Role of claudin interactions in airway tight junctional permeability

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THE TIGHT JUNCTION (TJ) is a key regulator of ion and solute homeostasis, which is of particular importance in the airway, where airway surface liquid height and ionic composition are necessary for lung defense. Airway TJs also form a rate-limiting barrier to the penetration of inhaled pathogens into the interstitial compartment. The functional characteristics of airway TJs have suggested that they display relatively low transepithelial resistance \( R_t \), \( \sim 100 \ \Omega \text{cm}^2 \), and hence are described as moderately leaky (20). Such properties may be important for the alternation between absorption and secretion observed in the epithelium.

Epithelia and endothelia display highly diverse TJ barrier properties, which is likely indicative of the varied requirements for ion and solute regulation by tissue type. For example, \( R_t \) of the blood-brain barrier (BBB) is relatively high, 1,500–2,000 \( \Omega \text{cm}^2 \), where a decreased level of paracellular flow is imperative (6). Conversely, \( R_t \) in human placental endothelium is low, 22–52 \( \Omega \text{cm}^2 \), corresponding to an increase in the flow of nutrients to the fetus (19). That human airway \( R_t \) is of a moderate degree suggests that the relative leakiness of the junction is key in the regulation of normal airway functioning.

Several studies have investigated the role for airway epithelial TJs in the development of chronic disease (3, 5, 11, 18, 27). These studies have largely focused on TJ ultrastructure in diseased lungs, and most work investigating the correlation between the diseased airway and TJ ultrastructure has been conducted in patients with cystic fibrosis (CF) (7, 16). Although these studies identified differences in junctional ultrastructure in CF patients, these changes most likely occurred as a result of an acquired response due to the high degree of inflammation associated with the disease. Previous data have shown that exposure of primary airway cultures to cytokines upregulated in CF leads to modulation of airway barrier function with alterations in the expression and localization of junctional-associated proteins, which is consistent with an acquired response (10).

Regional differences in TJ function may also occur along the bronchial tree. Several studies have sought a correlation between location in the upper or lower airway and TJ morphology, but none of these studies found significant differences in strand morphology between the two pulmonary regions (12, 15). However, electrophysiological data have suggested that there may be inherent differences in the TJ along the human airway, with the junction becoming leakier with each successive branch (1, 2).

The claudin family of transmembrane proteins localized to the TJ now includes at least 20 members. The existence of such a large number of claudin family members might explain the disparity in TJ barrier function among epithelia. Analysis of claudin mRNA has shown a wide disparity in claudin expression...
among various tissue types (22), which may suggest that the inherent properties of TJs may be determined by their claudin expression pattern.

The variability of TJ barrier function combined with the unique pattern of claudin gene expression has led to speculation that claudin polymerization may affect the relative barrier of the TJ. Some data have suggested that a leaky junction could be formed when opposing cells express different claudins that do not form an interaction, thereby forming a pore in the junction and increased solute or ion flow (14). We explored the hypothesis shown that claudins may form such interactions in vivo by investigating the expression of claudin family members in freshly excised human airways of normal donor and diseased lungs by Western blot analysis and immunofluorescence microscopy. After identifying the claudins expressed at high levels in the airway TJ, we overexpressed these claudins alone or in various combinations in two cell lines, NIH/3T3 mouse fibroblast cells and the human airway epithelial cell line IB3.1. We then determined the effect of this expression on R, paracellular permeability coefficients (P_app), morphology of TJ strands by freeze-fracture analysis, and protein interactions among claudin species, to correlate airway claudin expression with airway TJ function.

MATERIALS AND METHODS

Plasmid constructs, transfections, and cell culture. Full-length human claudin-1, -3, and -5 were amplified with Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) from human airway cDNA isolated as previously described (10). The product was then cloned into the pCR blunt vector (Invitrogen) and subcloned into either the pcDNA3.1/Zeo− (Invitrogen) for claudin-3 and -5 or pIRES (Clontech) vector for claudin-1. All constructs were verified using DNA sequence analysis. Plasmids were transfected into cells using Geneporter 2 (Gene Therapy Systems) according to the manufacturer’s protocol. NIH/3T3 and IB3.1 cells were maintained as previously described (9, 29). Cells were placed under selection in the appropriate antibiotic (G418, Zeocin, or G418 and Zeocin) for 2–3 wk, and clones were screened for expression of the appropriate transgene by Western blot analysis (25–75 clones were screened per stable cell line).

Primary airway cells and freshly excised airways from human subjects were obtained in accordance with guidelines approved by the Institutional Committee on the Protection of the Rights of Human Subjects. Primary airway cells were cultured as described previously (17).

Transepithelial cell permeability. Permeation of FITC-dextran of 10 and 2,000 kDa across cells was measured at 5 days after plating of cells (2 × 10^5 cells/12 mm; 0.4-μm pore size) on Transwell-Col inserts as previously described (10). R_t measurements were also performed at this time point as previously described (9).

