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Molecular aspects of tight junction barrier function

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Abstract

In complex multicellular organisms, epithelia lining body cavities regulate absorption and secretion of ions, organic molecules, and water. Proper function of epithelia depends on apically and basolaterally situated ion channels as well as tight junctions which seal the apical intercellular space. Without tight junctions, transepithelial concentration gradients of ions and nutrients would be dissipated through the paracellular space. Elevated tight junction permeability is a feature of many diseases of multiple organs, including the gastrointestinal tract [1,2,3*,4*], kidney [5,6], and lungs [7,8]. In the intestines, epithelial barrier dysfunction is a major contributor to diarrhea and malnutrition and is associated with significant morbidity and mortality worldwide.

Introduction to tight junction proteins

In 1986, the first tight junction protein, zonula occludens-1 (ZO-1), was discovered [9], and since that time, many additional proteins and interactions have been discovered. These include a large number of transmembrane proteins such as claudins, junctional adhesion molecules (JAMs), coxsackie adenovirus receptor (CAR), and members of the tight junction associated marvel protein (TAMP) family, including marvelD3, occludin, and tricellulin. These proteins are situated at the apical intercellular space with the extracellular domains interacting between adjacent cells to form the paracellular barrier. In addition to the these transmembrane tight junction proteins, cytoplasmic scaffolding proteins including members of the ZO family, cingulin, and related proteins, which provide coupling to the cytoskeleton (Figure 1a) and a means of interactions with multiple cellular signaling pathways which regulate paracellular flux.

Extracellular stimuli modulate tight junction barrier function

Multiple signaling pathways have the capacity to regulate tight junction barrier function. Physiologic mechanisms of tight junction barrier regulation include cross talk between plasma membrane ion channels and transporters and the tight junction. For example, in the

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intestine, ingested glucose and amino acids modulate paracellular permeability by interacting with the plasma membrane sodium glucose cotransporter (SGLT1), which activates myosin light chain kinase (MLCK) through activating another apical membrane transporter, Na^+/H^+ antiporter (NHE3), to promote contraction of the apical actin cytoskeleton [10]. This form of barrier regulation is thought to promote increased passive paracellular flux of ions and water soon after a meal. Other plasma membrane ion channels and transporters, including the Na^+/K^+ ATPase, and the chloride channel ClC-2, have also been reported to regulate the tight junction barrier. The Na^+/K^+ ATPase regulates tight junction permeability by inhibiting protein phosphatase 2A activity to induce occludin phosphorylation [11]. ClC-2 channels influence tight junction permeability via caveolar trafficking of occluding to the tight junction [12]. Thus physiologic tight junction regulation depends not only on tight junction protein expression and localization, but also on the expression of plasma membrane channels and transporters.

In addition to these physiological regulators of tight junction barrier function, pathological stimuli such as enteric pathogens [13], or basolateral inflammatory cytokines [7,14–16] mediate changes in tight junction conductance. Many signaling pathways including protein kinase C, mitogen activated protein kinases, and Rho GTPases have been shown to regulate tight junctions in cytoskeleton dependent and independent mechanisms [17]. Recent studies have highlighted novel roles of non-coding micro RNAs (miRNAs) in tight junction maintenance and regulation [18]. MiR-21 is upregulated in patients with ulcerative colitis, induces barrier dysfunction, and decreased occludin protein *in vitro*. These effects are correlated with degradation of RhoB mRNA [19]. Another miRNA, MiR-122a, directly controls occludin expression by degrading occludin mRNA [20]. Thus, numerous physiological and pathophysiological signaling pathways converge at the tight junction for fine-tuned regulation of paracellular flux. We now understand that the full capacity of barrier regulation is not achieved by simple paracellular tightening and loosening, but via regulation of multiple dynamic paracellular charge and size selective permeability pathways.

