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Mutation of Aspartate 555 of the Sodium/Bicarbonate Transporter SLC4A4/NBCe1 Induces Chloride Transport

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To understand the mechanism for ion transport through the sodium/bicarbonate transporter SLC4A4 (NBCe1), we examined amino acid residues, within transmembrane domains, that are conserved among electrogenic Na/HCO₃ transporters but are substituted with residues at the corresponding site of all electroneutral Na/HCO₃ transporters. Point mutants were constructed and expressed in Xenopus oocytes to assess function using two-electrode voltage clamp. Among the mutants, D555E (charge-conserved substitution of the aspartate at position 555 with a glutamate) produced decreasing HCO₃⁻ currents at more positive membrane voltages. Immunohistochemistry showed D555E protein expression in oocyte membranes. D555E-induced Na/HCO₃-dependent pH recovery from a CO₂-induced acidification. Current-voltage relationships revealed that D555E produced an outwardly rectifying current in the nominally CO₂/HCO₃⁻-free solution that was abolished by Cl⁻ removal from the bath. In the presence of CO₂/HCO₃⁻, however, the outward current produced by D555E decreased only slightly after Cl⁻ removal. Starting from a Cl⁻-free condition, D555E produced dose-dependent outward currents in response to a series of chloride additions. The D555E-mediated chloride current decreased by 70% in the presence of CO₂/HCO₃⁻. The substitution of Asp⁵⁵⁵ with an asparagine also produced a Cl⁻-current. Anion selectivity experiments revealed that D555E was broadly permissive to other anions including NO₃⁻. Fluorescence measurements of chloride transport were done with human embryonic kidney HEK 293 cells expressing NBCe1 and D555E. A marked increase in chloride transport was detected in cells expressing D555E. We conclude that Asp⁵⁵⁵ plays a role in HCO₃⁻ selectivity.

The electrogenic Na/HCO₃ cotransporter NBCe1 (SLC4A4) is one of the SLC4A gene family members transporting HCO₃⁻ across the plasma membrane (1–3). NBCe1 plays a role in transepithelial HCO₃⁻ movement and pH regulation in many tissues (4–6). NBCe1 is responsible for HCO₃⁻ reabsorption in the proximal tubules of the kidney (7). The proximal tubule cells reclaim HCO₃⁻ from the lumen through a series of reactions involving titration of HCO₃⁻ by H⁺ secretion via the apical Na/H exchanger, production of CO₂, and regeneration of HCO₃⁻ and H⁺ in the tubule cells. HCO₃⁻ then moves to the interstitium via the basolateral NBCe1. The essential feature driving this basolateral Na⁺/HCO₃⁻ exit is the stoichiometry of 1:3 Na⁺:HCO₃⁻, which makes the equilibrium potential for NBCe1 more positive than the resting membrane potential of the proximal tubule cells (8). The stoichiometry of 1Na⁺:1HCO₃⁻ or 1Na⁺:2HCO₃⁻ causes both ions to move into cells in other tissues such as pancreas, brain, and cardiovascular tissues (9, 10).

Despite the importance of NBCe1 for basolateral HCO₃⁻ reabsorption in the proximal tubules, the mechanism of electrogenic Na/HCO₃ transport via the transporter is not well understood. Ion movement depends on loading ions at their translocation or binding sites that likely reside within the membrane field at some distance from the bath solution (11). This implies that the transmembrane domains (TM)² of NBCe1 and amino acid residues within TMs play critical roles in ion transport.

Sequence analysis of different SLC4A proteins shows similar hydropathy plots, predicting that these proteins share structural elements of transport function (12). Such similarities have facilitated structure/function studies to define molecular domains or motifs responsible for conferring Na/HCO₃ transport of NBCe1. Abuladze et al. (13) performed a large scale mutagenesis on acidic and basic amino acids in non-TMs and found many residues affecting Na⁺-dependent base flux. McAlear et al. (14) identified amino acids in TM8 involving ion translocation. By a systematic approach of chimeric transporters between NBCe1 and the electroneutral Na/HCO₃ cotransporter NBCn1 (SLC4A7) (15), we and our colleagues (16) demonstrated that electronegative Na/HCO₃ transport of NBCe1 requires interactions between the regions TM1–5 and TM6–13 of the protein. Zhu et al. (17) recently proposed TM1 as a domain lining the ion translocation pathway. On the other hand, Chang et al. (18) reported that the cytoplasmic N-terminus of the protein might contribute to HCO₃⁻ permeation.

In the present study, we searched amino acid residues that are highly conserved among electrogenic Na/HCO₃ transporters but not among electroneutral Na/HCO₃ transporters and examined their role in electronegative Na/HCO₃ transport. Nine candidate residues in human renal NBCe1-A (5, 19) were selected and mutated by replacement with the amino acids at the corresponding sites of NBCn1. Mutant transporters were expressed.
Asp$^{555}$ of NBCe1 Contributes to HCO$_3^-$ Selectivity

in *Xenopus* oocytes and assessed via two-electrode voltage clamp. Our data show that Asp$^{555}$ of NBCe1 plays an important role in HCO$_3^-$ selectivity.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The plasmids containing human renal NBCe1-A (GenBank$^\text{TM}$ accession number: NM_003759) (19) and rat NBCn1 (GenBank$^\text{TM}$ accession number: NM_058211) (15) served as templates for site-directed mutagenesis. Mutagenic primers were designed to replace the codons for candidate amino acids (primer sequences are presented in the supplemental data). Mutations were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). PCR was carried out with 16 cycles of 95°C for 1 min, 55°C for 0.5 min, and 68°C for 10 min. The products were sequenced.

**Transporter Expression in Xenopus Oocytes and Two-electrode Voltage Clamp**—Oocytes of *Xenopus laevis* (stages V and VI) were prepared as described (20). Defolliculated oocytes were injected with 15–20 ng of NBCe1 or mutant RNAs that were prepared by linearization with NheI and *in vitro* transcription kit (Ambion, Austin, TX). Control oocytes were injected with 46 nl of sterile water. The oocytes were maintained for 3–5 days (18°C) before use. For two-electrode voltage clamp, the oocytes were impaled with two microelectrodes filled with 3 M KCl (a resistance of <1.5 MΩ) and clamped at −60 mV using a voltage-clamp amplifier OC-725C (Warner, Hamden, CT). A staircase voltage command (−120 to +60 mV with 20-mV increments; 100 ms) was applied in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES, pH 7.4) and then in a solution buffered with 5% CO$_2$/25 mM HCO$_3^-$ (pH 7.4). NaHCO$_3$ was substituted for NaCl. The voltage command was applied before and 1 min after switching solutions. The difference between the current in CO$_2$/HCO$_3^-$ solution and the current in ND96 solution was calculated to obtain the HCO$_3^-$-dependent current and plotted as a function of voltage. The same voltage command was applied in ion depletion experiments. Na$^+$-free solutions were made by substituting with N-methyl-D-glucammonium and Cl$^-$-free solutions were made by substituting with gluconate. For anion substitution experiments, recordings were made in solutions that replace 25 mM Cl$^-$ with equimolar concentrations of Cl$^-$, Br$^-$, I$^-$, SCN$^-$, HCO$_3^-$, or NO$_3^-$ at a constant pH of 7.4. Voltage signals were sampled by an A-D converter Digidata 1322 (Molecular Devices, Sunnyvale, CA). The data were acquired using pClamp 8 (Molecular Devices). Experiments were done at room temperature.