Western blotting. Lysates of NIH/3T3 and IB3.1 cells were prepared with 0.1% Triton X-100 extraction buffer containing PMSF and DTT. Equal amounts of protein (30 μg) were loaded onto Tris-glycine gels (Novex, San Diego, CA). After electrophoresis for 1 h at 200 V, protein was transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane at 33 V and blocked in 5% fat-free milk. Membranes were probed at antibody dilutions of 1:1,000 in Tris-buffered saline-Tween 20. Antibodies against claudin-1, -2, -3, -4, -5, -15, and -16 were purchased from Zymed Laboratories, and those against claudin-6, -7, -9, -10, and -11 were purchased from Santa Cruz Biotechnology. Protein was visualized with a peroxidase-conjugated secondary antibody (1:20,000) by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Human bronchi from donor lungs were dissected immediately after removal from patients undergoing clinical lung transplantation (freshly excised) and placed in ice-cold PBS, and epithelium was removed with a scalpel. Protein was isolated, and Western blotting was performed as previously described (10).

Perfluorooctanoic acid- and native PAGE. Samples were harvested from NIH/3T3 cells grown on Transwells for 3–5 days in either perfluorooctanoic acid (PFO) lysis buffer [25 mM NaH_2PO_4, 200 mM NaCl, and 4% (wt/vol) PFO] or 0.1% Triton X-100. For PFO-PAGE of freshly excised tissue, epithelium was removed and protein was harvested in PFO lysis buffer with sonication. PFO-PAGE was performed as described previously (21, 25).

Briefly, whole cell lysate (30 μg) was resuspended in sample buffer (100 mM Tris base, 8% PFO, 20% glycerol, and 0.005% bromphenol blue). Samples were loaded onto 4–20% Tris-glycine gels (Invitrogen) that had been preelectrophoresed at 50 V for 45 min in running buffer [25 mM Tris base, 192 mM glycine, and 0.5% (wt/vol) PFO (pH 8.0)]. Electrophoresis was performed at 80 V and transferred to PVDF for 1 h at 33 V. Protein bands were analyzed by Western blot analysis. For determination of separation of known standards, albumin for nonadenating PAGE and the protein mass marker kit (Sigma) were subjected to the above conditions.

For native PAGE, proteins isolated as described above were subjected to electrophoresis on 4–20% Tris-glycine gels according to the manufacturer’s protocol for native gel electrophoresis (Invitrogen).

Immunofluorescent labeling and confocal microscopy. Cells were washed and then permeabilized with 1% Triton X-100 for 10 min at room temperature, rinsed, and blocked with 1% BSA in PBS for 30 min at 37°C. Cells were then incubated with rabbit polyclonal antibodies against claudin-1, -3, or -5 (Zymed) for 1 h. After being washed, anti-rabbit Texas red antibody was incubated in 10% goat serum/PBS for 1 h at room temperature. All cells were postfixed with 4% paraformaldehyde. Images were captured with a confocal laser-scanning microscope (Leica, Exton, PA).

Freshly excised human bronchi from nondiseased (ND) subjects were embedded in optimum cutting temperature compound, and frozen sections (10 μm) were cut and stained as previously described (10).

Coimmunoprecipitation. NIH/3T3 cells grown for 5 days on Transwell inserts were washed briefly in PBS, the cell-impermeable, cleavable, 3,3’-dithio bis(sulfosuccinimidylproprionate) (DTSSP) cross-linker (2 mM, Pierce) was added to the apical compartment for 2 h at room temperature, and then 1 M Tris-HCl (pH 7.4) was added for 15 min at room temperature to quench the reaction. Cells were washed with PBS and lysed in 0.1% Triton X-100 lysis buffer containing PMSF by sonication and freeze-thawing. After a bicinechonic acid protein assay (Pierce), 30 μg of total protein were incubated with the indicated primary antibody for 1 h at 4°C, followed by addition of protein G conjugated to sepharose beads for an additional 1 h at 4°C. Beads were washed with PBS, SDS-sample buffer containing 5% 2-mercaptoethanol, and 10 mM DTT, added to the sample, and incubated for 15 min at 37°C and for an additional 10 min at 100°C. Western blotting was performed as we described earlier.
For coimmunoprecipitation experiments with freshly excised human airway, epithelium was removed as described above. After being removed, epithelium was incubated with 2 mM DTSSP for 2 h at room temperature, and immunoprecipitation and Western blotting was performed as described in Western blotting.

Freeze-fracture electron microscopy. NIH/3T3 cells grown to confluence for 5 days on Transwell inserts were fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C overnight. The cells were gently removed from the Transwell-Col with a scalpel and rinsed in phosphate buffer containing 0.2 M sucrose at room temperature, followed by a 25% glycerol cryoprotectant solution. Freeze-fracture was performed as described previously (10).

Statistics. Data are presented as means ± SE. A one-way analysis of variance and Bonferroni’s correction for multiple comparisons were used to determine statistical significance (P < 0.05).