A Dynamic Model of Tight Junction Function

Historically, the tight junction was often assumed to be a simple static paracellular seal, but evidence supports that tight junctions are far more complex with more than one distinct permeability pathway and the capacity to dynamically regulate paracellular flux in a size and charge selective manner. Early evidence favoring a more complex mechanism of barrier regulation comes from freeze fracture scanning electron microscopy studies. Using this approach, the lipid bilayer at the apical intercellular space is fractured along hydrophobic planes providing a lateral view of the tight junction. These studies showed that the tight junction is composed of multiple parallel branching stands encircling the cell at its apical surface (Figure 1b). Leaky epithelia, like the kidney proximal convoluted tubule and gallbladder, generally have 1–2 strands, and tight epithelia like the bladder contain 5 or more strands [21]. Remarkably, it was shown that the number of tight junction strands does not correlate linearly with transepithelial resistance, as would be expected of resistors arranged in series. Rather, transepithelial resistance follows a logarithmic function of strand number [22]. Such non-linear behavior could be explained by a model in which each strand is populated by conductive pathways which can open and close [22]. In such a model,

conductance depends not only on the number of strands, but also the number of open pores in each strand.

To date it has been impossible to directly test this model through direct measurements due to limitations in our ability to study tight junction function at the molecular level. Instead, we have relied on temporally and spatially averaged measurements of barrier function across small areas of epithelium containing thousands of epithelial cells and intercellular spaces.

Additional insight into the dynamics of tight junction structure can be obtained by studying the diffusion of fluorescent tagged tight junction proteins. Using an approach known as fluorescence recovery after photobleaching (FRAP), a small (e.g., several microns) area of fluorescently tagged tight junction protein is transiently bleached. The rate and extent of fluorescence recovery in this area is followed over time. This provides both a measurement of the rate of recovery (i.e. $t_{1/2}$) as well as percent recovery (i.e. mobile fraction). Using this approach and a similar approach, fluorescence loss in photobleaching (FLIP), it was discovered that the interactions between tight junction proteins and the actin cytoskeleton are labile and undergo continuous remodeling at steady state [23]. ZO-1, occludin, and claudin-1 each have distinct recovery kinetics. Specifically, claudin-1 and occludin both diffuse within the plasma membrane but differ dramatically with respect to their mobile fraction. In contrast, the scaffolding protein ZO-1 does not diffuse within the plasma membrane, but rather exchanges with intracellular pools. Its exchange depends on interactions with the actin cytoskeleton and MLCK activity. Remarkably, the mobile fraction of ZO-1 correlates with MLCK activity and tight junction barrier function [24]. Thus, these data support a dynamic model of tight junction barrier in which tight junction mobility is reflective of tight junction electrical conductance.

Pore and Leak Pathways of tight junction conductance

Measurements of tight junction barrier function usually rely on either electrical measurements of tight junction conductance and/or flux assays of molecular tracers. These two approaches have historically been used as interchangeable methods to study the tight junction barrier. However, examples where electrical conductance and flux are not correlated suggest a more complicated model in which the barrier to macromolecules is distinct from the barrier to small ions [25,26]. To better understand this phenomenon, additional assays of barrier function to measure tight junction size selectivity and charge selectivity are required [27]. Tight junction size selectivity can be assessed by measuring the permeability of a graded series of uncharged polyethylene glycol oligomers as a function of their radius [14]. A second approach is to substitute different sized monovalent cations for Na^+ and to measure relative permeabilities electrically from shifts in equilibrium potential [28]. From such measurements, it is clear that the relationship between tracer size and tight junction flux is biphasic. There is a high permeability for molecules smaller than ~4 Å radius and relatively low permeability for larger molecules [27,29]. Tight junction charge selectivity can be assessed from the relative permeabilities of Na^+ and Cl^- determined from the equilibrium potential measured after reducing the concentration of NaCl on one side of the epithelium.

From such measurements, it was determined that expression of claudins are the principal determinants of tight junction charge selectivity and define tight junction permeability to small molecules [30]. Stimuli that affect ZO-1, occludin, or tricellulin were shown to modulate flux of larger molecules irrespective of charge [31]. Based on these data, the prevailing hypothesis is that there are at least two distinct pathways of trans-tight junction flux: a low capacity ZO-1- and occludin-dependent “leak” pathway that defines the permeability of macromolecules and a high capacity claudin-dependent “pore” pathway that regulates permeability of the tight junction to ions and small molecules.

Further data support that these two pathways are differentially regulated by pathophysiological stimuli. For example, activation of SGLT1, which regulates tight junction permeability via communications through the actin cytoskeleton [10], results in increased tight junction conductance to small ions (i.e. reduced transepithelial resistance). However, when one assesses macromolecular flux using tracer molecules, it becomes apparent that permeability is not always increased by activation of SGLT1. There is a size cutoff such inulin (radius = 11.5 Å) cannot cross the tight junction, while the smaller molecule mannitol (radius = 3.6 Å), can pass. These data are consistent with activation of small tight junction pores which would be expected to augment paracellular ion and water absorption without allowing significant paracellular loss of larger proteins and nutrients.

