Claudin interactions in and out of the tight junction

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Claudins form the paracellular tight junction seal in epithelial tissues. Although there is still limited information on how these proteins are organized at the junction, a number of recent studies have provided useful insights both into claudin-claudin interactions and into interactions between claudins and other proteins. The focus of this review is to summarize recent information about claudin interactions and to identify critical unanswered questions about claudin organization and tight junction structure which will be required to understand claudin function.

Introduction

Half a century ago, Farquhar and Palade described the ultra-structure of the apical junctional complex of epithelial cells.[1] They divided this region into three neighboring structures: the apical zonula occludens or tight junction, the adherens junction, just below the tight junction, and the macula adherens or desmosomes that form spot-like contacts on the lateral membranes. In transmission electron microscopy, the tight junction was distinguished by close apposition of the plasma membranes and several spots, or "kisses," where the membranes of the adjacent cells appear to fuse. Freeze-fracture electron microscopy of the same region revealed a series of strands that encircle the apical end of the lateral membrane;[2] these strands correspond to the regions of membrane contact detected in transmission electron microscopy and represent a series of barriers to the diffusion of material across the epithelium between adjacent cells. Of note, when tissues are fractured without prior fixation with aldehyde the strands appear as rows of discrete 10 nm particles.[3] It is tempting to speculate that the particles represent a basic structural unit of claudin interacting proteins although there is currently no biochemical evidence to support this notion. In 1998, Furuse and Tsukita demonstrated that expression of claudins, small four-TM proteins they had recently localized to the tight junction, could recapitulate similar freeze fracture strands when expressed in non-epithelial cells.[4] They and others have subsequently clearly demonstrated that claudins are the critical sealing proteins of the tight junction. Variable tissue-specific expression of different combinations of the 23+ members[5] of the claudin family of proteins is likely to explain the observed physiologic differences in the sealing properties of tight junctions in different epithelia.[6] Much progress has been made in understanding the contributions of different claudins to barrier functions. In addition, many other proteins have been localized to tight junctions and several of these are thought to interact with claudins directly.

In spite of our insights into the protein components of the barrier, we still lack a detailed understanding of the molecular structure and protein interactions of tight junctions. However, below we review a number of recent studies that better define claudin interactions and provide some organizational clues. Both microscopic and biochemical techniques have allowed identification of critical regions in transcellular claudin-claudin interactions. Physiologic and genetic analyses of claudin mutants have suggested regions important in the sealing properties of claudins and thus offer hints about the structural organization of their extracellular domains. Other studies have demonstrated the requirement for heterologous oligomerization of some claudins for tight junction targeting, which may lead to identification of domains involved in both localization at the barrier and into regions required for specific claudin-claudin interactions.

Several recent studies have also examined the interactions of claudins with other tight junction proteins, including occludin and other members of the Marvel-domain containing family. In addition, along with the initial recognition of claudin interactions with the tight junction scaffolding proteins, ZO-1, -2 and -3, there have been several studies on the interaction of claudins with other PDZ-domain containing proteins. Finally, some claudins have been shown to interact with non-tight junction proteins, including the cell adhesion proteins EpCam and tetraspanins and the signaling proteins, ephrin A and B and their receptors, EphA and EphB. The role of these and other proteins interacting with lateral membrane claudins is likely to provide important information about how the non-tight junction, non-barrier, pool of claudin is organized and regulated.