RESULTS

Claudin expression in human airways. To determine the expression pattern of members of the claudin family in freshly excised human airways, we performed immunofluorescence staining followed by confocal microscopy of claudin-1, -2, -3, -4, -5, -6, -7, -9, -10, -11, -15, and -16. To investigate whether the expression of claudins was unique between the large and small airways, bronchi and bronchioles were isolated from seven patients. The characteristic epithelium of large bronchi is pseudostratified columnar, ciliated epithelium containing mucous-secreting goblet cells. As the bronchi undergo reduction in diameter, the cartilage support is lost, and there are few goblet cells, at which point the airway is termed a bronchiole. Both bronchi and bronchioles expressed claudin-1, -3, -4, -5, and -7, but none of the other claudin species screened (Fig. 1A).

The individual pattern of claudin localization in human airways was also unique. Staining of claudin-1 and -4 revealed increased localization to the apical-TJ region. However, there was also staining detected throughout the lateral intercellular junction, with staining surrounding basal cells that anchor the columnar epithelium to the basal lamina and have no contact with the luminal compartment. In contrast, claudin-3 and -5 were localized exclusively to the apical-most region of the TJ, with no staining below the level of the apical junction. Claudin-7 was localized primarily to the lateral intercellular junction, with little to no staining in the area of the TJ. Costaining with zonula occludens-1 (ZO-1) confirmed the localization of claudin-7 primarily below the level of the apical TJ complex (Fig. 1B). These data show that individual claudin species have unique localization in the airway.

Isolation and characterization of claudin transfectants. To characterize the functional consequences of claudins expressed in airway epithelium in vivo, we expressed them in stable clones alone or in various combinations, focusing on claudins localized at the apical-most region of the junctional complex. Because claudin-4 has been well characterized as a claudin conferring selective ion flow with no changes in paracellular solute permeability, we did not study the re-
sults of its stable expression on TJ permeability (26). Because claudin-7 localized exclusively to the lateral membranes, we did not generate stable transfectants expressing this member.

Claudin-1, -3, or -5 were stably expressed in 1) a cell line that expresses low levels of ZO-1 but that lacks any organized TJ, mouse fibroblast (NIH/3T3) cells, and 2) the CF human airway epithelial IB3.1 cell line, which expresses several TJ components, including ZO-1 and occludin (data not shown), and claudin-1 and -3 (Fig. 2). Stable clones of NIH/3T3 cells expressing claudin-1 (CL1), claudin-3 (CL3), or claudin-5 (CL5) alone or in combinations of claudin-1 and -3 (CL1.3) or claudin-1, -3, and -5 (CL1.3.5), were constructed. In IB3.1 cells, we overexpressed either CL1, CL3, or CL5 alone. Because claudin-1 and -3 are endogenously expressed in IB3.1 cells, the claudin-5-expressing clones expressed claudin-1, -3, and -5. Three clones expressing high-, intermediate-, and low-level claudin expression were selected (Fig. 2). In Fig. 2A, left, three high-expressing clones isolated from NIH/3T3 stable transfectants were probed with an antibody to the indicated claudin. In Fig. 2B, three high-expressing clones isolated from IB3.1 stable transfectants were probed with the indicated antibody. There was a low level of endogenous expression of claudin-1 and -3 in these cells.

To determine whether overexpression of claudins in NIH/3T3 and IB3.1 cells resulted in the localization of these proteins to sites of cell contact, we performed immunofluorescent localization of stable transfectants expressing high levels of the indicated claudin. We found that claudins expressed in both NIH/3T3 (Fig. 3A) and IB3.1 (Fig. 3B) cells concentrated at cell borders when grown to confluence.

Strand morphology in NIH/3T3 claudin-stable transfectants. We performed freeze-fracture analysis to determine the TJ strand morphology of NIH/3T3 stable transfectants expressing claudins singly or in various combinations. Claudin-1 transfectants displayed TJ strands composed largely of chains of particles forming random linear strands with numerous connections between strands (Fig. 4). Conversely, NIH/3T3 cells expressing claudin-3 formed nonlinear strands with few connections between strands. Although CL1 and CL3 strand morphology was random, the appearance of strands in CL1.3 transfectants was similar to those associated with polarized epithelia (Fig. 4). Quantification of CL1.3 strands revealed that TJs were composed of 3.4 ± 0.5 strands and that the junction itself displayed an average junctional depth of 0.402 ± 0.117 μm. These data correlate with previously published data correlating the degree of junctional leakiness with TJ strand morphology in epithelia of various types (8). Of note, the localization of TJ strands in all stable transfectants was random, as expected in nonpolarized cells.

Strands of NIH/3T3 cells expressing claudin-5 were distinct from all other claudin-stable transfectants. Whereas CL1, CL3, and CL1.3 transfectants formed TJ strands, CL5 transfectants formed TJ strands composed primarily of particles and particle arrays (PAs), similar to those seen in gap junctions (Fig. 4, see arrows). Western blot analysis and immunofluorescent microscopy did not show any increase in either the expression or localization of connexins known to be
expressed in NIH/3T3 cells, indicating that the PAs were formed due to claudin-5 (unpublished data). Although frequent areas were observed where PAs were expressed without accompanying TJ strands (Fig. 4), in all cases the appearance of TJ strands correlated with colocalization with PAs.