**Na$^+$ and HCO$_3^-$ Dependence of Transporter Currents**—The oocytes were superfused with Na$^+$-free 5% CO$_2$/25 mM HCO$_3^-$ until CO$_2$-induced acidification reached steady state (~15 min). The oocytes were then exposed to a series of test solutions containing different concentrations of Na$^+$ ([Na$^+$])$_o$. Each test solution was separated by the Na$^+$-free CO$_2$/HCO$_3^-$ solution to maintain steady state between test solutions. Varying [Na$^+$]$_o$ was accomplished by substitution with N-methyl-D-glucammonium or Li$^+$ (at constant pH 7.4). All of the solutions contained 21 mM Cl$^-$. For HCO$_3^-$ dependence experiments, the oocytes were superfused with modified Cl$^-$-free ND96 until the basal current reached steady state and then exposed to a series of Cl$^-$-free solutions containing different concentrations of HCO$_3^-$ ([HCO$_3^-$])$_o$ at constant pH 7.4. The pH was maintained by varying P$_{CO_2}$ according to the Henderson-Hasselbalch equation. Each solution was bracketed with Cl$^-$-free ND96.

Simultaneous Measurement of pH$_i$ and Current—The oocytes were impaled with three electrodes: one for measuring membrane potential, one for measuring current, and one for measuring pH$_i$. The pH electrode was made with borosilicate fiber capillaries, silanized, filled with the proton ionophore 1 mixture B (Sigma-Aldrich), and back-filled with a pH 7.0 phosphate buffer. The current and voltage electrodes were filled with 3 M KCl (a resistance of <1.5 MΩ) and connected to the OC-725C clamp. The pH electrode was connected to a high impedance electrometer FD 223 (World Precision Instruments, Sarasota, FL). The voltage electrode signal was subtracted from the pH electrode signal using a subtraction amplifier (model V3.1, Yale University, New Haven) and amplified for input into a Digidata 1322. The data were acquired with customized software (Cell and Molecular Physiology, Yale University). The slope of pH to voltage was obtained by placing the three electrodes in the chamber filled with standards at pH 6.0 and 8.0. The measurements were done in ND96 and then in 5% CO$_2$/25 mM HCO$_3^-$ at a holding potential of 0 mV.

**Transfection of HEK 293 Cells**—Cells (70–80% confluent) were plated on coverslips in a 35-mm dish and incubated overnight in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, and 50 µg/ml streptomycin. The cells were transfected with pcDNA3.1/NBCe1 or pcDNA3.1/D555E using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cells were incubated in the reduced serum medium OptiMEM (Invitrogen) for 6 h and then in Dulbecco’s modified Eagle’s medium for 48 h before use.

**Immunocytochemistry**—Oocyte sections (1 µm in thickness) were prepared as described previously (21). The sections were treated with 1.2% H$_2$O$_2$ for 30 min to inhibit endogenous peroxidase activity. After washes with the phosphate-buffered saline (PBS), sections were preincubated with PBS containing 10% normal goat serum and then incubated for 1 h with the antibody (1:500) that recognizes the C-terminal end of rat renal NBCe1 (22). After washes, the sections were incubated with the goat Alexa 488 anti-rabbit IgG (Invitrogen). The sections were examined using a Zeiss Axiosview 135 microscope (Oberkochem, Germany) with a Plan-Neofluar 40× lens (numerical aperture of 0.75). For immunofluorescence of HEK 293 cells, the cells on coverslips were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.02% Triton X-100 (PBT), and blocked in PBT plus 10% normal goat serum for 1 h. The coverslips were then incubated with the NBCe1 antibody (1:500) overnight and treated with the goat Alexa 488 anti-rabbit IgG (Invitrogen).

**Immunoblot**—Transfected HEK 293 cells were collected and homogenized in an ice-cold buffer containing 300 mM mannitol, 0.1 mg/ml phenylmethanesulfonyl fluoride, 1× protease inhibitor mixture I (Calbiochem; San Diego, CA) and 5 mM HEPES (pH 7.2). The cells were centrifuged at 810 × g for 10 min. The resulting cell homogenate was centrifuged at 25,000 × g at 4°C for 30 min and the supernatant was used for immunoblot analysis. Proteins were separated by SDS-PAGE according to the method of Laemmli (23). The gels were then stained with Coomassie blue to reveal the presence of NBCs. The NBC bands were visualized using a ChemiDoc XRS system and quantified using Quantity One software (Bio-Rad). The samples were loaded with 5 µg of protein. The proteins were run without stacking gel and 4–20% gradient gel (Bio-Rad). The membrane was scanned and analyzed using Quantity One software (Bio-Rad).
Asp<sup>555</sup> of NBCe1 Contributes to HCO<sub>3</sub><sup>-</sup> Selectivity

min, and the supernatants were collected and assayed to determine protein concentration using the Bradford reagents (Sigma-Aldrich). Equal amounts of total proteins from the samples were separated on a 7% SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. The blot was incubated with the NBCe1 antibody (1:500) in PBS containing 0.05% Tween 20 and 5% nonfat dry milk for 2 h. The blot was washed for 40 min and then incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon) for 1 h. The blot was washed and visualized by ECL chemiluminescence (GE Healthcare).

**Fluorescence Measurement of Chloride Transport**—Chloride transport in HEK 293 cells expressing NBCe1 and D555E was measured using the protocol (23) with a slight modification. Briefly, 48 h after transfection, the cells on coverslips were incubated for 4 min in the 1:3 mixture of nitrate buffer (135 mM NaNO<sub>3</sub>, 2.4 mM K<sub>2</sub>HPO<sub>4</sub>, 0.6 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM CaSO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 10 mM HEPES, 10 mM d-glucose, pH 7.4) and water containing 50 mM 6-methoxyl-N-(3-sulfopropyl) quinolinium (SPQ). The coverslip was mounted to a chamber affixed on the stage of a Zeiss Axiovert 135 inverted microscope with a Plan-Neofluar 40× lens (numerical aperture of 0.75). The microscope was equipped with a Lambda 10-2 filter wheel controller (Sutter Instrument, Novato, CA) and a multi-wavelength filter set. Fluorescent dye was excited at 355 nm, and the emission light at 450 nm was captured. Excitation light was reflected by a 400-nm dichroic mirror. Images (exposure time of 200 ms) were acquired every 1 min with a digital camera Regita Exi (Q-Imaging) and analyzed by using MetaMorph software (Molecular Devices). At the end of the experiment, the cells were treated with 150 mM KSCN and then incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon) for 1 h. The blot was washed and visualized by ECL chemiluminescence (GE Healthcare).

