proteins were depicted as a percent of NBCe1-A-5C− flux for comparison, and sensitivity of mutants to MTSEA treatment were depicted as percentage of activity in the absence of MTSEA. At least seven different experiments were performed in each protocol.

**Biotin Maleimide Labeling and Immunoprecipitation**—Whole cell labeling with biotin maleimide was proceeded as described previously (20, 21). Briefly, transfected HEK 293 cells were incubated with 0.2 mM biotin maleimide in 1 ml of PBS/CM (PBS containing 0.1 mM CaCl2 and 1 mM MgCl2, pH 7.0) for 10 min at room temperature. Subsequently, reaction was stopped by adding 5-fold molar excess of dithiothreitol and cells were lysed with 500 μl of IPB buffer 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 pre-cleared with preimmune rabbit serum and protein A-Sepharose resin, and then NBCe1-A protein was immunoprecipitated by a rabbit anti-human NBCe1-A N terminus polyclonal antibody (22). For detailed protocols see Ref. 21.

**SDS-PAGE and Immunoblotting**—Protein samples were resolved on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Biotinylated proteins were detected by incubation of blots with 1:10,000 diluted streptavidin-biotinylated horseradish peroxidase (GE Healthcare) in TBST buffer (TBST buffer containing 0.1% (v/v) Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.5), containing 0.5% (w/v) bovine serum albumin). The protein expression level was assessed by probing the blot with anti-NBCe1 C terminus polyclonal antibody (17) at 1:3,000 dilutions in TBSTB buffer (TBST buffer containing 5% (w/v) nonfat milk).

**Membrane Isolation and Na2CO3 Treatment**—Membrane treatment with Na2CO3 was performed as previously described (23). 48 h post-transfection, cells (10-cm plates) were collected and washed once with TBS buffer (140 mM NaCl, 10 mM Tris, pH 7.4). Following a 30-min incubation in homogenization buffer (10 mM Tris, 5 mM EDTA, pH 7.4 with Roche protease inhibitors) on ice, cells were lysed by Dounce homogenization. Cell debris were removed by low-speed centrifugation (4,000 × g, 5 min, 4°C) and the membrane fractions were collected by high speed centrifugation (35,000 × g, 30 min, 4°C). Membrane pellets were first re-suspended in 100 μl of 0.3 M sucrose, and then mixed with 2 ml of ice-cold 100 mM Na2CO3 (pH 11.5) and incubated for 30 min on a rotating shaker at 4°C. Membranes
were collected by high speed centrifugation, washed twice with PBSCM, and then re-suspended in 1.0 ml of PBSCM followed by biotin maleimide labeling.

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 a 3-min incubation, cells were washed twice with PBS and subsequently subjected to analysis under a microscope (Olympus BH2) at an excitation wavelength of 545 nm and emission wavelength of 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 UN-SCAN-IT™ gel version 6.1 software. Biotinylation levels were calculated according to Ref. 20.

Statistical Analysis—Mean ± 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

NBCe1-A pRTA Residues and the Substrate Translocation Pore

Cellular Localization of Cysteine-substituted NBCe1-A pRTA Residues—The substituted cysteine accessibility method requires the target protein free of endogenous reactive cysteines (21, 24). Replacement of the 5 cytoplasmic cysteines with serines (Cys120, Cys389, Cys399, Cys992, and Cys1035) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is known that 3 pRTA missense mutations (R510H, L522P, and R881C) completely eliminates the endogenous free reactive cysteines in the NBCe1-A protein (NBCe1-A-5C−) (5). This 5 cysteine-less construct is fully functional as previously characterized (5). By using NBCe1-A-5C− as the mutagenesis template, 8 pRTA residues were individually substituted with cysteines. It is know...
greater than 50%, and R486C has 30–40% the transport function of NBCe1-A-5C−.

