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The first extracellular domain of claudin-7 affects paracellular Cl^- permeability

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Abstract

Tight junctions (TJ) constitute paracellular diffusion channels regulating the passage of ions and solutes across epithelia. We recently demonstrated that overexpression of the TJ membrane protein claudin-7 in LLC-PK1 cells decreases paracellular permeability to Cl^- and increases paracellular permeability to Na^+. To investigate the effect of charged amino acid residues in extracellular domains (ED) of claudin-7 on paracellular charge selectivity, we created claudin-7 mutants by replacing negatively charged amino acids on ED with positively charged amino acids. Immunofluorescence light microscopy showed that these mutant proteins were correctly targeted to the cell junction. Ultrastructure examination of TJ morphology did not reveal any difference between cells expressing wildtype (WT) and mutant claudin-7. However, electrophysiological studies showed increased Cl^- permeability in cells expressing first extracellular domain (ED1) mutants, but not second extracellular domain (ED2) mutants, compared to that of WT claudin-7. Our results demonstrate that negatively charged amino acids in ED1 of claudin-7 are involved in modulating paracellular Cl^- permeability.

Keywords: Claudin-7; Tight junctions; Paracellular charge selectivity; Dilution potentials; Cl^- permeability; LLC-PK1 cells

TJ regulate passive diffusion of ions and small neutral molecules between cells [1–4]. Three major types of integral membrane proteins localize at TJ: occludin, junctional adhesion molecules (JAM), and claudins [5,6]. Among these TJ proteins, claudins are the major structural and functional components of TJ [7–10].

The role of claudins in paracellular transport has been examined by several studies [11–18]. For instance, Furuse et al. [13] demonstrated that introducing claudin-2 cDNA into MDCK I cells that have high transepithelial electrical resistance (TER) caused a 20-fold decrease in TER. Amashen et al. [14] reported that claudin-2 induces cation-selective channels in TJ of kidney epithelial cells. In addition, Van Itallie et al. [15] showed that expression of claudin-4 in MDCK II cells increased TER by selectively decreasing paracellular Na^+ permeability. Other studies reported that expression of claudin-8 in MDCK II cells reduced the paracellular conductance and Na^+ permeability [16,17]. Site-directed mutagenesis has been employed to examine the role of charged amino acid residues on ED1 of claudins in paracellular charge selectivity [18]. These studies demonstrate that claudins are the major component of charge-selective paracellular ion channels or barriers between epithelial cells.

Our previous studies have shown that claudin-7 modulates paracellular Cl^- and Na^+ permeability in LLC-PK1 cells [19]. In this study, we tested the role of charged amino acids in ED of claudin-7 on ion selectivity by substituting negatively charged amino acids on ED1 and ED2 with positively charged amino acids. Claudin-7 mutants were stably expressed in LLC-PK1 cells and ion transport was evaluated by measuring transepithelial dilution potentials across cell monolayers. Our results demonstrate that mutations of charged amino acids in ED1 increased paracellular conductance

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to Cl^- without a significant change in paracellular conductance to Na^+ when compared to that of cells expressing WT claudin-7. In contrast, mutations of charged amino acids in ED2 had little effect on paracellular Cl^- and Na^+ permeability. The current study suggests that the ED1 of claudin-7 has a major role in paracellular charge selectivity.

Materials and methods

Construction of claudin-7 mutants. Site-directed mutagenesis was performed on the mouse claudin-7 cDNA using a Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specific HPLC purified oligonucleotide primer pairs (Integrated DNA Technologies, Inc., Coralville, IA) are listed as follows: D38R sense 5′-GAG CTC CTA TGC GGG C**AG** **A**AA CAT CAT CAC AGC CCA G-3′, D38R antisense 5′-CTG GGC TGT GAT GAT GTT **TCT** GCC CGC ATA GGA GCT C-3′; E53 sense 5′-GGG CTC TGG ATG **AAG** TGC TGC ACG CAG-3′, E53 antisense 5′-CTG CGT GAC GCA **CTT** CAT CCA GAG CCC-3′; D147R sense 5′-GGT CAT CAG ATT GTC ACA **AGG** TTT TAT AAC CCC TTG ACG C-3′, D147R antisense 5′-GCG TCA AGG GGT TAT AAA **ACC** **T**TG TGA CAA TCT GAT GAC C-3′; E160K sense 5′-CCC ATG AAC GTT AAG TAC **AAG** TTT GGA CCT GCC ATC-3′, E160K antisense 5′-GAT GGC AGG TCC AAA **CTT** GTA CTT AAC GTT CAT GGG-3′. ED1 mutations were made by replacing aspartate with arginine at position 38 (D38R) and glutamate with lysine at position 53 (E53K). ED2 mutants were created by substituting aspartate with arginine at position 147 (D147R) and glutamate with lysine at position 160 (E160K).