**Statistical Analysis**—The data were reported as the means ± S.E. The level of significance was assessed using the unpaired, two-tailed Student t test for comparison between NBCe1 and point mutants and the paired, one-tailed test for comparison of single transporters in two different solutions. The one-way analysis of variance was used for the comparison of currents produced by mutant transporters proposed by Romero et al. (3). The electrochemical measurements of the currents produced by mutant transporters were performed using the following protocol (23): NBCe1 and mutant transporters were clamped at an injection voltage of 70 mV, with a step voltage command (∼120 mV and 20-mV increments, 100 ms) applied before and after switching solutions from CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>-free ND96 (pH 7.4) to a comparable solution buffered with 5% CO<sub>2</sub>/25 mM HCO<sub>3</sub><sup>-</sup> (pH 7.4). The electrochemical HCO<sub>3</sub><sup>-</sup> current (I<sub>Elec</sub>) was computed by subtracting the current in ND96 from the current in CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. A current-voltage (<i>I</i>-<i>V</i>) plot was made, from which the slope conductance (<i>g</i><sub>Elec</sub>) and reversal potential (<i>E</i><sub>rev</sub>) were computed at the zero current voltage. The numbers of oocytes/group were four to seven mutants, five NBCe1, and three water-injected controls.

**RESULTS**

**Selection of Candidate Amino Acids**—To identify the candidate residues responsible for electrogenic Na/HCO<sub>3</sub> transport of NBCe1, we set three guidelines. First, amino acids in TMs of Na/HCO<sub>3</sub> transporters are conserved for sequence comparison. In defining TM boundaries, we used the 13-TM topology model (3) proposed based on extensive studies of the topology model for the Cl/HCO<sub>3</sub> exchanger AE1 (SLC4A1) (24–27). Second, the candidate amino acids should be conserved in electroneutral Na/HCO<sub>3</sub> cotransporters NBCn1 and NBCn2 (SLC4A10), the electroneutral Na<sup>+</sup>-driven Cl/HCO<sub>3</sub> exchanger NDCBE1 (SLC4A8), and the Cl/HCO<sub>3</sub> exchanger AE1. Third, the amino acid difference between electrogenic transporters and electroneutral transporters should be significant enough to predict a change in function.

Fifteen amino acid residues of human renal NBCe1-A (19) were selected according to the above criteria (selected residues are available in supplemental data; NBCe1-A is referred to NBCe1 in this study). We examined nine of these residues (Fig. 1A): Ser<sup>127</sup> and Asn<sup>439</sup> (TM1), Phe<sup>161</sup> (TM2), Ser<sup>483</sup> (TM3), substiuated with amino acids that are conserved in electroneutral Na/HCO<sub>3</sub> cotransporters NBCn1 and NBCn2 (SLC4A10), the electroneutral Na<sup>+</sup>-driven Cl/HCO<sub>3</sub> exchanger NDCBE1 (SLC4A8), and the Cl/HCO<sub>3</sub> exchanger AE1. Third, the amino acid difference between electrogenic transporters and electroneutral transporters should be significant enough to predict a change in function.
Asp\textsuperscript{555} of NBCe1 Contributes to HCO\textsubscript{3}\textsuperscript{−} Selectivity

![Image of J-\textit{V} relationships for NBCe1, N439P, and D555E.](image)

**FIGURE 2.** \(J\)-\(V\) relationships for NBCe1, N439P, and D555E. \(J\)-\(V\) relationships were determined for oocytes injected with water (A) or RNAs for NBCe1 (B), N439P (C), or D555E (D) by using the voltage command as described in the legend to Fig. 1. The recordings were made first in ND96 (open circles) and then in 5% CO\textsubscript{2}/25 mM HCO\textsubscript{3} (closed circles). The difference between two mean currents is \(i_{NBC}\) (red line). The numbers of oocytes per group are given in Fig. 1. E, paraffin sections of oocytes were labeled with the antibody to the C-terminal domain of rat renal NBCe1 (22). Tetramethylrhodamine fluorescence was detected on the plasma membrane of oocytes expressing NBCe1 or D555E, but not N439P and water-injected controls.

Asp\textsuperscript{555} (TM5), Phe\textsuperscript{695} and Cys\textsuperscript{696} (TM7), Ala\textsuperscript{793} (TM9), and Ala\textsuperscript{871} (TM11). Five of these residues are located in the region TM1–5, and four are in the region TM6–13. Point mutant transporters were constructed by substituting these residues with aspartic acids present at the corresponding sites of NBCn1.

Low HCO\textsubscript{3}\textsuperscript{−}—Conductance of D555E—Mutant transporters were expressed in \textit{Xenopus} oocytes and assessed via two-electrode voltage clamp. The oocytes were clamped at \(-60\) mV (close to the average resting membrane potential of \(-62 \pm 4\) mV for 8 uninjected oocytes), and a voltage command from \(-120\) to \(+60\) mV was applied in CO\textsubscript{2}/HCO\textsubscript{3}−-free ND96 solution (pH 7.4) and then again 1 min after switching to a solution buffered with 5% CO\textsubscript{2}, 25 mM HCO\textsubscript{3}− (pH 7.4). The difference between the current in CO\textsubscript{2}/HCO\textsubscript{3}− and the current in ND96 is a good estimate of the current mediated by the transporter \((i_{NBC})\). Slope conductance \((g_{NBC})\) and reversal potential \((E_{NBC})\) were calculated at zero current voltage or at voltages close to zero current, from \(i_{NBC}-V\) plots.

Most of the mutant transporters had \(g_{NBC}\) and \(E_{NBC}\) values comparable with those for wild type NBCe1, except N439P (substitution of Asn\textsuperscript{439} with a Pro) and D555E (substitution of Asp\textsuperscript{555} with a Glu) (Fig. 1, B and C). N439P had basal \(g_{NBC}\) and \(E_{NBC}\) values, whereas D555E had a negative \(g_{NBC}\) and a positive \(E_{NBC}\) markedly distinct from values for NBCe1. Fig. 2 shows \(i_{NBC}-V\) plots for NBCe1, N439P, D555E, and controls. The application of 5% CO\textsubscript{2}, 25 mM HCO\textsubscript{3}− produced a large outward current in oocytes expressing NBCe1 (Fig. 2B). This change is due to the cotransport activity of NBCe1 because there is net movement of HCO\textsubscript{3}− ions across the membrane (1 Na\textsuperscript{+} and 2 HCO\textsubscript{3}−) (28). The slope in CO\textsubscript{2}/HCO\textsubscript{3}− (closed circles) was significantly larger than the slope in ND96 (open circles) (13.3 ± 2.5 \(\mu\)S versus 5.7 ± 1.3 \(\mu\)S, \(n = 5\) for each, \(p < 0.01\)). Thus, \(i_{NBC}\) (red line) was larger at more positive voltages. The slope in ND96 was higher than that for water-injected controls (Fig. 2A), because of a HCO\textsubscript{3}−-independent basal current of NBCe1 as reported by Sciortino and Romero (28).

