Channel formers and barrier builders: functional characterization of claudins in epithelial cells

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Tight junction proteins as channel formers and barrier builders:
claudin-2, -5, and -8

Salah Amasheh¹, Susanne Milatz¹, Susanne M. Krug¹, Alexander G. Markov³,
Dorothee Günzel¹, Maren Amasheh², Michael Fromm¹

¹Institute of Clinical Physiology;
²Department of Gastroenterology, Infectious Diseases, and Rheumatology,
Charité, Campus Benjamin Franklin, Berlin, Germany;
³Faculty of Biology, St. Petersburg State University, St. Petersburg, Russia

Correspondence: Dr. Salah Amasheh, Institute of Clinical Physiology, Charité, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany, Tel: +49-30-8445 2500, FAX +49-30-8445 4239, email: salah.amasheh@charite.de

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Abstract

Tight junctions (TJ) form the paracellular barrier for ions and uncharged solutes not only in "tight" but also in "leaky" epithelia. Therefore in the pre-molecular era of tight junction research this was believed to be achieved in a perfect or less perfect way, depending mainly on the amount of horizontally oriented TJ strands. During the past decade, it emerged that tight junction molecules like claudin-1 and many others strengthen the barrier, while a few claudins like claudin-2 or -10 weaken it. This report focuses on three claudins, one channel former and two barrier builders. Claudin-2 represents the prototype of a paracellular channel-forming tight junction protein responsible for specific transfer of solutes across the epithelium without entering the cells. This channel is selective for small cations but nearly impermeable to anions and uncharged solutes of any size. In contrast, claudin-5, a tight junction protein typical for all endothelia but also found in some epithelia, was characterized as a potent barrier builder. Claudin-8, another barrier builder, was demonstrated to be regulated by Na⁺ uptake in surface epithelial cells of human colon. Here, aldosterone enhanced Na⁺ uptake...
absorption by dual action, transcellularly by inducing the epithelial sodium channel ENaC and paracellularly by preventing back-leakage of absorbed Na⁺ by up-regulating claudin-8.

Introduction

Epithelia and endothelia line all surfaces and cavities of the body and form barriers. By this they produce compartments with different solute compositions on both sides. This barrier function becomes effective only due to presence of the characteristic organelle of epi- and endothelial tissues, the tight junctions. As the name Zonula occludens indicates, the sealing function of the tight junction was known from the beginning and from the observation that Lanthanum does not penetrate it was even believed that these structures are completely impermeable.

Then, in the early seventies, it was learned from electrophysiological measurements that tight junctions can also be very leaky structures,¹ giving rise to the concept of "leaky" and "tight" epithelia,² located e.g. in the nephron or the intestinal tube in the respective proximal and distal segments. A tissue is by definition "leaky" if its paracellular pathway exhibits a lower resistance than the transcellular one (R_{para} < R_{trans}), and "tight" means the opposite.³ These properties do not in all cases correlate with common transepithelial resistance (TER, R_{t}), although leaky epithelia often are low-resistance tissues and vice versa.

In the "pre-molecular" era of tight junction research it was widely accepted that tight junctions form the paracellular barrier only in a more or less perfect way depending above all on the amount of horizontally oriented tight junction strands providing the boundary. During the past decade, however, it emerged that there are tight junction molecules like claudin-2 or claudin-10 which, instead of strengthening the barrier, weaken it.

For claudin-2 it has been demonstrated that overexpression of this claudin turned Madin-Darby canine kidney I cells from high-resistance to low-resistance epithelia.⁴ On the other hand, it was a similar surprise that a knock out of occludin, the first-discovered and probably most abundant tetraspan tight junction protein,⁵ did not impair the paracellular barrier at all.⁶

For several other claudins a clear-cut barrier function has been established. The first proof of a direct influence of a claudin in the selective control of paracellular ion permeability was demonstrated by Van Itallie et al. for claudin-4.⁸ The most spectacular example for a barrier forming single claudin was claudin-1 which leads in knockout mice to a fatal breakdown of the tightening function of the zonula occludens.⁹

For functional characterization of claudins, two general strategies have prevailed, analyses of single claudin deficiencies (or knockouts), and expression in epithelial cell lines which do
not show endogenous expression of the respective claudin. A combination of both e.g. recently resulted in a detailed characterization of claudin-16 function and hereditary disease linked to mutations of the claudin-16 gene, namely familial hypomagnesemia with hypercalciuria and nephrocalcinosis.\textsuperscript{10, 11, 12}

This report focuses on three claudins, one channel former and two barrier builders, which are not only of outstanding biological importance in health and disease, but also represent excellent models for basic studies on functional mechanisms and signal transduction.