Cell culture and generation of stable cell lines. LLC-PK1 cells (a porcine kidney epithelial cell line kindly provided by Dr. J.A. Marrs, Indiana University Medical Center) were grown in DMEM/F12 medium containing 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in humidified air-5% CO_2 at 37 °C. *Claudin-7*-GFP, *Claudin-7*-GFP D38R, *Claudin-7*-GFP E53K, *Claudin-7*-GFP D147R, *Claudin-7*-GFP E160K, and empty GFP vectors were transfected into LLC-PK1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Following the geneticin selection, GFP positive cells were selected using a high speed Flow Cytometry Instrument (Becton–Dickinson).

Electrophysiological measurements. Dilution potential measurements were performed using cell monolayers grown on Snapwell membranes [19]. The cell monolayer was mounted on a slider that can be inserted into the EasyMount chamber (Physiologic Instruments, San Diego, CA). The left (apical) and right (basal) chambers were filled with one of the following buffers (in mM): 140 NaCl; 70 NaCl, 140 mannitol; 35 NaCl, 210 mannitol. Mannitol was added to the solutions with 70 and 35 mM NaCl in order to maintain the same osmolarity as that of the buffer with 140 mM NaCl. In some experiments, NaCl in the buffers was replaced by lysine–Cl or Na–aspartate. All buffers contained (in mM) 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 glucose at a pH of 7.3. During experiments, the buffer was maintained at 37 °C and bubbled constantly with 95% air–5% CO_2.

To measure the dilution potential of monolayers, Ag/AgCl electrodes were inserted into pipette tips and connected to the buffer in each chamber with a 3 M KCl agar bridge. Voltage and current signals were displayed by a VCC MC6 Voltage/Current Clamp instrument. Dilution potential is defined as $V_{dp} = V_2 - V_1$, where V_2 is the apical voltage and V_1 is the basal voltage.

Electron microscopy. Cells grown on coverslips were fixed in 2.5% glutaraldehyde and 1% tannic acid in 0.1 M sodium cacodylate buffer (pH 7.4). Cells were washed in 0.1 M cacodylate buffer and postfixed in OsO_4. The coverslips were en bloc stained with 1% uranyl, dehydrated with ethanol, and flat embedded in Spurr embedding media. Sections were stained with lead citrate and viewed with a JEOL 1200-EX transmission electron microscope. Images were recorded using a SIS MegaView III CCD camera.

Results

Claudin-7 mutant proteins were correctly localized at the cell junction and did not alter the ultrastructure of TJ in LLC-PK1 cells

Claudin-7 ED1 (D38R and E53K) and ED2 (D147R and E160K) mutants were generated by site-directed mutagenesis (Fig. 1A). These mutants as well as WT *claudin-7* constructs were stably transfected into LLC-PK1 cells. The cellular localization of WT and mutants was examined by immunofluorescence microscopy. As shown in Fig. 1B, all four claudin-7 mutant proteins, D38R, E53K, D147R, and E160K, were localized at cell junction in a pattern similar to that of the WT protein (a–e). Both WT and mutant proteins co-localized with the TJ associated protein ZO-1 (f–j) as revealed by their merged images (k–o). To determine whether the expression of claudin-7 mutants affects TJ structure, we examined TJ morphology by electron microscopy. Fig. 2 demonstrated the presence of TJ (arrows) at the apical surface of cells transfected with vector (a), WT (b), and D38R mutant (c).

ED1 mutants, but not ED2 mutants, attenuate the effect of WT claudin-7 in reducing paracellular Cl^- permeability without affecting Na^+ permeability

Previously, this laboratory reported that overexpression of claudin-7 in LLC-PK1 cells decreased paracellular permeability to Cl^- and increased paracellular permeability to Na^+. To study the role of the extracellular domains of claudin-7 on paracellular charge selectivity, electrophysiological experiments were performed using LLC-PK1 cells expressing claudin-7 with non-mutated extracellular domains (WT) or mutations in extracellular domain (ED) 1 or 2. A dilution potential is produced when the ionic gradient is created by decreasing the concentration of ion(s) of interest in one of the two chambers. The magnitude of dilution potential depends on the concentration gradient of ions in the buffer and the charge selectivity of the cell monolayer.