In contrast, there was a complete absence of PAs in CL1.3.5 transfectants (Fig. 4). TJ strands in CL1.3.5 transfectants associated primarily with the P face and were composed largely of single strands with repeated areas of breakage within the strands (Fig. 4). These data indicated that coexpression of claudin-1 and -3 with claudin-5 inhibited the formation of PAs.

Effect of claudin expression on $R_t$ and permeability. To determine whether claudin-stable transfectants could confer increased resistance to the flow of ions and solutes, we analyzed their effects on $R_t$. Both NIH/3T3 and IB3.1 cells have very low baseline $R_t$ values. Although CL1 and CL3 NIH/3T3 clones displayed a trend toward an increase in $R_t$ by 5 days after plating, only CL1.3 cells displayed a significant increase in $R_t$ over wild-type controls (Fig. 5A). A trend toward an increase in $R_t$ was detected in CL1 and CL3 IB3.1 clones that did not reach significance (Fig. 5B).

We also investigated the effects of claudin expression on $P_{app}$ to dextran. We chose three sizes of dextrans (10, 70, and 2,000 kDa) to determine whether there were changes in the relative pore size of the intercellular space induced by claudin overexpression. In NIH/3T3 cells, CL1 and CL3 clones did not cause significant changes in $P_{app}$ to the 10-, 70-, or 2,000-kDa dextrans (Fig. 5C). However, when both claudin-1 and -3 were coexpressed (CL1.3 cells), there was a significant decrease in $P_{app}$, with a 24, 42, and 70 ± 9% reduction in $P_{app}$ to the 10-, 70-, and 2,000-kDa dextrans, respectively, compared with wild type. In contrast, claudin-5-expressing clones showed significant increases in $P_{app}$, with a 75 ± 5, 509 ± 17, and 728 ± 78% increase in $P_{app}$ over wild type to the 10-, 70-, and 2,000-kDa dextrans, respectively. Of note, the level of increase in $P_{app}$ in cells expressing claudin-5 was reduced in clones also expressing claudin-1 and -3 (CL1.3.5). A decrease in $P_{app}$ of 22 ± 7% to the 10-kDa dextran compared with wild type was detected in CL1.3.5 cells, and an increase in $P_{app}$ to the 70- and 2,000-kDa dextrans of 30 ± 8% and 51 ± 7%, respectively, was detected. Therefore, expression of claudin-5 into CL1.3 cells is consistent with the reversal of the tighter CL1.3 phenotype to that of a leakier epithelium (Fig. 5C).

Transient expression of claudin-4 into NIH/3T3 stable transfectants did not change $P_{app}$ from wild type (unpublished data).

In IB3.1 cells, overexpression of claudin-1 and -3 caused a significant decrease in $P_{app}$ to all dextrans (Fig. 5D). Claudin-1-expressing clones decreased $P_{app}$ to 51 ± 3%, 57 ± 9%, and 64 ± 10% to the 10-, 70-, and 2,000-kDa dextrans, respectively, compared with wild type. Claudin-3 transfectants showed an even more pronounced decrease in $P_{app}$, 66 ± 8.8%, 76 ± 7.9%, and 86 ± 8.4% less than wild-type controls to increasing sizes of dextran. Similar to NIH/3T3 clones expressing claudin-5, IB3.1 transfectants expressing claudin-5, which also express claudin-1 and -3 endogenously at low levels, showed a significant increase in
Claudin localization and expression in diseased airways. Excised airway epithelia exhibit low $R_t$ values (~65–70 $\Omega$cm$^2$), whereas by comparison, well-differentiated primary cultures derived from human airways display high $R_t$ values (4) (Table 1). We have found that although freshly excised human airways express claudin-1, -3, -4, -5, and -7, primary cultures of these airways express only claudin-1 and -4 (10).

Because published data have shown upregulation of claudin-5 after exposure of primary rat alveolar cells to an agent that modulates the TJ (methanandamide) (28), we evaluated whether localization and expression of claudin-5 were altered in diseased airways. Immunofluorescent localization of claudin-5 was performed in excised human airways from ND, primary ciliary dyskinesia, primary pulmonary hypertension (PPH), $\alpha_1$-antitrypsin deficiency, and CF patients. The localization of claudin-5 was not altered between ND and diseased airways (Fig. 7A). Furthermore, there were no differences in fluorescence intensity between ND and diseased airways (data not shown). We also found that the expression of all claudins screened was not different between non-CF (including ND and PPH) and CF patients by Western blot analysis (Fig. 7B).

Oligomerization of claudin species. Because TJ strands seen by freeze-fracture analysis resemble the relative size (10 nm) of other multimeric protein complexes, such as gap junctions and aquaporin-4 arrays, $P_{\text{app}}$ to all dextrans compared with wild-type controls. Transient transfection of claudin-4 into any IB3.1 stable transfectants did not alter the relative change in $P_{\text{app}}$ vs. wild-type controls (unpublished data).

