Claudins create charge-selective channels in the paracellular pathway between epithelial cells

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Claudins create charge-selective channels in the paracellular pathway between epithelial cells. Am J Physiol Cell Physiol 283: C142–C147, 2002; 10.1152/ajpcell.00038.2002.—Epithelia separate tissue spaces by regulating the passage of ions, solutes, and water through both the transcellular and paracellular pathways. Paracellular permeability is defined by intercellular tight junctions, which vary widely among tissues with respect to solute flux, electrical resistance, and ionic charge selectivity. To test the hypothesis that members of the claudin family of tight junction proteins create charge selectivity, we assessed the effect of reversing the charge of selected extracellular amino acids in two claudins using site-directed mutagenesis. Claudins were expressed in cultured Madin-Darby canine kidney cell monolayers under an inducible promoter, and clones were compared with and without induction for transmonolayer electrical resistance and dilution potentials. Expression and localization of Claudins were determined by immunoblotting, immunofluorescence microscopy, and freeze-fracture electron microscopy. We observed that substituting a negative charge at position 65 in the first extracellular domain of claudin-4 increased paracellular Na+ permeability. Conversely, substituting positive charge at position 65 in the first extracellular domain of claudin-15, singly and in combination, reversed paracellular charge selectivity from a preference for Na+ to Cl−. These results support a model where Claudins create charge-selective channels in the paracellular space.

Claudin-4; claudin-15; dilution potential; intercellular junction

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However, these studies did not prove that claudin-4 was directly responsible. This led us, in the present study, to test whether claudins might behave like paracellular channels and directly determine ion permeability through the electrostatic charges on their extracellular amino acid residues. The extracellular domains of claudins contain regions of highly conserved residues and intervening positions where the charge can be positive, negative, or neutral. In this study we have focused on variable residues in the larger first extracellular domain (see Fig. 1, A and B).

MATERIALS AND METHODS

Plasmid constructs and cell lines. Claudin-4K65D (K65D) was generated using the Stratagene (La Jolla, CA) QuikChange site-directed mutagenesis kit. Full-length human claudin-15 was amplified by PCR with the use of the human kidney cDNA (Quick-clone cDNA; Clontech Laboratories, Palo Alto, CA) as a template and primers 53988 (5'-CCACATGGATCGCCATATGTC-3') and 53989 (5'-CAGAGCTGCTACCGTAGGC-3'). The amplified product was cloned into the TOPO-TA vector (InVitrogen, San Diego, CA) and subcloned into the pTRE vector (Clontech Laboratories). Claudin-15 mutations were made using the Quik-Change site-directed mutagenesis kit (Stratagene). All wild-type and mutant claudin constructs were verified by DNA sequencing in both directions. Clonal cell lines of MDCK II Tet-Off cells (Clontech Laboratories) were derived by standard transfection and selection techniques; regulated expression of the transgene products was accomplished by varying doxycycline levels in the culture media as previously described (22).

Immunoblots and immunofluorescence. Anti-human claudin-4 mouse monoclonal antibody (Zymed, South San Francisco, CA) was used for immunoblots at a dilution of 1:4,000. Immunofluorescence for claudin-4 and K65D was performed using established protocols (22). Anti-human claudin-15 mouse monoclonal antibody (Zymed) was used for immunoblots at a dilution of 1:2,000 and for immunofluorescence on 1.0% paraformaldehyde-fixed cells at a 1:100 dilution. Immunoblots and immunofluorescence for ZO-1, occludin, and claudin-2 were performed using established protocols (22). Autofluorograms were scanned (Afga Duoscan T1200; Agfa-Gevaert, Mortsel, Belgium), and the integrated optical densities of constant areas at a specific molecular mass range were determined using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD). Local area background-integrated optical density was subtracted from each band.

Freeze-fracture electron microscopy. Freeze-fracture electron microscopy was carried out using established protocols (13).

Electrophysiology. Electrophysiological characterization of MDCK II monolayers was carried out according to published methods (22). Stable MDCK II Tet-Off cell lines (Clontech Laboratories) transfected with wild-type or mutant claudins were grown on Snapwell (Costar; Corning Life Sciences, Acton, MA) for 4 days, induced without doxycycline or noninduced with doxycycline-repressible promoter (1). Properties of monolayers induced to express claudin-4 or the K65D mutant were compared with noninduced controls of the same clone; at least three clones were examined for each example.