For experiments shown in Fig. 3, the apical buffer contained 140 mM NaCl and the basal buffer contained either 70 or 35 mM NaCl. In this condition, positive dilution potentials were produced on LLC-PK1 cells expressing a GFP-vector since LLC-PK1 cells are anion-selective and more permeable to anions than cations (Fig. 3). The dilution potential measured for cells expressing a construct containing WT claudin-7 was less than that of the vector control cells (Fig. 3) and in agreement with previously reported data [19]. In contrast, the dilution potential in cells expressing the D38R claudin-7 mutation increased 150% and 100% when measured in 70 and 35 mM NaCl, respectively, compared to that of WT expressing cells (Fig. 3A). Similar experiments conducted with cells expressing mutant E53K showed a moderated increase in dilution potential (40% in 70 mM and 35% in 35 mM

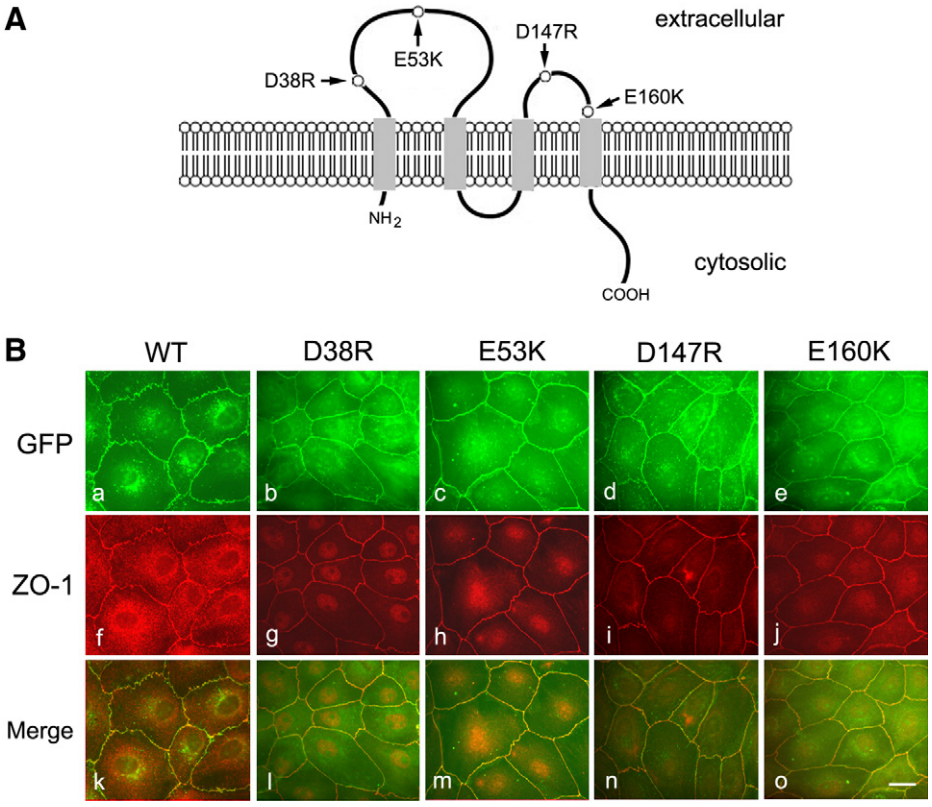

Fig. 1. (A) Claudin-7 is a tetraspan integral membrane protein. Claudin-7 D38R and E53K point mutations were created in ED1, and D147R and E160K mutations were generated in ED2. (B) GFP-tagged WT, D38R, E53K, D147R, or E160K proteins localized to the cell junction region of stably transfected LLC-PK1 cells (a–e). This staining pattern was similar to that for anti-ZO-1 rabbit polyclonal antibody (f–j) (1:100 dilution, Zymed). Co-localization of WT and mutant proteins with ZO-1 is demonstrated by the merged images (k–o). Bar: 15 μm.

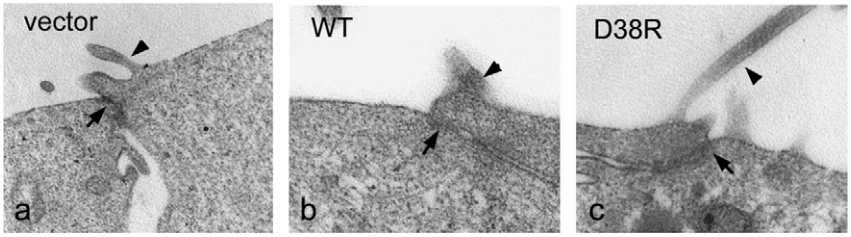

Fig. 2. LLC-PK1 cells stably transfected with vector, WT, or D38R construct were fixed in 2.5% glutaraldehyde and processed for electron microscope examination. Arrows in a–c indicated the tight junction formed at the apical surface of adjacent cells. Arrowheads in (a–c) pointed to the microvili. 50,000×.

