Disruption of Claudin-Made Tight Junction Barriers by CpE

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Claudins are a family of integral membrane proteins that enable epithelial cell/cell interactions by localizing to and driving the formation of tight junctions. Via claudin self-assembly within the membranes of adjoining cells, their extracellular domains interact, forming barriers to the paracellular transport of small molecules and ions. The bacterium Clostridium perfringens causes prevalent gastrointestinal disorders in mammals by employing an enterotoxin (CpE) that targets claudins. CpE binds to claudins at or near tight junctions in the gut and disrupts their barrier function, potentially by disabling their assembly or via cell signaling means—the mechanism(s) remain unclear.

claudins | tight junctions | cell/cell interactions | Clostridium perfringens enterotoxin | membrane proteins | structural biology

1. Introduction to Tight Junction Barriers

For metazoans, the dense packing of epithelial and endothelial cells helps to compartmentalize tissue-specific functions. Although packing greatly reduces intercellular spacing, spaces nonetheless remain prevalent. Cell junctions act as intercellular bridges, providing adhesion between adjacent cells. Tight junctions are the most apical cell junction, with adherens junctions, desmosomes, and gap junctions residing sub-apically [1][2]. While tight junctions help cells adhere, their primary function is regulating the molecular transport of small molecules, solutes, and ions between cells through their paracellular spaces [3][4]. This molecular gatekeeping maintains tissue homeostasis and can be used to fine-tune the molecular properties of a given tissue or cell type. Tissue-specific functions can, thus, be imparted by tuning the permeability—i.e., leakiness—of tight junctions.

Tight junction structure was first observed through electron microscopy (EM) as membrane fusions or “kissing points” between cells [5]. Freeze-fracture EM (FF-EM) later revealed the detailed structure of tight junctions, wherein they appear as anastomosing networks of strands connected to membrane-embedded plaques [6][7]. Cell biology showed that tight junctions are composed of several families of integral membrane proteins, such as the claudins; the tight junction-associated marvel proteins (TAMPs) occludin, tricellulin, and Marvel-D3; the angulins; and the junctional adhesion molecules (JAMs), which work in concert to direct tight junction form and function in the presence of many scaffolding proteins [4]. The complexity of tight junction strand networks observed by FF-EM does not occur in the absence of claudins, making them the major architects of tight junction structure and function [8][9].

It is now understood that claudins create the barriers or charge- and size-selective pores that tight junctions use to govern paracellular transport and fine-tune molecular homeostasis in tissues [3][4]. To control the formation of barriers or pores, claudins self-assemble both laterally in the plane of one cell's plasma membrane (cis) and with other claudins on neighboring cells across the paracellular space (trans) [10][11]. Various mechanical and chemical insults can disrupt these claudin self-assemblies, either through intra- or paracellular means, which result in a cornucopia of disease states that all stem from losses in tight junction barrier or pore function [12][13][14][15][16]. The Gram-positive bacterium *Clostridium perfringens* produces an enterotoxin (CpE) that is perhaps the most effective natural molecule at breaking down tight junction barriers. CpE disruption of gut homeostasis causes common antibiotic-associated and foodborne illnesses in domesticated animals [3][17][18].

2. Claudins

Tight junctions were discovered in 1963, yet it took 30 more years to identify the first membrane protein that contributes to their formation, occludin [19][20]. In 1998, Tsukita and co-workers performed experiments on occludin and found its reduction did not affect the formation of tight junctions, that tight junctions could be formed in tight junction-less fibroblasts by claudins, and that claudins recruit occludin to tight junctions [19][20]. These pioneering experiments were the first to reveal that claudins are solely responsible for tight junction strand formation and that occludin is an accessory to tight junction function. Although other membrane protein families have since been identified that localize to tight junctions, it remains clear that claudins are the backbone of tight junction form and function [21][22][23][24].