Finally, several recent papers that better define the ultrastructural anatomy of the tight junction suggest that the overall organization of junctions is likely to be more complex than commonly thought, with differences in protein composition and structure in different subdomains of a single tight junction.[7,8] All of this new information is helpful in understanding the tight junction, but better molecular models require structural information of the
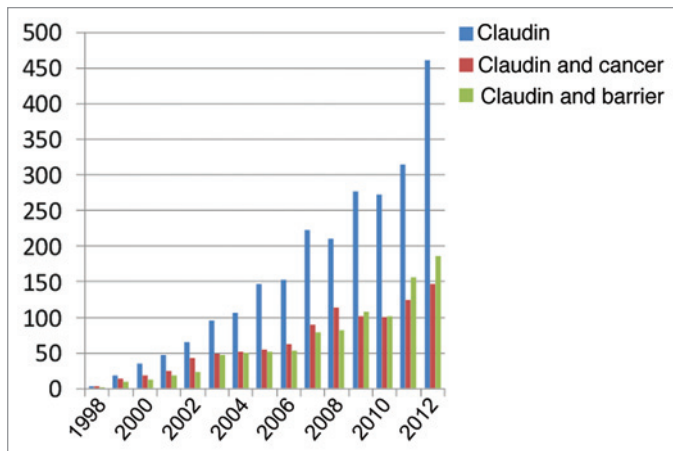
Figure 1. Citations per year in PubMed determined by searching with MeSH (Medical Subject Headings) terms "Claudin," "Claudin and cancer" or "Claudin and barrier."

component proteins and better understanding of protein-protein interactions, which are still lacking.

Although the focus of this review and this journal is on epithelia barriers it is worth noting that there has been significant interest in the role of claudins in cancer. **Figure 1** displays the number of publications in PubMed each year since claudins were first described in 1998 by searching with MeSH terms "Claudin," "Claudin and cancer" or "Claudin and barrier." Overall publications on claudins continue to grow at a nonlinear rate and with approximately equal focus on their role in cancer and in forming the tight junction barrier. In this review we provide a survey of protein-protein interactions which should inform the function of claudins in both cancer and in forming the paracellular barrier. We focus on cases where there is published evidence of a direct protein interaction with a claudin and acknowledge there is suggestive evidence for indirect interactions with more.

Claudin-Claudin Interactions

As mentioned above, soon after claudins were identified as tight junction constituents, Furuse et al.[4] demonstrated that expression of either claudin-1 or claudin-2 in fibroblasts resulted in the formation of continuous linear polymeric rows that were similar to the fibrils seen in cultured epithelial cells and epithelial tissues by freeze fracture electron microscopy. Since then, exogenous expression of many different claudins in either fibroblasts or cultured epithelial cells has been demonstrated to result in tight junction-like fibrils.[9-15] In biochemical assays, claudins have been demonstrated to form multimers, but the structure and subunit number of polymerized claudins is unclear.[16,17] To better define the ability of individual claudins to polymerize into freeze fracture fibrils, Yamazaki et al.[18] expressed several different claudins in an epithelial-like cell line, S7 cells, which lacks most claudins and does not normally contain tight junction strands. These authors found that in these cells, claudin-7, -14, and -19, but not most other claudins, could polymerize and form strands when stably expressed. Several other transfected claudins were

distributed on the lateral membrane, but did not polymerize to form strands. Since some of the same claudins (claudins 1, 2, 3, and 10 as examples) that were studied by Yamazaki et al. can form strands in other non-epithelial cells,[4,19] it appears that the polymerization of claudins may be affected by the cell background, perhaps requiring cell-specific post-translational modification or interaction with a cell-specific protein(s). In this context, it is notable that although in the study by Yamazaki et al., claudin-7 had among the strongest polymerization propensity, in many epithelial cells, claudin-7 is localized not at the tight junction, but on the lateral call membrane, without associated lateral strands.[20] Together, these results support the notion that although claudins likely oligomerize to form the tight junction strands, this process can be influenced by other cellular components.

One of the most dramatic pieces of evidence supporting the role of other cellular proteins in the organization of the freeze fracture strands was also demonstrated by Ikenouchi et al.[21] In this study, expression of claudin-1 in L cell fibroblasts resulted in linear, non-branching strand arrays, while co-expression of tricellulin with claudin-1 resulted in a highly branched network of strands. Expression of tricellulin alone does not result in strand formation. The morphology of freeze fracture stands varies widely among epithelia and it seems likely that morphology is regulated by the profile of claudins as well as interacting proteins expressed in each cell type.

