Crystal structures of claudins: insights into their intermolecular interactions

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Claudins are four-transmembrane proteins that constitute the backbone of tight junction strands via self-polymerization in the apicolateral membranes of epithelial cells. Together with their cell–cell adhesion function, claudin proteins form the paracellular barrier and/or channels through epithelial cell sheets whose permeability is primarily dependent on the claudin subtype. Recently determined crystal structures of several claudins revealed the unique claudin fold of four transmembrane helices in a left-handed helical bundle with an extracellular β-sheet domain. Here, we focus on the structural basis of the intermolecular interactions between claudin molecules and between the Clostridium perfringens enterotoxin and its receptor claudins.

Keywords: claudin-4; claudin-15; claudin-19; C-CPE; crystal structure

Introduction

In epithelial and endothelial cells, tight junctions (TJs) function as cell adhesion structures and as paracellular barriers to restrict the permeation of solutes and small molecules through cell sheets. The adhesion and meshwork properties of TJ strands are mainly attributed to the claudin membrane protein family.1 In mammals, there are 27 known claudin subtypes, some of which have splice variants.2 The combination of claudin subtypes expressed in different tissues determines the specific properties of the paracellular permeability.

On the basis of their sequence homology, all claudin subtypes are considered to possess a common membrane-spanning topology comprising four transmembrane (TM) segments, a very short intracellular N-terminus (1–3 residues), a large first extracellular loop that contains a consensus W–LW–C–C motif, a shorter second extracellular loop, and an intracellular C-terminal tail whose sequence varies and ends in a scaffold-binding motif in most mammalian claudins.3,4 Claudin molecules, the polymers of which form the backbone of TJ strands, have two distinct potential interaction sites. One of the intermolecular interactions is head-to-head adhesion in opposing lateral cell membranes (trans-interactions), and the other is side-by-side polymerization within the same membrane (cis-interactions).5 How claudins assemble into paired TJ strands to form the putative paracellular pathways selective for size and charge is an intriguing question.

The first crystal structure of a claudin was recently solved,6 and two more structures of different claudin subtypes in complex with a toxin fragment were subsequently revealed.7,8 The structural information provides clues toward understanding the molecular architecture of TJs and the mechanism of their barrier/channel functions in different epithelial tissues. Here, we describe recent advances in knowledge of the crystal structures of claudin molecules. We then discuss the possible intermolecular interactions underlying their arrangement in TJ strands and the susceptibility of claudins to bacterial toxins based on the structures of toxin–claudin complexes.

Overall structure of the claudin fold

The overall structures of mouse claudin-15 (mCldn15, PDB: 4P79)6 protein as well as mouse...
Figure 1. Overall structures of claudins in complex with or without C-CPE, viewed parallel to the membrane plane. (A) Structure of the mCLDN15 monomer [PDB: 4P79]. (B) Structure of mCldn19 in complex with C-CPE [PDB: 3X29]. (C) Structure of hCLDN4 in complex with C-CPE [PDB: 5B2G]. The claudin molecule is colored in a gradient ranging from blue at the N terminus to red at the C terminus. C-CPE is shown as the wheat-colored structure. The consensus motif W–LW–C–C is shown in yellow stick representation. (D) Secondary structure diagram of mCLDN15. The orange line represents a disulfide bond. Red star symbols indicate the conserved residues critical for lateral interactions observed in the crystal and suggested to be present in TJ strands. Residues of the consensus motif in ECS1 are shown as yellow circles labeled with a one-letter code.