A correlation between the level of claudin expression and the percent change in $P_{\text{app}}$ was also detected. Although NIH/3T3 stable transfectants expressing high levels of claudin-5 induced significant increases in $P_{\text{app}}$ to all sizes of dextrans, this increase was directly related to the level of claudin-5 expression (Fig. 6A). Similarly, IB3.1 stable transfectants expressing low levels of claudin-3 exhibited $P_{\text{app}}$ values similar to wild-type controls, whereas cells expressing low levels of claudin-5 exhibited $P_{\text{app}}$ 50% over wild-type controls (Fig. 6B). Overexpression of high levels of claudin-3 in IB3.1 cells correlated with a significant decrease in $P_{\text{app}}$ values compared with wild type, which was less prominent when claudin-3 was expressed at low levels (Fig. 6F). In contrast, expression of claudin-5 in IB3.1 cells at high, intermediate, or low levels increased $P_{\text{app}}$ to 338 ± 13.5, 202 ± 8.8, or 155 ± 10.1, respectively. These data indicate that the changes in $P_{\text{app}}$ exhibited in NIH/3T3 and IB3.1 stable transfectants were due solely to the expression of the exogenous protein.

The discrepancy in the fold increase in $P_{\text{app}}$ induced by overexpression of claudin-5 between CL5 and CL1.3.5 cells led us to investigate whether coculturing of NIH/3T3 cells singly expressing claudin-1, -3, and -5 could prevent the significant increase in $P_{\text{app}}$ in CL5 cells. Coculturing CL1 and CL5 cells (CL1.CL5) had no effect on the increase in $P_{\text{app}}$, whereas a partial inhibition of this increase was measured in CL3 and CL5 cells (CL3.CL5). $P_{\text{app}}$ in CL5 and CL1.CL5 to the 10-, 70-, and 2,000-kDa dextrans was ~180, 350, and 800% over wild type, whereas $P_{\text{app}}$ in CL3.CL5 cells was 112 ± 8.8%, 222 ± 10.2%, and 377 ± 16.1%, respectively (Fig. 6C). These data indicate that the inhibition of the increase in $P_{\text{app}}$ induced by overexpression of claudin-5 in CL1.3.5 cells may be due to possible interaction(s) between claudin-3 and -5.
we investigated whether claudins oligomerize to form multimeric complexes capable of inserting into the membrane and forming pores. Recent data have shown that purified claudin-4 forms oligomers, with the largest proportion in a hexameric configuration, and that this may contribute to its formation of cation-selective pores (21). Whether claudins form oligomers in a cell system remains undetermined.

To investigate this possibility, we performed native gel electrophoresis of NIH/3T3 claudin-stable transfectants. In claudin-1-expressing cells subjected to native gel electrophoresis, protein migrated as a diffuse band with an apparent molecular mass of ~30–40 kDa. In contrast, claudin-3 and -5 each migrated between 55 and 130 kDa (Fig. 8A, left). The slower migration of claudin-3 and -5 in the native gel than in heated, denatured samples, which typically migrate between 22 and 23 kDa, indicated the presence of complexes. These data also indicated that claudin-3 and -5 formed oligomeric structures, which were either absent or undetectable in cells expressing claudin-1. However, the difficulty in determining the exact molecular weight of bands by native gel electrophoresis prevented a determination of the stoichiometry of the oligomeric configuration.

In contrast, PFO-PAGE allows for the retention of multimeric complexes while allowing for accurate determination of molecular weight (25). No significant oligomerization of claudin-1 or -3 was detected by PFO-PAGE except for minor formation of pentamer and hexamer in claudin-3-expressing cells. In contrast, cells expressing claudin-5 exhibited banding patterns consistent with the formation of monomer (23.1 kDa), dimer (46.2 kDa), trimer (69.3 kDa), tetramer (92.4 kDa), pentamer (115.5 kDa), and hexamer (138.6 kDa) (Fig. 8A). Moreover, while claudin-1 and -3 appeared to exist predominately as monomer, claudin-5 distributed equally into monomeric, trimeric, or tetrameric config-

Table 1. Transepithelial resistance properties of human airway epithelium

<table>
<thead>
<tr>
<th>Epithelium</th>
<th>Transepithelial Resistance, Ωcm²</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Excised airways</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-cystic fibrosis</td>
<td>66.7 ± 2.7</td>
<td>(4)</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>78.9 ± 5.0</td>
<td>(4)</td>
</tr>
<tr>
<td>Well-differentiated human airway epithelial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-cystic fibrosis</td>
<td>892 ± 86</td>
<td></td>
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<tr>
<td>Cystic fibrosis</td>
<td>867 ± 92</td>
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Values are means ± SE. For well-differentiated human airway epithelial cells, n = 6.
The oligomeric structures of Claudin-5 were maintained when cells were lysed with either Triton X-100 or 4% PFO (unpublished data). When subjected to SDS-PAGE, there was partial retention of the tetrameric, pentameric, and hexameric conformations of Claudin-5, indicating that SDS was incapable of completely dissociating Claudin multimeric complexes at this concentration (Fig. 8). The Claudin-5 oligomers present by PFO-PAGE fit a log-linear relationship, confirming correct migration of complexes (Fig. 8B).