In epithelial cells and tissues, it appears that most tight junctions contain more than one claudin type. Several studies have demonstrated that multiple claudins are present in a single tight junction strand, suggesting that these different claudin gene products can co-polymerize. However, the site and mechanisms of this side to side (cis) interaction within the same cell are unclear (these and other interactions are presented schematically in **Figure 2**). Recent studies by Piontek et al.[22] have begun to catalog the ability of different claudins to form heteropolymers. These authors have focused on those claudins that are expressed in cerebral vasculature, including claudin 1, 2, 3, 5, and 12. Fluorescently-tagged claudins were transfected pairwise into HEK293 cells and interactions detected by fluorescence resonance energy transfer (FRET). They found that most claudins that they studied (but not claudin-12) could interact in cis, although not equally well. The distance between the CFP and YFP tags used for the assay was less than 7 nm,[23] which does not prove direct interaction, but is consistent with close proximity.

Two special cases of cis heterotypic claudin interactions have recently been identified by Hou et al.[24,25] These very interesting studies demonstrated a requirement for cis interactions between claudin-16 and claudin-19 and between claudin-4 and claudin-8 for proper targeting. Exogenously expressed claudin-19 is localized to tight junctions but claudin-16 is not. However, when co-expressed with claudin-19, claudin-16 was efficiently incorporated into tight junction strands. Direct interaction between these two proteins (and in a similar study, between claudin-4 and claudin-8) was demonstrated by yeast-2-hybrid analysis; the results suggested that interactions between the different claudins might be mediated by their transmembrane domains and that this is required to recruit or guide some claudins to the tight junction.

Claudins associate not only in cis, but also across the paracellular space, in trans. Early studies demonstrated that claudin-1, -2, and -3 could interact homotypically in trans, and that only claudin-1 and -3 but not -2 could interact heterotypically with claudins on adjacent cells.[15] Both extracellular domains have been reported to contribute to this binding interaction.[26-29] In spite of considerable sequence conservation among different claudins, heterotypic trans interactions between different claudins appear to be relatively rare. For example, claudin-3 and -4 share considerable sequence identity in the first extracellular domain, but do not interact in trans. However, mutation of a single amino acid in the first extracellular domain of claudin-3 was sufficient to promote interaction with claudin-4.[29] Similarly, mutational analysis of a single critical tyrosine residue in the second extracellular domain can prohibit interactions between claudins-1 and -5.[22] Overall, the published data suggest that the sequence requirements for trans interactions across the cells are much stricter than cis interactions in the strands of a single bilayer.

Although in vitro studies suggest that the trans interactions for individual claudins are weak,[28] several pieces of evidence suggest that polymerized claudins are likely to form very stable interactions. First, exogenous expression of claudins in fibroblasts resulted in increased calcium independent cell-cell adhesion.[30] Second, when cells singly expressing claudins labeled with different fluorescent tags were co-cultured, microscopic analysis of endocytosed claudins revealed vesicles contained both fluorescent tags, suggesting that the tight junction containing membranes from the apposed cells were co-endocytosed, referred to by the authors as "eat each other endocytosis".[31] Finally, FRAP analysis of claudins in epithelial cells revealed a very low level of mobility, consistent with the ability of these proteins to form stable polymers with low diffusion within the membrane.[32] However, it is worth noting that when fluorescent (GFP-fused) claudins were expressed in fibroblasts, the paired strands formed between cells were dynamic in that they broke and reformed continuously.[33] Although this dynamic behavior has not been visualized in epithelial cells, it is possible that it occurs at a slower time frame. This behavior could allow the tight junction seal to be maintained as adjacent epithelial cells shift position relative to each other or divide and apoptose.