Claudin-19 (mCldn19, PDB: 3X29) and human claudin-4 (hCldn4, PDB: 5B2G) proteins in complex with a C-terminal fragment of the bacterial Clostridium perfringens enterotoxin (C-CPE) are currently available. Although the crystallization of mCLDN15 could only be achieved in the lipidic cubic phase, the other two claudins bound to soluble C-CPE were crystallized using the vapor diffusion method. All claudin proteins share the same overall structure (Fig. 1): TM segments 1–4, which form a tight left-handed four-helix bundle, and an extracellular β-sheet domain comprising two extracellular loops (ECL1 and ECL2), which we renamed as extracellular segments 1 and 2 (ECS1 and ECS2, respectively) from a structural point of view. The hydrophobic W–LW residues in the consensus motif protrude from the beginning of the β3 strand and the tip of the β2–β3 loop and are located adjacent to each other. The side chains are embedded in a crevice formed by the top of the four-helix bundle and are partially exposed to the lipid environment, anchoring the entire β-sheet domain close to the membrane surface (Fig. 1A–C). Because these residues are required for hepatitis C virus entry, it is likely that hydrophobic residues are critical for stabilizing the conformation of the extracellular domain. The crystal structures demonstrate that the cysteine residues in the β3 and β4 strands, which are in the consensus motif sequence (W–LW–C–C), form an intramolecular disulfide bond (Fig. 1), as was suggested by mutagenesis studies. A recent review updated the consensus sequence as a claudin family signature sequence, including an arginine/glutamine residue (Arg79 in mCLDN15) located downstream of the conserved cysteine residues. In the structures, the highly conserved arginine residue extends from the intramembrane part of TM2 to the membrane surface, interacting with the membrane-proximal region of the extracellular domain. At the end of ECS1, an additional short extracellular helix (ECH) runs almost parallel to the membrane plane and connects the β4 strand to TM2 in the mCLDN15 structure, and the helix kink induced by a Gly/Pro hinge residue is likely to be a conserved conformation that is stabilized by the conserved arginine/glutamine residue in TM2 and clustered hydrophobic residues (Phe65, Leu69, and Val75 in
mCLDN15) distributed around the ECH.\(^6\) The corresponding region in both mCLDN19 and hCLDN4 is disordered or unwound because of the binding of C-CPE. All of the conserved residues mentioned above are likely involved in stabilizing the extracellular β-sheet domain, which could function as the most exposed interface to cell-to-cell junctions and the paracellular space.

For crystallization, the C-terminal fragments of claudins are truncated in all of the expression constructs.\(^5–8,18\) The flexible C-terminus is dispensable for forming TJ-like strand polymers \textit{in vitro},\(^6\) although the majority of claudins have a PDZ-binding motif (–YV) at the C-terminus that is critical for binding to many kinds of scaffolding proteins \textit{in vivo}\(^3,19\) as well as for proper recruitment of claudins at TJ regions.\(^4\) In addition, none of the three claudins contained any posttranslational lipid modifications owing to the deficient mutations in the expression constructs or properties of the cell-free protein synthesis method. Some membrane-proximal cysteines at the cytoplasmic regions just after TM2 and TM4 could be subjected to \(S\)-palmitoylation, which may be required for the efficient localization of claudins into TJs, but not for strand assembly.\(^20\)

The four-TM helices are tightly intertwined by residues with small side chains, in clear contrast with the recently solved tetraspanin CD81, whose four TM domains are widely exposed to the extracellular side.\(^21\) Mutations at small/hydrophobic residues throughout the TM helices of claudin-14 and claudin-16 are reported to cause inherited human diseases,\(^22,23\) suggesting the potential importance of the tight helix bundle for the global folding of claudin proteins, as well as their functions.

**Regions with variable sequences among claudin subtypes**

Various epithelial cell tissues express multiple claudin subtypes,\(^2\) and coexpression studies in cultured cells suggest that different claudin subtypes are incorporated into the same TJ strands.\(^24,25\) Multiple subtypes can homo- and heterotypically interact with specific claudin subtypes, although the residues involved in heterotypic compatibility are unclear. On the basis of the sequence alignment among members of the claudin family, the crystal structures revealed that poorly conserved sequences are located at the most exposed regions in the extracellular segments, defined as variable regions V1 and V2 (Figs. 2 and 3). Some of the nonconserved residues at V2 in claudin-3 and claudin-5 are suggested to affect head-to-head interactions, either directly or indirectly.\(^26\) In the crystal of mCLDN15, no loop structure in V1 was observed owing to disorder likely caused by the conformational flexibility (Fig. 2A). On the other hand, a density was clearly observed in the V1 region of both mCLDN19 and hCLDN4 in complex with C-CPE (Fig. 2B and C), making direct contact with the C-CPE surface, as described below. The variable regions provide flexibility that might contribute to subtype-specific interactions induced by their conformational changes from a nonadhesive state to an adhesive state.

