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The Cytoplasmic Tails of Claudins Can Influence Tight Junction Barrier Properties through Effects on Protein Stability

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Abstract. The tight junction seal formed between epithelial cells varies among tissues in both tightness and ionic charge selectivity. We recently demonstrated that the extracellular domains of the claudin family of proteins are determinants of both characteristics, but in that study other unidentified domains in the claudins clearly contributed to their physiological potency. To investigate the importance of the cytoplasmic carboxyl-terminal domains in determining the degree to which a claudin can influence barrier properties, we constructed chimeras by exchanging the tails of claudin-2 and -4 and expressing them in MDCK II cells. Although swapping these domains had little effect on claudin localization, we found that the tail of claudin-2 could stabilize claudin-4, with a concomitant increase in both protein level and physiologic influence. This difference in stability was not an artifact of their chimeric structure, since metabolic radio-labeling experiments revealed that the half-life of endogenous claudin-2 is more than three times longer than claudin-4 (>12 h and ~4 h respectively). Further, half-life was not affected by removing the carboxyl-terminal three amino acids, which form a PDZ-binding motif. The finding that cytoplasmic tails of claudins strongly influence stability reveals a potential mechanism by which cells can establish their tight junction protein composition and thus function.

Key words: Claudin — Claudin-2 — Claudin-4 — Tight junctions — Paracellular permeability

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Introduction

The tight junction forms an intercellular barrier between epithelial cells, regulating the flux of small solutes and ions through the paracellular space. The barrier is created by transmembrane proteins from adjacent cells, which interact to form continuous adhesive contacts encircling the apical end of the lateral membrane. The physiologic properties of the barrier vary among different epithelia, both in the magnitude of tightness (as measured by the transepithelial electrical resistance, TER) and in ionic charge selectivity [17]. There are at least three types of tight junction transmembrane proteins: occludin, a 60–65 kDa tetraspan product of a single gene [7]; at least 4 members of the IgG superfamily of junctional adhesion molecules (JAMs) [2]; and the claudins, small (20–24 kDa) tetraspan proteins of which there are more than 20 members [9, 15]. Expression of claudins in fibroblasts, cells without tight junctions, results in formation of freeze-fracture fibrils reminiscent of those seen in epithelial cells [8] and also confers Ca^{2+}-independent adhesiveness [13]. These results, coupled with the finding that occludin knockout mice have apparently normal tight junctions [20], has led to the concept that claudins are the primary components of the barrier.

Claudins are differentially distributed among tissues, with a single claudin in some epithelial cells and several in others [12, 18, 19]. The pattern and level of claudin expression likely explains the physiologic paracellular characteristics of each epithelial cell type. For example, Madin-Darby canine kidney (MDCK) II cells express at least 5 different claudins (1, 2, 3, 4, and 5) [1]. Monolayers of these cells normally have low resistance and highly cation-selective tight junctions. When these cells are transfected and express exogenous claudin-4, TER increases about

2- to 3-fold and they become less permeable to Na^+ ions [24].

Each claudin appears to have unique physiologic characteristics. For example, expression of exogenous claudin-8 in MDCK II cells results in a higher increase in TER than expression of claudin-4 but in a similar discrimination against Na^+ [26], while expression of claudin-2 results in a very small [5] or no [25] effect on either TER or charge selectivity. We [5] recently used chimeras in which the first or both extracellular domains were interchanged between claudin-2 and -4 to demonstrate that the first extracellular domain of claudin 4 is sufficient to determine its unique effects on TER and charge selectivity. In contrast, the transmembrane and cytoplasmic domains did not influence the direction of change in either TER or selectivity. Unexpectedly, chimeras with the extracellular domains of claudin-4 combined with the four transmembrane and cytoplasmic sequences of claudin-2 had more profound effects on TER and charge selectivity than that seen following expression of wild-type claudin-4. These results led us to ask if the carboxyl-terminal domains of claudins might differentially affect their extracellular functions. This is by analogy to the "inside-out" signaling function of integrins [10] and the requirement for the cytoplasmic domain of cadherin in inducing strong adhesion by the extracellular domain [3]. Claudin-2 and -4 share ≈40% amino-acid sequence identity overall, and are most different in their cytoplasmic carboxyl tails. The tail of claudin-2 is twice the length of that of claudin-4 (45 compared with 23 amino acids) and has no obvious regions of identity (*see* Fig. 1*A*). To test the role of the cytoplasmic tails, we created chimeras in which the carboxyl domains of claudin-2 and -4 were interchanged. In addition, claudins have been shown to bind through their extreme carboxyl-termini to the PDZ domains of scaffolding proteins ZO-1, -2 and -3 [11] although it remains unclear whether different claudins have differing affinities for the PDZ-binding proteins. Of interest is the finding that myelinating Schwann cells express at least 3 different claudins, which are found in different subcellular locations, and each interacts with a different set of PDZ-containing proteins [16]. To test the role of the PDZ-binding domains on claudin function, we created claudin-2, -4 and chimeric claudins that lacked the last three amino-acid residues [11].

Our results demonstrate that cells expressing claudin-4 with the cytoplasmic carboxyl domain of claudin-2 display a more profound increase in TER and decrease in Na^+ permeability than seen with induction of wild-type claudin-4. This differential effect can be explained by an increase in the protein stability and consequently higher level of the chimeric protein. Half-lives were independent of the carboxyl-terminal three amino acids, suggesting that PDZ-interactions do not influence protein stability. Finally, we determined that endogenous claudin-2, which is normally more abundant in the Tet-Off MDCK II cells than is claudin-4, has a longer half-life than does claudin-4.