Claudin-Occludin/Tricellulin/Marvel D3 Interactions

Occludin was much heralded as the first identified tight junction transmembrane protein,[34] but occludin gene deletion was subsequently shown to have little effect on the paracellular barrier or on tight junction strand formation.[35] Although exogenous expression of occludin does not result in tight junction strand formation, occludin is recruited to the tight junction strands when co-expressed with claudins.[4] Cording et al.[36] have recently extended these observations and used a combination of techniques to study interactions between tight junction members of the occludin family (TAMPs, for tight junction-associated Marvel domain proteins) occludin, tricellulin[37] and Marvel D3.[38] Cording and coworkers demonstrated that expression of either claudin-1 or claudin-5 in HEK293 cells resulted in increased recruitment of tagged
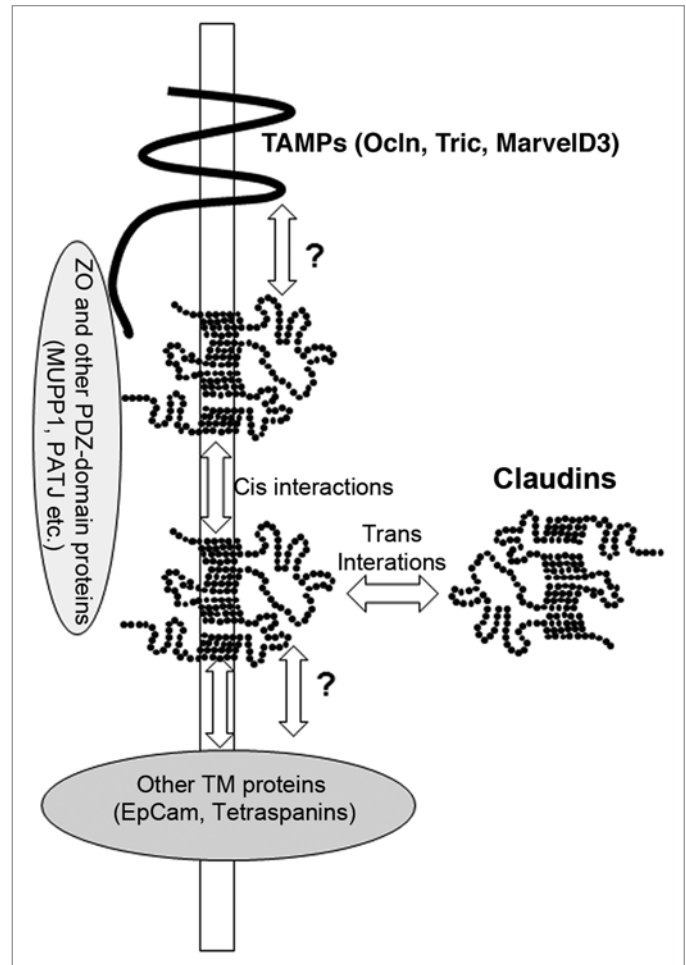
Figure 2. Model of claudin interactions with other proteins. The question marks refer to the possibility that interactions between claudins and TAMPs and claudins and tetraspanins may in part be mediated by the extracellular domains (see text).

occludin, tricellulin and Marvel D3 to sites of cell contact as measured by FRET. FRAP analysis demonstrated that co-expression with claudin-1 stabilized TAMPs at the sites of cell-cell contact. Additionally, this group found that co-expression of claudin-1 with any of the TAMPs resulted in differing freeze fracture strand patterns. Co-expression of claudin-1 with occludin or Marvel D3 increased the number of parallel freeze fracture strands, while Marvel D3/claudin-1 co-expression also increased the overall complexity of cross-bridges among tight junction strands. In contrast, co-expression of tricellulin with claudin-1 dramatically and specifically increased the cross-bridging of the strands. This group noted that there were differences in the strength of interactions between different members of the claudin and TAMP families. For example, the interaction between occludin and claudin-1 was stronger than the interaction between tricellulin and claudin-1. Reasoning that more stable claudin-1/occludin interaction might dominate over the tricellulin/claudin-1 interaction, they knocked down claudin-1 in Caco-2 cells and noted redistribution of tricellulin to bicellular border. They suggested that higher affinity interactions between claudin-1 and occludin than between claudin-1

and tricellulin normally result in the exclusion of tricellulin from bicellular contacts and thus may in part explain its restriction to tricellular cell contacts.