Oocytes expressing N439P had negligible currents in either ND96 or CO\textsubscript{2}/HCO\textsubscript{3}−, thus producing no measurable \(i_{NBC}\) (Fig. 2C). In contrast, oocytes expressing D555E displayed outwardly rectifying currents in ND96 (Fig. 2D). This rectification is due to Cl\textsuperscript{−}, as discussed later. The application of CO\textsubscript{2}/HCO\textsubscript{3}− produced the outward currents decidedly larger than the currents in ND96. However, the difference between those two currents became progressively smaller at positive voltages that should be favorable for electrogenic Na/HCO\textsubscript{3}− transport. Thus, \(i_{NBC}\) did not increase at positive voltages but instead decreased. \(i_{NBC}\) was estimated to be 85 ± 10 mV and \(g_{NBC}\) was \(-2.6 ± 0.2\ \mu\text{S}\) (\(n = 7\) for both). Immunochemistry with the anti-NBCe1 antibody (22) revealed prominent fluorescence on plasma membranes of oocytes expressing NBCe1 or D555E (Fig. 2E). For oocytes expressing N439P, immunofluorescence on membranes was negligible, indicating that N439P has a defect in targeting to membranes.

**FIGURE 3.** Simultaneous measurements of pH and \(i_{NBC}\) in oocytes expressing NBCe1 or D555E under voltage clamp conditions. A, representative pH and \(i_{NBC}\) traces of an oocyte expressing NBCe1; pH recovery (arrow) from a CO\textsubscript{2}−-induced acidification and an outward \(i_{NBC}\) (arrowhead) upon CO\textsubscript{2}/HCO\textsubscript{3}− application are hallmarks for electrogenic Na/HCO\textsubscript{3}− transport. The immediate short peak in pH upon CO\textsubscript{2}/HCO\textsubscript{3}− application is a solution delivery artifact. The measurements were done at the holding potential of 0 mV. One of four experiments is shown. B, representative pH and \(i_{NBC}\) traces of an oocyte expressing D555E. One of six experiments is shown.

Decrease in Na/HCO\textsubscript{3}− Transport of D555E—To assess the transport characteristics of D555E, we simultaneously measured the pH and \(i_{NBC}\) values of oocytes under voltage clamp conditions. For this experiment, the oocytes were clamped at 0 mV, where electrogenic Na/HCO\textsubscript{3}− transport should favorably occur. Fig. 3 shows representative pH and \(i_{NBC}\) traces of an oocyte expressing NBCe1 or D555E. Upon the application of 5% CO\textsubscript{2}, 25 mM HCO\textsubscript{3}−, the pH in an oocyte expressing NBCe1 decreased as CO\textsubscript{2} entered into the cytosol and generated H\textsuperscript{+} after its hydration (Fig. 3A; the immediate, short peak is a solution delivery artifact). The pH then quickly recovered from a CO\textsubscript{2}−-induced acidification (arrow) because HCO\textsubscript{3}− was transported into the oocyte via NBCe1. The mean rate of pH change per time (dPH/dt; computed at the initial rising phase of the recovery from acidification) was \(48.4 ± 13.4 \times 10^{-4} \text{pH/s}\) (\(n = \ldots\))

![Image of representative pH and \(i_{NBC}\) traces in oocytes expressing NBCe1 and D555E.](image)
Asp^{S555} of NBCe1 Contributes to HCO₃⁻ Selectivity

![Diagram A](image1.png)

**FIGURE 4. I_{NBC} responses to Na⁺ and HCO₃⁻.** A and B, representative I_{NBC} response to [Na⁺]₀ in an oocyte expressing NBCe1 or D555E. The oocyte was superfused with Na⁺-free 5% CO₂/25 mM HCO₃⁻ until the basal current reached steady state (~15 min) and then applied with 5–96 mM [Na⁺]₀ in the continued presence of CO₂/HCO₃⁻. For NBCe1, the delivery of Na⁺ was stopped after the peak response to minimize time to return to the basal level between test solutions. One of four experiments for NBCe1 and one of five experiments for D555E are shown. C and D, representative I_{NBC} response to [HCO₃⁻]₀ in an oocyte expressing NBCe1 or D555E. The oocyte was superfused with modified Cl⁻-free ND96 until the basal current reached steady state and then applied with CO₂/HCO₃⁻ solutions containing 5–96 mM [HCO₃⁻]₀. Gluconate replaced Cl⁻. One of four experiments for NBCe1 and one of five experiments for D555E are shown. All of the experiments were done at the holding potential of ~60 mV.

4). The application of CO₂/HCO₃⁻ also produced an outward I_{NBC} (1.91 ± 0.01 μA, n = 4; arrowhead). Na⁺ removal in the continued presence of CO₂/HCO₃⁻ reversed the direction of pH recovery and I_{NBC}. In an oocyte expressing D555E (Fig. 3B), the application of CO₂/HCO₃⁻ caused more robust acidification. This large acidification corresponds to a relatively small HCO₃⁻ influx via D555E. Consistent with this, the dpH/dt was 17.0 ± 1.4 × 10⁻⁵ pH/sec (n = 6). The application of CO₂/HCO₃⁻ also produced less I_{NBC}. The mean amplitude was 0.48 ± 0.07 μA, corresponding to ~25% of I_{NBC} mediated by NBCe1. Na⁺ removal reversed the pH recovery and decreased I_{NBC}.

To examine the Na⁺ and HCO₃⁻ dependence of D555E, we then clamped oocytes at ~60 mV, superfused with Na⁺-free 5% CO₂, 25 mM HCO₃⁻ for ~15 min to reach steady state and then exposed to 5–96 mM [Na⁺]₀ in the continued presence of CO₂/HCO₃⁻. NBCe1 produced a peak I_{NBC} with progressively larger amplitudes at higher [Na⁺]₀ (Fig. 4A). In contrast, D555E produced a steady state I_{NBC} with relatively small amplitudes at all [Na⁺]₀ (Fig. 4B). I_{NBC} mediated by D555E appeared to be nearly saturated at 5 mM [Na⁺]₀. At 96 mM [Na⁺]₀, the mean I_{NBC} mediated by D555E corresponded to ~27% of the mean value for NBCe1. In other experiments, the oocytes were superfused with modified Cl⁻-free ND96 until the basal current became steady state (~10 min) and then exposed to Cl⁻-free solutions buffered with 5–96 mM [HCO₃⁻]₀. Similar to the responses to [Na⁺]₀, NBCe1 produced a peak I_{NBC} with progressively larger amplitudes at higher [HCO₃⁻]₀ (Fig. 4C), whereas D555E produced a steady state I_{NBC} with relatively small amplitudes at all [HCO₃⁻]₀ levels (Fig. 4D). I_{NBC} was nearly saturated at 5 mM [HCO₃⁻]₀, although it appeared to be slightly enhanced at higher [HCO₃⁻]₀.

