Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins

Christina M. Van Itallie,1 Alan S. Fanning,2 and James M. Anderson2

Departments of 1Medicine and 2Cell and Molecular Physiology,
University of North Carolina, Chapel Hill, North Carolina 27599-7545

Submitted 24 March 2003; accepted in final form 30 July 2003

Van Itallie, Christina M., Alan S. Fanning, and James M. Anderson. Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins. Am J Physiol Renal Physiol 285: F1078–F1084, 2003.—Tight junctions (TJ) regulate paracellular ionic charge selectivity and conductance across epithelial tissues and cell lines. These properties vary among epithelia, and recent evidence implicates the claudins, a family of TJ transmembrane proteins, as important determinants of both characteristics. To test the hypothesis that each claudin contributes a characteristic charge discrimination to the TJ, we expressed claudins-2, -4, -11, and -15 in both cation-selective Madin-Darby canine kidney (MDCK) II cells and in anion-selective LLC-PK1 cells and examined changes in transepithelial electrical resistance (TER) and paracellular charge selectivity. Regulated expression and localization were verified by immunoblot analysis and immunofluorescence microscopy, respectively. Expression of claudin-4 increased TER in both cell lines, whereas effects of the others on TER were variable. Claudin-4 and -11 decreased paracellular permeability for Na+ in MDCK II cells, whereas neither claudin-2 nor -15 had an effect. Conversely, in LLC-PK1 cells, claudin-2 and -15 increased the permeability for Na+, whereas claudin-4 and -11 were without effect. We conclude that the contribution of each claudin is most easily detectable when it reverses the direction of monolayer charge selectivity. These results are consistent with a model in which exogenous claudins add new charge-selective pores, leading to a physiological phenotype that combines endogenous and exogenous contributions. Additionally, it is possible to rationalize the direction of charge selectivity conferred by the individual claudins on the basis of electrostatic effects of the charged amino acids in their first extracellular loops.

IONS AND SOLUTES move across epithelial cells by either the transcellular or paracellular route. The paracellular route is governed by the tight junction (TJ), a continuous band of intercellular contacts encircling the cell at the apical end of the lateral membrane. The TJ barrier is quite variable among different epithelia in terms of electrical resistance and charge selectivity. For example, transepithelial electrical resistance (TER) varies from $>100,000 \ \Omega \cdot \text{cm}^2$ in urinary bladder to $<10 \ \Omega \cdot \text{cm}^2$ in the renal proximal tubule (reviewed in Ref. 19). Paracellular charge selectivity varies from cation-selective (permeability ratio: $P_{\text{Na}}/P_{\text{Cl}} = 10$) in rat jejunum (19) to anion-selective ($P_{\text{Na}}/P_{\text{Cl}} = 0.14$) in the Necturus proximal tubule (14). In low-resistance epithelia, the paracellular pathway is the major route for ion permeation and thus has a major influence on transepithelial conductance and charge discrimination.

The TJ is a network of intercellular contacts that appear as strands of transmembrane particles when analyzed by freeze-fracture electron microscopy. The number and complexity of the TJ strands largely (2), but not completely, correlate with the tightness of the epithelial barrier. Many TJ protein components have been defined over the last 10 years, including multiple cytoplasmic plaque proteins, for example, the ZO proteins, and at least four types of transmembrane proteins (reviewed in Refs. 5 and 10). Some of these components, like ZO-1, are apparently invariant components of the TJ; others, including the transmembrane proteins, are present in varying combinations in different epithelial tissues. These transmembrane proteins include occludin, junctional adhesion molecules, and the claudin family of proteins.