Sensitivity of Cysteine-substituted NBCe1-A Residues to MTSEA—Although some of the pRTA-missense mutations have been reported to cause intracellular retention or membrane mistargeting in the polarized cells (14, 16), the structural importance of these mutations is unknown. Given the fact that 6 of the cysteine-substituted constructs processed to the plasma membrane and retained significant transport function in HEK 293 cells, we analyzed their sensitivity to the inhibition of a small sulfhydryl reactive reagent, MTSEA. MTSEA is a membrane-permeable reagent that can enter the substrate translocation pore of a transport protein from either the extracellular or intracellular side. The inhibitory mechanism of MTSEA that is caused by it forms a covalent adduct to a reactive cysteine lining the substrate translocation pathway and subsequently sterically blocks ion translocation (21). If the substituted cysteine is in the lipid bilayer or in an aqueous inaccessible conformation, BM will not be able to react and subsequently the protein will not be labeled. Fig. 5A shows NBCe1-A-5C− is minimally labeled with BM, whereas NBCe1-A-C1035 (positive control with only one free reactive cysteine) (5) is strongly labeled. Surprisingly, none of the cysteine-substituted pRTA residues could be labeled with BM, suggesting that none of the pRTA residues line the ion translocation pore.

**NBCe1-A pRTA Residues Are Buried in the Protein Complex/Lipid Bilayer**

Accessibility of Cysteine-substituted NBCe1-A pRTA Residues to Biotin Maleimide and MTS-TAMRA—Currently, the only available experimentally determined topology model of NBCe1-A is from an in vitro glycosylation mapping analysis (17). In this model, the missense mutations causing pRTA, T485S, G486R, R510H, and R881C are located in intracellular surface loops 1 and 4, respectively (Fig. 1A). It is known that the in vitro glycosylation mapping technique identifies the TMs that carry a stop transfer signal. However, the technique may miss the regions having no stop transfer signal that fold back into the protein complex in the later stage of protein folding. To address this difficulty and clearly locate the pRTA residues in NBCe1-A, we performed substituted cysteine accessibility analysis on the 8 cysteine-substituted pRTA residues by examining their accessibility to a membrane permeable cysteine-specific reagent, biotin maleimide (BM).

The cysteine-substituted constructs were transiently expressed in the HEK 293 cells and subjected to whole cell labeling assay with BM. BM is a cysteine-specific membrane permeable reagent that reacts with the free cysteines that are exposed to medium on the surface of a protein. The chemical basis for BM labeling is a covalent reaction of the maleimide group with the ionized sulfhydryl group in a protein via the thio-ether bond (21, 24). If the substituted cysteine is in the lipid bilayer or in an aqueous inaccessible conformation, BM will not be able to react with it and subsequently the protein will not be labeled. Fig. 5A shows NBCe1-A-5C− is minimally labeled with BM, whereas NBCe1-A-C1035 (positive control with only one free reactive cysteine) (5) is strongly labeled. Interestingly, none of the cysteine-substituted pRTA residues could be labeled with BM, suggesting that the residues are not exposed on the surface of the NBCe1-A protein in the cellular environment. To test if the lack of labeling is caused by the residues are in an aqueous inaccessible conformation shielded by peripheral proteins, we isolated the plasma membrane from cultured cells expressing mutant proteins and stripped with 100 mM sodium carbonate, an established method to remove peripheral protein without affecting membrane protein function (23). As shown in Fig. 5B, NBCe1-A-5C− (negative control) remains unlabeled, whereas NBCe1-A-C1035 (positive control) is strongly labeled after
membrane stripping. Again, we did not observe any significantly increased labeling of the cysteine-substituted pRTA residues except R298C (Fig. 5B).

Our previous study showed that MTS-TAMRA, a cysteine-specific chemical with a small reactive group, can reach cysteine residues deeper in the protein complex than BM can label (5). To further confirm that the pRTA residues are not exposed to the aqueous medium, we performed MTS-TAMRA labeling on HEK 293 cells grown on tissue culture plates expressing the mutant NBCe1-A proteins. Fig. 6A shows that the positive control, T442C, is strongly labeled with MTS-TAMRA, whereas the construct with a free reactive intracellular cysteine, NBCe1-A-C1035, and all the pRTA residues could not be labeled (Fig. 6B). These observations clearly demonstrated that the amino acids in NBCe1-A whose mutations cause pRTA are not located on the surface of NBCe1-A, but buried in the protein complex/lipid bilayer.

