Regulation of Heterotypic Claudin Compatibility*

Brandy L. Daugherty, Christina Ward, Tekla Smith, Jeffrey D. Ritzenthaler, and Michael Koval

From the Division of Pulmonary, Allergy and Critical Care Medicine, Emory University School of Medicine, Atlanta, Georgia 30322

Tissue barrier function is directly mediated by tight junction transmembrane proteins known as claudins. Cells that form tight junctions typically express multiple claudin isoforms which suggests that heterotypic (head-to-head) binding between different claudin isoforms may play a role in regulating paracellular permeability. However, little is known about motifs that control heterotypic claudin compatibility. We found that although claudin-3 and claudin-4 were heteromerically compatible when expressed in the same cell, they did not heterotypically interact despite having extracellular loop (EL) domains that are highly conserved at the amino acid level. Claudins-1 and -5, which were heterotypically compatible with claudin-3, did not heterotypically bind to claudin-4. In contrast, claudin-4 chimeras containing either the first EL domain or the second EL domain of claudin-3 were able to heterotypically bind to claudin-1, claudin-3, and claudin-5. Moreover, a single point mutation in the first extracellular loop domain of claudin-3 to convert Asn44 to the corresponding amino acid in claudin-4 (Thr) produced a claudin capable of heterotypic binding to claudin-4 while still retaining the ability to bind to claudin-1 and -5. Thus, control of heterotypic claudin-claudin interactions is sensitive to small changes in the EL domains.

The presence of multiple claudins in a single cell can confound the analysis of head-to-head (heterotypic) interactions between claudins on adjacent cells. Given this, initial studies of claudin-claudin associations were performed using fibroblasts that do not express endogenous claudins. Claudins expressed by transfected fibroblasts assemble into structures reminiscent of tight junction strands as analyzed by freeze-fracture immunogold electron microscopy. Co-cultures of L-cells or 3T3 fibroblasts transfected to express different claudins have been used to demonstrate that claudin-1 heterotypically binds to claudin-3 but not claudin-2 or claudin-5 (13, 14). Conversely, claudin-2 and claudin-5 heterotypically bind to claudin-3 but not claudin-1. Thus, the compatibility of claudins for head-to-head binding is not easily predicted.

We have found that HeLa cells are claudin-null yet express several other tight junction proteins which modulate tight junction integrity, including occludin, junction adhesion molecule-A (JAM-A)2, and scaffold proteins such as Zona Occludens (ZO)-1, ZO-2, and ZO-3. Although some of these proteins are also expressed by fibroblasts, fibroblasts do not express occludin (15). Although occludin expression is not required for epithelial barrier function (16, 17), it does modulate tight junction formation when expressed (18–20) and could potentially alter claudin-claudin interactions. We used HeLa clones stably expressing single or multiple claudins to examine the ability of claudin-1, claudin-3, claudin-4, and claudin-5 to interact with each other. Although the extracellular loop domains of claudin-3 and claudin-4 are highly conserved, claudins that interact with claudin-3 did not heterotypically bind to claudin-4. Furthermore, we found that although claudin-3 and claudin-4 did not heterotypically bind to each other, they did form heteromeric complexes. Using a series of claudin-3/4 chimeras, we found that the specificity of heterotypic claudin compatibility was sensitive to changes in either extracellular loop domain.

**EXPERIMENTAL PROCEDURES**

Recombinant DNA—Human claudin cDNAs were obtained from the American Type Culture Collection (ATCC). The constructs were amplified by PCR using primers containing either EcoRI and XhoI restriction sites or, in the case of claudin-3, HindIII and BamHI sites and then inserted into pcDNA3 and pcDNA3.1/hygro by enzyme digestion and ligation. Claudin-3/-4 chimeras were produced by two-step PCR amplification using established protocols (21). Chimeras produced were claudin-3e2-4 (claudin-31–29 + claudin-431–81 + claudin-381–222), claudin-3e3-4 (claudin-31–143 + claudin-4145–160 + claudin-

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1 To whom correspondence should be addressed: Emory University School of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, Whitehead Biomedical Research Building, 615 Michael St, Suite 205, Atlanta, GA 30322. Tel.: 404-712-2976; Fax: 404-712-2974; Email: mhkova@emory.edu.