The cellular localization of the K65D mutant protein, like both endogenous and experimentally induced wild-type claudin-4, was predominantly at apicolateral cell membranes (Fig. 1C). The immunolocalization of claudins was compared with that of the cytoplasmic TJ protein ZO-1. In all cases the focused TJ location of ZO-1 was unaffected by forced expression of the claudins (Fig. 1C). Likewise, induction of either wild-type claudin-4 or the K65D mutant did not affect the levels of several other TJ proteins (Fig. 1D), suggesting that changes in permeability can be interpreted as a specific effect of the claudin transgene product.

We next determined whether either claudin-4 or the K65D mutant altered paracellular charge selectivity by comparing transepithelial dilution potentials (22) with and without induction of protein expression. In the low-resistance monolayers used for our studies, the majority of ionic conductance is paracellular and the transmonolayer electrical potential immediately following imposition of a NaCl gradient reflects the relative permeabilities of Na+ vs. Cl− ions through TJs. Previously reported evidence in support of a paracellular source of the dilution potential in MDCK cells includes unchanged net dilution potentials upon reversing the orientation of the NaCl gradient (22), the lack of current saturation in current-voltage curves (22), and the unchanged dilution potential upon application of inhibitors of major transcellular transporters (22). MDCK cells were grown on porous membranes, transgene expression was induced, and the membranes were removed and placed in modified Ussing chambers (22). Transmonolayer resistance (>20 Ω·cm²) was

RESULTS

A charge-reversing mutation of claudin-4, K65D, eliminates its ability to discriminate against Na+. Claudin-4 contains a single basic residue, Lys65, in a charge-variable region of the first extracellular domain (Fig. 1, A and B). To test whether electrostatic repulsion from Lys65 of claudin-4 discriminates against the paracellular movement of Na+ (22), we reversed the charge at this position by replacement with aspartic acid (K65D) using site-specific mutagenesis (Fig. 1A). The K65D mutant was expressed in stably transfected clones of MDCK cells under a tightly regulated doxycycline-repressible promoter (1). Properties of monolayers induced to express claudin-4 or the K65D mutant were compared with noninduced controls of the same clone; at least three clones were examined for each example.

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used to verify the presence of intact TJs. MDCK cells have an unknown claudin profile; however, cells used for our studies have TJs that are approximately four- to five times more permeable for Na⁺ than Cl⁻ at baseline (2). As previously demonstrated, we observed that induction of claudin-4 significantly decreased the dilution potential (Fig. 1E), reflecting a decrease in the relative permeability of Na⁺ compared with Cl⁻. In contrast, expression of the K65D mutant did not decrease the dilution potential. This implies that replacement of a positive for negative residue (K65D) considerably diminished the ability of claudin-4 to discriminate against Na⁺ ions (Fig. 1E). These data are consistent with a direct electrostatic effect of the charged residue at position 65 in defining charge selectivity.

Claudin-15 creates Na⁺-selective paracellular channels. To test the generality of the hypothesis that charged residues on the extracellular domains of clau-
dues establish paracellular charge selectivity, we performed charge-reversing mutations in the opposite direction, from acidic to basic residues, and on a different claudin. Claudin-15 was selected for study because it has three acidic residues at positions that are typically variable among the claudin family members. Furthermore, their combined reversal creates a first extracellular domain with only positive charges (Fig. 2A). We reasoned that if the electrostatic channel model were correct, this extreme case might reverse the junction selectivity from a preference for Na\(^+\) to a preference for Cl\(^-\) ions.