Identification of the first two claudins by Tsukita and co-workers led to subsequent classification of the claudin family by cloning and sequence analyses, which showed that all claudins share a conserved WG/NLWCC motif that defines the fold and predicted that all have four transmembrane helices (TMs) [19][20][25][26][27]. Since then, 27 claudins have been annotated in humans that range in size from 23 to 34 kDa. Each subtype localizes specifically to various organs at distinct plasma membrane domains to impart the tissues they inhabit with unique physiological functions [4][11]. Claudins are classified into three categories based on their barrier properties and sequence similarity. The claudins that form barriers are claudin-1, -3, -5, -11, -14, -18, and -19. Claudin-2, -10, -15, and -17 form paracellular pores, while claudin-4, -6, -9, -12, -13, -16, and 20–27 form barriers or pores depending on their tissue localization, expression levels, or binding partners [4][11]. When focusing on sequence similarity, claudins are classified as "classic" (claudin-1–10, -14, -15, -17, and -19) or "non-classic" (claudin-11, -12, -13, -16, -18, and 20–27) [4][11].

3. *Clostridium perfringens* Enterotoxin (CpE) and the Identification of Claudins as CpE Receptors

Clostridium perfringens is a pathogenic Gram-positive bacterium responsible for both antibiotic-associated and foodborne gastrointestinal diseases in domesticated animals [3][28][29]. Type F strains produce an enterotoxin, CpE, upon sporulation in the animal gut that is required for virulence [3][28][29]. CpE is a 35 kDa protein containing 319 or

325 amino acids. Of these, the CpE gene, with 319 amino acids, is the best known, with the 325 variant only being recently discovered and having unknown clinical relevance [30][31]. In 1997, Katahira et al. determined that CpE bound to two tight junction membrane proteins in humans and mice that were not yet identified as claudins, which they called RVP1 and CpE-R [32]. Later, after the identification of claudin-1 and -2, RVP1 became known as claudin-3, while CpE-R was called claudin-4 [25][26][27]. Importantly, one study determined that CpE receptor capacity, i.e., binding, was lower for claudin-3 than for claudin-4 [27].

The identification of claudins as CpE receptors still left the question of what claudin's native function was unanswered. In the absence of structure, biochemical evidence had suggested that the C-terminal domain of CpE (cCpE) was responsible for receptor binding activity [33]. Using cCpE, Sonoda et al. discovered that it removed claudins from tight junction strands while reducing tight junction barrier integrity; however, cells did not die [34]. This work with cCpE provided the first evidence that claudins impart barrier function to tight junctions and that cCpE is able to disrupt this function. Later, it was shown with CpE that its incidence in the small intestine also disabled the tight junction barrier and caused morphological damage to gastrointestinal cells and tissues [35]. Taken together, these findings hinted that CpE was a multi-domain protein whose cCpE functioned to bind claudins and whose N-terminal domain (nCpE) functioned to kill epithelial cells in the gut [33].

4. Structures of Claudins in Complex with cCpE

The classification of claudins that are receptor or non-receptors for CpE was being determined using biochemical, biophysical, or cell-based assays that qualified cytotoxic effects. Veshnyakova et al. provided an exhaustive account of the methods and findings from work conducted prior to 2010 [18]. In short, these efforts showed that claudin-3, -4, -6, -7, and -9 were high-affinity receptors and claudin-1, -2, -5, -8, -14, and -19 were low-affinity receptors, while all other claudins were true non-receptors and do not bind cCpE [18][34][36][37][38][39][40][41]. However, ambiguous and sometimes contradictory findings were obtained due to differences in the claudin orthologs or cCpE or CpE constructs and the amounts of each used in the assay as well as the assay's sensitivity. Specifically, claudins -1, -2, and -5 were found to be both insensitive or sensitive to CpE toxicity [32][34][37][38][40]; claudin-4 and -19 were found to bind or not bind CpE [32][37][42]; there was disparity in claudin-3 and claudin-4 affinities for CpE [32][43][44]; and there was discord in what interaction types and residues direct enterotoxin binding [37][38]. To confound progress, claudin subtypes from different species (orthologs), such as claudin-4, did not bind enterotoxin identically [37]. Even the types of interactions enterotoxins form with claudins were difficult to pinpoint, as it was found that both electrostatic and hydrophobic interactions via the $NPLVA^{153}$ motif could influence cCpE binding [37][45][38]. Prior to structures, a comprehensive understanding of CpE receptors and the details of their interactions remained elusive.