2 The abbreviations used are: JAM-A, junction adhesion molecule-A; ZO, Zona Occludens; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; PBS, phosphate-buffered saline; EL, extracellular loop.
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![Image](60x523 to 396x733)

**FIGURE 1. HeLa cells do not express claudins.** a, by immunoblot, HeLa cells (H) were compared with claudin-expressing epithelial cells (+, usually T84 cells, see "Experimental Procedures") for expression of claudin-1, -2, -3, -4, -5, -7, -8, and -18. In contrast to controls, HeLa cells did not express any of these claudins. b, in contrast, HeLa cells were found to express several tight junction proteins, including occludin, ZO-1, ZO-2, ZO-3, and JAM-A.

3\(^{161–222}\), claudin-3e1,2-4 (claudin-3\(^{1–29}\) + claudin-4\(^{31–81}\) + claudin-3\(^{81–143}\) + claudin-4\(^{145–160}\) + claudin-3\(^{160–222}\), claudin-4e1-3 (claudin-4\(^{1–30}\) + claudin-3\(^{30–80}\) + claudin-4\(^{82–209}\)), claudin-4e2-3 (claudin-4\(^{1–144}\) + claudin-3\(^{144–159}\) + claudin-4\(^{161–209}\)), claudin-4e1,2-3 (claudin-4\(^{1–144}\) + claudin-3\(^{30–80}\) + claudin-4\(^{82–144}\) + claudin-3\(^{144–159}\) + claudin-4\(^{161–209}\)). The claudin-3N44T and -3N44R constructs were produced using the Qiagen QuikChange kit, and N-terminal-tagged CFP and YFP-tagged claudin-5 were prepared using standard restriction digestion and ligation reactions to insert the claudin-5 coding sequence into pECFP-C3 or pYFP-C3. All cDNA constructs were digested and ligated reactions to insert the claudin-5 coding sequence into pECFP-C3 or pYFP-C3. All cDNA constructs were then transfected into bacteria using the Qiagen Miniprep kit according to the manufacturer’s instructions.

**Antibodies**—Monoclonal antibodies recognizing claudin-4 and -5 and polyclonal antibodies recognizing claudin-1, -2, -3, -4, -5, -7, -8, and -18 JAM-A, occludin, and ZO-1 were from Invitrogen/Zymed Laboratories Inc. (San Francisco, CA). Rabbit anti-ZO-2 and ZO-3 were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Cell Culture**—HeLa cells were cultured in minimum essential medium containing Earle’s salts, l-glutamine, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Madin-Darby canine kidney cells were cultured in Dulbecco’s modified Eagle’s medium containing 1000 mg/liter d-glucose, l-glutamine, pyridoxine hydrochloride, 110 mg/liter sodium pyruvate, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. FuGENE 6 transfection reagent from Roche Diagnostics was used to obtain cells stably expressing various claudin constructs. Cells were plated 1 day before transfection in 35-mm culture dishes in media lacking antibiotics to achieve a density of 60% confluence for transfection. Briefly, 6 μl of FuGENE 6 was diluted dropwise to 100 μl of Opti-MEM followed by the addition of 2 μg of plasmid DNA. Tubes were incubated for 30 min at room temperature to allow for DNA-FuGENE 6 complex formation, and tube contents were then added to cells. The cells were incubated 48 h with the DNA complexes, the transfection efficiency was examined using immunofluorescence, and stably transfected cells were selected in culture medium containing 2 mg/ml Geneticin (Invitrogen). Doubly transfected cells were also selected using 2 mg/ml hygromycin. For co-culture studies cells were plated together and then passed three times as a co-culture population before analysis.