Oligomerization of Claudin species in a cell line with high levels of stable expression suggested that the organization of Claudins into multimeric complexes may occur in vivo. To determine whether homooligomerization of Claudin-1, -3, and -5 occurred in human airway epithelium, we harvested protein from human bronchi after lung transplant and subjected protein to PFO- and SDS-PAGE. Claudin-1 and -3 existed predominantly in monomeric form, whereas Claudin-5 existed predominantly in pentameric and hexameric configurations, with less significant formation of monomer, dimer, and trimer, and no significant formation of tetramer (Fig. 9A). These oligomeric protein complexes detected in protein isolated from freshly excised bronchial specimens also migrated in the appropriate log-linear fashion (Fig. 9B).

Coimmunoprecipitation of Claudins. To determine whether Claudin species could form heteromultimers, we coimmunoprecipitated Claudins coexpressed in NIH/3T3 cells. To compare these interactions with what occurs in the airway in vivo, we also performed coimmunoprecipitations in protein isolated from freshly excised human airways. To stabilize the potential interactions between Claudins, a cross-linker was added to the cells before lysing and subsequent immunoprecipitations in some experiments.

In CL1.3 NIH/3T3 stable transfectants, Claudin-1 and -3 were coimmunoprecipitated with antibodies to either protein (Fig. 10A). There was also coimmunoprecipitation of these proteins in CL1.3.5 NIH/3T3 cells (Fig. 10A) but to a lesser extent than in CL1.3 cells. Coimmunoprecipitation of Claudin-3 and -5 was also detected in CL1.3.5 cells (Fig. 10B), but Claudin-1 and -5 did not coimmunoprecipitate (Fig. 10C). Addition of cross-linker to cells had no effect on the degree of coimmunoprecipitation, indicating that either the interactions formed between Claudin species were of sufficiently high affinity to be stable during immunoprecipitation, that the high level of expression in stable transfectants negated the use of a cross-linker, or that the interactions occurred independent of their extracellular domains, suggesting possible intracellular interactions.

When CL1 and CL3 and CL3 and CL5 cells were cocultured, there was coimmunoprecipitation of heterogeneous Claudins, suggesting extracellular domain-specific interaction (data not shown). When protein harvested from singly expressing clones was added at equal concentrations and subjected to the same protocol, there was a small level of coimmunoprecipitation of Claudin-3 and -5, but not Claudin-1 and -3 or -1 and -5, indicating that there were interactions between Claudin-3 and -5 during the coimmunoprecipitation process. Because the amount of coimmunoprecipitation between Claudin-3 and -5 was small under these con-

![Fig. 7. Expression and localization of Claudin species in freshly excised nondiseased (ND) and diseased human airway epithelium.](http://ajplung.physiology.org/)

**A:** bronchi from ND, primary ciliary dyskinesia (PCD), primary pulmonary hypertension (PPH), α1 antitrypsin deficiency (A1AD), and cystic fibrosis (CF) patients were isolated and prepared for staining as described in MATERIALS AND METHODS. Blue, DAPI-stained nuclei; red, positive protein staining. **Left:** DIC image. Images are representative of bronchi isolated from 7 ND, 1 PCD, 2 PPH, 1 A1AD, and 7 CF patients. **B:** Western blot analysis protein isolated from 7 non-CF (5 donor and 2 PPH, asterisks represent the 2 PPH) and 7 CF patients.
ditions, the majority of claudin-3 and -5 interactions likely occurred in situ.

To determine whether the heterophilic associations occurring between claudin species in NIH/3T3 stable transfectants also occurred in the human airway in vivo, we performed the same coimmunoprecipitation experiments in excised human airway epithelium. We detected coimmunoprecipitation of claudin-1 and -3

Fig. 8. Western blot analysis of claudins by native-PAGE, perfluorooctanoic acid (PFO)-PAGE, and SDS-PAGE. A: Western blot analysis of 30 μg of protein harvested from the indicated NIH/3T3 stable transfectants subjected to either native-, PFO-, or SDS-PAGE. Whereas claudin-1 and -3 exist primarily as monomers, there are 6 immunoreactive bands identified in stable transfectants expressing claudin-5 (labeled ×1–×6). B: relative migration of multimeric species in cells expressing claudin-3 or -5 (in mm) plotted against the logarithm of the predicted oligomer molecular mass (in kDa).
Airway TJs are a critical component in the regulation of airway paracellular permeability to ions and pathogens. To investigate the molecular components that confer permeability properties to the airway TJ, we analyzed the expression of the claudin family of transmembrane proteins in bronchi and bronchioles and then constructed stable cell lines expressing claudins expressed in airway epithelium. Although expression of claudins in L-fibroblasts can form TJ strands by freeze-fracture analysis, the effects of claudin expression on TJ properties, such as $R_t$ and $P_{app}$, have not been reported in detail (13, 14), and interactions between claudin family members in these cells have not been extensively studied (14). However, coexpression of claudin-1 and -3 in these cells led to the formation of unique TJ strand morphology by freeze-fracture analysis that suggested an interaction between these two claudin species (14).

Expression of claudin-1, -3, -4, -5, and -7 was detected by immunofluorescent localization in both bronchi and bronchioles (Fig. 1A). The claudins expressed (Fig. 10D) and -3 and -5 (Fig. 10E), but not -1 and -5 (Fig. 10F), similar to the results found in the NIH/3T3 stable transfectants. In contrast to NIH/3T3 stable transfectants, cross-linking of human airway epithelium increased the level of protein coimmunoprecipitation, indicating stabilization of the protein interaction(s).

