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Setting up a selective barrier at the apical junction complex

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Across the animal kingdom the apical junction complex of epithelial cells creates both a permeability barrier and cell polarity. Although based on overlapping and evolutionarily conserved proteins, the cell–cell contacts of nematodes, flies and mammals appear to differ in morphology and functional organization. Emerging evidence shows that the selective pore-like properties of vertebrate and invertebrate barriers are created by the claudin family. Similarly, assembly of the barriers requires a conserved set of polarity-generating protein complexes, particularly the PAR protein complexes.

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Abbreviations
AJ adherens junction
TJ tight junction

Introduction

The defining characteristics of epithelia include their ability to create selective barriers between tissue spaces and to generate polarity of cellular structure and function. The first characteristic allows tissues to regulate paracellular movements of solutes down their electro-osmotic gradients. The second allows the apical and basolateral membrane surfaces to recognize signals directionally or to transport material across the epithelium. The apical junctional complex, which is composed of the tight junction (TJ) and the adherens junction (AJ), is intimately involved in both permeability and polarity. In this short review we will focus on advances in understanding control of the paracellular barrier by the claudin family of transmembrane proteins [1•]. We briefly highlight the similarities and differences across phyla in creating the barrier and polarity. Recent excellent reviews have focused on the molecular components [1•,2] and regulation [3,4] of TJs.

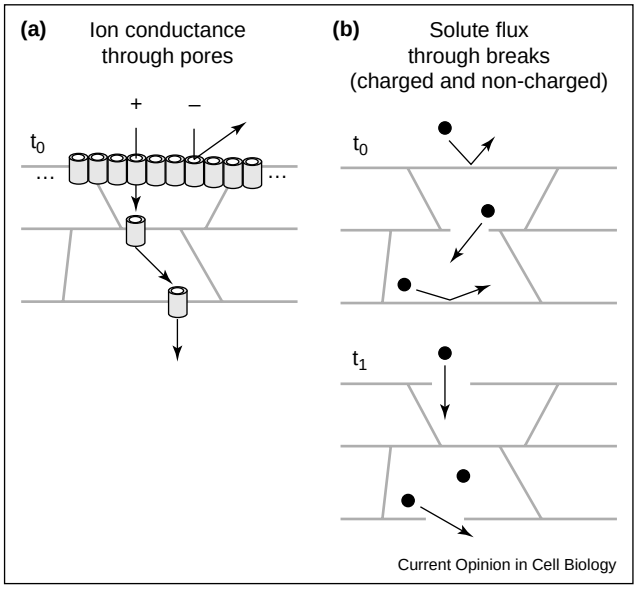
Claudins create the barrier and its selective pore properties

The paracellular–TJ pathway across epithelia behaves like a barrier perforated with selective pores [3]. Together with transcellular transport (e.g. channels, pumps, carriers and exchangers), tissue-specific TJ characteristics determine the overall epithelial absorption and secretion. The defining ultrastructural features of vertebrate TJs are strands of transmembrane protein particles that adhere to similar strands on adjacent cells to create a series of barriers in the paracellular pathway [1•] (Figure 1). The strands are composed of claudins, which are tetraspan proteins with two extracellular loops. They comprise a gene family in mammals with at least 24 members ([5•] and personal database search). Recent studies support the hypotheses that claudins create the TJ barrier, that each claudin may have unique selectivity characteristics, and that discrimination against charged and non-charged solutes is controlled by distinct mechanisms (Figure 1).

Several groups have reported changes in TJ characteristics following expression of individual claudins through transfection in epithelial MDCK cell monolayers. An emerging model is that the fixed charges on the extracellular loops of claudins line aqueous pores and electrostatically influence the passage of soluble ions. For example, replacing negative with positive residues in the first extracellular loop of claudin-15 converts it from a cation- to an anion-selective pore [6•]. Expression of claudin-8 reduces monolayer electrical conductance [7]. A more detailed characterization by Yu and colleagues of the reasons underlying these observations [8•] suggests that claudin-8 discriminates strongly against cations and forms pores of low conductance, replacing the pores that are normally present, which are formed by leakier claudins. Claudin-8 having this selectivity is consistent with its expression in the distal renal tubule segments, where it maintains high cation gradients by limiting paracellular electro-diffusion. Models of how pores might be organized within strands are well reviewed by Yu [3].