Claudin-15 was cloned from a human kidney cDNA library and expressed in MDCK cells, again under the control of a doxycycline-regulated promoter. Studies on claudin-15 have not been previously reported, so we first documented by RT-PCR that the protein is widely expressed in rodent tissues and by immunomicroscopy that it is located in TJs in human colon (not shown). Immunoblot analysis of MDCK cells expressing human claudin-15 showed no alteration in the levels of ZO-1 or claudin-2; however, there was a decrease in the level of occludin (Fig. 2B). The relevance of this decrease is not obvious, because occludin has a striking absence of charged extracellular amino acids except immediately adjacent to the membrane, and expression of exogenous occludin by itself in MDCK cell monolayers has little effect on paracellular charge selectivity (not shown). Freeze-fracture electron microscopy revealed an increase in the number, depth, and complexity of TJ brils (not shown) in cells induced to express claudin-15 compared with noninduced cells. These changes in fibril architecture confirm the appropriate localization of human claudin-15 into TJ fibrils. Some protein is also obvious by immunofluorescence microscopy on the lateral cell surface and in intracellular puncta (not shown). Expression of human claudin-15 resulted in a significant increase in transmonolayer resistance (see legend to Fig. 2); however, dilution potentials from monolayers expressing human claudin-15 were not significantly different from either nontransfected or noninduced monolayers. This indicated that TJs with exogenous wild-type claudin-15 protein are more permeable for Na\(^+\) than Cl\(^-\), similar to the normal MDCK cell background (Fig. 2E).

Charge-reversing mutations on the first extracellular domain of claudin-15 create Cl\(^-\)-selective paracellular channels. To determine whether all three acidic residues in the first extracellular domain of claudin-15 influence paracellular charge selectivity, we replaced them with basic residues, singly and in combination (Fig. 2A). Immunoblot analysis demonstrated that expression of all mutant proteins could be tightly regulated and that, except for occludin, their induction did not alter levels of other TJ proteins (Fig. 2B). Freeze-fracture electron microscopy performed on a subset of the mutants revealed an increase in TJ fibril complexity (Fig. 2D) in a pattern that was indistinguishable from that of cells expressing exogenous wild-type claudin-15. Immunofluorescence microscopy of all mutants revealed localization similar to that seen with wild-type claudin-15 (Fig. 2C). Dilution potentials revealed that reversing the charge at the first acidic position, m1 (E46K), had no effect on paracellular charge selectivity. In contrast, mutation of either position m2 (D55R) or m3 (E64K) resulted in a significant decrease in the ratio of \(P_{Na}\) to \(P_{Cl}\) (Fig. 2E). Introducing a positive charge at position m2 actually reversed the overall permeability ratio to favor Cl\(^-\) ions. We conclude that some, but not all, charged positions in claudin-15 influence paracellular charge selectivity.

To determine whether the influence of specific residues is additive, we measured dilution potentials after reversing charges at the first and second position, m1,2 (E46K, D55R), and at all three positions, m1,2,3 (E46K, D55R, E64K). Mutant m1,2 had the same effect on charge selectivity as m2 alone, consistent with lack of any contribution from position m1. Mutant m1,2,3 created a significantly Cl\(^-\)-selective paracellular pathway, the magnitude of which appeared to result from the addition of effects from positions m2 and m3 (Fig. 2E). Induction of wild-type claudin-15 and all mutants resulted in monolayers with significantly higher resistance (30–278%) compared with nontransfected or noninduced cells. We interpret this to mean that TJs remained intact and the observed changes in discrimination do not result from loss of paracellular selectivity.

**DISCUSSION**

The results presented here provide the first conclusive evidence that claudins directly regulate charge selectivity of the paracellular pathway. Their extracellular sequences share a core of conserved residues and presumably a similar tertiary structure. We speculate that the extracellular adhesive contacts between claudins bring them together in a manner that creates aqueous channels lined by the variably charged positions. This charged-channel model is consistent with the observation that TJs in all epithelia have a relatively similar size discrimination for uncharged solutes but have variable charge selectivity and overall electrical resistance (17). A similar model was proposed almost four decades ago based on elegant physiological experiments (25), but the physical basis has remained unknown.

The paracellular pathway has been characterized as having large aqueous spaces and unique charge selectivities. Unlike transmembrane ion channels, the paracellular pathway shows selectivity for net charge rather than specific ions (3, 14). Parallels to the paracellular pathway can be found in the properties of gap junctions, where connexons provide large aqueous cell-to-cell channels and show moderate charge and solute selectivity (16). Similar to our work on claudins, an extracellular domain of connexins with charged residues has been characterized to influence gap junction charge selectivity (20).