The results obtained from the above two sets of experiments indicate that D555E produces a small I_{NBC} in response to [Na⁺]₀ and [HCO₃⁻]₀. The amplitude of the D555E-mediated I_{NBC} is not greatly enhanced at higher concentrations of those ions.

Cl⁻ Current Mediated by D555E—Fig. 5A illustrates I-V relationships for NBCe1 and D555E in ND96. The current mediated by D555E was outwardly rectified at positive voltages. The zero current voltage was ~36.3 ± 0.7 mV (n = 7). The major components of ND96 were 96 mM Na⁺ and 100.8 mM Cl⁻. To test whether Na⁺ was responsible for this outward rectification, we examined I-V relationships before and after Na⁺ removal in ND96 (Fig. 5B). Na⁺ removal had negligible effect on I-V relationships for both NBCe1 and D555E. The slope conductance remained unaffected (p > 0.05 for both; paired and two-tailed Student t test).

We then tested whether Cl⁻ was responsible for the outward rectification by analyzing I-V relationships before and after changing the solution from normal ND96 to Cl⁻-free ND96 (gluconate replaced Cl⁻). Cl⁻ removal markedly reduced the outward rectification (Fig. 5C). The slope conductance (measured between 20 and 60 mV using the linear least square fitting) decreased from 35.2 ± 5.3 to 12.8 ± 2.3 μS (n = 6, p < 0.01). In the presence of 5% CO₂, 25 mM HCO₃⁻ (Fig. 5D), Cl⁻ removal slightly decreased the slope conductance (from 27.7 ± 3.5 to 22.3 ± 2.9 μS, n = 6, p < 0.01), but zero current voltages were similar (~36.1 ± 4.1 mV in normal CO₂/HCO₃⁻ versus ~67.1 ± 5.7 mV in Cl⁻-free CO₂/HCO₃⁻, p > 0.1). Fig. 5E summarizes the results. Cl⁻ significantly contributes to the current mediated by D555E in the absence of HCO₃⁻ but contributes considerably less in the presence of HCO₃⁻.

Fig. 5F shows I-V relationships under Cl⁻-free conditions. The oocytes were superfused with Cl⁻-free ND96 until the basal current reached steady state and then exposed to Cl⁻-free solutions containing 10–96 mM [HCO₃⁻]₀ (~1 min). Each test solution was bracketed with Cl⁻-free ND96. Outward currents were observed at positive voltages and progressively increased at higher [HCO₃⁻]₀. The slopes among different [HCO₃⁻]₀ were relatively linear and parallel. Nevertheless, these plots did not cross the I-V plot for 0 mM [HCO₃⁻]₀ and remained separated at negative voltages. This separation at negative voltages is most likely due to the combination of Cl⁻ efflux when intracellular [HCO₃⁻] is very low and HCO₃⁻ efflux when intracellular [HCO₃⁻] is high.
Asp\textsuperscript{555} of NBCe1 Contributes to HCO\textsubscript{3}\textsuperscript{-} Selectivity

*FIGURE 5. Chloride current produced by D555E. A, I-V relationships for NBCe1 and D555E in ND96. I-V relationships for NBCe1 (n = 5; open squares) and D555E (n' = 7; closed squares) were obtained using the voltage command as described in Fig. 1. B, effect of Na\textsuperscript{+} removal on the conductance associated with NBCe1 or D555E in ND96. The slope conductance was measured between 20 and 60 mV. For both NBCe1 and D555E, the conductances were similar in solutions with and without Na\textsuperscript{+} (p > 0.05, paired and one-tail Student \(t\) test). The oocytes expressing D555E are represented in C–F. C, I-V relationships in ND96 with and without Cl\textsuperscript{-} (n = 6). The recordings were done before (closed circles) and after switching to Cl\textsuperscript{-}-free solution (open circles). D, I-V relationships in CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} with and without Cl\textsuperscript{-}. The oocytes in C were used to record I-V in 5% CO\textsubscript{2}/25 mM HCO\textsubscript{3}\textsuperscript{-} (closed circles) and then 1 min after switching to Cl\textsuperscript{-}-free CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} (open circles). E, summary of the conductance associated with D555E in the presence and absence of Cl\textsuperscript{-}. The asterisk represents p < 0.05 (paired and one-tail Student \(t\) test). F, D555E-mediated \(I_{\text{Na}}\) at different [HCO\textsubscript{3}\textsuperscript{-}] in either ND96 or CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} (Fig. 6A). In contrast, D555E produced \(I_{\text{Na}}\) in response to Cl\textsuperscript{-} in ND96 (Fig. 6B); the immediate and short peak is a solution delivery artifact. The same application of chloride in CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} produced a smaller \(I_{\text{Na}}\). The reduction was 68% based on the calculation of the mean \(I_{\text{Na}}\) (398.7 ± 69.2 nA in ND96 versus 127.8 ± 26.1 nA in CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}, n = 5) (Fig. 6C). \(I_{\text{Na}}\) was inhibited by anion channel blockers niflumic acid and 4,4'-disothiocyanato-2,2'-disulfonate stilbene (DIDS) (Fig. 6D). The inhibition was 55% by 100 μM niflumic acid (n = 4) and 70% by 100 μM DIDS (n = 4).

In other experiments in which oocytes were exposed to 5–96 mM [Cl\textsuperscript{-}]\textsubscript{o}, NBCe1 produced negligible response at all [Cl\textsuperscript{-}]\textsubscript{o} (Fig. 7A). However, D555E produced \(I_{\text{Cl}}\) with progressively larger amplitudes at higher [Cl\textsuperscript{-}]\textsubscript{o} (Fig. 7B). The D555E-mediated \(I_{\text{Cl}}\) was plotted as a function of [Cl\textsuperscript{-}]\textsubscript{o} (Fig. 7C). The data did not fit a Michaelis-Menten plot but appeared to include a Michaelis-Menten and a linear component comparable with the combined fit for the HCO\textsubscript{3}\textsuperscript{-} current of rat NBCe1 (10, 29).

Selectivity of D555E to Other Anions—The above data demonstrate that the charge-conserved substitution of Asp\textsuperscript{555} with a Glu induces \(I_{\text{Cl}}\). To examine other anion currents that might be mediated by D555E, we superfused oocytes with solutions replacing 25 mM Cl\textsuperscript{-} with equimolar concentrations of HCO\textsubscript{3}\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-}, SCN\textsuperscript{-}, Br\textsuperscript{-}, I\textsuperscript{-}, or Cl\textsuperscript{-} (all solutions had pH 7.4). Recordings were made before and after switching to CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} solutions containing 10–96 mM [HCO\textsubscript{3}\textsuperscript{-}]. Test solutions were bracketed with Cl\textsuperscript{-}-free ND96.