**Electrostatic potential of the extracellular surface**

Mutagenesis and physiological studies elucidated the residues responsible for the paracellular ion selectivity of the TJs, which are charged and mostly located in the C-terminal half of ECS1.\(^16,27–30\)
Especially in the structure of mCLDN15, the negatively charged residues (Asp55 and Asp64) are located on β3 to β4, facing upward toward the extracellular side. This upward/downward orientation of the side chains, which is restricted by the β-sheet fold, determines the electrostatic potential of the extracellular surface according to the amino acid sequence of each claudin subtype. The mCLDN15 structure and homology models of other claudins with different charge selectivity exhibit a distinct charge distribution at the distal part of the β-sheet domain, forming a palm-shaped structure, which could be the pore-lining surface of the putative paracellular ion pathways.

**Proposed structural models for TJ architecture**

Based on the nonsymmetric linear arrangement of molecules packed in the mCLDN15 crystal lattice, the crystal structure revealed side-by-side tandem intermolecular interactions mediated by highly conserved residues (Met68, Phe146, Phe147, Glu157, and Leu158) between adjacent juxtamembrane domains on the extracellular side. Some of the corresponding residues in claudin-5 (Phe147, Tyr148, and Glu159) are also reported to contribute to the intermolecular interactions and the formation of TJs. Because mutagenesis experiments indicated that these residues are required for mCLDN15 to form TJ-like strands, the linear arrangement in the crystal likely represents a cis-arrangement of claudin molecules in TJ strands in vivo, and thus the conservation of the residues among claudin subtypes suggests that different claudin subtypes could comingle in the same TJ strand. As previously reported for claudin-2, a residue in ECS1 (Asp65) could locate to a narrow part of the putative paracellular pore where cysteine-crosslinking dimerization by mutagenesis occurs, and cysteine crosslinking at a similar position (Asn61) in claudin-15 led to a model of antiparallel double-row arrangement of the linear polymers in a TJ strand (Fig. 4A and B). This model could result in the formation of continuous β-barrel–like pores along the strand by docking between two opposing cells (Fig. 4B and C), provided that the V1 and V2 regions change their conformation appropriately for head-to-head interactions. The proposed architecture of the β-barrel–type pores is a favorable explanation of two TJ properties, either increasing the tightness of the barrier or forming paracellular channels. The number of β-strands and the conformation of side
Figure 4. Proposed arrangement of claudin molecules in TJ strands. (A) Model of an antiparallel double row of claudin molecules viewed from the extracellular side. The mCLDN15 monomers are shown in cartoon representation. Broken lines in the red circles indicate the V1 region, including the disordered region of ECS1. The V2 parts are indicated in blue circles. Magnified view of the modeled dimer interface of mCLDN15. (B) The side view of the double row arrangement shown in (A). A solid half oval indicates the extracellular “half-pipe” structure formed by a claudin dimer, and the broken line indicates another putative half-pipe structure in an adjacent cell to complete the channel. (C) Model of paracellular TJ channels shown in cartoon representation. Disordered loops and regions causing steric clashes in the model are not shown. The filled gray oval indicates a putative channel pore among these pores.

chains sticking into the pore would define the pore diameter as well as the surface charge and thus could contribute to the permeability of solutes through the modeled TJs. Some of the residues predicted to line the claudin-2 pore in TJs, however, do not face the interior of the proposed β-barrel–like pore. All of the experimental studies together cannot be fully explained by the proposed model, and therefore further structure–function studies are required to properly tune the models for each subtype.

Findings from a recent fluorescence resonance energy transfer (FRET)-based study support the notion of a cis-arrangement of claudins having at least two different interfaces. The intermolecular interactions in cis at two sites in claudins may differentiate cis-dimerization and cis-polymerization and are likely to complicate interpretations of the
results from site-directed mutagenesis studies of Tyr147 and Tyr148 in claudin-3 and claudin-5, respectively, because there could be mutations that affect only cis-polymerization (e.g., by diminishing only side-by-side tandem interactions) and not cis-dimerization and consequently diminish trans-interactions. Other mutagenesis studies suggest that TJ strands contain intramembrane cis-interactions between TM helices of claudins, such as TM2 in claudin-2 \(^{27}\) or TM3 in claudin-3/5, \(^{26}\) which are not prominent in the antiparallel double-row model. \(^{32}\) It is also unclear how TJ strands achieve the morphological dynamics in the junctional membranes and the winding flexibility of the intramembrane polymers that are visualized by fluorescence microscopy and freeze–fracture electron microscopy. \(^{38,39}\) In addition, intermolecular interactions of claudins in membranes with other TM proteins, such as occludin and junctional adhesion molecules, must be included when considering the higher-order assembly of the TJ strands and their molecular dynamics in TJs. \(^{40,41}\) Therefore, further biochemical and physiological studies of the structure–function relationship and further structural analyses of strand-forming claudins in the junctional state are needed.