TJs also show size discrimination, with cut-offs reported to range between ~4–40 Å depending on the tissue [9]. The molecular basis for size selectivity is obscure; however, the Tsukita group has now provided the first evidence that claudins influence size-selectivity [10••]. Brain endothelia express claudins 5 and 12, possibly together with other claudins, and effectively exclude even small solutes from entering brain tissue. To test the role of claudins in the blood–brain barrier they created claudin-$5^{-/-}$ mice. These are born with TJs of normal appearance but die within several hours. When the vascular space is

Figure 1



Models of the TJ that might explain why the barriers for ions and solutes behave differently. The barrier strands are formed by rows of charge-selective claudin pores. **(a)** shows strands formed by cation-selective claudins, which permit instantaneous transjunctional passage when measured at t_0. Anions experience relatively lower permeability. **(b)** By contrast, non-charged solutes that cannot pass through pores as readily as the ions must wait for breaks in the strands to pass. Their step-wise progression takes much longer. One break pattern is shown at t_0 and another at t_1.

perfused with a range of size markers, brain endothelia from wild-type animals retain markers with sizes ranging from 68 kDa (albumin) down to 562 Da (the Hoechst dye 33258). Intriguingly, the endothelia of claudin-$5^{-/-}$ mice become leaky to the 562 Da marker yet still restrict the next largest (1,862 Da). Although these studies confirm a role for claudins in size discrimination, the molecular mechanism remains unclear.

It has become increasingly clear that the physical barriers for ion conductance and solute flux are different. Ionic permeability is measured by the instantaneous electrical conductance of soluble ions (predominantly Na^+ and Cl^-). This seems to be defined by the sum of the selectivities of the different claudins in the strands (Figure 1). On the other hand, solute flux is measured over timescales of minutes to hours. If the stands break and are resealed, then solutes (noncharged and changed) could move across the barriers in a step-wise fashion; the kinetics and control of this pathway could differ greatly from those of instantaneous ion conductance. In a seminal paper, Tsukita and colleagues provide evidence that claudin-based stands are dynamic [11]. This study used real-time imaging of strands formed by GFP-tagged claudin expressed in fibroblasts. Paired strands form at cell contacts and are seen to break and reanneal within minutes in both an end-to-end and an end-to-side fashion. We must wait to see if results gleaned from fibroblasts apply to epithelial TJs. A second concern is that the claudin's C-terminal PDZ-binding motif was blocked by GFP or an epitope tag, presumably preventing the claudin from binding to PDZ-containing scaffolding proteins like ZO-1 and MUPP1. Nevertheless, if this dynamic behavior occurs in epithelial TJs it might explain the observed dissociation between electrical conductance and solute flux.