*FIGURE 6. Effect of HCO\textsubscript{3}\textsuperscript{-} on D555E-mediated \(I_{\text{Cl}}\). A and B, representative \(I_{\text{Cl}}\) of an oocyte expressing NBCe1 or D555E under Cl\textsuperscript{-} conditions. The oocyte (clamped at 0 mV) expressing NBCe1 or D555E was exposed to 71 mM [Cl\textsuperscript{-}]\textsubscript{o} before and after switching to 5% CO\textsubscript{2}/25 mM HCO\textsubscript{3}\textsuperscript{-}. The immediate, short peak in B is a solution delivery artifact. C, summary of \(I_{\text{Cl}}\) produced by NBCe1 and D555E. The data were obtained from four oocytes expressing NBCe1 and five oocytes expressing D555E. \(I_{\text{Cl}}\) was calculated after subtraction of the current in Cl\textsuperscript{-}-free solutions from the current in Cl\textsuperscript{-}-containing solutions. D, effect of niflumic acid (NFA) and DIDS on \(I_{\text{Cl}}\). Oocytes (n = 5) were applied with 71 mM [Cl\textsuperscript{-}]\textsubscript{o} in Cl\textsuperscript{-}-free ND96 to induce \(I_{\text{Cl}}\). The experiments were then repeated in the presence of 100 μM niflumic acid or 100 μM DIDS. The asterisk represents p < 0.05 (one-way analysis of variance).
Asp\textsuperscript{555} of NBCe1 Contributes to HCO\textsubscript{3}\textsuperscript{-} Selectivity

Asp\textsuperscript{555} of NBCe1 Contributes to HCO\textsubscript{3}\textsuperscript{-} Selectivity

![Diagram](https://example.com/diagram.png)

**FIGURE 7.** Response of D555E-mediated $I_{\text{Cl}}$ to Cl\textsuperscript{-}. A and B, representative $I_{\text{Cl}}$ of an oocyte expressing NBCe1 or D555E at different [Cl\textsuperscript{-}]. The oocyte expressing NBCe1 or D555E was superfused with modified Cl\textsuperscript{-}-free ND96 until the basal current reached steady state and then applied with 5–96 mM [Cl\textsuperscript{-}]. One of four experiments for NBCe1 and one of five experiments for D555E are shown. C, effect of Cl\textsuperscript{-} on $I_{\text{Cl}}$. $I_{\text{Cl}}$ was plotted as a function of [Cl\textsuperscript{-}]. The data were obtained from the experiments in A and B.

**FIGURE 8.** Selectivity of D555E to other anions. A and C, representative $I-V$ relationships for different anions in an oocyte expressing NBCe1. The recordings were made in modified ND96 that replaced 25 mM Cl\textsuperscript{-} with equimolar concentrations of HCO\textsubscript{3}\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-}, SCN\textsuperscript{-}, Cl\textsuperscript{-}, or Br\textsuperscript{-} (constant pH 7.4). One of six experiments is shown. B and D, representative $I-V$ relationships for different anions in an oocyte expressing D555E. The recording protocol was identical to that in A and C. One of six experiments is shown.

![Diagram](https://example.com/diagram.png)

**FIGURE 9.** HCO\textsubscript{3}\textsuperscript{-} conductance associated with Asp\textsuperscript{555}-related mutants. A, $g_{\text{NBC}}$ in the presence of Cl\textsuperscript{-}. D555N is the substitution of Asp\textsuperscript{555} with an asparagine, and E742D and E742Q are the substitutions of Glu\textsuperscript{742} with an aspartate and a glutamine, respectively, at the corresponding sites of NBCn1. The numbers of oocytes were eight control, three NBCe1, eight NBCn1, and seven to eleven mutants. B, $g_{\text{NBC}}$ in the absence of Cl\textsuperscript{-}. The recording protocol was identical to that in A, except that experiments were done under Cl\textsuperscript{-}-free conditions. The numbers of oocytes were two controls, three NBCe1, three NBCn1, and four to five mutants.

Cating that D555E is electrogenic. To further examine the role of Asp\textsuperscript{555} in the electrogenicity of the transporter, we constructed additional mutant transporters: D555N (charge-neutralizing substitution of Asp\textsuperscript{555} with an asparagine), E742D (reciprocal substitution of Glu\textsuperscript{742} at the corresponding site of NBCn1 with an aspartate), and E742Q (charge-neutralizing and reciprocal substitution of Glu\textsuperscript{742} with a glutamine). The electrogenicity of these mutants was evaluated by analyzing their $g_{\text{NBC}}$ values.

In the presence of Cl\textsuperscript{-} (Fig. 9A), both D555E and D555N had negative $g_{\text{NBC}}$ (−4.0 ± 0.8 μS for D555E, $n = 7$ and −3.7 ± 1.1 μS for D555N, $n = 9$). These are different from a positive $g_{\text{NBC}}$ for NBCe1 ($p < 0.01$). On the other hand, NBCn1 and its mutants E742D and E742Q had negligible $g_{\text{NBC}}$. No measurable $I_{\text{NBC}}$ was produced by these mutants. In the absence of Cl\textsuperscript{-} (Fig. 9B), whereas D555E had a positive $g_{\text{NBC}}$ (4.1 ± 1.1 μS, $n = 4$), D555N had only a small positive $g_{\text{NBC}}$ (0.6 ± 0.4 μS, $n = 5$). For D555N, the zero current voltage was negatively shifted by CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} (from −3.8 ± 2.1 mV to −22.9 ± 1.2 mV; figure not shown). Thus, a small positive $g_{\text{NBC}}$ represents a near parallel, negative shift in $I-V$ plot. These results suggest that D555N is HCO\textsubscript{3}\textsuperscript{-} permeable, but the HCO\textsubscript{3}\textsuperscript{-} conductance is very small. A similar D555N mutation made by Abuladze et al. (13) reduces Na\textsuperscript{+}-mediated base influx by 40% compared with NBCe1. The electroneutral transporter NBCn1 and its mutants E742D and E742Q had negligible conductance in the absence of Cl\textsuperscript{-} (Fig. 9B). From these experiments, we conclude that, despite its unique presence in both NBCe1 and NBCe2, Asp\textsuperscript{555} is not associated with the electrogenicity of the transporters. The residue instead offers HCO\textsubscript{3}\textsuperscript{-} selectivity.

Chloride Transport of HEK 293 Cells Expressing D555E—Despite the above results, it is possible that the chloride current we observed might not be intrinsic to the mutant transporter but rather derived from endogenous chloride channels in frog oocytes. To address this issue, we expressed NBCe1 and D555E in HEK 293 cells and measured chloride transport using the Cl\textsuperscript{-}-sensitive fluorescence dye SPQ (23). The expression of the transporters were confirmed by immunocytochemistry and immunoblot (Fig. 10, A and B). Immunofluorescence of NBCe1 or D555E was detected on plasma membranes of transfected cells. An immunoreactive band with the expected size of 120
Asp<sup>555</sup> of NBCe1 Contributes to HCO<sub>3</sub><sup>-</sup> Selectivity

Furthermore, the chloride transport of D555E-expressing HEK 293 cells (Fig. 10) supports the conclusion that D555E-mediated I<sub>C</sub> is not caused by endogenous oocyte channels.