The first claudin portrayed here is claudin-2 which represents the prototype of a paracellular channel-forming tight junction protein responsible for specific transfer of solutes across the epithelium without entering the cells. This channel is selective for small cations but almost impermeable for anions, even small ones, and for uncharged solutes of any size.\textsuperscript{13}

The second claudin described, claudin-5, was known to be primarily expressed in tight junctions of endothelia. Knockout of this protein in mice was reported to result in a selective increase in paracellular permeability of the blood-brain barrier for molecules smaller than 800 Da.\textsuperscript{14} However, we detected genuine claudin-5 expression also in epithelial tight junctions, namely in the human colon cell line HT-29/B6. Claudin-5 proved to be a potent barrier builder in these cells. By contrast, claudin-5 was absent in the human colonic cell line Caco-2 and in Madin-Darby canine kidney cells, subclones C7 and C11.\textsuperscript{15}

Finally, the function and regulation of claudin-8 is characterized. In MDCK cells claudin-8 forms a barrier to cations, including protons, but also to ammonium and bicarbonate ions.\textsuperscript{16, 17} These effects are, in part, explained by replacement of endogenous claudin-2.\textsuperscript{18} As a clinical consequence, already in mild to moderately active Crohn’s disease an upregulation of pore-forming claudin-2 and a downregulation and redistribution of sealing claudins -5 and -8 take place which lead to an altered tight junction ultrastructure and pronounced barrier dysfunction.\textsuperscript{19} Recently, a regulatory mechanism of claudin-8 expression was described: It was demonstrated that enhanced Na\textsuperscript{+} influx into human colon epithelium caused an up-regulation of claudin-8 so that it tightens the epithelium against back-leakage of absorbed Na\textsuperscript{+}.\textsuperscript{20}

\textbf{Functional characterization of claudins}

Prior to transfection approaches, endogenous presence of claudins in the human intestinal cell lines Caco-2 and HT-29/B6 and in MDCK subclones was analyzed. MDCK subclone C7 clone has “tight” tight junctions, whereas the MDCK-C11 clone exhibits “leaky” tight junctions.\textsuperscript{21} In immunostainings comparing respective claudins with occludin detection, occludin has emerged as a reference marker for localization of the tight junction complex. Like occludin, claudin-1 and claudin-3 have been detected in the cell types examined here. These
Claudins appear to belong to the basic pattern of epithelial tight junction proteins, as both are found in combination in many organs, such as liver, lung, kidney, colon, and skin. Whereas claudin-1 is known to be crucial for the “sealing” of the barrier, a clear functional contribution of claudin-3 has not been shown yet.

Transepithelial resistance, $R_t$, and tight junction protein expression patterns of untransfected cells are depicted in Fig. 1. $R_t$ ranged from $58\pm1$ Ω·cm$^2$ of MDCK-C11 cells which represent a leaky epithelium, to $1396\pm67$ Ω·cm$^2$ of MDCK-C7 cells, representing a tight epithelium ($n=12$, respectively). Values for HT-29/B6 and Caco-2 cells ranged in between these two cell types ($603\pm6$ and $251\pm3$ Ω·cm$^2$, respectively ($n=15$ each, Fig. 1A). To correlate functional and structural parameters, the presence of tight junction proteins occludin and claudin-1, -2, -3, and -5 was analyzed (Fig. 1B). Whereas HT-29/B6 cells represent the most comprehensive expression of claudins, Caco-2 cells lack expression of claudin-2 and -5, and MDCK-C7 cells do not express endogenous claudin-2, giving a first hint on functional contribution of this protein (Fig. 1B). Moreover, both, MDCK-C7 and -C11, lack expression of claudin-5, which was confirmed by PCR and experiments employing immunization peptide used for generation of the claudin-5 antibody. Apparently conflicting, a stronger expression of the sealing tight junction protein claudin-1 was detected in low-resistance MDCK-C11 versus tight MDCK-C7 cells. This apparent discrepancy was soon explained by a comparison of immunofluorescent staining of claudin-1 in MDCK-C7 and -C11 cells. In low resistance MDCK-C11 cells, the strong claudin-1-signal was detected in subjunctional areas, speaking against a direct functional contribution in this cell type (Fig. 2). Cells were transfected with cDNA encoding endogenously missing claudins, namely MDCK C7 with claudin-2, and MDCK-C7, -C11, and Caco-2 cells with claudin-5 cDNA. Expression was analyzed by means of Western blots (Fig. 3A) and confocal laser scanning microscopy. After stable transfection, both, claudin-2 and FLAG-claudin-5 were detected within the tight junction complex in colocalization with occludin. Expression of claudin-2 in MDCK cells resulted in a decrease of transepithelial resistance, whereas expression of claudin-5 resulted in an increase in transepithelial resistance in Caco-2-cells. In MDCK-C7 cells and MDCK-C11 cells, expression of claudin-5 did not result in changes of transepithelial resistance (Fig 3B).