Claudins appear to be the main structural component of the freeze-fracture strands of TJs, since expression them in fibroblasts leads to de novo strand formation (8). More than 20 different claudins have been identified, but their distributions and functions are still incompletely defined. Although in Sertoli cells claudin-11 is apparently the sole claudin (11, 17), in other tissues and cell lines multiple claudins are expressed (9, 12, 20, 23). Mouse kidney has been reported to express at least 12 claudins (12, 21) in varying combinations along the nephron and in renal endothelial cells. Although lack of reagents has meant the complete claudin profile of Madin-Darby canine kidney (MDCK) II and LLC-PK1 cells has not yet been enumerated, both cell lines express at least claudins-1, -2, and -4. Studies by Furuse and co-workers (9) demonstrated copolymerization of different claudins in a single TJ strand, suggesting that the overall barrier is defined by contributions from an ensemble of different

Address for reprint requests and other correspondence: C. M. Van Itallie, 6312 MBBB, CB#7545, Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7545 (E-mail: vitallie@med.unc.edu).
claudins coexpressed and incorporated into the same strands.

Recent studies demonstrate that expression of specific claudins in MDCK I or II cells can alter both TER and paracellular charge selectivity. Expression of claudin-4 (22) and -8 (25) in MDCK II cells increased TER and decreased paracellular cation permeability, whereas expression of claudin-2 in high-resistance MDCK I cells decreased TER (7) and increased cation permeability (1). On the other hand, expression of claudin-2 (4) had no effect on the ionic selectivity of MDCK II cells. Although the claudin-2 results appear at first inconsistent, MDCK II but not MDCK I cells are already cation selective. Based on these limited published observations, we hypothesized that claudins that decrease cation permeability might only have detectable effects in cells that were highly cation permeable, whereas measurement of the effects of claudins that increase permeability for cations might require an anion-selective cell background.

To test this hypothesis, we expressed four different claudins in two cell lines that have opposite charge selectivity and compared the resulting changes in charge selectivity and TER. We predict, based on the pattern of their charged extracellular amino acids, that two of the claudins, claudin-2 and -15, will be cation selective and the other two, claudins-4 and -11, anion selective. At baseline, one cell line is very leaky and cation selective (MDCK II) and the other slightly anion selective and has a higher resistance (LLC-PK1). Our results are consistent with the idea that each claudin may have its own intrinsic paracellular selectivity, but that the overall selectivity depends on a combination of the cell background and the expressed claudin.

MATERIALS AND METHODS

Plasmid constructs and cell lines. Plasmids encoding claudins-2, -4, and -15 have been described previously (3, 4, 22). A plasmid containing mouse claudin-11 was provided by Alexander Gow (Wayne State; see Ref. 11) and subcloned into the pTRE vector (Clontech Laboratories, Palo Alto, CA). Clonal cell lines of MDCK II Tet-off cells (Clontech Laboratories) were derived and selected with hygromycin B, as described previously (22). Clonal cell lines of Tet-off LLC-PK1 cells(18), generously provided by James Mullin (Lankenau Medical Research Center, Wynnewood, PA), were derived after cotransfection of the various claudin vectors with 1/20th the amount of the pSZevo vector (Invitrogen Life Technologies, Carlsbad, CA) and selection with 1 mg/ml Zeocin (Invitrogen). Stable cell lines were screened for transgene expression by immunoblot and homogeneous expression verified by immunofluorescent analysis. At least four stable cell lines were generated for each construct. All cell lines were maintained in the presence of 50 ng/ml doxycycline to repress transgene expression. For experiments, all cells were plated on removable filters (Snapwell; Corning Life Sciences, Acton, MA) in doxycycline-containing (uninduced) or doxycycline-free (induced) media, cultured for 4 days, and then used for physiological measurements and subsequent immunoblot analysis. For immunofluorescent analysis, cells were treated identically but cultured on clear filters (Transwell Clear; Corning) and processed as described below.