**DISCUSSION**

Airway TJs are a critical component in the regulation of airway paracellular permeability to ions and pathogens. To investigate the molecular components that confer permeability properties to the airway TJ, we analyzed the expression of the claudin family of transmembrane proteins in bronchi and bronchioles and then constructed stable cell lines expressing claudins expressed in airway epithelium. Although expression of claudins in L-fibroblasts can form TJ strands by freeze-fracture analysis, the effects of claudin expression on TJ properties, such as $R_t$ and $P_{app}$, have not been reported in detail (13, 14), and interactions between claudin family members in these cells have not been extensively studied (14). However, coexpression of claudin-1 and -3 in these cells led to the formation of unique TJ strand morphology by freeze-fracture analysis that suggested an interaction between these two claudin species (14).

Expression of claudin-1, -3, -4, -5, and -7 was detected by immunofluorescent localization in both bronchi and bronchioles (Fig. 1A). The claudins expressed.
in the airway exhibited a unique pattern of localization. Both claudin-1 and -4 localized not only to the area corresponding to the columnar cell TJ, but also throughout the lateral intercellular space, with localization also to basal cells. Basal cells of airway epithelia are pluripotent cells that have no known role in barrier function. In contrast, both claudin-3 and -5 localized exclusively to the apical-TJ region in both bronchi and bronchioles. Moreover, claudin-7 was localized to the lateral intercellular junction, with no staining in the region of the TJ (Fig. 1B). Despite data that has shown regional differences in $R_t$ along the bronchial tree, we found the same pattern of expression of the claudins in both bronchi and bronchioles (Fig. 1A) (1, 2). Although these data do not exclude inherent differences in the TJs between large and small airways, they do confirm that the pattern of expression of many claudins is retained along the bronchial tree.

To determine the functional significance of the pattern of expression of claudin species in airway epithelium, we constructed cell lines stably expressing claudin-1, -3, and -5 (Fig. 2, A and B). These claudins localized to the sites of cell contact in stable cell lines, and freeze-fracture analyses revealed unique strand morphologies between claudin-stable transfectants. Whereas CL1 and CL3 cells formed unorganized strands, CL1.3 cells formed strands that appeared to be similar in depth and strand number to data published in leaky epithelia (Fig. 4) (8). Claudin-5-expressing transfectants displayed TJ strands composed of both particles and PAs (Fig. 4). Although previously published data have shown the formation of TJ strands in fibroblasts expressing murine claudin-5, there was no evidence of PA formation, indicating that the formation of PAs may be unique to the human form (23).

We next determined whether claudin-stable transfectants displayed changes in permeability properties of the TJ, as assessed by $R_t$ and $P_{app}$ to dextrans of 10, 70, and 2,000 kDa. We found a significant increase in $R_t$ on day 5 in NIH/3T3 cells coexpressing claudin-1 and -3, which suggested that coexpression of these claudins led to heterophilic interactions, thereby conferring increased adhesiveness between neighboring cells (Fig. 5A). This change in $R_t$ was maximal at 5-day postplating, at which point NIH/3T3 and IB3.1 cells on Transwells were multilayered, indicating that the increase in $R_t$ may be due largely to the increased interactions between claudin-expressing cells throughout the cell layers. Although CL1.3 NIH/3T3 cells caused an increase in $R_t$, $R_t$ in CL1.3.5 cells was not different from wild type, suggesting that expression of claudin-5 altered the interaction between claudin-1 and -3 that increased $R_t$ or reduced $R_t$ independently.

Measurements of $P_{app}$ to dextrans showed that CL1.3 NIH/3T3 cells exhibited a significant decrease in $P_{app}$ to the 70- and 2,000-kDa dextrans (Fig. 5C), similar to the change induced in $R_t$. CL3 IB3.1 cells also displayed a slight decrease in $P_{app}$ to all sizes of dextrans. However, in both NIH/3T3 and IB3.1 cells, there was a significant increase in $P_{app}$ in CL5 stable transfectants (Fig. 5, C and D). Whereas claudin-1 and -3 coexpression increased $R_t$ and decreased $P_{app}$ in NIH/3T3 cells, expression of claudin-5 in CL1.3 cells reversed the decrease in $P_{app}$ to levels greater than or equal to those measured in wild-type cells. Coculture of NIH/3T3 cells singly expressing claudin-3 or -5 also partially inhibited the increase in $P_{app}$ induced by claudin-5 expression, suggesting a possible functional interaction between claudin-3 and -5 to decrease permeability. The regulation of permeability by coculturing of CL3 and CL5 clones was confirmed as arising from protein-protein interactions by coimmunoprecipitation studies in CL1.3.5 cells and in freshly excised human airways (Fig. 10).