Flux measurements employing $[^3]$H]-mannitol (182 Da) showed a decrease in paracellular permeability of Caco-cld5 to about 50% compared to controls, whereas no significant changes were observed for MDCK-C7-cld5 or -cld2, respectively.

Compared to MDCK-C7 vector controls, paracellular resistance ($R_{para}$) was dramatically reduced in MDCK-C7-cld2 cells, indicating that claudin-2 has formed a conductive channel across the tight junction (Fig. 3C). This effect has recently been verified by two-path impedance spectroscopy in its current configuration.
To characterize the charge preference of claudin-2, dilution and biionic potential measurements were performed. Partial ion conductivities were calculated from relative paracellular permeabilities of Na\(^+\), K\(^+\), NMDG\(^+\), Cl\(^-\) and Br\(^-\) (Fig. 3D). Clone C7-cld2 showed a selective increase of Na\(^+\) and K\(^+\) conductance compared with MDCK-C7 control (Na\(^+\): 1.74±0.06 mS/cm\(^2\) vs. 0.21±0.01 mS/cm\(^2\); K\(^+\): 0.069±0.003 mS/cm\(^2\) vs. 0.008±0.001 mS/cm\(^2\)), whereas Cl\(^-\) conductance was not significantly changed (0.18±0.01 mS/cm\(^2\) vs. 0.16±0.01 mS/cm\(^2\)).

**Claudin-2 and -5 in health and disease**

Claudin-2 typically is expressed in leaky epithelia as in proximal renal tubules,\(^2^4\) intestinal crypts,\(^2^5\) as well as choroid plexus.\(^2^6\) Pathophysiological relevance of claudin-2 recently has been highlighted by the finding of an elevated expression of claudin-2 in colon epithelia of patients suffering from inflammatory bowel diseases (IBD; for review, see Mankertz et al., 2007).\(^2^7\) In both main disorders, Crohn’s disease (CD) and ulcerative colitis (UC), claudin-2 appears to aggravate and sustain inflammation.\(^2^8,2^9\)

Moreover, in CD a decrease in claudin 5 was reported,\(^1^9\) which is in accordance with the tightening function of the protein. A significant role for claudin-5 was also found by identification of hereditary diseases, which have been attributed to mutations of the genomic sequence. These include glioblastoma multiforme and velo-cardio-facial syndrome.\(^3^0,3^1\)

**Claudin-2 forms a channel for small cations**

Claudin-2 has been shown to convert "tight" tight junctions into "leaky" ones and it was identified as a cation-selective paracellular channel.\(^4,1^3\) Expression of claudin-2 resulted in an increased permeability of the paracellular pathway for small cations in MDCK-C7 cells, which lack endogenous claudin-2, allowing tight barrier properties. As also shown in that study, claudin-2 also is not endogenously expressed in Caco-2 cells, demonstrating that solely the lack of claudin-2 does not necessarily result in a "tight" tight junction, but is dependent on the presence of “tightening” proteins.

As was confirmed in this study, exogenous claudins assemble into tight junctions in addition to existing ones, since other claudins were not changed within the tight junction after stable transfection with claudin-2 cDNA. Another possibility is that exogenous claudins may replace endogenous claudins. As an example, TetOff-induced expression of claudin-8 in MDCK II cells caused claudin-2 to down-regulate and by this to decrease cation selectivity.\(^1^8\)

Identification of the specific channel-forming function of claudin-2 was confirmed recently by experiments employing a continuous series of noncharged polyethylene glycols (PEGs) across monolayers of cells expressing claudin-2. These experiments resulted in a selective
increase in pore number but not size and had no effect on the permeability for PEGs that are larger than the pores. Incidentally, claudin-2 had no effect on barrier properties, if expressed in MDCK II cells, because these cells already are very conductive and cation-selective.