**Localization of the pRTA Residues in NBCe1-A, in Situ Topology Determination**

Construction and Cellular Localization of NBCe1-A-introduced Cysteine Mutants—Our aforementioned findings indicated that none of the pRTA residues is exposed on the intra- or extracellular surface of NBCe1-A, suggesting the proposed topology models based on in vitro glycosylation mapping and AE1 homology are not accurate. To address this question, we performed a topology analysis of NBCe1-A using the substituted cysteine accessibility method in living cells. The substituted cysteine accessibility method has two major advantages over other topology determination methods: 1) single residue substitution minimally disturbs the structure and function of the target protein, and 2) the target protein is analyzed in its
pRTA Causing Mutations in NBCe1-A

**FIGURE 6. MTS-TAMRA labeling of cysteine-substituted pRTA residues in NBCe1-A.** A, representative images of MTS-TAMRA labeling. HEK 293 cells expressing various NBCe1-A cysteine-substituted constructs were incubated with MTS-TAMRA in PBS and imaged at an excitation wavelength of 545 nm and emission wavelength of 590 nm. Fluorescence images were acquired by a PXL charge-coupled device camera coupled to a Nikon Microphot-FXA epifluorescence microscope. a, cells expressing mutant T442C; b, cells expressing Cys1035; c, cells expressing mutant G486C stained with Ab-162 in intact cells. B, summary of MTS-TAMRA labeling of the cysteine-substituted pRTA residues. Constructs T442C and Cys1035 were used as experimental controls. +, positive labeling; −, negative labeling; N/A, not analyzed.

native environment (21). To determine the topology of the transmembrane region of NBCe1-A, we individually introduced 55 cysteine codons into the NBCe1-A-5C cDNA at positions selectively located in the intra- and extracellular loops of the proposed NBCe1-A topology model based on in vitro glycosylation (Fig. 1A). The region that has been demonstrated to be glycosylated was intentionally excluded because it unambiguously was shown to be an extracellular loop. Membrane processing of all the constructs was assessed by immunocytochemistry with anti-NBCe1-A antibody 162 on intact cells. Of the 55 introduced cysteines, only 3 constructs were not processed to the plasma membrane (Y506C, R538C, and S804C). Further analysis of methanol-permeabilized cells showed that the membrane unprocessed mutants are well expressed but not analyzed.

To determine whether the introduced cysteine mutations were on the extra- or intracellular surface of NBCe1-A, we performed whole cell labeling with a membrane impermeant sulphydryl-specific chemical MTS-TAMRA. MTS-TAMRA carries a sulphydryl reactive MTS group and a highly charged tetramethylrhodamine group that can be localized optically (fluorescence excitation 545 nm/emission 590 nm). Fig. 6A shows that cells expressing T442C can be clearly labeled with MTS-TAMRA, whereas the cells expressing NBCe1-A-C1035 had no surface labeling indicating that only the extracellular facing residues can be labeled with MTS-TAMRA. Of the 55 introduced cysteines, 8 were labeled with both BM and MTS-TAMRA (A450C, T451C, D452C, N503C, E714C, T718C, R722C, and M858C), 4 were only labeled with BM (E814C, A819C, Q823C, and D960C), and 3 were only labeled with MTS-TAMRA (D449C, T926C, and V927C) (Table 2). Labeling of the cysteine mutants with MTS-TAMRA indicates their extracellular localization, whereas lack of labeling indicates their intracellular location. Interestingly, 3 mutants, D449C, T926C, and V927C, are accessible to MTS-TAMRA but not to BM labeling, suggesting they are in a region that restricts access of the bulky BM. On the basis of these results, we propose that NBCe1-A contains 14 TMs in the native cellular environment (Fig. 9).