The data from the measurements of $R_t$ and $P_{app}$ in NIH/3T3 and IB3.1 stable transfectants suggest that coexpression of claudin-1 and -3 decreased $P_{app}$, creating a tighter epithelium, whereas expression of claudin-5 singly or in combination with claudin-1 and -3 reversed the epithelium to a leakier phenotype. To address the physiological significance of this observation, we compared the electrophysiological characteristics of normal and diseased airways compared with well-differentiated cultured models. The airway epithelium in vivo is a moderately leaky epithelium displaying $R_t$ $>$ 100 $\Omega$cm$^2$ in studies of excised tissues (4).

In contrast, well-differentiated primary cultures derived from this tissue display a significantly greater $R_t$, $>$ 800 $\Omega$cm$^2$ (Table 1). Although excised airways express claudin-5 at a high level, the expression of this claudin is absent in the well-differentiated primary airway cell culture model (10). This finding is consistent with the observation that expression of claudin-5 in cells coexpressing claudin-1 and -3 led to a reversal of the decrease in $P_{app}$. Furthermore, when claudin-5 was expressed in IB3.1 cells, which endogenously express claudin-1 and -3, there was an increase in $P_{app}$. These findings agree with recently published data showing that increased expression of claudin-5 in alveolar epithelium correlated with an increase in permeability induced by methanandamide (28). Thus claudin-5 in epithelia may be responsible for the creation of a relatively leaky epithelium permitting the passive transduction of solutes, like albumin, to airway surfaces as a normal component of airway homeostasis.

The observation in IB3.1 cells and alveolar epithelial cells that expression of claudin-5 induced an increase in $P_{app}$ to low- and high-molecular-weight dextrans differs from its expression in the tight endothelium of the BBB (23, 24, 28). That claudin-5-deficient mice exhibit an increase in $P_{app}$ to small solutes ($<$ 800 Da) is suggestive of a loss of heterophilic interactions between claudin species, presumably between claudin-5 and -12 (24).

The appearance of particles within TJ strands by freeze-fracture analysis has led to speculation that the oligomeric assembly of claudins into multimeric complexes forms the backbone of the TJ. Recently, purified claudin-4 isolated from S9 insect cells was shown to oligomerize by PFO-PAGE and sucrose velocity centrifugation (21). We performed native gel electrophoresis
to determine whether claudins oligomerized in NIH/3T3 stable transfectants expressing claudin species. Our data show that whereas claudin-1 does not form oligomers in our cell system by PFO-PAGE, cells expressing claudin-3 and -5 do (Fig. 8). These data may indicate either that claudin-1 does not form oligomers or that the formation of these complexes is less stable than those in the other claudin species we analyzed and may dissociate under the conditions employed. Claudin-3 was found predominantly in monomeric form but did form oligomers. Claudin-5 was found as a monomer, dimer, trimer, tetramer, pentamer, and hexamer, with the largest proportion associated with the monomeric and tetrameric forms.

That claudin-5 homooligomers were present in freshly excised human epithelium supports the formation of claudin multimeric configurations in vivo. Although claudin-5 expressed in NIH/3T3 cells existed predominantly as a monomer, trimer, and tetramer, the configuration in human airway epithelium was concentrated almost exclusively in the pentameric and hexameric configurations. These data may indicate that the lack of polarization and expression of junctional-associated proteins in NIH/3T3 cells may inhibit the proper oligomerization of claudin-5.

The disparity in the permeability between CL5 and CL1.3.5 cells, the unique morphology of CL5 cells compared with CL1.3.5 cells, and the partial inhibition of the increase in \( P_{\text{app}} \) by coculturing cells, led us to speculate that there were heterophilic interactions between claudin-1, -3, and -5 that were altering the appearance of TJ strand morphology. To investigate this possibility, we performed coimmunoprecipitation experiments with CL1.3- and CL1.3.5-expressing cells. We found that in both cell types, there was interaction between claudin-1 and -3 (Fig. 10A). Although CL1.3 and CL1.3.5 cells express similar amounts of both claudin-1 and -3, there was a distinct decrease in the claudin-1 and -3 interaction in CL1.3.5 cells. Although claudin-1 and -5 did not coimmunoprecipitate (Fig. 10C), there was a strong interaction between claudin-3 and -5 (Fig. 10B). Coimmunoprecipitations of claudin-1, -3, and -5 in protein harvested from freshly excised human airways confirmed that the heterophilic interactions detected in NIH/3T3 transfectants also occurred in the human airway in vivo (Fig. 10, D–F).

Thus we have evaluated the claudin species responsible for conferring the selective flow of solutes through the airway TJ. Claudin-1 and -3 interact to decrease permeability to tighten the epithelium, whereas claudin-5 expression increases permeability, creating a leakier epithelium. Heterophilic interactions between claudin-1 and -3 and claudin-3 and -5 appear to limit the relative degree of change in solute permeability induced by claudin-5 expression. No significant alterations in claudin-5 expression appeared to occur in supplicative airway disease from human subjects, but we do not know whether claudin-5 levels change in conditions such as asthma and chronic obstructive pulmonary disease. Nevertheless, these data from stable cell lines and freshly excised human airways indicate that the relative permeability properties of airway TJs under basal conditions are mediated by direct interactions among claudin species.

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DISCLOSURES

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