NaCl) compared to that of WT cells (Fig. 3B). This data suggested that modifications of charged amino acids in ED1 significantly impacted the function of WT claudin-7. On the other hand, expression of either ED2 mutant, D147R or E160K, did not significantly affect the ability of WT claudin-7 to decrease the dilution potential as indicated by Fig. 3C and D.

To determine the contribution of Cl^- and Na^+ on measured dilution potentials in cells expressing ED1 mutants, NaCl in the buffer was replaced either by lysine–Cl to eliminate the effect of Na^+ (Fig. 4A and C) or by Na–aspartate to remove the effect of Cl^- (Fig. 4B and D). Expression of WT claudin-7 decreased dilution potential values relative to vector controls in the presence of 70 and 35 mM lysine–Cl (Fig. 4A and C) and increased dilution potential values relative to vector controls in the presence of 70 and 35 mM Na–aspartate (Fig. 4B and D). These results indicate that cells expressing WT claudin-7 are less permeable to Cl^- and more permeable to Na^+ as reported previously [19]. Dilution potentials measured for cells expressing the D38R mutation on ED1 increased 80% in 70 mM and 50% in 35 mM lysine–Cl compared to those measured for cells expressing WT claudin-7 (Fig. 4A), indicating that cells expressing the D38R mutant are more permeable to Cl^- (negative ion flow from apical to basal chamber). In the presence of Na–aspartate, dilution potentials were not different between D38R and WT cells (Fig. 4B), suggesting that the D38R mutation had no effect on Na^+ permeability.

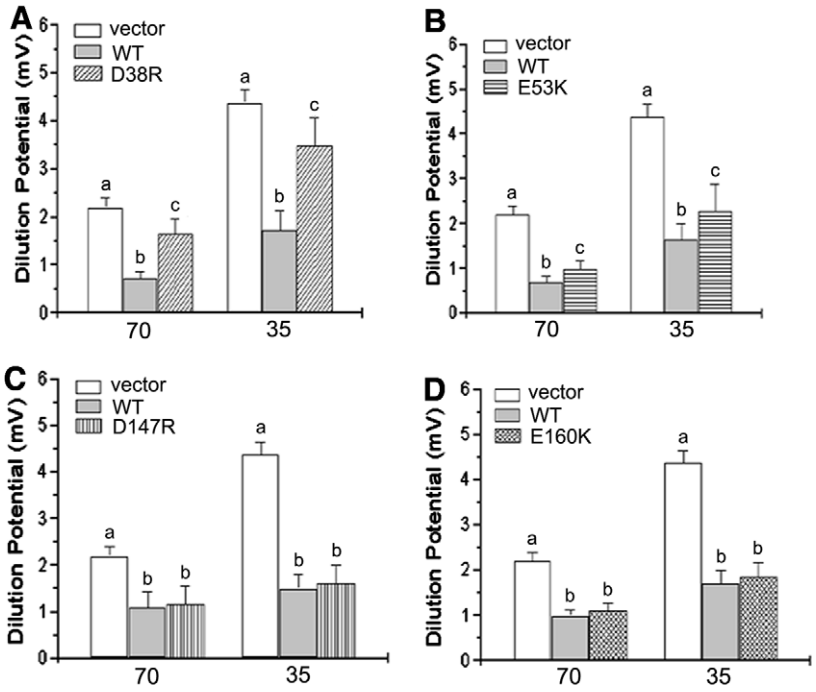

Fig. 3. Dilution potentials were determined on cells stably transfected with vector or WT compared to that of D38R (A), E53K (B), D147R (C), and E160K (D). Measurements were achieved upon switching 140 mM NaCl to 70 mM (70) or 35 mM (35) in the basal chamber. Statistical analysis was performed using Origin50. These data represent means ± SEM from three independent experiments ($n = 9$). Differences between means of each group were tested for statistical significance ($P < 0.05$) using one-way ANOVA followed by Bonferroni post-test. Letters of a, b, and c indicate differences between groups.