**Structural Importance of the T485S pRTA Mutation**

Sequence alignment shows that the pRTA residues in NBCe1-A, Arg298, Gly486, Arg510, Ala799, and Arg881 are highly conserved among all the bicarbonate transporters, whereas Ser127, Thr485, and Leu522 are less conserved (Fig. 1B). Of the latter 3 amino acids, Thr485 is particularly interesting. It is conserved among all the Na+/HCO3 cotransporters, but not in the well characterized Na+-independent anion exchangers, where it is replaced with serine. Therefore, it is surprising that substitution of threonine with serine would reduce NBCe1-A transport function to ~50%, considering that the two amino acids only have subtle structural differences: serine lacks a -CH3 group compared with threonine. We hypothesized that the -CH3 group of threonine at this unique amino acid position may have important structural significance, and therefore sub-
stituted it with amino acids with a similar structure that lack the -CH₃ group (Cys) in wild-type NBCe1-A, or have two -CH₃ groups (Val). Fig. 10 shows that cysteine substitution of Thr485 also impaired transport function by 50%, whereas valine substitution only decreased NBCe1-A function to 75% that of the control. The 25% loss of the transport function of T485V likely indicates that the -OH group at this position is also required, therefore we subsequently substituted Thr485 with isoleucine, an amino acid with a -CH₃ group at the corresponding position of the -OH group in threonine. Functional assays show that this substitution impaired NBCe1-A transport activity by 50%.

To further determine the structural requirement of threonine at the amino acid position of 485, we substituted it with alanine, an amino acid that lacks both corresponding -CH₃ and -OH groups in threonine. Fig. 10 shows that this substitution impaired NBCe1-A function to 30% of control. These results indicate that the -CH₃ and -OH groups at position 485 are structurally important for maintenance of the NBCe1-A function.

DISCUSSION

In this study, we determined the topology of NBCe1-A and explored in detail the role of NBCe1-A residues involved in causing pRTA (pRTA residues). We showed for the first time that NBCe1-A is composed of 14 TMs in its native cellular environment. The N-terminal transmembrane region has 8 TMs homologous to AE1, whereas the C-terminal transmembrane region has 6 TMs that significantly differ from AE1. Our results complement previous studies of the N-terminal R298S mutation (15) and suggest that all identified pRTA residues are likely located in the protein complex/lipid bilayer, and importantly, may not line the ion translocation pore. This novel finding provides a new basis for understanding how the seemingly subtle structural alteration of Thr485 to serine mutation causes pRTA. Specifically, both -CH₃ and -OH groups at position 485 appear critical for maintaining the proper conformation of NBCe1-A required for normal transport function.

Our new NBCe1-A topology model localizes pRTA residues Ser⁴²⁷ to TM 1, Thr⁴⁸⁵, Gly⁴⁸⁶ to TM 3, Arg⁵¹⁰ and Leu⁵²² to TM 4, Ala⁷⁹⁹ to TM 10, and Arg⁸⁸¹ to TM 12. The membrane-embedded location of these residues was demonstrated by the observations that none of the cysteine-substituted pRTA residues were accessible to the labeling by either biotin maleimide or MTS-TAMRA, and these residues remain unlabeled even...
after the plasma membrane was stripped with Na₂CO₃ (to remove peripheral proteins). Considering the significant structural change or charge alteration for most of the missense mutations involved in pRTA, it is conceivable that these TM residing mutations would affect protein folding/helix packing in the lipid bilayer, which could lead to significant loss of mutant protein transport function. Other than R510H, L522P, and R881C, which cause protein intracellular retention, the remaining pRTA causing mutations (T845S, G486R, A799V, and S427L) process to the plasma membrane and retain 10–50% transport function (10–12). In this regard, cysteine substitution of the pRTA residues also produced similar cellular and functional effects, except that S427C and L522C traffic to the plasma membrane at a similar level of NBCe1-A-5C and become more than 50% functional. These findings essentially rule out the possibility that the reduced transport function of the membrane-processed pRTA causing mutations is caused by dramatic protein misfolding. One potential disease causing mechanism is that missense pRTA mutations line the translocation pore, thereby impairing ion translocation. However, upon treatment of cysteine-substituted pRTA residues with MTSEA, a cysteine reactive reagent that can sterically block the substrate translocation pore, none of the membrane-processed...
constructs was functionally inhibited. The most likely explanation for these findings is that these residues do not line the NBCe1-A substrate translocation pathway, however, we cannot rule out the possibility that certain residues may be sterically inaccessible to MTSEA.