Structural and functional zones along the apical junctional complexes

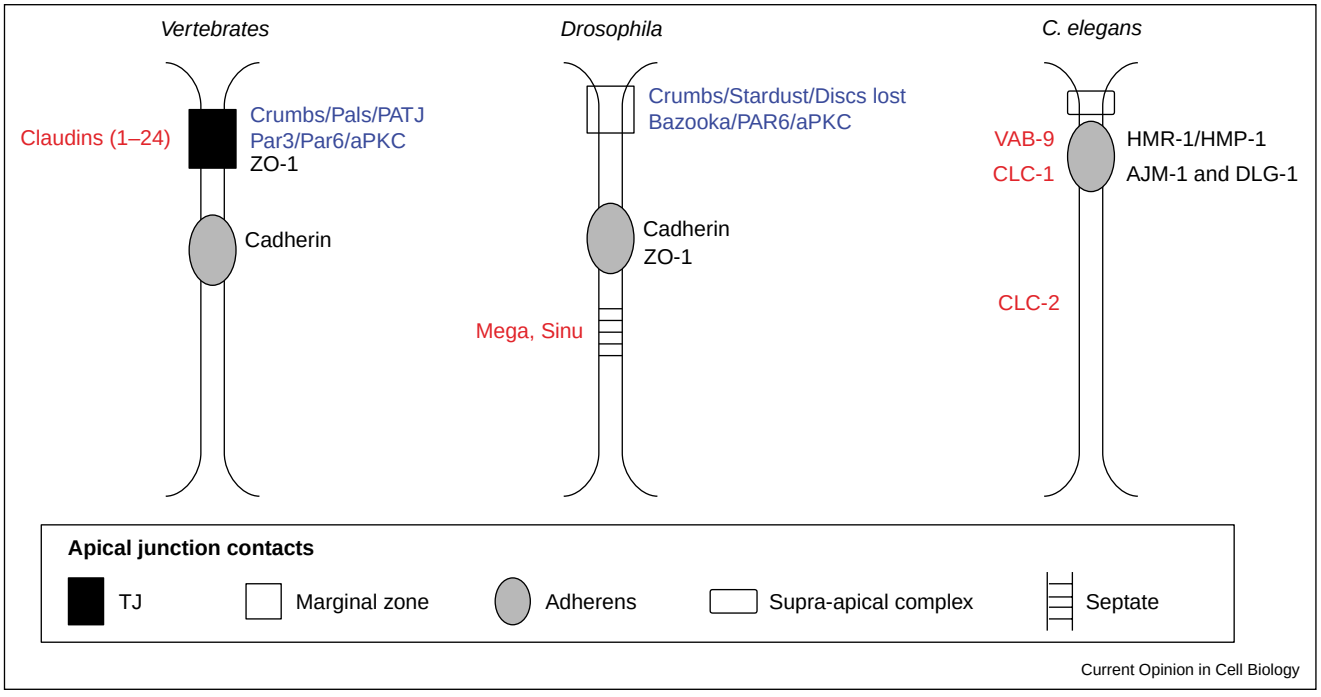
From nematodes to flies to mammals, the apical junctional complex controls permeability, adhesion, cell growth and polarity. Despite this, its morphological details vary among the groups and their protein sets only partially overlap (reviewed in [12,13]; see Figure 2). In vertebrate epithelial cells, the apical-most contact is the barrier-forming TJ, followed by the cadherin-based AJ (Figure 2). In *Drosophila*, the complex begins at the apical end with the so-called apical marginal zone, followed by the AJ and finally the barrier-forming septate junctions. In *C. elegans*, zonal gradations remain less well defined and a single electron-dense structure promotes both adhesion and regulates permeability.

Claudins in *Drosophila*

In *Diptera*, the epithelial barrier is functionally located at septate junctions, which are ultrastructurally very distinct from vertebrate TJs [14]. Further obscuring their comparison, most of the *Drosophila* homologs of TJ proteins were previously documented to be in the AJ or in marginal zone (reviewed in [15••]). Now, the recent demonstration [15••,16••] of the presence of two claudins in septate junctions provides the first definitive evidence of a common molecular basis for the barrier in insects and vertebrates. The claudin homologs, Megatrachea (Mega) [16••] and Sinuous (Sinu) [15••], localize to septate junctions, but, whereas Mega is required for septate junction formation, in sinuous mutants the septate junctions are present but discontinuous. In any case, both claudins are required to form a barrier (as defined by fluorescently labeled 10 kDa dextran permeability studies) and both are essential for normal tracheal development, but they have differing effects on cell shape. Four other *Drosophila* claudins have been identified by sequence analysis [13,15••] and it will be interesting to investigate their localization and role in barrier formation.

Notably, vertebrates also display septate-like junctions in the paranodal region of axons. Their morphological and biochemical similarities to *Drosophila* septate junctions raise the question of whether claudins are also expressed in vertebrate septate junctions. Different claudins and different PDZ proteins have been localized to distinct

Figure 2



Representations of diffusion barrier proteins (red labels), polarity proteins (blue labels) and other proteins (black labels) creating the apical junction complex of vertebrates, *Drosophila* and *C. elegans*, showing their relative locations within the complex.

TJs in mesaxons and paranodal loops and within the node of Ranvier [2], where they probably define different types of interactions, but the role of claudins in the septate junction in this region is unknown.

Claudins in *C. elegans*

Although apical junctions in *C. elegans* appear as single electron-dense structures, immunofluorescent analysis of various proteins reveals subdomains, with polarity proteins and cadherin/catenins (HMR-1/HMP-1) localized apical to AJM-1 and DLG-1 [17]. Provocatively, Tsukita and colleagues [18••] recently identified a potential new intercellular junction apical to the AJ. Although clearly not a TJ, this structure is characterized by more closely opposed plasma membranes than are seen in AJs; its molecular components remain unknown (Figure 2). Five claudin-like proteins are described in *C. elegans*: CLC-1, -2, -3, and -4 [18••] and the more distantly related VAB-9, which is more similar to members of the PMP-22 superfamily [19••]. Within the junctional region, VAB-9 colocalizes with HMR-1 and CLC-1 with AJM-1. CLC-2 has a more lateral and diffuse distribution. Despite the absence of morphological TJs, RNAi experiments using a high-molecular-weight (10 kDa) tracer reveal a role for CLC-1 in pharyngeal barrier formation and CLC-2 in hypodermis barrier formation. In contrast, VAB-9 contributes to cell adhesion through interactions with the cytoskeleton. The CLCs do not form the strands seen in vertebrate TJs and how they organize to form a barrier across the intercellular space remains unknown.