**Immunofluorescence**—Cells were plated on glass coverslips (Fisher) for immunofluorescence staining. Cells were rinsed 3 times with phosphate-buffered saline, pH 7.4, containing 2 mM CaCl\(_2\) and 1 mM MgCl\(_2\) and fixed for 2 min at room temperature with equal volumes of methanol and acetone. Cells were then rinsed twice with PBS lacking divalent cations (PBS) followed by a 5-min incubation in PBS + 0.5% Triton X-100 (PBS-Tx) and a subsequent 5-min incubation in PBS-Tx + 2% goat serum (Sigma-Aldrich). Cells were incubated with the appropriate primary antibody diluted in PBS containing 2% goat serum for 1 h at room temperature. Cells were washed 3 times with PBS containing 2% goat serum (PBS-GS) and then incubated for 1 h at room temperature with the appropriate secondary antibody diluted in PBS-GS. Cells were subsequently rinsed once with PBS containing 2% goat serum and 3 times with PBS alone before mounting in Mowiol-488 (Calbiochem). For simultaneous staining of claudin-1 and claudin-3, cells were blocked with PBS containing 0.5% Triton X-100 and 2% donkey serum, incubated with rabbit anti-claudin-1, washed, incubated with excess goat anti-rabbit IgG Fab fragment, washed, incubated with Cy3-conjugated donkey anti-goat IgG, washed, incubated with rabbit anti-claudin-3, washed, incubated with Cy2-conjugated donkey anti-rabbit IgG, washed, and mounted. A negative control demonstrating that this approach did not show cross-reactivity is shown in supplemental Fig. 1.

Immunofluorescence was visualized by fluorescence microscopy using an Olympus X-70 microscope system and imaged with a Hamamatsu Orca-1 CCD camera and acquired and quantified using Image Pro image analysis software (Media Cybernetics, Silver Spring, MD). Claudin co-localization was scored as the fraction that showed regions with a minimum of 100 contiguous pixels of cell-cell interfaces between cells expressing different claudins. Each experiment was quantified from at least five fields from 2 or 3 independently prepared samples and a minimum of 80 heterologous cell-cell interfaces.

**Immunoprecipitation**—Cells were plated in 100-mm tissue culture dishes and grown to ~90% confluence for co-immunoprecipitation experiments. Cells were rinsed twice with chilled...
PBS. Cells were subsequently incubated for 5 min on ice with
PBS containing 1 mM phenylmethylsulfonyl fluoride (Pierce),
harvested using a cell scraper, and centrifuged at 200 × g for 5
min at 4 °C through chilled PBS. Cells were then resuspended in
lysis buffer (PBS containing 1 mM phenylmethylsulfonyl fluo-
ride, 1:100 protease inhibitor mixture (Sigma-Aldrich), and
0.1% Triton X-100) and lysed for 10 min on ice. Cell lysates were
sonicated on ice to shear the nuclear material and centrifuged
for 10 min at 4 °C at 16,000 g. A portion of each post-nuclear
lysate was transferred to a tube with an equal volume of 2
sample buffer (100 mM Tris, pH 6.8, 200 mM dithiothreitol, 4%
SDS, 0.2% bromphenol blue, 20% glycerol), incubated for 5 min
at 70 °C, and stored at −20 °C for later use as an indicator of the
amount of claudin protein present in the cell lysate. The
remaining post-nuclear lysate was precleared with protein
A-agarose (Invitrogen), incubated with primary antibody at
4 °C overnight, and subsequently incubated with protein
A-agarose for 1 h at 4 °C. Immunoprecipitates were washed 5
times with wash buffer (PBS containing 0.1% Triton X-100) and
once with low salt wash buffer (50 mM NaCl, 5 mM Sodium
phosphate, pH 7.4) before the addition of 2 sample buffer,
incubation at 70 °C, and storage at −20 °C.

**Electrophoresis and Immunoblot**—Claudins and junction
adhesion molecule were resolved using 12% SDS-PAGE, occlu-

**FIGURE 2.** Claudins transfected into HeLa cells localize to the plasma
membrane. HeLa cells stably transfected with claudin-1 (a and e), claudin-3
(b and f), claudin-4 (c and g), and claudin-5 (d and h) were analyzed by immu
nofluorescence (a–d). Phase contrast images are shown in e–g. In each case, the
transfected claudins localized to the plasma membrane at sites of cell-cell
contact. Bar, 10 μm.

**FIGURE 3.** Preferential heterotypic claudin co-localization. To determine
whether different claudins were compatible for head-to-head binding, we
examined co-cultures of stably transfected HeLa cells. As a control, HeLa/CFP-
cldn-5 cells were co-cultured with HeLa/YFP-cldn-5 cells (a–c). HeLa/cldn-1
cells co-cultured with HeLa/cldn-3 cells (d–f), and HeLa/cldn-3 cells co-cul-
tured with HeLa/cldn-5 cells (g–i) showed claudin immunofluorescence co-
localization at areas of cell-cell contact and in intracellular vesicles containing
both claudins (arrowheads). Claudins did not co-localize in co-cultures of
HeLa/cldn-1 cells and HeLa/cldn-5 cells (j–l), HeLa/cldn-4 cells and HeLa/
cldn-5 cells (m–o), or HeLa/cldn-1 cells and HeLa/cldn-4 cells (p–r). Bar, 10 μm.