Unlike the other known pRTA causing mutations, the T485S mutation is particularly interesting based on the following considerations: 1) mutation of Thr485 to serine/cysteine impairs NBCe1-A function by 50%; 2) the function of cysteine-substituted Thr485 is insensitive to MTSEA treatment; 3) serine and threonine both belong to the same amino acid category (nucleophilic) and have the same pK_a; and 4) structurally, serine closely resembles threonine, but only lacks a -CH_3 group. These observations suggest that the -CH_3 group of threonine at position 485 may have a unique role in maintaining the structure of NBCe1-A for normal ion translocation. This hypothesis was confirmed by the functional study of valine substitution that restored NBCe1-A transport activity to 75% that of the wild-type, despite the fact that it is a hydrophobic amino acid. Moreover, functional analysis of Thr485 with isoleucine substitution (impairs 50% transport function) also highlighted the structural requirement of the -OH group at the 485 position. Based on these findings, we propose that Thr485 may reside in a space confined position involving both -CH_3 and -OH chemistry that is critical for maintaining NBCe1-A in a conformation required for normal transport function. Indeed, when both -CH_3 and -OH groups are removed at this particular amino acid position (alanine substitution), the transport function is decreased to 30% of the wild type. We suspect this mechanism may also be applicable to the G486R mutation, which resides adjacent to Thr485.

Residue Arg298 in the cytoplasmic domain of NBCe1-A was previously predicted to reside in an aqueous inaccessible conformation based on homology modeling to the crystallized cytoplasmic domain structure of AE1 (15). Our BM labeling assay of R298C in living cells provides direct support for this prediction. Substitution of Arg298 with cysteine did not impair the mutant protein membrane processing. However, it reduced NBCe1-A transport to ~50% in comparison with the functional reduction caused by the R298S mutation (~25%) (15). Arg298 was proposed to form hydrogen bonds with adjacent residues (15), yet disruption of these putative bonds by cysteine substitution did not expose this residue to BM labeling, indicating it resides deep in the protein complex. However, this residue becomes ~20% BM labeled following Na_2CO_3 treatment suggesting that the N-terminal cytoplasmic domain is partially unfolded by alkalinization.

Mutation of Ser427 to leucine induced a surprising functional effect: unidirectional transport at 10% of wild-type NBCe1-A (10). However, following the substitution of Ser427 with threonine (10), alanine (7), or cysteine (5) NBCe1-A retains substantial transport function. Therefore, Ser427 has been proposed to be involved in helix interaction and leucine mutation may disrupt NBCe1-A "voltage sensing" (10), affect a Na^+ coordination site (10), or alter the local conformation required for normal function (7). In our new topology model, Ser427 is located in TM 1 adjacent to Ala428, a residue that lines the substrate translocation pore (5). Our current and previous studies (5) both showed that S427C is functionally insensitive to the inhibitions by MTSEA, MTSET, and MTSES treatments. These observations suggest that the bulky side chain of leucine may significantly alter the geometry of the ion translocation pore, and thereby impair transport function.

Of the 8 cysteine-substituted NBCe1-A pRTA residues, 2 (R510C and R881C) were not processed to the plasma membrane in HEK 293 cells as revealed by immunocytochemistry. Accordingly, surface labeling with sulfo-NHS-SS-biotin and immunoblot analysis with an anti-NBCe1-A antibody failed to detect any glycosylated forms of R510C and R881C compared with the remaining cysteine-substituted constructs, implying significant misfolding of the mutant proteins. Although R881C was partially expressed on the plasma membrane in the *Xenopus* oocytes and had significant transport activity (14), it was fully retained intracellularly in Madin-Darby canine kidney cells (14). Our new topology model assigns Arg510 and Arg881 to TM 4 and 12, respectively. These residues do not reside in surface or re-entrant loops as previously thought (1, 14). Misfolding of NBCe1-A caused by these two mutations suggests that the arginine at positions 510 and 881 is involved in forming ionic interactions within the TMs to maintain the overall folding of the protein.