Immunoblots and immunofluorescence microscopy. Immunoblots were performed on cells grown on filters after induc- tion of protein and measurement of physiological properties. Filters were excised and placed directly in 150 μl SDS-sample buffer, incubated at 23°C for 10 min, and stored at -80°C until used for analysis. Equal volumes of lysate were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted, as described previously (3, 4, 22). Claudin-1, -2, -4, and -15, ZO-1, and occludin were detected using antibodies from Zymed Laboratories (South San Francisco, CA). Claudin-11 was detected with a monoclonal antibody generously supplied by A. Gow. Detection was performed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) after incubation in horseradish peroxidase-coupled secondary antibodies (anti-mouse from Chemicon International, Temecula, CA, and anti-rabbit from Amersham Biosciences). Immunofluorescent analysis of cell lines was performed as previously described (3, 4). Briefly, cells were fixed in ice-cold 100% ethanol and blocked in PBS plus 2% goat serum. ZO-1 was detected using a rat monoclonal antibody, R40.76, followed by Cy-3-labeled secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA); claudins-2, -4, and -15 were detected with Zymed antibodies, and claudin-11 (1:250) was detected by a monoclonal antibody from A. Gow followed by Cy-2-labeled secondary antibodies (Jackson Immunoresearch). Images were captured on a Zeiss LSM510 Meta laser scanning confocal microscope using a ×40 (numeric aperture 1.3) Plan-Neofluor objective lens. Images were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA).

Electrophysiological measurements. Electrophysiological measurements on the MDCK and LLC-PK1 cell monolayers were performed as described previously (3). Briefly, after transgene induction, filters were placed in a modified Ussing chamber with a microcomputer-controlled voltage-current clamp (Harvard Apparatus, Holliston, MA), and transmonolayer resistance was determined in a (apical and basal) solution (buffer A) containing 120 mM NaCl, 10 mM HEPES, pH 7.4, 5 mM KCl, 10 mM NaHCO3, 1.2 mM CaCl2, and 1 mM MgSO4. Dilution potentials were then determined after replacing the apical solution with a solution (buffer B) containing 60 mM NaCl, 10 mM HEPES, pH 7.4, 5 mM KCl, 10 mM NaHCO3, 1.2 mM CaCl2, 1 mM MgSO4, and 120 mM mannitol. The Henderson diffusion equation for univalent ions (15) was used to calculate liquid junction potentials under dilution potential conditions. Dilution potentials were identical in magnitude in both the apical and basal directions.

Because doxycycline repression of transgene expression was incomplete in the LLC-PK1 cells (see RESULTS) and because there were no statistical differences among uninduced MDCK cell clones, all induced cells are compared with parental cell lines. Statistical analysis was performed using Instat and graphs generated using GraphPad Prizm (GraphPad Software for Science, San Diego, CA).

RESULTS

Induced expression of claudins does not alter levels of other TJ proteins. Immunoblot analysis was used to verify inducible claudin expression in transfected cell lines (Fig. 1). Both MDCK and LLC-PK1 cells normally express claudin-2 and -4. Induction of transfected claudins resulted in large (∼10-fold) increases in the amount of these claudins in both MDCK (Fig. 1A) and LLC-PK1 (Fig. 1B) cells. Note that, because the relative
expression level of the claudin-2 transgene was so high in the LLC-PK1 cells, it was difficult to detect the endogenous claudin-2 expression on the same exposure. Neither claudin-11 nor claudin-15 was detected in untransfected cells, although it has not been established whether the available antibodies cross-react with the canine and porcine Claudins. Nevertheless, induction of the transgene proteins was easily verified in transfected cell lines. As was previously demonstrated in MDCK Tet-Off cells, each of the individual transgenes was tightly regulated by doxycycline (Fig. 1A and Refs. 3, 4, 22). In contrast, in all clones of LLC-PK1 Tet-Off cells, there was some transgene expression, even in the presence of doxycycline (Fig. 1B). In the case of claudin-15, this low level of transgene expression, although significantly less than in induced cells, was still able to confer a maximal change in the paracellular selectivity. Because of the leaky expression in the LLC-PK1 cells, LLC-PK1 cells induced to express maximal amounts of the transgenes were compared with the parental LLC-PK1 cells in both immunofluorescent and physiological analyses.

As we have seen previously (3, 4, 22), there was little reproducible effect of transgene induction on the expression levels of other Claudins, occludin or ZO-1. However, we only measured a small subset of known TJ proteins (5, 10) and cannot rule out effects on other components of this complex structure.