**FIGURE 4.** Heterotypic claudin binding specificity. Co-cultures of cells
expressing different claudins were solubilized in Triton X-100 and examined
for the ability to co-immunoprecipitate (IP) as determined by immunoblot (IB). Shown are representative immunoblots from co-cultures containing HeLa/
cldn-1 + HeLa/cldn-5 (a), HeLa/cldn-3 + HeLa/cldn-5 (b), HeLa/cldn-4 + HeLa/cldn-5 (c), HeLa/cldn-1 + HeLa/cldn-3 (d), or HeLa/cldn-1 + HeLa/
cldn-4 (e). Arrowheads denote claudin pairs that did not co-immunoprecipi-
tate. f, relative immunoprecipitation was quantified as described under
“Experimental Procedures.” Bars represent the average ± range (n = 2).
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din, ZO-2, and ZO-3 were resolved using 8% SDS-PAGE, and ZO-1 was resolved using 6% SDS-PAGE. Positive controls for claudin expression in Fig. 1 included T84 cells (claudin-1, -3, -4, -5, -8, and -18), SKHep-G2 cells (claudin-7) and Madin-Darby canine kidney cells (claudin-2). Proteins were transferred to Immobilon-P membranes and blocked overnight at 4 °C in 40 mM Tris, pH 7.4, 5% nonfat dried milk, and 0.1% Tween 20 (blocking buffer). Membranes were incubated for 1 h at room temperature with blocking buffer containing the appropriate primary antibody, washed 3 times with blocking buffer, and incubated for 1 h with the appropriate horseradish peroxidase-conjugated-secondary antibody diluted into blocking buffer. Membranes were then washed three times with blocking buffer and three times with PBS, and proteins were visualized using ECL Western blotting detection reagents (GE Biosciences).

Immunoblots were quantified using a Kodak EDAS system. The co-immunoprecipitation index was calculated by first normalizing the amount of immunoprecipitated claudin to the total amount of claudin present in the samples and then calculating the ratio of the amount of co-immunoprecipitated claudin divided by the amount of directly precipitated claudin. Statistical significance was determined using Student’s t test.

RESULTS
To analyze paracellular claudin interactions, we sought to identify an epithelial-like cell line expressing most tight junction proteins but lacking claudin protein expression. Affymetrix microarray analysis of HeLa cells from an unrelated study indicated that HeLa cells do not transcribe claudin genes (22). To confirm that HeLa cells lack claudin protein expression, we examined HeLa and cell lysates for the presence of claudins. As seen in Fig. 1, HeLa cells did not express claudin-1, -2, -3, -4, -5, -7, -8, or -18 at
the protein level. In contrast, several other tight junction proteins were detected in HeLa cell lysates, including occludin, ZO-1, ZO-2, ZO-3, and JAM-A. Thus, we used HeLa cells as a claudin-null background for further studies of claudin-claudin interactions.

HeLa cell clones stably expressing human claudins were generated. Immunofluorescence analysis of transfected HeLa cells revealed that exogenous claudins expressed by HeLa cells accumulate predominantly at cell-cell contact sites with weaker staining at cell surfaces that are not in contact with an adjacent cell (Fig. 2). Based on this observation we concluded that that claudin-1, claudin-3, claudin-4, and claudin-5 are likely to be stabilized at cell-cell contact sites through homotypic head-to-head binding.

Co-cultures of HeLa cells expressing different claudins were then examined using immunofluorescence co-localization and co-immunoprecipitation as assays to determine whether pairs of different claudins were compatible for heterotypic binding. (Figs. 3 and 4). By immunofluorescence, compatible claudins co-localized at the plasma membrane at areas of cell-cell contact. As a positive control, we included co-cultures of cells expressing CFP-claudin-5 and cells expressing YFP-claudin-5, here pseudocolored red and green, respectively. Compatible claudins also showed co-localization in intracellular vesicles, which likely were due to internalization of claudins from the adjacent cell (23). Consistent with previous reports using transfected fibroblasts, claudin-1 and claudin-3 were heterotypically compatible, as were claudin-3 and claudin-5 (13, 14). Furthermore, claudin-1 and claudin-5 were not compatible, again consistent with a previous report (14). Based on quantification of heterotypic co-localization, we found that the homotypic pair of tagged claudin-5 constructs had the highest level of co-localization, although the other combinations of compatible claudins also showed significantly more co-localization than incompatible controls.