5. Mechanisms of CpE Disruption of Claudins and Tight Junctions

One of the first models for claudin cis assembly came from the observation by Suzuki et al. that mCLDN-15 in LCP crystals packed in linear arrangements [46]. In crystals, claudin/claudin interactions were driven by the Met68 in one protomer binding within a hydrophobic cavity created by Phe 146, Phe 147, and Leu 158 of the adjacent protomer (cis1). Mutations of these residues to smaller or charged side chains disrupted tight junction strand formation on insect cell plasma membranes as assessed by FF-EM, giving in vivo validation that LCP linear arrangements may be physiological. Suzuki et al. would use this structure-based model as the impetus to model a larger complex of cis- and trans-interacting mCLDN-15 that form paracellular pores [47]. Here, the Asn61 in β4 is also involved in the cis interface and with the other residues, arranging claudins in the linear antiparallel double-rows. They propose that cis-assembled double-rows from one cell could interact with similar complexes on adjoining cells to form a "β-sheet channel" in paracellular space. Another model by Zhao et al. suggested that other cis interfaces were possible for mCLDN-15 [48]. In this model, which was derived computationally then tested in silico and in vivo, the interface consists of residues Ser67, Arg79, Phe146, Phe147, Leu158, and Glu157 (cis2). Cis1 and cis2 are distinct but overlap partially in the residues involved.

The structures of claudins and claudins bound to cCpE were critical to understanding their trans and cis assembly as well as CpE's mechanisms for disrupting tight junction barriers by offering important new insights that generated many testable hypotheses. Whether CpE binding occurs outside of tight junctions before claudins integrate into polymerized strands, whether this occurs at the apical or basolateral compartments (or both), and whether CpE can directly and actively break claudin/claudin interactions at polymerized tight junctions remains to be established. Although the structures of claudin/cCpE complexes were unable to answer such questions unequivocally, they were nonetheless foundational for narrowing down the hypotheses that best explain decades worth of findings from biochemical, biophysical, and cell-based analyses.

6. Applications of CpE for Therapeutic Use

The use of claudins as diagnostic and prognostic biomarkers or therapeutic targets has gained traction over the last decade, especially for treatments of human cancers. A recent review by Li et al. highlights the current state and availability of potential clinically relevant claudin-targeting agents [49]. Claudins play roles in nearly all aspects of tumor development and are known to be tumor suppressors or promotors [50]. Recent studies have demonstrated the overexpression of claudins in many cancers, including pancreatic, uterine, breast, gastric, and ovarian cancer [49][51]. Because several of the claudin subtypes that are overexpressed in human cancers are also CpE receptors, CpE is being actively investigated for translational applications and therapies in cancer. These applications include the targeted destruction of claudin-expressing cancer cells by CpE and the in vivo visualization of claudin-expressing cancers using radio or fluorescently labeled CpE. This normally gut-specific interaction is being exploited to target claudin-expressing cancer cells for CpE-mediated cytotoxicity in these cells. Recent work has employed CpE to induce cytolysis in cancerous breast, ovarian, colon, prostate, and gastric cells, most of which express claudin-3 and claudin-4 [52][53][54][55][56][57][58][59][60]. Moreover, other novel approaches for cancer treatment include the use of non-cytotoxic cCpE fused to antitumor reagents for targeted drug delivery to claudin-expressing tumors. Tumor necrosis factor was fused to cCpE and shown to be efficiently delivered and more cytotoxic to

ovarian cancer cells than un-fused tumor necrosis factor [61]. Moreover, gold nanoparticles conjugated to cCpE have been shown to bind claudin-expressing tumors and then be selectively destroyed via nanoparticle-mediated laser perforation [62].

7. Conclusions

In less than eight years, nine structures of five unique claudin subtypes have been determined by X-ray crystallographic methods, whereas no structural information existed for the 15+ years after the discovery of claudins as the structural backbone of tight junctions. Eight of the nine structures attribute their successful structural determination to cCpE. Not only have these structures illuminated the claudin fold and an individual claudin's function but together with the knowledge gained by CpE structures they all have contributed to a rapid, comprehensive, and mechanistic understanding of the structural and functional biology of CpE and its claudin receptors at sub-molecular levels. The insights provided by this structural information have synthesized several decades worth of functional data into a cohesive but still incomplete understanding of tight junction barriers. It is expected that continued progress will be made by answering currently unanswered questions of which structural biology will likely play an indelible role. The role of structural biology is already apparent in the advancements of cCpE- and CpE-based diagnostics and therapeutics. Hence, future innovations will require research groups from multidisciplinary fields to be interdependent to enable treatments for claudin-linked diseases and improve human health. Even in the absence of translational applications, CpE will continue to be an increasingly powerful tool to examine the structure and function of tight junctions.

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