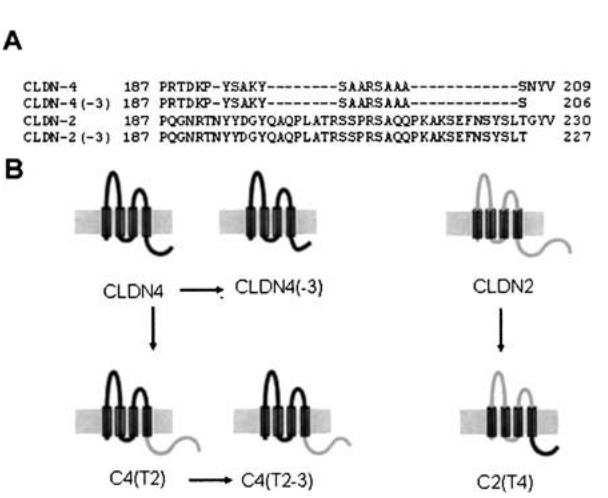

Fig. 1. Construction of claudin tail chimeras. (*A*) Amino-acid sequence alignment of the cytoplasmic tails of claudin-2, -4 and the (-3) mutants (lacking the PDZ-binding domain) sequences. Tails were defined using a program predicting transmembrane domains [21] at http://www.cbs.dtu.dk/services/TMHMM. (*B*) Schematic of predicted membrane topology of claudin-2 and -4 and the following chimeras: Cldn4; C4(T2); C4(-3); C4(T2-3); Cldn-2 and C2(T4).

Materials and Methods

Plasmid Constructs and Cell Lines

The carboxyl-terminal cytoplasmic domains of mouse claudin-2 and human claudin-4 are presented in Fig. 1*A*. Claudin-2 and -4 chimeras were constructed by first creating a *Bsi*WI site at amino acids 189 (claudin-2) and 188 (claudin-4), using a PCR-based mutagenesis strategy (Quik-Change, Stratagene, La Jolla, CA). Sequences encoding the cytoplasmic domains were cut at the engineered site and in the multiple coding region of pTRE. The tails were then ligated into the reciprocal vectors creating the chimeric constructs. Introduction of the *Bsi*WI site and ligation of the C-terminus of claudin-2 onto claudin-4 altered the amino-acid sequence and site-directed mutagenesis was employed to correct the sequences as follows: $R^{188}T^{189}$ to $Q^{188}G^{189}$. The (-3) mutants, lacking the PDZ-binding motifs, were constructed by amplification using oligonucleotides (for the tail of claudin-2) 5′-GCTCTAGATCAAGTCAGGCTGTATGAGTT-3′ and (for the tail of claudin-4) 5′-GCTCTAGATTAGCTGGCAGCAGCAGAGCGGGC-3′ along with the pTRE 5′ sequencing primer. All constructs were verified by DNA sequencing in both directions. Clonal cell lines of tightly regulated MDCK II Tet-Off cells (BD Biosciences Clontech, Palo Alto, CA) were derived by standard transfection and selection techniques in 200 μg/ml hygromycin; regulated expression of the transgene products was accomplished by addition or removal of doxycycline. Stable cell lines were screened by immunoblot analysis and uniformity of expression was verified by immunofluorescence; induced claudins are easily distinguished from native claudins by the high level of expression.

At least 3 highly regulated cell lines were generated for each construct.

Bacterial Fusion Protein Constructs

The cytoplasmic tails of claudin-2 and -4 were amplified by PCR using for claudin-2 the primers 5′-GGAATTCGGCCCCCAGG-GCAATCGTACC-3′ and 5′-GCCAAGCTTTCACACATACC-CAGTGAG-3′ and for claudin-4 5′-GGAATTCGGCCCACCCC GCACAGACAAG-3′ and 5′-CGCAAGCTTTTACACGTAGTT-GCTGGC-3′; PCR products were digested and cloned into pMAL CM vector and constructs were verified by DNA sequencing. Fusion protein was induced and then purified using Talon resin (BD Biosciences Clontech); the amount of purified protein was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL).

Immunoblots and Immunofluorescence Microscopy

Immunoblots were performed as previously reported [5, 24] using the following antibodies available from Zymed Laboratories (South San Francisco, CA): anti-human claudin-4 mouse monoclonal antibody; anti-human claudin-2 mouse monoclonal antibody; anti-human claudin-1 rabbit polyclonal antibody; anti-human occludin mouse monoclonal antibody; anti-human ZO-1 mouse monoclonal antibody and anti-γ-tubulin mouse monoclonal antibody at the dilutions previously reported [5]. Detection was by HRP-coupled secondary antibodies (anti-rabbit, Amersham Biosciences, Piscataway, NJ and anti-mouse, Chemicon International, Temecula, CA) using ECL (Amersham Biosciences). Band densities were quantified using either GelPro (Media Cybernetics, Silver Spring, MD) or ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Immunofluorescence microscopy of claudin-2, -4, chimeras and ZO-1 was performed as described previously [5]. The antibodies used did not distinguish between endogenous proteins and the chimeras. In immunoblots, the high level of expression of the induced proteins relative to endogenous protein allowed quantification of induction, but in immunofluorescent analysis, images reported both the endogenous plus the induced protein.

Electrophysiological Measurements

Electrophysiological characterization of MDCK II monolayers was carried out as previously described [5, 24]. Briefly, stable cell lines expressing wild-type or chimeric proteins were cultured on Snapwell (Costar, Corning Life Sciences, Acton, MA) filters for 4 days in the presence (noninduced) or absence (induced) of 50 ng/ml doxycycline. In some experiments, graded levels of doxycycline were used to achieve a range of protein induction. Transmonolayer resistance and dilution potential were determined as previously described, using a modified Ussing chamber with a microcomputer-controlled voltage/current clamp (Harvard Apparatus, Holliston, MA).