Compared with the aforementioned pRTA causing mutations, much less attention has been paid to L522P and A799V. L522P was found not processed to the plasma membrane in *Xenopus* oocytes, ECV 304, and Madin-Darby canine kidney cells (12), whereas in the present study, L522C did not impair membrane processing. This suggests that it is the proline residue rather than the loss of leucine that causes intracellular retention of NBCe1-A. Considering that Arg510 and Leu522 both reside in TM 4, a helix that carries signal anchor and stop transfer sequences (17), and has several residues whose mutation cause protein intracellular retention, TM 4 in NBCe1-A may act as a scaffolding helix that is important for the second stage folding of the transporter. Therefore, it is predictable that a helix disruption mutation (L522P) would significantly misfold the transporter protein. In our new topology model, Ala799 is located close to the extracellular end of TM 10. Although A799V and A799C process to the plasma membrane normally, they impair NBCe1-A transport function more than 70%. Our study shows that Ala799 may not line the ion translocation pore.

NBCe1-A has been proposed to have at least 10 TMs based on *in vitro* glycosylation mapping studies (17), and to have 13 TMs based on the topology of AE1 because of their high amino acid sequence homology (40% in the TM region) (3, 18). Our results demonstrated that the N-terminal transmembrane region of NBCe1-A has 8 TMs that correspond to the first 8 TMs of AE1. The conclusion is based on the observation that 8 of the introduced cysteines in the N-terminal transmembrane region of NBCe1-A are accessible to the extracellular medium, together with the two N-glycosylated residues (Asn597 and Asn617), which unambiguously shows there are 8 TMs in this region.

Q. Zhu, L. Kao, R. Azimov, D. Newman, W. Liu, A. Pushkin, N. Abuladze, and I. Kurtz, unpublished observations.
Topologic analysis in the C-terminal region of NBCe1-A was surprising in several aspects. First, we observed that 3 residues after TM 8 are strongly labeled with BM, but inaccessible to TAMRA labeling, suggesting that they are located in an intracellular loop. Second, with 20 introduced cysteine residues in the region Gly827–Phe950, only 1 residue, Met858, was labeled with BM and TAMRA, indicating it is in a region facing the extracellular medium. Third, none of the 4 selected residues in the corresponding AE1 re-entrant loop are labeled with BM, although the protein sequence in the region is about 42% homologous to AE1. Fourth, 2 introduced cysteines in the predicted last extracellular loop are accessible to TAMRA labeling, but not BM, suggesting this loop is buried deeper in NBCe1-A facing the extracellular medium. Fifth, Asp960 is only weakly labeled with BM, but not BM, suggesting this loop is buried deeper in NBCe1-A facing the extracellular medium. Third, none of the 4 selected residues in the predicted intracellular boundary of the last TM, indicating that the last TM may be longer than predicted. These observations demonstrated that the C-terminal transmembrane region of NBCe1-A has 6 TMs that differ significantly from AE1.

We observed that some residues in the predicted intracellular loops were not accessible to BM labeling in intact cells, which may indicate that these residues are shielded from being labeled due to the interaction of these intracellular loops with the cytoplasmic region of NBCe1-A or other cytoplasmic proteins. Although we cannot rule out the possibility that the secondary structure of the C-terminal region of NBCe1-A might be similar to AE1, the tertiary structure of the two transporters may be different based on the fact that they employ two distinct substrate transport mechanisms: NBCe1-A is a symporter that electrogenically co-transport 1 Na\(^+\) and 3 HCO\(_3\)\(^-\), whereas AE1 is an antiporter that electroneutrally exchanges 1 Cl\(^-\) for 1 HCO\(_3\)\(^-\) (1–3).

In summary, our data show for the first time that NBCe1-A and by inference NBCe1-B and -C have 14 membrane spanning segments. The residues involved in causing pRTA are not exposed on the surface of the NBCe1-A protein. Our results suggest that the involved residues may have important structural roles. An intriguing explanation for our findings is that pRTA mutations impair the function of NBCe1-A by either altering the static conformation of the cotransporter or by perturbing the dynamic conformational changes that the protein undergoes during its transport cycle.