Interestingly, neither claudin-1 nor claudin-5 was compatible to heterotypically bind to claudin-4. This was unexpected, since the extracellular loop (EL) domains of claudin-3 and claudin-4 are highly conserved at the amino acid level (EL1, 94% identical, 100% identical or conservative; EL2, 71% identical, 94% identical or conservative; Fig. 5i). Given this, we tested whether claudin-3 and claudin-4 could form heterotypic complexes. As shown in Fig. 5, claudin-3 and claudin-4 were not heterotypically compatible. Co-cultured HeLa/cldn-3 and HeLa/cldn-4 cells showed little immunofluorescence co-localization, and claudin-3 did not co-immunoprecipitate with claudin-4. In contrast, when HeLa cells were doubly transfected to express both claudin-3 and claudin-4, these two claudins co-localized and formed a complex isolatable by co-immunoprecipitation (Fig. 5). This is significant since it suggests that claudin-3 and claudin-4 were heteromerically compatible despite their inability to form a heterotypic complex.

To further investigate the role of EL domains in regulating heterotypic claudin-claudin binding, we produced a series of claudin chimeras. As shown in Fig. 6, the only pair that was not heterotypically compatible was wild type claudin-3 and wild type claudin-4. Each of the other combinations was able to form a co-immunoprecipitable, heterotypic complex. These results were also confirmed by immunofluorescence co-localization (supplemental Fig. 2). In particular, the “double loop swap” experiment (Fig. 6, c and d) was surprising, since this represents the same combination of EL domains that restrict heterotypic interactions between wild type claudin-3 and -4. This suggests that regulation of heterotypic claudin compatibility requires protein motifs beyond the EL domains. Note that a precedent for this type of regulation was also observed for two gap junction proteins, connexin40 and connexin43, where heterotypic interactions are regulated in part by the cytoplasmic C-terminal domain of connexin43 (24).

We also examined whether an EL domain point mutation could have an effect on heterotypic compatibility. To do this we substituted the asparagine residue with threonine, the corresponding amino acid in claudin-4, to create a claudin-3-N44T mutant. As shown in Fig. 7, claudin-3-N44T was heterotypically compatible with claudin-4 and also retained compatibility with claudin-1, claudin-5, and the claudin-3e1,2-4 loop chimera. Thus, the claudin-3N44T mutant had a compatibility profile distinct from both wild type claudin-3 and claudin-4. Similar results were obtained with claudin-4 chimeras containing entire EL loop swaps tested for heterotypic compatibility with claudin-1 and claudin-5 (supplemental Fig. 3). However, changing asparagine 44 to arginine did not alter heterotypic compatibility, since claudin-3N44R did not bind claudin-4 but still heterotypically interacted with claudin-5. This suggests that amino acid 44 in the EL1 domain has a critical role in regulating heterotypic compatibility between claudin-3 and claudin-4.

To further test the effect of altering the EL1 domain on claudin compatibility, we produced two claudin-4 chimeras containing the EL1 domain of either claudin-1 or claudin-5. As shown in Fig. 8, in contrast to wild type claudin-4, both of these chimeras were heterotypically compatible with claudin-1, claudin-3, and claudin-5. Note in particular that claudin-1 interacts with claudin-4e1-5, and claudin-5 interacts with claudin-4e1-1. Thus, three mutually incompatible claudins could be recombined to produce a chimera and a wild type claudin now able to heterotypically interact.
The specificity of claudin heterotypic binding was highly sensitive to EL domain composition. Because most changes to individual EL domains induced heterotypic compatibility as opposed to incompatibility, this suggests that regulating heterotypic claudin-claudin interactions requires the simultaneous recognition of motifs in both pairs of EL domains on the proper protein backbone.