**Immunolocalization demonstrates induced Claudins are expressed at cell borders.** Indirect immunofluorescent confocal analysis of Claudin-expressing cell lines was used to determine the subcellular localization of the induced Claudins (Fig. 2). The localization of induced Claudins-2, -4, and -15 in MDCK II cells has been reported previously (3, 4, 22). Because of this, and because claudin-11 localization in MDCK II cells was similar to that in LLC-PK1 cells, only immunofluorescent images of induced Claudins in LLC-PK1 cells are shown (Fig. 2) and compared with localization of ZO-1. The localization of endogenous Claudins (-1, -2, and -4) was not affected by overexpression. In all Claudin-expressing LLC-PK1 cells, Claudins colocalized with ZO-1 at the apical junction, and there was also considerable intracellular localization. This is similar to that previously reported for Claudins-2, -4, and -15 in MDCK cells (3, 4, 22). In MDCK cells, all these Claudins increase the number of freeze-fracture fibrils (3, 4, 22; data for claudin-11 not shown). We have not determined the fibril patterns in LLC-PK1 cells but would expect on the basis of immunofluorescent analysis that this would also be true in this cell line.

**Effects of Claudin expression on TER varies between MDCK and LLC-PK1 cells.** Overexpression of the four Claudins in MDCK and LLC-PK1 cells did not confer a consistent pattern of change on the TER (Fig. 3A). At baseline, both the Tet-off MDCK II and LLC-PK1 cells are considered “leaky” monolayers, with the resistance of LLC-PK1 cells (100 Ω·cm²) about two times as high as that of MDCK cells (40–45 Ω·cm²). Expression of Claudin-4 in both cell lines resulted in significant increases in transmonolayer resistance (Fig. 3A; 3-fold in...
the MDCK cells and more than a 1.6-fold increase in the LLC-PK1 cells). Expression of either claudin-11 or -15 resulted in small increases in TER in the MDCK II cells but significant decreases in the LLC-PK1 cells. This pattern was similar to that for claudin-2 expression, which resulted in a very small (4) or no significant (Fig. 3A) increase in the MDCK II cells, but caused a significant decrease of TER in the LLC-PK1 cells. The decreases in TER in the LLC-PK1 cells, although marked, do not represent a loss of the TJ, since the resistances remained well above background and were in the range of the uninduced MDCK II cells.

Claudin expression alters charge selectivity in MDCK and LLC-PK1 cells. The effect of claudin expression on dilution potentials in MDCK II and LLC-PK1 cells is easier to rationalize than their effects on TER (Fig. 3B). As previously demonstrated, MDCK cells normally form a very cation-selective paracellular barrier, giving rise to dilution potentials of up to +8–11 mV. This is consistent with a higher permeability for Na+ than Cl− of ~3–4:1. When claudin-4 is expressed in this cell line, the paracellular pathway becomes less Na+ permissive, as evidenced by dilution potentials of about +2.5–4 mV (Fig. 3B and Ref. 22). When claudin-11 is expressed in MDCK II cells, the dilution potentials of the induced cells are very close to those seen when claudin-4 is expressed. In contrast to the MDCK II cells, LLC-PK1 cells normally form a Cl−-selective paracellular barrier that is slightly Cl− selective. When claudin-4 and -11 are expressed in the already Cl−-selective LLC-PK1 background, there is no detectable deviation from the baseline dilution potential. Conversely, claudin-2 and -15, which appear to form Na+ selective pores, do not appreciably change the dilution potentials of normally Na+-selective MDCK cells. However, in the Cl−-selective LLC-PK1 cells, expression of claudin-2 and -15 reverses the paracellular barrier, which now becomes permissive for Na+, with dilution