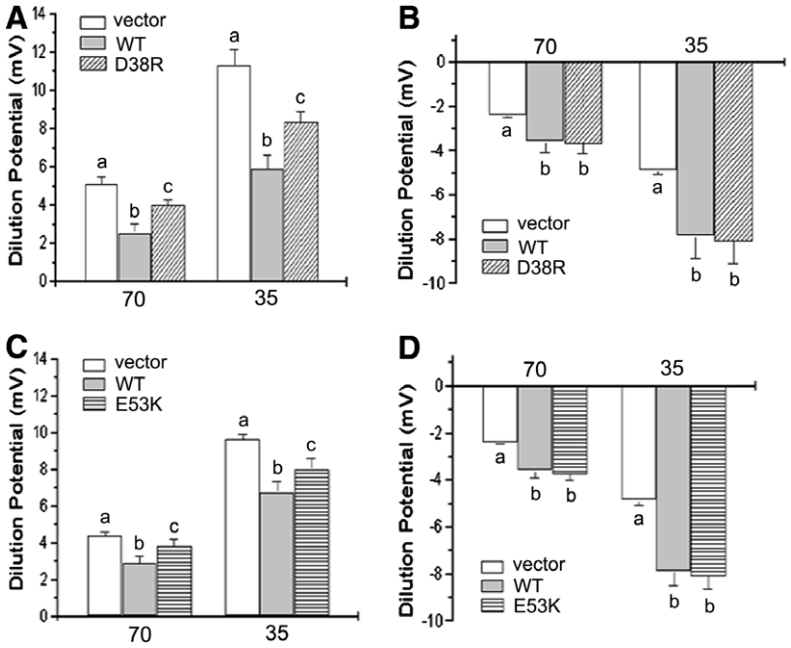

Fig. 4. Dilution potentials were measured on D38R (A,B) and E53K (C,D) expressing cells compared to that of vector and WT expressing cells in the presence of lysine–Cl (A,C) or Na–aspartate (B,D). Both D38R and E53K mutants attenuated the ability of WT cells to reduce dilution potentials compared to that of control cells (vector) in 70 and 35 mM lysine–Cl (A,C). There were no differences in dilution potentials between D38R and WT cells (B) or E53K and WT cells (C) in the presence of 70 and 35 mM Na–aspartate (B,D). These data represent means ± SEM from three independent experiments ($n = 9$). Letters of a, b, and c indicate significant differences between groups ($P < 0.05$).

A similar effect on dilution potential was observed with cells expressing the E53K mutation. Dilution potentials for these cells increased 30% and 20% compared to WT cells when measured in 70 and 35 mM lysine–Cl, respectively (Fig. 4C). In the presence of Na–aspartate, dilution potentials were not different between E53K and WT cells

suggesting that the E53K mutation had no effect on Na^+ permeability (Fig. 4D). Together, these data indicate that the negatively charged amino acids at position 38 and 53 of claudin-7 ED1 are important for the paracellular charge selectivity and that they increase the paracellular diffusion of Cl^- but not Na^+ ions.

Discussion

The objective of the present study was to determine whether negatively charged amino acid residues on ED of claudin-7 play a role in paracellular ion selectivity. Our results indicate that expression of claudin-7 ED1 mutants D38R and E53K increased the paracellular conductance to Cl^- without affecting the paracellular conductance to Na^+. Conversely, mutations on ED2 of claudin-7, D147R and E160K, showed little effect on the paracellular conductance to Cl^- or Na^+. Thus, we speculate that aspartate at position 38 and glutamate at position 53 on ED1 of claudin-7 contribute to the barrier properties against Cl^- in the paracellular pathway. In contrast, aspartate at position 147 and glutamate at position 160 on ED2 of claudin-7 do not seem to be involved in controlling paracellular ion permeability.

Recently, Hou et al. [20] reported that knockdown of claudin-7 expression by small interfering RNA against claudin-7 depressed the permeation of Cl^- in LLC-PK1 cells and elevated the permeation of Na^+ in MDCK II cells. Their data suggest that claudin-7 functions as a paracellular barrier to Na^+ in MDCK II cells and a paracellular channel to Cl^- in LLC-PK1 cells. Their data from LLC-PK1 cells is different from our results. This discrepancy could be caused by different claudin–claudin interactions in knockdown or overexpression conditions. We have tagged the claudin-7 with myc instead of GFP and the results were similar (data not shown). Therefore, GFP tag is less likely the cause for the difference.

Immunofluorescence microscopy studies indicate that claudin-7 ED1 and ED 2 mutants were properly targeted to the membrane and co-localized with TJ protein ZO-1. The TJ ultrastructure among control, WT, and mutant cells showed no obvious difference when revealed by electron microscopy. The fact that only ED1 mutants D38R and E53K showed an increased paracellular conductance to Cl^- compared to WT, suggests that ED1, but not ED2 of claudin-7, is involved in regulating the paracellular charge selectivity.

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