Metabolic Labeling to Determine half-life

MDCK II Tet-Off cells were plated into 60 mm dishes at 80% confluence. After 24 h, cells were changed into methionine-free medium (Gibco/InVitrogen, Carlsbad, CA) containing 10% dialyzed fetal bovine serum for 1–2 h. Cells were changed into labeling medium (methionine-free medium supplemented with 1.5 mg/L methionine and cysteine, 10% dialyzed fetal bovine serum, 10 mM HEPES, pH 7.4, 2 mM glutamine) containing 0.8–1 mCi/mL Tran ^{35}S-label (ICN Biomedicals, Inc., Irvine, CA). Cells were incubated in labeling medium for 24 h, washed 3 times in pre-warmed DMEM supplemented with 10% fetal bovine serum and collected 0, 3, 6, 12 and 24 h or at 0, 8 and 24 h). At collection, cells were washed twice with ice-cold PBS and extracted with immunoprecipitation buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton-X 100, 0.05% SDS, 0.2% sodium deoxycholate, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF and protease inhibitor cocktail [14]. Insoluble material was pelleted at 10,000 × *g* and supernatants were incubated for 3–6 h with 30 μL claudin-2 or claudin-4 antibodies coupled to CNBr-activated sepharose. Beads were washed 4 times with immunoprecipitation buffer and once with 25 mM HEPES (pH 7.4), 150 mM NaCl; and then 30 μL of 4 × SDS-sample buffer was added; samples were stored at −80°C until analysis. Ten μL of sample was run on SDS-PAGE gel for analysis of radiolabeled proteins; 5 μL for immunoblot. Gels for quantifying incorporation of ^{35}S-amino-acid analysis were fixed (10% acetic acid, 20% methanol, 3% glycerol), dried and exposed to a Molecular Dynamics (Amersham) LE storage phosphor screen and scanned using a Storm PhosphoImager (Molecular Dynamics). Band densities were quantified with ImageQuant software (Molecular Dynamics) and relative specific activities [^{35}S/protein] determined by normalizing to the ECL signal scanned into Adobe Photoshop (Adobe Systems, San Jose, CA.) and also quantified using ImageQuant software. All data sets were fit to single exponential decay curves to generate graphs and protein half-lives (GraphPad Prism 4) and statistically analyzed using Instat, both from GraphPad Software, Inc. (San Diego, CA).

Results

Construction of Claudin Chimeras

Previously we observed that expression in MDCK II cells of claudin chimeras containing the first or both extracellular loops of claudin-4 on a background of the transmembrane and cytoplasmic domains of claudin-2 resulted in a larger changes in TER and ion selectivity than expression of wild-type claudin-4 [24]. We hypothesized that the cytoplasmic tail of claudin-2 was responsible for the exaggerated physiologic phenotype of the chimera. To test this, we constructed two chimeric claudins, in which the tails of claudin-2 and -4 (Fig. 1*A*) were interchanged by creating unique restriction sites in the DNA sequence encoding the junction of the fourth transmembrane domain and the carboxyl-terminal tail. The carboxyl-terminal tails were then cut at the engineered site and at a second unique site in pTRE, and DNA fragments were ligated into the reciprocal vectors. A second set of constructs lacking the last three amino acids, and thus the PDZ-binding domains, was generated by PCR and subsequent ligation and subcloning into pTRE (Fig. 1*B*). Antibody epitopes for immunoblot and immunofluorescence microscopy are located in the C-terminal tails, proximal to the PDZ-binding domains.

Chimeric Claudins Are Inducible and Localize to Cell Borders

Clonal lines of MDCK II cells were produced capable of regulated expression of the chimeric claudins. A representative immunoblot of two clonal cell lines, claudin-2 with the tail of claudin-4 [C2(T4)] and claudin-4 with the tail of claudin-2 [C4(T2)] is shown

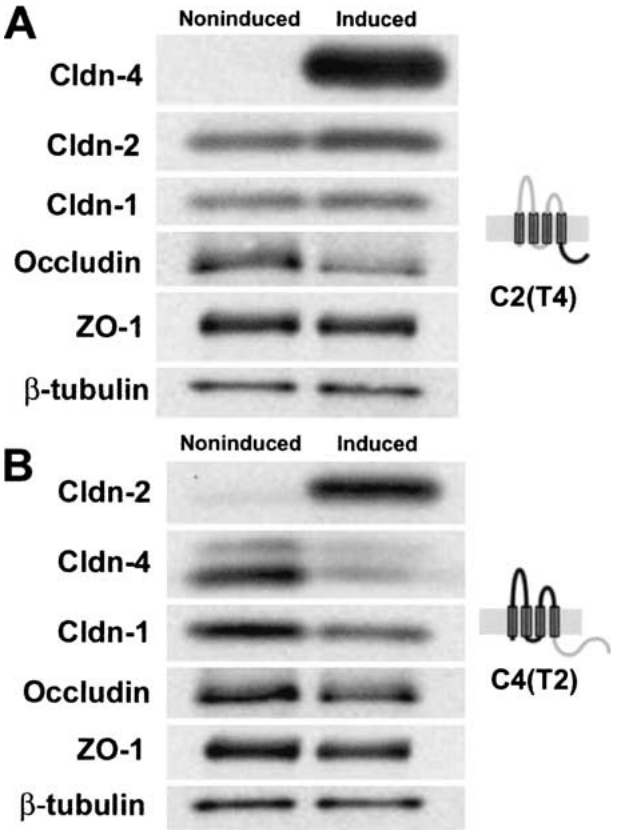

Fig. 2. Claudin chimeras are inducible in Madin-Darby canine kidney (MDCK) II Tet-Off epithelial cells. Stable cell lines were grown on Snapwell filters and not induced or induced for transgene expression. Total cell lysates were immunoblotted for claudin-2, -4, -1, occludin, ZO-1 and β-tubulin (loading control) expression. (*A*) Expression of claudin-2/tail4 (detected with antibody to claudin-4); (*B*) expression of C4(T2), detected with antibody to claudin-2. Similar to results seen for claudin-2 [5], C2(T4) expression results in decreased occludin expression; C4(T2) results in decreased expression of endogenous claudin-4. Although only one cell line is shown for each construct, results are representative of those from all cell lines tested (3–5 clones for each construct).