Fig. 2. Immunofluorescent analysis of LLC-PK1 cells expressing claudins-2, -4, -11, and -15. Cells were plated on filters and cultured for 4 days in the absence of doxycycline and stained for each claudin (left) and ZO-1 (middle). Images on right show the overlap of localization of the expressed claudins and ZO-1. Both claudin-2 and -4 were detectable at lower levels in untransfected LLC-PK1 cells (data not shown), and the localization was identical to that seen in transfected cells, except that the transfected cells have more intracellular accumulations of claudins. The localization of all claudins was very similar in MDCK cells (3, 4, 22) to that shown in LLC-PK1 cells, with claudin-4 on the lateral cell surface more prominent than at the apical cell junction. Bar = 20 μm for all images.
We had hypothesized that each claudin has its own properties. We expressed four different claudins in two cell lines that have opposite paracellular selectivity. This begins to address the question of whether claudins form pores, which like transmembrane channels have their own unique electrophysiological signature, or whether the cell or tissue background controls the pore properties. We expressed four different claudins in two cell lines that have opposite paracellular selectivity. We had hypothesized that each claudin has its own unique selectivity and that overexpression would overwhelm the endogenous claudins, thus making the final paracellular selectivity independent of the cell background. Instead, we found that the contribution of each claudin is only detected in a cell line with the opposite paracellular selectivity. In MDCK II cells, which have a highly cation-selective TJ, only the claudins that are less cation-selective, that is claudin-4 and -11, had detectable effects on the paracellular ion selectivity, whereas in the normally anion-selective LLC-PK1 cells only claudin-2 and -15 expression altered paracellular selectivity. These results support a model where each claudin has unique properties, and the overall physiological properties of the paracellular space result from the profile of different claudins expressed in that cell.

In these studies, the expression of exogenous claudins in MDCK II and LLC-PK1 cells appears to represent an addition rather than a substitution of claudin pores. This differs from recent findings by Yu and co-workers (25), who conclude that expression of very low levels of claudin-8 in MDCK cells resulted in substitution of claudin-8 for endogenous claudin-2. Although in our studies it was possible to induce large

**DISCUSSION**

In this study, we investigated how cell background influences the ability to observe the effects of exogenous claudins on paracellular charge selectivity. This results suggest that the final selectivity is influenced by both the exogenous claudin and the background pattern of other claudins, which would be unique to each cell type. Intermittent

potentials of about +5 mV. The charge selectivity properties of the exogenous claudins are therefore most easily seen in cell background that differs from the selectivity of the tested claudins. These results suggest that the final selectivity is influenced by both the exogenous claudin and the background pattern of other claudins, which would be unique to each cell type.

**DISCUSSION**

In this study, we investigated how cell background influences the ability to observe the effects of exogenous claudins on paracellular charge selectivity. This begins to address the question of whether claudins form pores, which like transmembrane channels have their own unique electrophysiological signature, or whether the cell or tissue background controls the pore properties. We expressed four different claudins in two cell lines that have opposite paracellular selectivity. We had hypothesized that each claudin has its own unique selectivity and that overexpression would overwhelm the endogenous claudins, thus making the final paracellular selectivity independent of the cell background. Instead, we found that the contribution of each claudin is only detected in a cell line with the opposite paracellular selectivity. In MDCK II cells, which have a highly cation-selective TJ, only the claudins that are less cation-selective, that is claudin-4 and -11, had detectable effects on the paracellular ion selectivity, whereas in the normally anion-selective LLC-PK1 cells only claudin-2 and -15 expression altered paracellular selectivity. These results support a model where each claudin has unique properties, and the overall physiological properties of the paracellular space result from the profile of different claudins expressed in that cell.
changes in the expression of all the transfected claudins, there was little effect on the level of any of the other TJ proteins that we measured (3, 4, 22). These proteins, including claudin-1, occludin, and ZO-1, are expected to interact both physically and functionally with claudins; thus, a lack of coincident increase in endogenous TJ proteins may represent aberrant organization of the new claudins. There is previous evidence for altered claudin organization in overexpression studies. For example, McCarthy and co-workers (16) found that in MDCK cells overexpressing claudin-1 tagged at the COOH terminus with a myc epitope, exogenous claudin was found in both apical TJ strands as well as the aberrant lateral TJ strands seen only in the overexpressing cells. Occludin, on the other hand, was only seen in normal TJ strands (16). In similar experiments, Kobayashi and colleagues (13) found that overexpression of myc-tagged claudin-1 resulted in large numbers of aberrant fibrils on the lateral cell membrane that by immunofreeze fracture were densely labeled with anti-myc antibody. The apical-most fibrils were much more sparsely labeled with anti-myc antibody. Although in both of these experiments the presence of the myc epitope at the COOH terminus would presumably prevent PDZ-dependent interactions with TJ plaque proteins, lowering the expression level of myc-tagged claudin-1 avoided aberrant fibril formation and resulted in its localization in the apical fibrils (13). It is likely in our MDCK and LLC-PK1 cells that high-level exogenous claudin expression could also lead to fibrils composed mostly of the transfected claudins without a normal complement of associated proteins.