**C-CPE binding**

The two independent structures of claudins in complex with C-CPE showed that both ECS1 and ECS2 are extensively involved in C-CPE binding and that, consequently, the entire extracellular region of claudin is covered by the toxin fragment \(^{7,8}\) (Figs. 1B, 1C, and 5A). It has long been thought that only the residues in ECS2 are responsible for binding C-CPE, \(^{38}\) but this interpretation was based on binding assays using chimeric constructs of CPE-sensitive/resistant claudin subtypes \(^{42–44}\) as well as an overlay assay with peptide arrays of ECS2 residues of various claudins. \(^{45}\) These studies definitely revealed the importance of ECS2 for binding the toxin, but the findings did not exclude the possible involvement of ECS1 in the interactions. Most of the mutational analyses focused on the binding site of ECS2 or around the regions called the “tyrosine pocket” and the “leucine triplet,” as these regions are surrounded by three tyrosine residues (Y306/Y310/Y312) and by a three-leucine triplet (L223/L254/L315), respectively, in C-CPE, \(^{46}\) which are critical for claudin binding. \(^{46–50}\) Instead, the structures of the claudin–toxin complexes reveal that the interface that interacts with ECS1 and ECS2 is evenly distributed on one side of C-CPE (Fig. 5A), which includes the tyrosine pocket and the leucine triplet. Similar to the predicted ECS2 conformation of murine claudin-3 and claudin-4 in the tyrosine pocket of C-CPE, \(^{46}\) the N–P–(L/S) motif in ECS2 snugly fits into the tyrosine pocket, and the other residues also form an extensive hydrophobic interface with C-CPE (Fig. 5A and B). In addition to ECS2, one side of the first two \(\beta\)-strands (\(\beta_1\) and \(\beta_2\)) broadly interacts with the other half of the C-CPE interface, where the leucine triplet is also located. The interacting motif sequence in ECS1 ((A/N/S/T)–(I/V)–(I/L/V)–(I/T/V)) is actually conserved in the subgroup of classic claudin subtypes \(^{51}\) (Fig. 3). Because the motif of ECS1 contains similar hydrophobic residues among all CPE-sensitive/resistant classic claudins, the ECS1 region could not determine the sensitivity, but rather would enhance the binding affinity for C-CPE by increasing the buried surface area.

As far as we tested with full-length mouse claudin subtypes from 1 to 27, screening with a combination of insect cell expression and fluorescence-detection size-exclusion chromatography showed that C-CPE could bind to mouse claudin-3, -4, -6, -7, -8, -9, -14, and -19 (unpublished data), consistent with previous reports, \(^{52,53}\) except for claudin-19. Because the affinity of full-length mCLDN19 is 30-fold weaker than that of mCLDN3, \(^{7}\) which is a typical CPE-sensitive subtype, the overlay assay with peptide arrays of ECS2 could not detect the binding of mCLDN19 to C-CPE. \(^{45}\) The extracellular domains of mCLDN19 and hCLDN4 adopt similar conformations after structural alignment at the C-CPE regions, but the ECS2 region (in particular TM3) of mCLDN19 interacts with C-CPE in a slightly different manner (Fig. 5C). On the basis of superposition at the TM helices of hCLDN4, the orientation of C-CPE seems to vary depending on the extent of the helical kink around Pro135 in TM3 owing to the different crystal contacts (Fig. 5D). The corresponding residue of mCLDN19 is alanine, and thus TM3 prefers a straight conformation. The lower flexibility of TM3 might be the reason for the low binding affinity for C-CPE. Claudin-7 and claudin-14 also have an alanine at this site, and all the other CPE-sensitive claudin subtypes have a proline residue in TM3. Claudin-3, -4, -7, and -8 are
Figure 5. The interface between claudins and C-CPE. (A) Surface representation of C-CPE with bound mCLDN19 shown in cartoon representation. C-CPE residues that interact with ECS1, ECS2, or both are labeled and colored in marine, salmon, or gray, respectively. (B) Hydrophobicity of the C-CPE surface viewed as in (A). Residues are colored from yellow (hydrophobic) to white (polar/charged), and the residues that interact with ECS1 and ECS2 of mCLDN19 are labeled. (C, D) Comparison of mCLDN19 and hCLDN4 in complex with C-CPE. (C) Superposition of the two unique C-CPE molecules (in green) from the mCLDN19 (in rainbow) complex and four C-CPE molecules (in yellow) from the hCLDN4 (in gray) complex. Most Cα traces of ECS1 and ECS2 are very similar except for the disordered loop between β3 and β4. (D) Superposition of mCLDN19 and hCLDN4 based on transmembrane (TM) helices. C-CPE molecules are located at different positions depending on the TM3 orientation (dashed line), while other TM helices assume a similar conformation. The Pro135 of hCLDN4 induces an increase in the bending angle (~10°) in TM3. The color code is the same as in (C). The sites of kinks induced by Pro and the corresponding position of claudin are indicated as Cα spheres in each main chain color. For clarity, each representative pair of C-CPE/hCLDN4 and C-CPE/mCLDN19 complexes is shown.