**FIGURE 6.** Heterotypic compatibility of claudin-3 and claudin-4 extracellular loop chimeras. a and b, co-cultures of cells expressing either wild type or chimeric claudins were solubilized in Triton X-100 and examined for the ability to co-immunoprecipitate as determined by immunoblot (IB). The line drawings represent the claudins tested, where regions in red are derived from claudin-3, and regions in gray are derived from claudin-4. Blots underneath the heading total reflect the total amount of claudin present in the samples; IP indicates the immunoprecipitated fraction using either anti-claudin-3 or anti-claudin-4 as indicated. All of the combinations of constructs assayed were heterotypically compatible except for co-cultures of HeLa cells expressing wild type claudin-3 and wild type claudin-4 (arrowhead). b, bars represent the average ± S.E. (n = 3), *, significantly less co-IP than compatible pairs of claudins (p < 0.05). c and d, the ability of two double loop swap chimeras, cldn-4e1,2-3 and cldn-3e1,2-4, were assessed for the ability to heterotypically bind to each other or to wild type claudins. In contrast to cldn-3 and cldn-4, cldn-4e1,2-3 and cldn-3e1,2-4 were heterotypically compatible, suggesting that residues beyond the EL domains are critical to control compatibility.
Claudins form the physical basis for the tight junction barrier (1–4). For this to occur, the EL domains of claudins on one cell must interact with those on adjacent cells. The nature of this interaction is not well characterized. Because most barrier-forming cells express several claudins, tight junction formation and function is likely regulated at least in part by whether or not individual claudins are compatible for heterotypic binding. We found that claudin-4 did not heterotypically bind to either claudin-1, claudin-3, or claudin-5 (Table 1). To date, all of the known heterotypic claudin-claudin interactions appear to involve claudin-3. However, since only a limited subset of the claudins has been examined, further analysis may reveal other potential heterotypic claudin-claudin interactions. In contrast, homotypic claudin-claudin interactions appear likely to be universal.

The inability of claudin-3 and claudin-4 to heterotypically interact was particularly surprising given that the EL domains of these two claudins are so highly conserved. We found that a specific single amino acid substitution in the first EL domain of claudin-3 was sufficient to enable a heterotypic interaction with wild type claudin-4. Similarly, the claudin-3e2–4 chimera, containing five amino acid changes, also heterotypically interacted with claudin-4.

Using a series of constructs, we found that chimeras containing a mixture of claudin-3 and claudin-4 EL domains could heterotypically bind to both wild type claudin-3 and wild type claudin-4. Furthermore, all of the chimeras could bind to all of the other chimeras containing a mixture of EL domains. In Fig. 6, the only combination that was not heterotypically compatible was wild type claudin-3 and wild type claudin-4. This suggests that the nature of heterotypic binding was not due to a simple binary interaction of EL-1 with EL-1, EL-1 with EL-2, or EL-2 with EL-2. Instead, heterotypic binding appears to be mediated by a simultaneous interaction involving both pairs of EL1 and EL2 domains.

Moreover, since the double loop swap claudin-3 and claudin-4 chimeras were heterotypically compatible (Fig. 6, c and d), the context of the EL domains can have an effect on their structure. The notion that motifs beyond the EF domains can also play a role in regulating heterotypic compatibility is consistent with the observation that heterotypic binding between connexin40 and connexin43 could be inhibited by changes restricted to the cytoplasmic C terminus (24).

The complex nature of claudin heterotypic interactions is underscored by the finding that claudin-1 and claudin-5 are heterotypically compatible with claudin-3 even though they have EL domains that are less homologous to claudin-3 than claudin-4. That claudin-1 and claudin-5 are incompatible yet both bind to claudin-3 further suggests that a complex interaction between both pairs of EL domains dictates the specificity of heterotypic claudin-claudin interactions. This complexity could underlie the observed asymmetry in heterotypic claudin binding. However, a complete understanding of the molecular nature for heterotypic claudin binding will require detailed structural information of claudins which to date has not been determined.

Although claudin-3 and claudin-4 were not heterotypically compatible, they did form a heteromeric complex when both claudins were expressed in the same cell. Whether claudins form heteromeric compatibility groups or are universally heteromerically compatible is not known. Although heteromeric binding likely requires lateral interactions in plane of the plasma membrane, it is also possible that heterotypic interactions may contribute to heteromer formation. For instance, EL domains of claudins on adjacent cells may help regulate heteromeric claudin-claudin interactions within tight junction strands. Also, the C-terminal domains may participate in regulating heteromeric interactions through interactions with ZO-1 and/or ZO-2 (25).