Because of I<sub>C</sub> produced in ND96, the I<sub>NBC</sub>-V relationship for D555E shows a negative shift in g<sub>NBC</sub> and a positive shift in E<sub>NBC</sub> compared with NBCe1 or other mutants (Fig. 1). Additional experiments reveal that these changes do not fully represent the properties of D555E. In different media, the currents are mediated by different ions: I<sub>C</sub> in ND96, but mostly I<sub>NBC</sub> in CO<sub>2</sub>/HCO<sub>3</sub>. Therefore, a clearer estimation of I<sub>NBC</sub> can be achieved in the absence of Cl<sup>-</sup>. Under Cl<sup>-</sup>-free conditions, D555E produces a positive g<sub>NBC</sub> (Fig. 9B). The positive g<sub>NBC</sub> would be expected to generate a negative shift in E<sub>NBC</sub>, similar to that seen in oocytes expressing NBCe1. We can predict a negative E<sub>NBC</sub> when a straight line is extrapolated from I<sub>NBC</sub>-V plots at positive voltages (Fig. 5F). Nonetheless, we were unable to compute the exact E<sub>NBC</sub> because of the Cl<sup>-</sup> efflux at negative voltages. This difficulty in estimating E<sub>NBC</sub> hinders our attempt to calculate the Na<sup>+</sup>:HCO<sub>3</sub> stoichiometry of D555E.

HCO<sub>3</sub><sup>-</sup> versus Cl<sup>-</sup> Selectivity of Asp<sup>555</sup>—The electrophysiological properties of D555E provide valuable insight into the mechanism of Na/HCO<sub>3</sub> transport via NBCe1. The fact that D555E changes to I<sub>NBC</sub> from I<sub>C</sub> when HCO<sub>3</sub> is available suggests that HCO<sub>3</sub> has more favorable access to its binding site in the mutant transporter than does Cl<sup>-</sup>. Cl<sup>-</sup> binds to this site when HCO<sub>3</sub> is unavailable as I<sub>C</sub> is produced in ND96. Thus, the presence or absence of I<sub>I</sub> determines whether the site becomes occupied with HCO<sub>3</sub>. The data in Fig. 5B illustrate that I<sub>C</sub> is produced under Na<sup>+</sup>-free conditions. Our unpublished observation also shows that I<sub>C</sub> can be produced even in Na<sup>+</sup>-free CO<sub>2</sub>/HCO<sub>3</sub>, implying that the site is not occupied with HCO<sub>3</sub> under Na<sup>+</sup>-free conditions. We speculate that HCO<sub>3</sub> does not bind alone and that its binding to the transporter may require the precondition of Na<sup>+</sup> binding.

The acquired ability of D555E to produce I<sub>C</sub> and other ionic currents raises the strong possibility that Asp<sup>555</sup> is part of the site for HCO<sub>3</sub> selectivity. On the other hand, this brings a question of the role of Glu at the corresponding site in electroneutral Na/HCO<sub>3</sub> transporters. If Asp<sup>555</sup> is responsible for anion selectivity, does this mean that the electroneutral transporters (i.e. NBCn1, NBCn2, and NDCBE1) containing a Glu have broader anion selectivity? Obviously, addressing this question requires additional studies, but we note that among electroneutral

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**DISCUSSION**

**Overview**—The major findings of the present study are the following. First, the mutation of Asp<sup>555</sup> in NBCe1 to Glu reduces transport activity and produces a small I<sub>NBC</sub>. Second, the mutation induces I<sub>C</sub> in CO<sub>2</sub>/HCO<sub>3</sub>-free ND96. Third, this Cl<sup>-</sup> current does not require Na<sup>+</sup>. Fourth, the mutation increases permeability for other anions in addition to HCO<sub>3</sub>. Fifth, the mutation does not abolish the electronegativity of the transporter. Our findings provide the first molecular evidence for an amino acid residue (Asp<sup>555</sup>) that is closely associated with HCO<sub>3</sub> selectivity/translocation of the Na/HCO<sub>3</sub> transporters.

**Cl<sup>-</sup> Current Mediated by D555E—Oocytes expressing D555E produce I<sub>C</sub>.** This raises the question of whether D555E expression has caused an activation of endogenous chloride channels in Xenopus oocytes (31). However, it is highly unlikely that D555E-mediated I<sub>C</sub> is due to endogenous channels because I<sub>C</sub> is markedly reduced in CO<sub>2</sub>/HCO<sub>3</sub>-free solutions from chloride buffer to nitrate buffer. Four NBCe1-expressing cells and five D555E-expressing cells were perfused with nitrate buffer containing 135 mM NaNO<sub>3</sub> and then with chloride buffer containing the equimolar concentration of NaCl. After fluorescence reached steady state, the cells were exposed to nitrate buffer to monitor Cl<sup>-</sup> efflux (arrows). The end of the experiments, the cells were treated with 150 mM KSCN and 5 µM valinomycin to quench all the fluorescence. E, summary of the rate of fluorescence change. The rate (i.e. relative fluorescence unit/min) was computed from the initial rising phase of fluorescence after switching solutions from chloride buffer to nitrate buffer. Four NBCe1-expressing cells and five D555E-expressing cells were used from two experiments.

**Fluorescence measurement of Cl<sup>-</sup> transport mediated by D555E in HEK 293 cells.** A, immunocytochemistry of HEK 293 cells transfected with NBCe1, D555E, or vector only. The cells were stained with the anti-NBCe1 antibody and the goat Alexa 488 anti-rabbit secondary antibody. B, immunoblot of crude membrane preparation from transfected cells. C and D, SPQ assay of chloride transport. The cells loaded with SPQ were perfused with nitrate buffer containing 135 mM NaNO<sub>3</sub> and then with chloride buffer containing the equimolar concentration of NaCl. After fluorescence reached steady state, the cells were exposed to nitrate buffer to monitor Cl<sup>-</sup> efflux (arrows). E, summary of the rate of fluorescence change. The rate (i.e. relative fluorescence unit/min) was computed from the initial rising phase of fluorescence after switching solutions from chloride buffer to nitrate buffer. Four NBCe1-expressing cells and five D555E-expressing cells were used from two experiments.