expressed in the gastrointestinal tract and are thus natural CPE receptors. The two distinct conformations of TM3 observed in the C-CPE–bound claudin structures might also be present in the gastrointestinal tract. These findings suggest that CPE-sensitive claudin subtypes can be classified based on the kink in TM3.

In addition to the presence of an alanine or proline residue in TM3, the differences in the conformation between mCLDN19 and hCLDN4 and in their binding affinity for C-CPE may also be due to the following. Instead of Leu151 in the high-affinity receptor hCLDN4, mCLDN19 contains a serine at the corresponding position. Because the residue at
this position strongly affects the claudin/C-CPE interaction.\textsuperscript{45,46,52} this particular difference could contribute to the difference in affinity between hCLDN4 and mCLDN19. Furthermore, the C-CPE used for the mCLDN19/C-CPE complex contains an S313A substitution, whereas the hCLDN4/C-CPE complex has a wild-type C-CPE. Substitution of S313 might affect the interaction with C-CPE-sensitive claudins.\textsuperscript{49,50,55}

Because no crystal structure of a CPE-free form of either hCLDN4 or mCLDN19 is available, homology models of the proteins were compared with the CPE-bound structures to determine the conformational changes induced by C-CPE.\textsuperscript{7,8} Binding of C-CPE may push down the extracellular $\beta$3 and $\beta$4 strands and dislocate the ECH region, where the highly conserved hydrophobic residue that could form the side-by-side intermolecular interaction mentioned above is located, and consequently inhibit cis-polymerization as well as the strand assembly of specific claudin molecules.

Other members of the four-TM protein superfamily

Claudin belongs to a large superfamily of four-TM domain proteins that share the same membrane-spanning topology and a large first extracellular loop with a W–LW–C–C signature motif.\textsuperscript{26–28} Member groups in the superfamily have similar but distinct functions in cell membranes, including cell adhesion, scaffolding, protein aggregation, regulation of channel activity, intracellular signaling, and dynamic changes in cell morphology. Within the superfamily, claudin is the only group whose crystal structures are available, and therefore these structures are used as templates for homology modeling,\textsuperscript{59} as well as for building structural models at near atomic resolution for members of other groups.\textsuperscript{60–62} Several structures of the superfamily were recently determined by electron cryo-microscopy (cryo-EM), which revealed various intermolecular interactions of the four-TM proteins with other TM components, such as the $\gamma$ subunit of a calcium channel Ca\textsubscript{v}1.1 complex\textsuperscript{50,63} and the transmembrane $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor regulatory protein (TARP) $\gamma$2 stargazin.\textsuperscript{61,62} They have distinct types of interaction interfaces: TM2/3 in the Ca\textsubscript{v}1.1 $\gamma$-subunit and TM3/4 in stargazin, which are likely to be mediated by hydrophobic residues, as well as additional regions outside the membrane that may affect the conformational changes dependent on the binding partner. A previous cryo-EM study of the Euglena four-TM protein IP39 suggested that it could form three distinct TM interactions and one cytoplasmic interaction between monomers at different dimer interfaces, which may provide the basis for forming two-dimensional arrays of the IP39 proteins in the cell membrane.\textsuperscript{64} Together with considerations of the findings from claudin structures, there are a variety of intermolecular interfaces for interaction between the TM helices and between the TM and soluble regions of four-TM proteins. Understanding the different styles of interactions is crucial for identifying the appropriate interacting partners for each superfamily member, including claudin.

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Competing interests

The authors declare no competing interests.

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