The nature of the interaction of claudin and occludin (or other TAMPs) is not well understood. Both proteins can separately interact with the tight junction scaffolding protein ZO-1 (described in more detail below). Raleigh and coworkers[39] showed a ZO-1 dependent interaction between the tail of occludin and claudin-1 and -2; this interaction was influenced by the level of occludin phosphorylation at serine 408. However, ZO-1 binding is not required for occludin localization, since occludin with the ZO-1 binding domain deleted is recruited to cell contacts in claudin-expressing fibroblasts but not untransfected cells (our unpublished results). In addition, in the above studies,[36] the claudins being investigated had fluorescent protein tags at their C-termini, blocking the PDZ-binding motif required for ZO-1 interaction but were still able to recruit TAMPs to cell contacts. Data published by Harris and coworkers[40] demonstrate that claudin-1-occludin interactions, as measured by FRET analysis can be abrogated by mutational changes at residues I32M or E48K in the first extracellular domain of claudin, suggesting that this region is important for interaction. Interestingly, they also showed that claudin-7, which they find does not normally associate with occludin, shows significant interaction when the corresponding positions in the first loop are mutated to the same residues, M32I, K48E found in wild-type claudin-1. These results support those published earlier by Mrsny and coworkers, who also suggested that claudin and occludin may interact via their extracellular domains; they found a claudin mimetic peptide to a region of the first extracellular domain could be cross-linked to both claudin-1 and occludin.[41] They interpreted this result to suggest that the claudin peptide bound to occludin and represents the natural contact region between on claudin for occludin.

The physiologic importance of interactions between occludin and claudins is not clear, since as mentioned above, occludin knockout animals do not show an appreciable barrier defect and have normal appearing tight junctions. However, there are data that suggest that occludin may be important for signaling at the tight junction.[39,42,43] One recent report demonstrates that occludin is required for apoptosis following disruption of claudin-claudin interactions.[44] In this study, disruption of claudin-4 interactions with a mimic peptide to the second extracellular domain stimulated caspase activation and apoptosis, but not in occludin deficient cells. These authors suggest that occludin may be acting to recruit FADD (Fas-associated protein with death domain) and caspase to a tight junction signaling domain. It seems likely that even if occludin is not required for tight junction formation, that occludin-claudin interactions are likely to be important in the regulated tight junction behavior.

Claudin-ZO Family Interactions

The three ZO scaffolding proteins of the tight junction each contain three PDZ-binding motifs in their N-terminal half (reviewed in ref. 45) and almost all claudins (claudin-12 being the exception) have a PDZ-binding motif at their carboxyl termini.[46] Tsukita and colleagues demonstrated[47] direct binding of GST-fusion proteins representing the carboxyl tails of claudins 1–8 to His-tagged PDZ-1 of ZO-1, ZO-2, and ZO-3. This PDZ-dependent interaction with ZO proteins is thought to promote proper targeting of claudins to the tight junction[12] and studies with ZO-1/ZO-2 knockdown cells show disruptions in claudin localization and in barrier function.[48,49] However, in some cases, in the presence of pre-formed tight junctions, claudins do not require PDZ-dependent interactions with ZO-1 for localization,[50,51] presumably due to their ability to oligomerize at the tight junction with non-truncated resident claudins. In contrast, a disease-associated human mutation that disrupts the ability of claudin-16 to interact with ZO-1 resulted in mistargeting to lysosomes and a renal transport defect manifest as childhood hypercalcuria,[52] suggesting that the importance of the PDZ interactions may be context-dependent.

Although almost all claudins terminate in the amino acids YV, the other amino acids within the PDZ-binding motif (~6 amino acid residues) that are likely to influence PDZ binding interactions are not well conserved. Comparison of the crystal structure of the first PDZ domain of ZO-1 and that of the adherens junction protein, Erbin[53] with synthetic peptide ligands suggests lower specificity for interaction with ZO-1 than for Erbin. This might explain the ability of the first PDZ of ZO-1 to interact with other proteins (adherens junction proteins, for example) along with the variety of claudins.