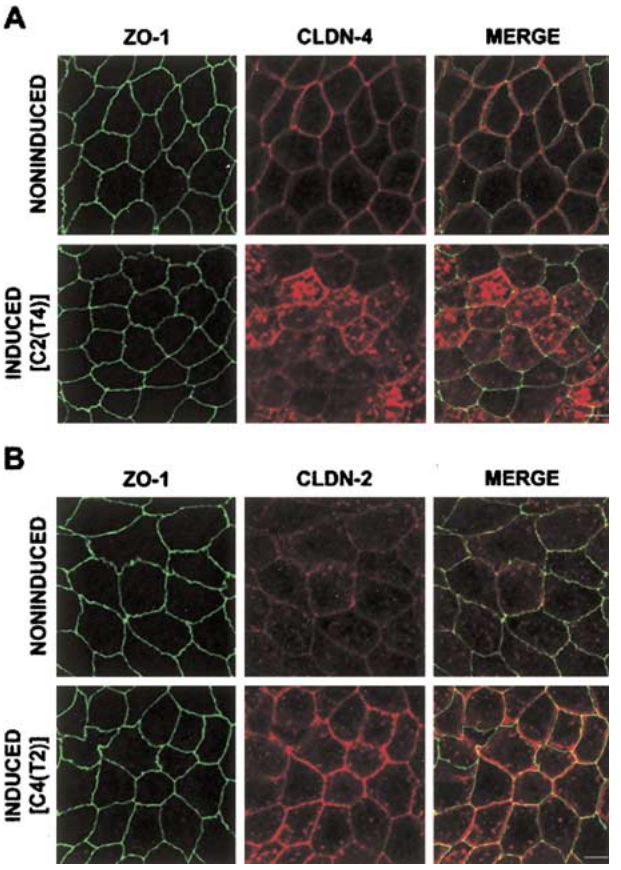

Fig. 3. Claudin chimeras localize to the cell borders of MDCK II Tet-Off cells. Stable clones of MDCK II Tet-Off cells were grown as in Fig. 2, with or without induction for chimera expression. Cells were analyzed by immunofluorescent confocal microscopy for ZO-1 (*left panels*, *A* and *B*), for claudin-4 (*A*, *middle panels*) and claudin-2 (*B*, *middle panels*) and the merged signals of ZO-1 and claudin-4 (right panels, *A* and *B*). (*A*) Claudin-4 shows the normal pattern of membrane localization in uninduced cells, while induction of the claudin-2/tail4 chimera shows the membrane staining with the intracellular staining characteristic of uninduced and induced claudin-2 expression (*see* Fig. 3*B*, [5]). (*B*) Endogenous claudin-2 shows membrane and intracellular staining, while induced C4(T2) shows enhanced membrane staining but little increase in intracellular staining. *Merge* shows superposition of immunofluorescences for ZO-1 and CLDN-4. Bar = 5 μm.

in Fig. 2*A* and 2*B*. Because of the high level of induction (~5-fold) of the transgenes, the immunoblot was deliberately underexposed for endogenous claudin-4 (panel *A*) and claudin-2 (panel *B*). Longer exposure revealed that endogenous claudin-2 and -4 are expressed normally in transfected, noninduced cells. Expression of the chimeras had little effect on the levels of other tight junction proteins measured, with the following exceptions. Like wild-type claudin-2 [6], expression of C2(T4) resulted in small decreases in the level of occludin. Expressing C4(T2) resulted in decreases in the levels of endogenous claudin-4; this was previously described in studies on the extracellular loops of these claudins [5]. The small decrease in claudin-1 expression in Fig. 2*B* was not reproducible. Similar results were seen with the constructs lacking the PDZ-binding motif.

Both chimeric claudins colocalize with their endogenous counterparts at cell contacts when induced in MDCK II cells (Fig. 3), suggesting they are functional proteins. As previously noted, in addition to the tight junctional localization [5] claudins-2 and -4 occupy additional and distinct subcellular locations. Claudin-4 also localizes to the entire lateral cell membrane (Fig. 3*A*), while claudin-2 is more focused at the junction but is also found in intracellular vesicles (Fig. 3*B*). These distinct localization patterns are characteristic of the normal endogenous proteins, thus not an artifact of over-expression. However, the intracellular pool seen with claudin 2 immunofluorescence is much expanded in cells expressing C2(T4).

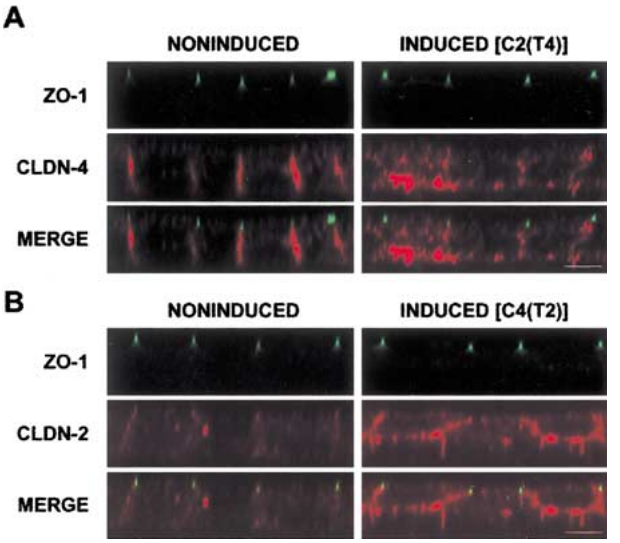

Fig. 4. Chimeric proteins localize to cell borders and to intracellular structures. Stable lines of MDCK II Tet-Off cell were grown with and without induction of C2(T4) and C4(T2) and labeled as in 3*A* and *B*. (*A*) Confocal XZ slices reveal that endogenous claudin-4 in non-induced monolayers localizes to lateral cell borders and overlaps with ZO-1 at the tight junction. However, expression of C2(T4) reveals a large increase in staining of claudin-4 in intracellular structures. (*B*) Confocal XZ slices reveal that endogenous claudin-2 is found in intracellular structures and at the tight junction colocalizing with ZO-1. C4(T2) induction increases the staining at lateral, apical and basal cell borders. Bar = 5 μm.