Claudin-5 is a sealing protein dependent to expression background

Claudin-5 first was detected in endothelial cells of blood vessels. In MDCK and Caco-2 cells, it was absent whereas it is expressed in native intestine. Claudin-5 could be detected in (and cloned from) HT-29/B6 cells, which originate from human colon and show properties of differentiated epithelial crypt cells. The lack of effects of claudin-5 expression on $R^t$ in MDCK cells might be interpreted due to the high endogenous transepithelial resistance in case of MDCK-C7, and to the predominant expression of claudin-2 in MDCK-C11 cells. A tightening effect of claudin-5, however, was reported in low resistance MDCK II cells. These results underline the notion that successful characterization of a tight junction protein is always dependent on the respective expression model used.

With regard to claudin-5 regulation it was shown that endothelial VE-cadherin at adherens junctions up-regulates the gene encoding claudin-5. This result explains why and how inhibition of VE-cadherin produces a decrease in resistance.

Claudin-8 expression is regulated by transmembranal Na$^+$ uptake

In the distal-most segments of tubular epithelia like intestine, nephron, and ducts of excretory glands, aldosterone controls ion transport in order to keep the composition of the body fluids constant. It is consensus that aldosterone does this by altering transcellular transport, mainly via the epithelial sodium channel (ENaC).

With increasing transepithelial Na$^+$ absorption rates high concentration gradients are built up which would be impaired by Na$^+$ back-leakage. We therefore investigated whether ENaC-mediated Na$^+$ absorption is paralleled by a tightening of the barrier to prevent breakdown of the Na$^+$ gradient. Tight junctions of the aldosterone-responsive segments of kidney and colon both contain claudin-8 which already indicates that this claudin may be involved in the regulation of Na$^+$ transport.

Short-circuit current of human sigmoid colon was monitored after adding 3 nM aldosterone for 8 hours. ENaC-mediated Na$^+$ transport was determined as the current sensitive to the ENaC-blocker amiloride. Subsequently, tissues were removed for molecular analysis of ENaC and TJ protein expression, localization, and regulation. Aldosterone stimulated Na$^+$
absorption by inducing ENaC, together with increased claudin-8 and occludin expression, but not of claudin 1 to 5, and 7 (Fig. 4A, B). Only claudin-8 was included into tight junctions, whereas occludin was localized outside the tight junction. ENaC as well as claudin-8 were induced in colon surface cells but not in crypts. Paracellular Na⁺ permeability, as determined from ⁴²Na⁺ fluxes in s-to-m direction, was reduced by half (Fig. 4C). The effect on claudin-8 was absent when Na⁺ uptake was permanently blocked by amiloride throughout the whole experiment, indicating that ENaC action, or more general, Na⁺ uptake, is part of the signaling pathway. Real-time PCR validated an increase in claudin-8 transcripts. Generality of the mechanism on functional and molecular level was evaluated in glucocorticoid receptor-transfected HT-29/B6 human colon cells. After stimulation with dexamethasone, these cells also showed increased claudin-8 expression and claudin-8 mRNA. As determined by means of two-path impedance spectroscopy, the paracellular resistance increased by a factor of 3, while the transcellular resistance was not altered significantly (Fig. 4D).

New concept of aldosterone action: ENaC-mediated Na⁺ absorption is protected against back-leakage by claudin-8 upregulation

Because claudin-8 provides a barrier for monovalent and divalent cations it can be assumed that not only back-leakage of Na⁺, but also that of K⁺, H⁺, Ca²⁺, and Mg²⁺ is reduced. The probably most significant effect of claudin-8, however, concerns Na⁺ if one considers its large transepithelial concentration gradient e.g. in large intestine. Rat distal colon in vivo (i.e. facing a plasma level of 141 mM) develops a luminal Na⁺ concentration as low as 22 mM under non-stimulated conditions. However, stress-induced aldosterone liberation results in a luminal Na⁺ concentration as low as 2.2 mM, yielding a zero-flux lumen-to-plasma ratio of 1:66. From a teleological point of view, the development of such large electrochemical gradient would require an increased tightness of the barrier in order to prevent Na⁺ back-leakage. Our data indicate that aldosterone indeed stimulates Na⁺ transport not solely by enhancing transcellular absorption, but in parallel also by tightening the paracellular pathway against immediate back-leakage of freshly absorbed Na⁺.