The PDZ binding motifs of claudins can and do interact with other PDZ domain proteins. Claudin-5, claudin-8, and claudin-4 can all interact with the multi-PDZ domain containing protein MUPP1.[54-56] Both MUPP1 and claudin-4 are upregulated by hypertonic stress and can be co-immunoprecipitated from an immortalized collecting duct kidney cell line. Claudin-1 colocalizes with the PDZ-domain containing protein PATJ in the paranodal loops of myelinating Schwann cells[56] and can interact with the 8th PDZ domain of PATJ in epithelial cells.[57] Given the large number of PDZ-domain containing proteins at the tight junction and lateral membrane, it seems likely that other interactions between claudins and some of these proteins can occurs as well. However, except for ZO-1, the relative importance of interactions of claudins and other PDZ domain-containing proteins remains to be determined.

Interactions with Other Proteins

EpCAM

Epithelial cell adhesion molecule (EpCAM) is a glycosylated single pass transmembrane protein found in epithelial cells where it forms homophilic calcium-independent bonds with partners on adjacent cells in the lateral plasma membrane. It is also frequently upregulated in cancer and recently implicated in stem cell signaling, reviewed in 58. Claudin-7, which is most often localized to the lateral membrane of epithelial cells, can be co-immunoprecipitated with EpCAM after crosslinking with a membrane-permeant crosslinking agent.[59] The EpCAM-claudin-7 complex is concentrated in glycolipid-enriched membranes; this domain

is also relatively enriched in phosphorylated claudin-7, suggesting that phosphorylation state might influence its interaction with EpCAM.[59] Recent data suggest that the EpCAM-claudin-7 complex is also associated with tetraspanin 8 (CO-029) and a form of CD44,[60] although the principal interaction appears to be between EpCAM and claudin-7. Detailed analysis of the interaction of these two proteins demonstrated a critical sequence in the transmembrane domain of EpCAM, and that the cytoplasmic domains of both proteins were dispensable for the interaction.[61] This group further showed that interaction with claudin-7 recruited EpCAM into tetraspanin-enriched membrane domains and that not EpCAM alone, but the EpCAM-claudin-7 complex strongly promoted tumorigenicity and accelerated tumor growth.[61]

EpCAM knockout mice show defects in intestinal barrier function, decreases in the levels of claudin-2, -3 and -15 and loss of claudin-7 along with disarranged tight junction fibrils.[62] In contrast, knockdown of EpCAM in intestinal cell lines promotes tight junction formation and results in a remarkable relocalization of claudin-7 from the lateral membrane to the tight junction.[63] Although TER and barrier function was increased and claudin-7 was now associated with the tight junction in the EpCAM knockdown cells, Wu and coworkers found a decrease in the levels of claudin-7. This was found to be due to increased degradation and further experiments demonstrated that EpCAM acted to stabilize claudin-7. Although these two studies show differing effects of reducing EpCAM levels on the tight junction, the importance of this EpCAM in claudin-7 localization on the lateral membrane and stability is consistent between the in vivo and in vitro analyses.

Tetraspanins

Tetraspanins are small integral 4TM membrane proteins with a similar topology but no sequence identity to the claudin family of proteins. Tetraspanins are postulated to organize a network of molecular interactions at the cell surface, referred to as a tetraspanin web, including integrins and other adhesive proteins, proteins with immunoglobulin domains, proteases and signaling molecules (reviewed in ref. 64). Although the interaction among claudin-7, EpCAM and tetraspanins described above is indirect, there are several reports of direct claudin-tetraspanin interactions. For example, OAP-1 forms a complex with claudin-11 and integrins[65] and chemical crosslinking reveals interaction between claudin-1 and the tetraspanin CD9.[66] However, by far the most attention has been focused on the interaction between CD81 and claudin-1, which along with several other proteins, are required for hepatitis C virus infectivity (reviewed in ref. 67). This interaction has been most carefully described in a recent study by Harris et al.[40] where using FRET analysis, they found mutation of two critical residues in the claudin-1 extracellular domains inhibited both CD81 interaction and binding of hepatitis C virus. In addition, they found that only claudin-1 at the basolateral surface of HepG2 cells was associated with CD81, not the tight junction pool of claudin-1, consistent with basolateral but not tight junction localization for virus infection.