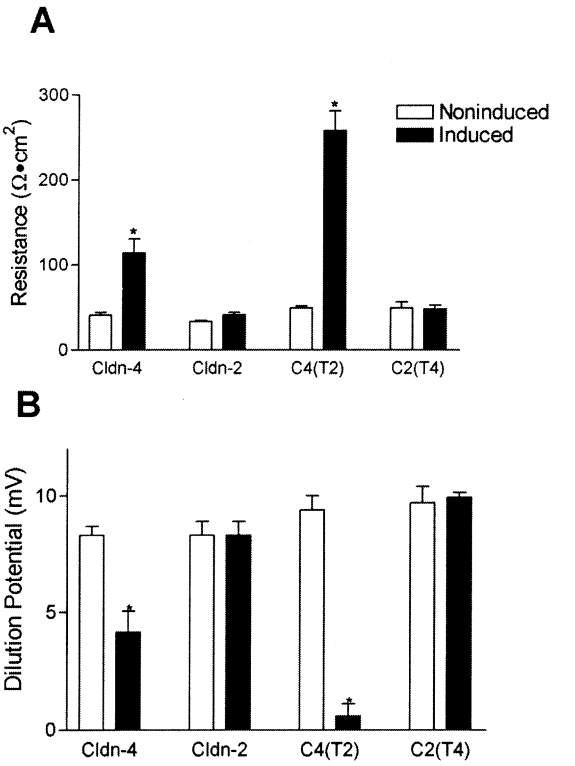

Fig. 5. The C-terminus of claudin-2 on claudin-4 increases the effects of claudin-4 on TER and paracellular charge selectivity. (*A*) Stable clones of MDCK II Tet-Off cells were plated with (*black bars*) and without (*white bars*) induction of claudins or chimeras as describe in the Methods section. Expression of wild-type claudin-4 increased MDCK II baseline TER by greater than 2-fold; induction of wild-type claudin-2 resulted in a 20% increase. C4(T2) increased TER by more than 5-fold, significantly greater than the increase from expression of exogenous claudin-4. In contrast, induction of C2(T4) does not result in a significant increase in TER. The TER before and after induction of wild-type and chimeric claudins were as follows: claudin-4, 40.9 ± 3.2 to 114.0 ± 16.7 Ωcm^2; claudin-2, 33.3 ± 1.2 to 41.1 ± 3.3 Ωcm^2; C4(T2), 49.2 ± 2.6 to 258.1 ± 23.0 Ωcm^2; C2(T4), 49.1 ± 7.6 to 48.0 ± 4.5 Ωcm^2. (*B*) C4(T2) decreases the dilution potential more than does wild-type claudin-4. Dilution potentials were compared between monolayers that were non-induced (*white bars*) or induced (*black bars*), as above. The dilution potentials before and after transgene induction were for claudin-4, 8.3 ± 0.4 mV to 4.15 ± 0.9 mV, for claudin-2, 8.3 ± 0.6 mV to 8.3 ± 0.6 mV; for C4(T2), 9.4 ± 0.4 mV to 0.6 ± 0.5 mV, for C2(T4), 9.7 ± 0.7 mV to 9.9 ± 0.2 mV. Dilution potentials of equal magnitude but opposite charge were generated following basal dilutions, confirming that the charge selectivity is paracellular. Data from both TER and dilution potentials represent the means and SE of determinations from duplicate Snapwells of 4–7 clones for the wild-type claudins and chimeras. Significance calculated by ANOVA followed by Dunnett's test; $^*P < 0.05$.

Interestingly, the localization patterns of the chimeric proteins follows their transmembrane and extracellular domains and not the cytoplasmic tails (Fig. 3*A* and *B*). This difference is more evident in XZ-series images of the same cells (Fig. 4*A* and *B*), in which the lateral membrane staining of endogenous claudin-4 is visible, but much of the induced C2(T4) appears in intracellular vesicles (Fig. 4*A*). Induction of C4(T2) leads to lateral plasma membrane staining reminiscent of wild-type claudin-4 staining (Fig. 4*B*). Removal of the PDZ-binding motif had no obvious effect on claudin localization (*data not shown*). Interpretation of the localization patterns is complicated by the fact that the antibodies recognize both the endogenous claudin and the tail of the respective chimera, since we chose not to tag the expressed claudins to avoid interfering with potential protein-protein interactions. However, at the resolution of immunofluorescent analysis, it appears as if the cytoplasmic tails of the claudins do not determine their unique subcellular distribution patterns.

The Physiologic Influence of Claudin-4 is Enhanced by Fusion to the Cytoplasmic Tail of Claudin-2

Stable clones of MDCK II Tet-Off cells were grown for four days on Snapwell filters induced or noninduced for the expression of C4(T2). As reported previously [24], expressing claudin-4 increases the TER two-fold above baseline, while expressing claudin-2 has little or no significant effect. Like claudin-2, expressing C2(T4) has no significant effect on TER [5]. In contrast, induction of C4(T2) increased TER

more than 5-fold over baseline (Fig. 5*A*). Parallel changes were seen on paracellular charge selectivity, with claudin-2 and C2(T4) having little or no effect on charge selectivity, as measured by dilution potential (Fig. 5*B*). Expressing wild-type claudin-4 decreased the dilution potential and paracellular Na^+ permeability by about half, as previously described [24]. However, expression of C4(T2) had a much stronger effect, changing the paracellular selectivity of the MDCK II cells from highly Na^+ selective to non-selective for Na^+ versus Cl^- (Fig. 5*B*). In summary, the cytoplasmic tail of claudin-2 significantly increased the efficacy of the extracellular and transmembrane domains of claudin-4 in affecting barrier properties.