The consequences of this lack of association with accessory proteins might explain the different effects of claudin expression on TER in the MDCK and LLC-PK1 cells. Overexpression of claudins has been reported previously to both increase (3, 16, 22) and decrease (1, 7) TER. In our hands, claudin-4 increased TER in both MDCK II and LLC-PK1 cells. As previously reported, claudin-2 had little effect on TER in MDCK II cells, whereas in LLC-PK1 cells it caused a decrease in TER similar to what has been described in MDCK I cells. However, expression of claudin-11 and -15, which both caused a small but significant increase in TER in MDCK cells, decreased TER in LLC-PK1 cells. Possible explanations include species differences or differences in the amount of accessory proteins that might result in differences in the organization of the exogenous claudins. Claudins apparently have the ability to polymerize into fibrils in the absence of a full complement of cytoplasmic binding partners (8). It is possible that without binding proteins the “open” or “closed” (2) state of the pores might be unregulated, leading to disruption of the TER. Claudin-4 may have a different set of interacting proteins, which could explain the fact that this claudin alone increases TER in both cell lines. Charge selectivity, which is thought to be the result of fixed charges lining the TJ pores (6, 14), would be insensitive to lack of regulation of the open or closed state of the new paracellular pores.

Recent results suggest that paracellular selectivity is primarily determined by the first extracellular domain of claudins (3, 4). Inspection of the amino acid sequence of the four claudins used in the present study reveals that the influential amino acids may lie in the second half of this loop. This corresponds to residue positions 52 to 77 of claudin-4 (Fig. 4A). Previous
mutagenesis analysis of claudin-15 pointed to the second and third acidic residues as being critical in defining the cation permeability characteristics of claudin-15, since charge reversal mutations at these positions changed this claudin from cation to anion permissive (3). Mutating a positive to negative residue on claudin-4 in the same region reduces its discrimination against paracellular movement of Na⁺ (3), again supporting the role of these charged residues in paracellular selectivity. Claudin-11, like claudin-4, has a positively charged residue at the same position; overexpression of this claudin produces a phenotype similar to that seen for claudin-4 in MDCK cells, namely discrimination against anions and vice versa. Although it is tempting to speculate that these residues are exposed to the extracellular selectivity of claudin-11, like claudin-4, has a positively charged residue at the same position; overexpression of this claudin produces a phenotype similar to that seen for claudin-4 in MDCK cells, namely discrimination against anions and vice versa. Although it is tempting to speculate that these residues are exposed to the extracellular space, it is not clear if they are indeed involved in paracellular selectivity.

The experiments described here indicate that, although expression of individual Claudins can change paracellular ionic selectivity, this effect is combined with the physiological characteristics of the expressing cell line. There are many Claudins expressed along the nephron in discrete and overlapping patterns. Presumably, their cell-specific profiles define the unique paracellular properties required to complement transcellular transport in each segment. We thank Dr. Oscar Colegio for innumerable helpful discussions.

DISCLOSURES

These studies were supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-45134 and DK-55389 and by the University of North Carolina at Chapel Hill.

REFERENCES