Conclusion

Tight junction strands are formed by a heteropolymer of different tight junction proteins contributing different properties to tight junction characteristics. This report highlights the identification of the paracellular cation channel claudin-2 and the sealing proteins claudin-5 and claudin-8. Exogenously expressed claudins generally can replace other endogenous claudins or can assemble with these claudins yielding different local collections of claudins which then determine the paracellular permeability in concerted action. Claudins are subject
to cellular regulation shown here for claudin-8 which is controlled by aldosterone-mediated Na⁺ uptake.

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References


Figure legends

Fig. 1 Transepithelial resistance measurements and detection of tight junction proteins. (A) $R_t$ of MDCK-C7, -C11, HT-29/B6, and Caco-2 cells revealed the highest values for MDCK-C7, representing a “tight” tight junction, and low values for MDCK-C11 and Caco-2 cells, representing relatively permeable to “leaky” tight junctions. (B) Membrane fractions of MDCK-C7, -C11, HT-29/B6, and Caco-2 revealed different expression profiles: occludin and claudin-1 were detected in all cell types, whereas the pore-forming tight-junctional protein claudin-2 was not detectable in MDCK-C7 and Caco-2 cells. Claudin-5 showed no signals in Caco-2 cells and only marginal signals in MDCK-C7 cells.

Fig. 2 Localization of claudin-1 in MDCK cells. Immunofluorescent staining and confocal laser-scanning microscopy showed subcellular distribution of occludin and claudin-1 in MDCK-C7, and -C11 cells. Focused colocalization within the tight junction complex was observed for occludin and claudin-1 in MDCK-C7 cells, whereas in C11 cells, Z-scans, as shown on the right or above the top, revealed that a marked expression of claudin-1 is detectable in subjunctional membrane areas (circles).

Fig. 3 Functional characterization of claudin-2 and -5. (A) Western blots detecting claudin-2 and claudin-5 in MDCK and Caco-2 clones stably transfected with claudin-2 (cld2), claudin-5 (cld5) or vector (v) cDNA alone, respectively. (B) Resulting changes of $R_t$. (C) Paracellular resistance ($R_{\text{para}}$) of MDCK monolayers. Compared to MDCK-C7 vector controls (C7-v), in claudin-2-transfected cells (-cld2) $R_{\text{para}}$ was substantially reduced. Data for MDCK-C11 cells genuinely expressing claudin-2 are given for comparison. (D) Partial conductivities of Na$^+$, K$^+$ and Cl$^-$ in C7-v, C7-cld2 and C11-v ($n=6$) calculated from dilution and bionic potential measurements. Na$^+$ and K$^+$ were almost equally permeable but Na$^+$ is 26-fold higher concentrated than K$^+$, leading to a 25-fold contribution of Na$^+$ to overall conductivity compared to K$^+$.

Fig. 4 Regulation of claudin-8 expression. (A) Expression of tight junction proteins in human sigmoid colon during ENaC induction. (B) Densitometry. Signals of occludin and claudin-8 were increased after stimulation with aldosterone (dark bars) ($n=4-7$, *$p<0.01$). Claudin-2 was not detectable in human colon. (C) Serosal-to-mucosal flux of $^{22}\text{Na}^+$ ($J_{\text{Na}^{\text{s-m}}}$) in controls and aldosterone-incubated tissues, measured at the period of final blockade with amiloride ($n=9$ and 10, **$p<0.01$). (D) Two-path impedance spectroscopy. Dexamethasone (dexa) induced an increase in epithelial resistance ($R^e$). This was caused by a strong in-
crease of paracellular resistance ($R_{\text{para}}$), reflecting sealing of the tight junction. The dexamethasone-induced increase in $R_{\text{para}}$ was prevented if amiloride was present continuously (amil + dexta; n=4-6, **p<0.01).
A

![Graph showing resistance (R') for different cell lines](image)

B

![Western blot images of various Claudin proteins](image)

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