Ephrins

The Eph receptors and their transmembrane ligands, the ephrins, regulate a number of cell-cell signaling pathways in epithelia, brain and the vascular system. Engagement of Ephrin B1 with the Eph receptor kinase on an adjacent cell can result in both downstream signal transduction on cells expressing Ephrin B1 and reverse signaling back through the Eph receptor kinase (reviewed in ref. 68). Ephrin B1 has been found to co-immunoprecipitate with and partially colocalize with claudin-1 and claudin-4 in both HT29 and MDCK cells; this interaction was demonstrated to depend on the extracellular domains of both proteins.[69] The cytoplasmic domain of Ephrin B1 is phosphorylated upon the formation of cell-cell contact and phosphorylation was shown to be dependent on the presence of the claudin extracellular domain identified as critical in the interaction between these two proteins. Overexpression of Ephrin B1 in MDCK cells results in small but significant changes in barrier function,[69] suggesting that this interaction may be a component of normal tight junction regulation. Evidence also points to a role for interaction between claudins and Ephrin B during normal development, since both claudin-1 and claudin-4 are exquisitely co-localized with phosphorylated Ephrin B at the point of septation in the tracheoesophageal foregut of developing mouse embryo.[70]

On the opposite side of the Eph receptor-Ephrin pair, claudin-4 is also known to interact with the extracellular sequences EphA2.[71] Interaction leads to phosphorylation of the cytoplasmic tail of claudin-4 at Tyr-208 within the PDZ binding motif and this correlates loss of claudin-4 from the junction and increased barrier leakiness. More work is required but this and similar cell culture studies suggest a mechanism by which tumors that overexpress Eph receptors result in junction breakdown which precedes metastasis.

Unanswered Questions

It is clear that claudins have multiple interactions with many proteins, but a number of critical unanswered questions remain obstacles in our understanding of claudin associations. These include: (1) What is the three dimensional structure of a claudin? To date claudin crystallization attempts for X-ray diffraction or cryo-EM studies have not been successful. However, a recent report of a cryo-EM structure of a claudin related protein from *Euglena gracilis*, IP39[72] may provide insight into claudin organization in the membrane. This protein forms trimeric units which polymerized into strands. The trimer was asymmetric and could interact with neighbors in multiple ways. The authors postulated that combinations of similar multiple different interactions might allow strand formation. This structure may provide a template for understanding claudin structure and inform mutagenesis studies of claudins. (2) What interactions create the 10 nM particle? Freeze fracture electron microscopy of unfixed tight junctions reveals not continuous tight junction strands, but rows of 10 nm particles.[3] A critical remaining question is what are the components of these particles (presumably claudins, but potentially other proteins as well) and how are they organized to form this fundamental particle unit and continuous barrier forming rows? (3) How do claudins interact in cis and trans to create charge-selective pores? Beyond our own early studies,[11,73] Yu and coworkers[74-77] in an elegant series of studies have begun to define critical residues in claudin-2 that influence paracellular

selectivity. Combined with structural studies, this type of analysis will help explain the organization of the pores. (4) How are claudin interactions regulated by post-translational modifications? Claudins are phosphorylated and palmitoylated (reviewed in 78) but how this might affect claudin interactions or regulation is incompletely understood. (5) Finally, it is highly probable that claudins interact with many other proteins than those described above, including of course signaling and trafficking proteins as well as tight junction and lateral membrane proteins. The rules regulating the associations of these proteins are likely to provide insights into the whole life cycle of claudin and tight junction regulation and behavior.

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