Replacing the Claudin-4 Tail with that from Claudin-2 Increases the Steady-State Level of Chimera Compared to Wild-Type Claudin-4

We have previously shown the physiologic effects of claudin-4 are proportional to its expression [24]. Thus, one explanation for the more dramatic phenotype conferred by the chimera compared with wild-type claudin-4 is that it accumulates to a higher steady-state level. In order to test whether claudin-4 and C4(T2) accumulate at different levels it was necessary to determine the specific sensitivities of their respective antibodies. This was achieved by simultaneously immunoblotting expressed protein and known quantities of bacterially expressed claudin-2 and -4 fusion proteins. Fusion proteins were generated by amplifying the C-terminal tails of claudin-2 and -4 by PCR and cloning them into a fusion protein vector (*see* Materials and Methods). Fusion proteins were induced, purified and quantified by BCA protein assay. Progressive dilutions of fusion proteins were run on SDS-PAGE gels and transferred to nitrocellulose along with progressive dilutions of cell lysate from induced claudin-expressing MDCK II cells. Comparison of immunoblot signals generated by the induced claudins compared with the fusion proteins allowed us to determine the amounts of the expressed protein. When TER or dilution potential was plotted as a function of the level of either claudin-4 or C4(T2) all values appear to fall on the same approximately sigmoidal curves (Fig. 6*A* and *B*), with C4(T2) reaching consistently higher protein levels than wild-type claudin-4. This relatively higher level of C4(T2) was true for all clones tested (3 of each). As previously observed, the physiologic parameters saturate at the highest protein levels [24]. We conclude the greater effect of C4(T2) on TER and dilution potential results from its ability to reach higher expression levels compared to wild-type claudin-4, although they both appear to have the same relationship between protein level and barrier effects. We presume this is because they share the same extracellular sequences.

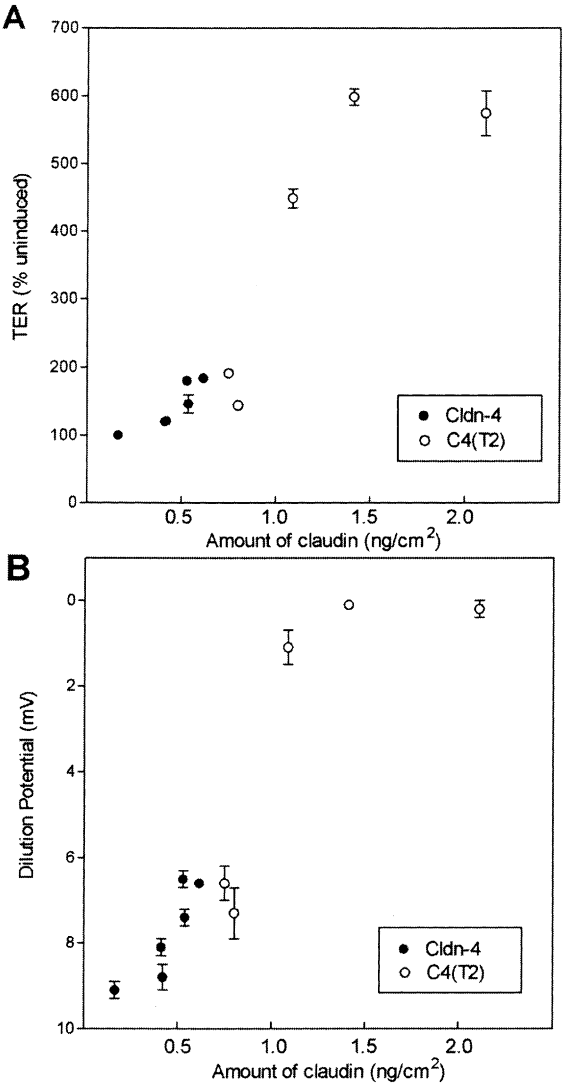

Fig. 6. Higher levels of C4(T2) than exogenous claudin-4 are expressed in MDCK II Tet-Off cell lines. Stable clones of claudin-4 and C4(T2) were induced to express variable amounts of transgene by plating cells in Snapwells in duplicate in 0, 0.005, 0.01, 0.05, 0.1 and 0.5 ng/ml doxycycline. After 4 days, filters were removed and TER and dilution potential were determined and then filters were excised and cells dissolved in SDS-sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and the amount of claudin protein per Snapwell (1 cm^2) was quantified by ECL by comparing with a standard curve of claudin fusion protein. The amount of endogenous claudin-2 was subtracted from the amount of induced protein. (*A*) TER or (*B*) dilution potentials were plotted against the amount of exogenous claudin-4 detected with the claudin-4 antibody, and against the amount of C4(T2), determined with the claudin-2 antibody. Two to three times as much C4(T2) was expressed in the maximally induced cells than was exogenous claudin-4.

The Tail of Claudin-2 Stabilizes Claudin-4 by Prolonging Half-life through a PDZ-independent Mechanism

Because the pTRE vectors for all of these constructs share the same regulated transcription sites and presumably the same synthesis rates, the most likely explanation for the difference in steady-state levels of claudin-4 and C4(T2) was that the two proteins had different decay rates, i.e. half-lives. To determine their half-lives, both constructs were induced for 4 days, as described previously, and further expression was repressed with doxycycline. Samples were collected at 0, 3, 6, 12 and 24 h, immunoprecipitated and processed for immunoblot analysis. As seen in an example Fig. 7*A*) and summarized in Fig. 7*B*, the half-life of induced claudin-4 was ~3 h, while C4(T2) had a half-life of ~9 h; these results were repeated with different claudin-4 and C4(T2) clones, with the same results (*data not shown*). Because PDZ-dependent interactions have been reported to stabilize proteins, we next determined if this effect on stability was dependent on the presence of the last three amino acids of claudin-2, which are required for binding to PDZ domains. Removal of these amino acids had no effect on the stability of the chimeric protein (Fig. 7*C*).