Barrier biogenesis and conserved polarity protein complexes

In both vertebrates and invertebrates, cell–cell junctions are associated with a cytosolic plaque that is enriched in multi-domain scaffolding proteins, including the ZO proteins (ZO-1, -2 and 3), MUPP-1 and MAGI (reviewed in [2]). Although it has long been speculated that interactions with these cytosolic proteins regulate the localization and function of the transmembrane barrier proteins, there is little direct evidence. Early studies suggested that transmembrane proteins like claudin and occludin localize to TJs even in the absence of ZO binding sites. However, a recent report demonstrates that expression of a fragment of ZO-3 functions to delay TJ assembly in cultured MDCK cells [20•]. In addition, loss of the ZO-1 binding site in JAM severely affects its localization to the TJ [21]. Thus, it remains unclear to what extent cytoplasmic proteins like ZO-1, -2 or -3 directly determine the subcellular localization and structural organization of TJ transmembrane proteins. Their role could alternatively be to serve as templates for recruiting other cytosolic regulatory proteins to cell–cell junctions.

One of the more exciting recent findings is that many of the TJ proteins are also associated with at least two

different macromolecular complexes that have distinct, but overlapping, roles in the biogenesis of epithelial polarity (Figure 2). These are the PAR3/PAR6/aPKC and the PAT-J/Pals-1/Crb-3 protein complexes (reviewed in [22,23]). These proteins all localize to the TJ, and altering their expression in flies or mammals results in a dramatic loss of polarity (reviewed in [22]) and, in cultured cell models, leads to mislocalization of junction proteins [24•,25••,26] and disruption of paracellular permeability [27,28••]. The molecular mechanism is poorly understood, but presumably involves direct interactions between members of these complexes and TJ proteins. Consistent with these predictions, both ZO-3 and claudin-1 bind to PATJ, and JAM-1 binds to PAR3 [29,30]. Interestingly, mutations in aPKC that disrupt activity of PAR3/PAR6/aPKC complex do not affect the localization of TJ proteins ZO-1, occludin and claudin-1 to early cell–cell contacts, but instead disrupt the physical continuity that is required to form an effective seal [31]. Thus, it appears that these polarity complexes may be involved in the later steps, or fine tuning, of junction assembly.

The sequential interaction of proteins during junction assembly (i.e. the assembly pathway) remains incompletely defined. Conceivably, cell–cell junctions could also feed back on the spatial assembly of PAR polarity proteins. However, to date there are no reports of polarity defects resulting from altered expression of ZO-1, -2, -3, occludin or claudin in vertebrate cultured cells. Furthermore, mutation of claudin-like genes in *C. elegans* and *Drosophila* has no discernable effect on cell polarity, although septate junctions are severely disrupted [16••,19••]. However, JAM proteins can recruit both PAR3 and ZO-1 to cell–cell contacts [21], and overexpression of JAM disrupts assembly of both Par3 and ZO-1 into cell–cell junctions [30]. Furthermore, both ZO-3 and claudin-1 bind PAT-J, and the ZO-3 binding site in PATJ is required for localization of PAT-J to TJs in MDCK epithelia [29]. Thus, it is possible that components of the barrier and polarity complexes are reciprocally regulated and interdependent.