REFERENCES

1. Pushkin, A., and Kurtz, I. (2006) *Am. J. Physiol. Renal Physiol* **290**, F580–F599
2. Cordat, E., and Casey, J. R. (2009) *Biochem. J.* **417**, 423–439
3. Boron, W. F., Chen, L., and Parker, M. D. (2009) *J. Exp. Biol.* **212**, 1697–1706
4. Kao, L., Sassani, P., Azimov, R., Pushkin, A., Abuladze, N., Peti-Peterdi, J., Liu, W., Newman, D., and Kurtz, I. (2008) *J. Biol. Chem.* **283**, 26782–26794
5. Zhu, Q., Azimov, R., Kao, L., Newman, D., Liu, W., Abuladze, N., Pushkin, A., and Kurtz, I. (2009) *J. Biol. Chem.* **284**, 8918–8929
6. McAlear, S. D., and Bevensee, M. O. (2006) *J. Biol. Chem.* **281**, 32417–32427
7. Yang, H. S., Kim, E., Lee, S., Park, H. J., Cooper, D. S., Rajbhandari, I., and Choi, I. (2009) *J. Biol. Chem.* **284**, 15970–15979
8. Lu, J., and Boron, W. F. (2007) *Am. J. Physiol. Cell Physiol.* **292**, C1787–C1798
9. Igarashi, T., Inatomi, I., Sekine, T., Cha, S. H., Kanai, Y., Kunimi, M., Tsukamoto, K., Sato, H., Shimadzu, M., Tozawa, F., Mori, T., Shiobara, M., Seki, G., and Endou, H. (1999) *Nat. Genet.* **23**, 264–266
10. Dinour, D., Chang, M. H., Sato, J., Smith, B. L., Angle, N., Knecht, A., Serban, I., Holtzman, E. J., and Romero, M. F. (2004) *J. Biol. Chem.* **279**, 52238–52246
11. Horita, S., Yamada, H., Inatomi, J., Moriyama, N., Sekine, T., Igarashi, T., Endo, Y., Dasouki, M., Ekim, M., Al-Gazali, L., Shimadzu, M., Seki, G., and Fujita, T. (2005) *J. Am. Soc. Nephrol.* **16**, 2270–2278
12. Suzuki, M., Vaisbich, M. H., Yamada, H., Horita, S., Li, Y., Sekine, T., Moriyama, N., Igarashi, T., Endo, Y., Cardoso, T., P., de Sá, L. C., Koch, V. H., Seki, G., and Fujita, T. (2008) *Pflugers Arch.* **455**, 583–593
13. Lin, S., Lo, Y., Yang, S., and Seki, G. (2009) *J. Am. Soc. Nephrol.* **20**, 33A
14. Toye, A. M., Parker, M. D., Daly, C. M., Lu, J., Virkki, L. V., Pelletier, M. F., and Boron, W. F. (2006) *Am. J. Physiol. Cell Physiol.* **291**, C788–C801
15. Chang, M. H., DiPiero, J., Sönntighsen, F. D., and Romero, M. F. (2008) *J. Biol. Chem.* **283**, 18402–18410
16. Li, H. C., Szigligeti, P., Worrell, R. T., Matthews, J. B., Conforti, L., and Soleimani, M. (2005) *Am. J. Physiol. Renal Physiol.* **289**, F61–F71
17. Tatishchev, S., Abuladze, N., Pushkin, A., Newman, D., Liu, W., Weeks, D., Sachs, G., and Kurtz, I. (2003) *Biochemistry* **42**, 755–765
18. Boron, W. F. (2006) *J. Am. Soc. Nephrol.* **17**, 2368–2382
19. Kurtz, I. (1987) *J. Clin. Invest.* **80**, 928–935
20. Zhu, Q., Lee, D. W., and Casey, J. R. (2003) *J. Biol. Chem.* **278**, 3112–3120
21. Zhu, Q., and Casey, J. R. (2007) *Methods* **41**, 439–450
22. Bok, D., Schibler, M. J., Pushkin, A., Sassani, P., Abuladze, N., Naser, Z., and Kurtz, I. (2001) *Am. J. Physiol. Renal Physiol.* **281**, F920–F935
23. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) *J. Cell Biol.* **93**, 97–102
24. Karlin, A., and Akabas, M. H. (1998) *Methods Enzymol.* **293**, 123–145