Both Induced and Endogenous Claudin-2 Are more Stable than Claudin-4

To determine if the difference in half-life seen between claudin-4 and C4(T2) was a function of the chimeric nature of C4(T2), we next compared half-lives of full-length transfected claudin-2 and -4. The half-life of claudin-2 was very similar to that of the chimeric C4(T2) protein (Fig. 8). However, because the tetracycline expression system allows the induction of large amounts of protein with possibly artifactual effects on half-lives, we went on to determine the half-lives of endogenous claudin-2 and claudin-4 by measuring decay in ^{35}S-pulse chase-labeled proteins (Fig. 8). Proteins were pulse-labeled, radioactive amino acids removed, then each claudin was individually immunoprecipitated and immunoblotted over time. The half-lives of endogenous and overexpressed claudin-2 were very similar, although induced claudin-4 was degraded somewhat faster than endogenous claudin-4. However, the longer half-life of claudin-2 relative to -4 remained conspicuous, consistent with an important role for the carboxyl-terminal domain in regulating half-life independently of protein level.

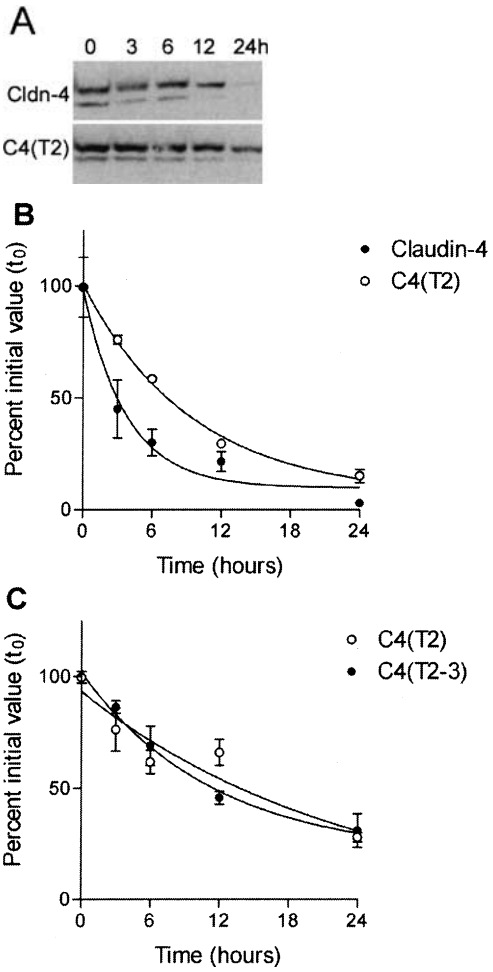

Fig. 7. The half life of C4(T2) is about three times as long as the half-life of exogenous claudin-4. Stable cell lines were grown on Snapwell filters and induced for transgene expression for 4 days. On day 4, cells were washed with warm media and transferred into media containing 50 ng/mL doxycycline. Cells were collected at 0, 3, 6, 12 and 24 h and processed for immunoblot. (*A*) Representative immunoblot of cells collected at the indicated times and probed for claudin-4 or C4(T2) expression. (*B*) Quantification of the decay of C4(T2) and exogenous claudin 4, mean ± SE from 3 separate experiments. In these experiments, the half-life of claudin-4 was approximately 3 h, while the half-life of C4(T2) was approximately 9 h. (*C*) To determine if the half-life of C4(T2) was dependent on the presence of the PDZ-binding motif, the same experiment was repeated using MDCK II Tet-Off cells expressing either C4(T2) or C4(T2-3); these cells were induced and processed as above. Both transgenes are detected equally well with the claudin-2 antibody; there was no apparent difference in half-life. Similar lack of difference in stability was seen between exogenous claudin-4 and claudin-4(-3).

Discussion

In this study, we demonstrate that the cytoplasmic tail of claudin-2 can increase the physiologic efficacy of the extracellular sequences of claudin-4 by stabilizing a C4/T2 chimeric protein and increasing its level. We further show that the half-life of both induced and endogenous claudin-2 in Tet-Off MDCK II cells is >three times as long as induced or endogenous claudin-4. Our findings support the idea that claudin turnover is differentially regulated within

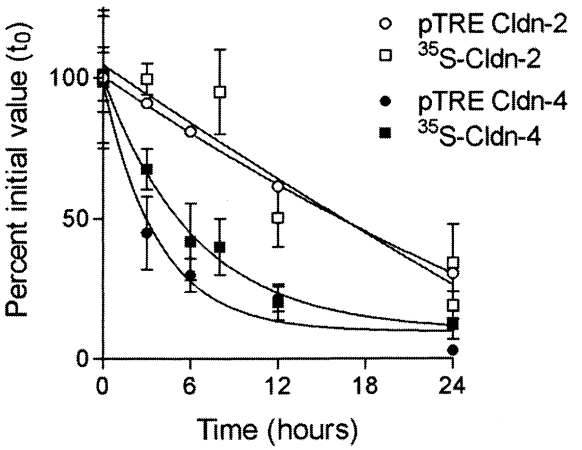

Fig. 8. The difference in stability between claudin-4 and C4(T2) is also present between wild-type and endogenous claudin-4 and –2. For the half-life experiment performed on cells induced to express exogenous claudin-2 and -4 (*empty and filled circles*), stable cell lines were grown on Snapwell filters and induced for transgene expression for 4 days. On day 4, cells were washed with warm media and transferred into media containing 50 ng/mL doxycycline. Cells were collected at 0, 3, 6, 12 and 24 h and processed for immunoblot analysis. For analysis of half-lives of endogenous claudin-2 and -4, (*empty and filled squares*), Tet-Off MDCK II cells were metabolically labeled for 24 h with 100 μCi Tran ^{35}S-label/mL and then chased with unlabeled methionine/cysteine with for the indicated periods. At the end of the chase, cell lysates were immunoprecipitated with claudin-4 or claudin-2 monoclonal antibody coupled to beads. The data was quantitated by densitometry and the half-lives calculated to be less than 6 h for claudin-4 and more than 12 h for claudin-2. Half-lives for endogenous and overexpressed claudin-2 were similar, while the half-life for induced claudin-4 was apparently shorter than for the endogenous protein.