We chose to study the larger first extracellular domain of claudins because it has greater numbers and variability of charges than the second domain. Fur-
Fig. 2. Replacing acidic (red) with basic (blue) residues on the first extracellular domain of claudin-15 reverses the paracellular selectivity from Na\(^+\) to Cl\(^-\) ions. A: amino acid sequence alignment of the first extracellular domain of claudin-15 and charge-reversal mutants: m1 (E46K); m2 (D55R); m3 (E64K); m1,2 (E46K, D55R); and m1,2,3 (E46K, D55R, E64K). The charges on wild-type claudin-15 and m1,2,3 are noted above and below the sequences.

B: expression of wild-type (WT) claudin-15 and mutants is inducible. Densitometric analysis revealed a decrease in the level of occludin between 45 and 65% upon induction of claudin-15 and all mutants. Immunoblots were performed as in Fig. 1D. C: claudin-15 and mutants localize to the apicolateral plasma membrane upon expression (m1,2,3 shown). Inducible transfected cell lines were plated and grown on filters as in Fig. 1C. Immunofluorescence microscopy for ZO-1 (a, c) and claudin-15 m1,2,3 (b, d) was performed on cells noninduced (a, b) or induced (c, d) for the expression of m1,2,3.

D: claudin-15 and mutants increase the number, complexity, and depth of TJ fibrils. Freeze-fracture electron microscopy was performed on cells noninduced (a) or induced (b) for the expression of m1,2,3. Inducible transfected cell lines were prepared as in Fig. 1C. Freeze-fracture electron microscopy was performed as previously reported (13). Bar, 0.2 \(\mu\)m.

E: charge-reversal mutants of claudin-15 reverse paracellular charge selectivity. Dilution potential experiments were performed as in Fig. 1E. Clonal lines are compared with themselves in the noninduced (open bars) and induced state (solid bars); results from 3–5 clonal cell lines are averaged for wild type and each mutant. The dilution potentials before and after induction of claudin-15 and mutants were as follows: claudin-15, 7.8 ± 0.6 to 7.1 ± 0.6 mV; m1, 7.8 ± 0.9 to 6.4 ± 1.1 mV; \(^*\)m2, 8.1 ± 0.2 to −1.2 ± 0.6 mV; \(^*\)m3, 8.5 ± 0.4 to 0.9 ± 1.3 mV; \(^*\)m1,2, 6.4 ± 0.6 to −2.5 ± 0.7 mV; and \(^*\)m1,2,3, 7.6 ± 0.6 to −7.1 ± 0.7 mV. Potentials of equal magnitude but opposite charge were generated following basal dilutions, confirming that the charge selectivity is paracellular. Transmonolayer resistances for noninduced clones were similar, with a range of 27.2–34.5 \(\Omega\)-cm\(^2\). A significant increase in transmonolayer resistance was observed following induction of claudin-15 and all mutants as follows: \(^*\)claudin-15, 60.6 ± 4.4 \(\Omega\)-cm\(^2\); \(^*\)m1, 53.8 ± 12.0 \(\Omega\)-cm\(^2\); \(^*\)m2, 105.5 ± 21.3 \(\Omega\)-cm\(^2\); \(^*\)m3, 56.7 ± 1.6 \(\Omega\)-cm\(^2\); \(^*\)m1,2, 50.0 ± 3.9 \(\Omega\)-cm\(^2\); and \(^*\)m1,2,3, 41.0 ± 3.8 \(\Omega\)-cm\(^2\). \(^* P < 0.05\) (Student’s t-test). Error bars represent SE.
thermore, consistent with a role for charge in the first extracellular domain influencing charge selectivity, indirect published evidence suggests that claudin-16, with 10 negative residues on its first extracellular domain, forms paracellular channels selective for divalent cations (19). Studies are currently in progress to characterize any contribution of the second extracellular domain in paracellular charge selectivity.

We speculate that paracellular ionic selectivities of different epithelia are determined by varying combinations and levels of different claudins. It is already known that claudins show unique expression patterns and that some TJs contain several different claudins (4, 8, 15, 18, 19). Physiological or pathological alterations in claudins are expected to have a significant impact on epithelial barrier properties. These could be manifest as changes in transport, antigen and pathogen entry, or, conceivably, tissue morphogenesis. Indeed, two recent reports of human diseases resulting from mutations in claudin-14 and 16 and the phenotype of a mouse knockout of claudin-11 (9, 19, 24) can be rationalized as defects in paracellular ionic charge selectivity. Understanding and manipulating the molecular basis of selectivity should also have therapeutic applications for enhancing the level and location of drug delivery.

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