**FIGURE 10.**

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**H. S. Yang, unpublished observation.**
Asp<sup>555</sup> of NBCe1 Contributes to HCO<sub>3</sub><sup>-</sup> Selectivity

Na/HCO<sub>3</sub> transporters, NBCn2 and NDCBE1 have the ability to transport Cl<sup>-</sup> or have an associated Cl<sup>-</sup> conductance. Even for NBCn1, its Cl<sup>-</sup> dependence has been determined by measuring pH<sub>i</sub> recovery upon Cl<sup>-</sup> removal in CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> (15). The Glu residue is also found at the corresponding sites in Cl/HCO<sub>3</sub> exchangers AE1–3. Thus, there is a possibility that the Glu residue in electroneutral transporters may play a central role in Cl<sup>-</sup> transport or conductance and/or ion selectivity. It will be interesting to test Cl<sup>-</sup> transport and anion selectivity of E742D and E742Q.

By what molecular mechanism does Asp<sup>555</sup> contribute to HCO<sub>3</sub><sup>-</sup> selectivity? D555E adds a single carbon to the side chain at the aspartate site, maintaining a net negative charge. Thus, charge alone at the site cannot account for the altered transport of Cl<sup>-</sup>. Our anion selectivity experiments suggest that steric properties of Asp<sup>555</sup> are more important for distinguishing HCO<sub>3</sub><sup>-</sup> from other anions. The effective radius of NO<sub>3</sub><sup>-</sup> is 1.89 Å, slightly larger than the molecular radius of 1.81 Å for Cl<sup>-</sup>. Nevertheless, NO<sub>3</sub><sup>-</sup> causes a negative shift in zero current voltage and produces a large conductance, comparable with the effect of HCO<sub>3</sub><sup>-</sup>. NO<sub>3</sub><sup>-</sup> and HCO<sub>3</sub><sup>-</sup> have similar structures, consisting of one central nitrogen or carbon atom surrounded by three identical oxygen atoms in a trigonal planar arrangement, except that a hydrogen atom is attached to one of the oxygens in HCO<sub>3</sub><sup>-</sup>. This implies that Asp<sup>555</sup> may serve as an anion selectivity filter that distinguishes HCO<sub>3</sub><sup>-</sup> from other polyatomic anions in a trigonal planar arrangement. The substitution of Asp<sup>555</sup> with a Glu containing a longer carbon side chain may thus disrupt this steric arrangement, allowing other polyatomic anions with similar structures to translocate via the altered site. D555E is also permeable to SCN<sup>-</sup>, but this polyatomic anion is linear in a space-filling model and has a smaller effective radius than HCO<sub>3</sub><sup>-</sup>.

Does the D555E mutation affect the electrogenicity of the transporter? D555E produces an outward current upon CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> removal in NBCe1 (Fig. 2). Thus, the D555E mutation does not abolish electrogenicity. There are two possible explanations for why I<sub>NBC</sub> mediated by D555E is small. First, it may be due to weak electrogenicity. Weak electrogenicity may remain if ion translocation or binding sites are affected but not completely lost, giving rise to an altered stoichiometry of 1 Na<sup>+</sup>/2HCO<sub>3</sub><sup>-</sup> (net charge between −1 and 0). This noninteger coupling ratio can occur when ion translocation involves cooperativity of multiple HCO<sub>3</sub><sup>-</sup>-binding sites and the mutation affects one of those binding sites. Nonetheless, we do not think that weak electrogenicity is responsible for causing a decrease in I<sub>NBC</sub> mediated by D555E. Lack of inducible I<sub>NBC</sub> in oocytes expressing E742D and E742Q (Fig. 9) supports our interpretation. Second, the D555E mutation may have no effect on the coupling ratio but instead reduce the rate of HCO<sub>3</sub><sup>-</sup> binding. It is possible that the D555E mutation not only affects anion selectivity, but it may also slow down the HCO<sub>3</sub><sup>-</sup> binding rate. In this case, we expect that D555E has reduced transport activity without losing the 1Na<sup>+</sup>/2HCO<sub>3</sub><sup>-</sup> coupling ratio. The exact stoichiometry of D555E warrants further investigation.

Asp<sup>555</sup> as a Residue in the Entry of the Pore-forming Unit—Our finding of Asp<sup>555</sup> as a site for HCO<sub>3</sub><sup>-</sup> selectivity provides valuable insights into the pore-forming region of NBCe1. Structural biology predicts that a protein will provide a close general structural model for other proteins if the core regions of those proteins have over 50% sequence homology (33). This suggests a similar topology of TMs for both NBC and AE. The 13-TM topology model was thus proposed on the basis of long and extensive studies of the AE topology (24–27). Asp<sup>555</sup> is 4 amino acids away from the DIDS-interacting motif Lys-Met-Ile-Lys (positions 559–602) (34), being located near Lys<sup>559</sup> on the same side of the α-helical TM5 structure. The DIDS-interacting sites are suggested to be close to the entry of the pore-forming unit in bicarbonate transporters, although those sites do not directly participate in ion binding/translocation (35). In AE1, the residues between Ser<sup>852</sup> and Leu<sup>857</sup> (TM13) are proposed to serve as an anion selectivity filter (36). These residues are located close to Lys<sup>551</sup>, which is another DIDS-binding site (37). Based on these, we propose that Asp<sup>555</sup> is located within the membrane field and near the entry of the pore region of the transporter.

Relations of Our Study to Natural NBCe1 Mutants—Among the mutant transporters we used in this study, it is worthwhile to briefly discuss S427A (substitution of Ser<sup>427</sup> with an alanine in TM1). Ser<sup>427</sup> is one of the sites for naturally occurring point mutations in NBCe1 (38). Functional characterization of S427L shows that it alters voltage- and Na<sup>+</sup>-dependent HCO<sub>3</sub><sup>-</sup> movement, resulting in ∼10% of I<sub>NBC</sub> compared with NBCe1. S427L has been proposed to contain a defect in the “voltage-sensing” processes of the transporter. In addition, S427L is targeted to the apical membrane of the polarized kidney cells, whereas NBCe1 is targeted to the basolateral membrane (39). All electroneutral Na/HCO<sub>3</sub> transporters possess an alanine at the corresponding site of Ser<sup>427</sup>. Our data show that S427A has g<sub>NBC</sub> and E<sub>NBC</sub> values comparable with those for NBCe1 (Fig. 1). Thus, the substitution of Ser<sup>427</sup> with a nonpolar residue leucine dramatically alters transporter function, whereas the substitution with another nonpolar residue alanine has no significant effect. This implies that the side chain polarity of Ser<sup>427</sup> is less involved in the altered function of S427L. We speculate that the functional defect of S427L may involve a conformational change caused by a larger leucine (molecular weight, 113) replacing a smaller serine (molecular weight, 87). The substitution with an alanine (molecular weight, 89) may not cause severe conformational change, thus maintaining the protein structure for function.

Summary—By structure/function analyses of NBCe-specific residues in TMs, we identified Asp<sup>555</sup> as a site playing a role in HCO<sub>3</sub><sup>-</sup> selectivity. This finding supports the conclusion that the functional specificity of Na/HCO<sub>3</sub> transporters is defined by amino acid residues within the transmembrane domains. The results obtained from this study are valuable for understanding the molecular mechanism of ion translocation or selectivity via Na/HCO<sub>3</sub> transporters.

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