the cell, and that at least part of this regulation is a function of the cytoplasmic carboxyl-terminal sequences. Additionally, although it is clear that PDZ-dependent interactions may be important in the ability of claudins to interact with tight junction scaffolding proteins [11], the presence of the PDZ-binding motif does not appear to be a major regulatory influence in determination of protein half-life or localization.

Like all of the claudins, both claudin-2 and -4 contain PDZ-binding sequences at their carboxyl termini: GYV for claudin-2 and NYV for claudin-4. Fusion proteins encoding the tails of claudins 1–8 have been demonstrated to bind to the first PDZ of ZO-1 in vitro, although it was not determined whether the binding constants for the individual interactions are different [11]. In addition, at least claudin-1 also interacts with PDZ domains in ZO-2 and ZO-3, and the PDZ interactions are dependent on the presence of the YV sequence at the carboxyl terminus [11]. It was recently demonstrated in myelinating Schwann cells that different claudins interact in different subcellular localizations with distinct PDZ-containing proteins, including multi PDZ-domain protein, MUPP1, and Pals-associated tight junction protein, PATJ, [16]. It seems likely therefore that although claudins can interact with ZO-1, different claudins might have distinct additional binding partners. In this study, we found that removal of PDZ-binding motif from exogenous claudin-2 and -4 did not decrease the half-lives of the expressed transgenes. Although PDZ-binding domains have been implicated in protein stability in some examples, in others, the removal of a PDZ-interaction domain had no effect on protein half-life [22]. Claudin degradation rate is apparently dependent on sequences present in the carboxyl terminus other than the PDZ-interaction motif.

That claudins might have different interacting proteins is highlighted by the differing localizations of claudin-2 and -4 within MDCK II cells. Claudin-2 is focused sharply at the tight junction and present in intracellular vesicles, while claudin-4 is at the tight junction and entire lateral plasma membrane. Our earlier work demonstrated that the extracellular domains of claudin-2 and -4 determined neither their intracellular localization nor the architecture of the freeze-fracture fibrils visualized by electron microscopy [5]. Results of immunofluorescence microscopy with the tail chimeras reveal that the carboxyl terminal domains are also unlikely to act as important determinants of localization, since these images show that the signal from the induced chimeric protein, C4/T2 for example, looks more like claudin-4 than induced claudin-2. These results suggest that claudin localization, and likely the freeze-fracture strand pattern as well, are determined by the transmembrane domains or possibly the short cytoplasmic domain that lies between the two extracellular loops.

With two exceptions, induction of the chimeric proteins had little effect on the levels of other tight junction proteins measured. One exception was that induction of the C4/T2 resulted in decreased endogenous claudin-4 levels; this result had also been demonstrated previously following expression of claudin-2 with the first extracellular loop of claudin-4 [5]. The decrease in endogenous claudin-4 levels could be due to decreased transcription and/or decreased half-life. In fact, induced full-length claudin-4 did have a somewhat shorter half-life than did endogenous claudin-4, although we can not exclude an additional feedback on transcription rates. This is different from the response for claudin-2, where there is no apparent influence of exogenous on the endogenous protein level. The second exception is that expression of C2/T4 resulted in a decrease in occludin levels; at present we have no explanation for either the mechanism or the consequences of this finding. Occludin knock-out mice show apparently normal tight junctions by morphologic and physiologic criteria [20], so it is unlikely that decreases in occludin level would have obvious effects on paracellular

physiology. The role of occludin in the tight junction remains undefined.

The dramatic difference in physiologic phenotypes between claudin-4 and C4/T2 was found to be a function of their expression levels. In our initial characterization of the effects of expression of claudin-4 in MDCK II cells [24], we varied claudin-4 protein levels and demonstrated a direct relationship between expression of the transgene and the change in both TER and charge selectivity. However, in that study we had not attempted to quantify this relationship. In the present analysis we determined apparent specific effects of the various claudin proteins by directly relating their physiologic effects to the protein level of each claudin. We first determined the immunoblotting detection sensitivities of the claudin-2 and -4 monoclonal antibodies and used this information to calculate the relative amounts of endogenous and exogenous claudin-2, -4 and the chimeric transgenes in the Tet-Off MDCK II cells. From these results, we determined that the endogenous level of claudin-2 molecules is about three times that of claudin-4. Additionally, C4/T2 transgene can be induced to about three times the level of exogenous claudin-4. We found that the discrepancy in maximal steady-state levels was explained by differences in half-life, since both endogenous claudin-2 and C4/T2 had half-lives about 3-fold longer than either endogenous or exogenous claudin-4.

There exists previous evidence that the half-lives of other TJ proteins are differentially regulated. Occludin, but not claudin-1, has been shown to interact with the E3 ubiquitin-protein ligase, Itch, and was demonstrated to have a very short (1.5 h) half-life [23]. ZO-1, on the other hand, is apparently a relatively stable junction component [4], although these authors also demonstrate a longer half-life for occludin than the previously cited paper. The finding that different TJ components, and in particular different claudins, have differing half-lives provides another locus for modulation of the biochemical composition and thus the physiologic characteristics of the complex. Differences in half-life may be a direct result of the primary amino-acid sequence or may be further modulated by sequence-specific post-translational modifications.

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