The TJ plaque is also rich in cytoskeletal proteins, and investigators have long speculated that the actin cytoskeleton regulates the barrier. The pharmacological disruption of F-actin (reviewed in [32]) and cytoskeletal effectors like the Rac and Rho GTPases (reviewed in [4]) clearly disrupt the structure and permeability of TJs, which suggests at least an indirect role for the cytoskeleton. However, recent evidence indicates that several of these cytoskeletal proteins also bind directly to TJ proteins, and that disruption of these interactions interferes with localization of TJ proteins and/or assembly of the barrier. For example, ZO-1 binds directly to F-actin and deletion of a 220-amino-acid binding site interferes with ZO-1 localization [32]. ZO-3 binds F-actin and cytoskeletal regulators AF-6 and p120 catenin. Expression of a fragment of ZO-3 that binds p120 catenin, but not AF-6, delays assembly of the TJ [20•]. Interestingly, it also disrupts the actin cytoskeleton and downregulates the activity of Rho GTPase in these cells. More recently, investigators have identified a guanine nucleotide exchange factor for Rho GTPase, GEF-H1, that is localized to TJs. Overexpression of GEF-H1 alters TJ structure and permeability [33]. These latter observations raise the intriguing possibility that scaffolding proteins like ZO-1 and ZO-3 not only link proteins to the cortical cytoskeleton but also regulate their activity and actin dynamics at the TJ.

Are tight junctions involved in differentiation and cell proliferation?

Theoretically, all junctions provide an opportunity for transfer of information across the plasma membrane. Cell differentiation and growth are often controlled by engaging molecules on adjacent cells or matrix. Surprisingly, until very recently there has been little suggestion that TJs influence differentiation and proliferation. Some evidence remains descriptive and we focus on a potential role for ZO-1 and claudin.

The most compelling example linking TJs with cell growth involves ZO-1. Balda and Matter identified a Y-box transcription factor, ZONAB [34], that binds ZO-1 in MDCK cells and localizes to both the nucleus and TJs. Reducing ZONAB levels in MDCK cells through RNAi methods reduces cell proliferation, as does sequestration of ZONAB in the cytoplasm by overexpression of ZO-1. Interestingly, ZONAB interacts with CDK4, a key regulator of cell proliferation, and manipulations that decrease nuclear levels of ZONAB also decrease nuclear CDK4 levels and proliferation. Cellular ZO-1 accumulates with increasing cell density, sequestering ZONAB and CDK4 outside the nucleus and suppressing growth. This system is reminiscent of the cadherin–catenin paradigm, whereby a cytoplasmic junction component has an additional role in regulating functional access of other proteins to the nucleus [35]. It remains to be determined how TJ contacts regulate the levels or location of ZO-1.

Claudin levels correlate with and may play a role in differentiation. Snail, a transcriptional repressor implicated in regulation of the epithelial–mesenchymal transformation, directly represses the expression of several claudins and of occludin in addition to its previously described inhibition of cadherin expression [36]. In addition, in the last one to two years many reports have documented changes in specific claudins in human epithelial cancers. Two examples includes a 30-fold decrease in claudin-7 mRNA in head and neck cancers [37] and elevated expression of claudins 3 and 4 in ovarian cystadenomas [38].

A loss of claudin with de-differentiation is not surprising and is reminiscent of the correlation between decreased

cadherin levels and increased metastatic potential. The SUIT-2 pancreatic cancer cell line is typically highly metastatic when injected into nude mice [39•]. If the cells are transfected to overexpress claudin-4, their metastatic potential is significantly reduced, as are their *in vitro* characteristics of transformation. Are claudins simply a 'glue' or they can they, like cadherin, induce differentiation? This question remains to be answered.

Conclusions

In spite of the varying morphologic features of the apical junctional complexes in different phyla, claudins appear to play a central role in creating all their barriers and selective properties. The machinery used to establish cellular polarity is also conserved across phyla and likewise is required to establish a competent paracellular barrier. Despite commonalties, there are curious differences at the detailed level between the different systems and continued comparison of all models is required.

Work on the claudins is expected to diversify. If they do create selective pores through the junction, then more work is needed to define their structure and their physical organization within the barrier strands. Presently this line of research is confined to the level of electron microscopy. What is their subunit composition and 3D structure? How is their function regulated by other proteins and by cellular signaling pathways? Several human diseases of epithelia are known to result from mutation of claudins [40,41] and one from ZO-2 [42•]. We can expect more examples of mutations in claudins that affect epithelial functions like transport, antigen and pathogen access and immune cell transmigration. The therapeutic manipulation of TJs for therapeutic purposes may be feasible, as a recent study targeting occludin showed [43•]. Another major unsolved question regarding the barrier is why ion conductance and solute flux appear to be controlled by different physical barriers. The revelation that strands are dynamic may explain how but not why the two barriers can change in opposite directions. Again, comparisons across phyla will ultimately explain how the barriers are assembled and controlled.

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- •• of outstanding interest

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