HeLa cells provided a claudin-null background to analyze claudin-claudin interactions. These cells offer the advantage that they express several other tight junction proteins, including occludin, JAM-A, ZO-1, ZO-2, and ZO-3. In particular,
HeLa cells enabled us to confirm that occludin does not alter claudin heterotypic compatibility, since our results were comparable with the heterotypic claudin-claudin compatibility profiles obtained using claudin-transfected fibroblasts (13, 14). The compatibility profile for claudin-1, claudin-3, and claudin-5 has also been observed for airway epithelia (14). Although occludin-null mice form functional tight junctions (17, 26), the ability of occludin EL peptides to disrupt epithelial barrier function suggests a role for occludin in regulating tight junctions (18, 19, 27). At present it is unclear whether heterotypic occludin-claudin interactions contribute to the ability of occludin to modulate barrier function. However, because claudin heterotypic compatibility is not altered by the presence of occludin and the EL loops of occludin and claudins show significant structural differences, such a scenario seems unlikely.

Heterotypic compatibility and/or incompatibility may help determine the characteristics of tight junction permeability. The up-regulation of several claudins, including claudin-2, claudin-5, and claudin-6, are associated with decreases in epithelial barrier function (28–31). However, these claudins are not necessarily inherently “leaky.” For instance, claudin-5 enhances barrier function in transfected Madin-Darby canine kidney cells and plays a critical role in maintaining blood-brain barrier function (32, 33). These apparently paradoxical results may reflect that function of a given claudin will vary depending upon which other claudins are present. For instance, if heterotypic incompatibility (e.g. between claudin-5 and claudin-4) is a dominant interaction in lung epithelia, then increased claudin-5 expression would be expected to compromise tight junction integrity, as previously observed (31). Conversely, in the endothelium, claudin-5 may be compatible with the other major endothelial claudin, claudin-12 (34). If this is the case, then up-regulation of claudin-5 might be expected to increase blood-brain barrier function. Alternatively, it is also possible that homotypic claudin-5 interactions dominate endothelial tight junctions and the junctions of some transfected cell models, in which case increased claudin-5 expression might also be expected to increase barrier function.

Heterotypic interactions may also have more subtle effects on barrier function by regulating claudin paracellular ion channel activity. Although claudin permeability is mainly regulated by the EL1 domain

**TABLE 1**

| Compatible | References          | Not compatible | References          |
|------------|---------------------|----------------|---------------------|
| Claudin-1  | Claudin-3            | Claudin-2      | 13                  |
| Claudin-2  | Claudin-3            | Claudin-4      | Current study       |
| Claudin-3  | Claudin-5            | Claudin-5      | 14 and current study|
|            | 14 and current study |                |                     |

**FIGURE 8.** EL1 domain substitutions alter claudin-4 heterotypic compatibility. Cells expressing claudin-1 (a, d, and g), claudin-3 (b, e, and h), or claudin-5 (c, f, and i) were co-cultured with cells expressing either claudin-4 (a–c), claudin-4e1-1 (d–f), or claudin-4e1-5 (g–i) and then solubilized in Triton X-100 and examined for the ability to co-immunoprecipitate (IP). None of these claudins co-immunoprecipitated with wild type claudin-4 (a–c and j, arrowheads). However replacement of the EL1 domain with either the claudin-1 or claudin-5 EL1 domain created a chimera capable of binding to all three claudins examined (k and l). IB, immunoblot.
(12), it is not known whether multiple EL1 domains contribute to net ion permeability. Also, whether the EL2 domain contributes to ion permeability is not known at present. We were unable to use HeLa cells for barrier function measurements, as these cells do not form a continuous monolayer able to cover a permeable support and instead form discrete colonies. Despite this limitation, transfected HeLa cells offered the ability to biochemically analyze binary claudin-claudin interactions in a claudin-null cell model that expresses other tight junction proteins. Note that claudin-transfected fibroblasts suffer from this limitation, transfected HeLa cells offered the ability to biochemically analyze binary claudin-claudin interactions in a claudin-null cell model that will allow defined combinations of claudins to be functionally examined in high resistance monolayers needed to better understand how EL1 and EL2 interact and to determine whether multiple claudins act in concert to regulate paracellular ion permeability.

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