Cytokines, which are produced by lamina propria inflammatory cells, also have the capacity to differentially regulate pore and leak pathways. Interleukin-13 (IL-13) [15,25,32,33] and tumor necrosis factor (TNF)-α [33–35], in particular, have been extensively studied because of their relevance to inflammatory bowel disease. Interestingly, these cytokines differentially regulate pore and leak pathways. IL-13 acts to specifically increase tight junction claudin-2 expression and specifically increases tight junction permeability to Na^+ and small cations, but does not significantly alter transepithelial permeability of negatively charged ions or macromolecules [25]. TNF-α causes increased permeability of small ions irrespective of their charge and increased flux of uncharged macromolecules and is associated with internalization of occludin [25,36*].

Thus, there are at least two distinct pathways of trans-tight junction conductance: pore and leak. We still do not fully understand the relative contributions of these pathways or significance of alterations to diseases. Bridging this gap in knowledge of molecular mechanisms of these pathways is expected to help with the development of more specific approaches to treat barrier dysfunction in the future.

Molecular mechanisms of pore pathway flux

There are 24 members of the claudin family of tight junction proteins. Claudin expression varies between types of epithelium, depending on physiological requirements [37–40], changes over the course of development [41], and is altered in a large number of different disease states [1–3,15,33,42,43]. The pattern of claudins expressed is the major determinant of pore pathway permeability. Some claudins form relatively tight paracellular barriers to ions [44], while others, such as claudin-2 and claudin-15 make tight junctions selectively permeable to small cations, but impermeable to anions and macromolecules [45]. It is

notable that knockout of claudins 2 and 15 in mice was recently shown to cause dramatic defects in paracellular Na^+ flux and this was coupled with defects in cellular sodium coupled nutrient transport [46**]. Other claudins (e.g. claudin-4, 10a, 17), induce anion selectivity [47*–49].

The crystal structure of a claudin-15 monomer was recently discovered, and agrees with mathematical predictions that claudins consist of four transmembrane alpha helices and two extracellular loops [50**]. The extracellular loops interact to form a beta sheet fold. Claudin monomers are believed to assemble into polymeric structures. The structure of these polymers is defined by conserved residues within transmembrane segment 3 of claudins, and this also contributes to strand ultrastructure [51*]. It appears that a disulfide bond between conserved extracellular cysteines is important to intermolecular interactions and pore formation [52*]. Although the structure of the claudin pore remains undefined, mutagenesis studies have pinpointed several amino acids in the first extracellular loop, which are important to defining pore ion selectivity properties [28,53]. Bulky covalent modifications in this region of the pore have the capacity to reduce paracellular conductance [54].

Molecules which interact with residues lining claudin pores are potential modulators of pore pathway function. Multivalent cations Ca^{2+} and La^{3+} are competitive inhibitors of the pore pathway function which compete with monovalent cations for binding to negatively charged residues within the pore [54]. However, since these ions interact with other cell membrane transporters, they are not specific modulators of paracellular flux. A possible pharmacologic approach to treat barrier dysfunction may be to design peptides that act as claudin binding partners. For example, a peptide derived from the claudin-1 extracellular loop has been shown to specifically disrupt barrier function [55]. However, this effect is associated with a drop in barrier function and as such, appears to be due to defects in tight junction assembly rather than pore blockade. Another claudin binder derived from Clostridium perfringens enterotoxin increases tight junction permeability to macromolecules [56,57]. Improved understanding of claudin pore structure is expected to aid in the development of agents to specifically block pore function.

Since tight junctions are dynamic structures that undergo continuous remodeling, stimuli which modulate the ability of claudins to form stable pore structures is another approach which can be used to reduce pore permeability. One such stimulus is casein kinase 2 (CK2). CK2-dependent phosphorylation of the occludin C-terminal tail favors the formation of a stable complex between claudin-1, claudin-2 and ZO-1, preventing formation of claudin-2 pores after IL-13-induced increases in permeability [58]. In addition to phosphorylation of occludin, other stimuli which directly phosphorylate of claudin-2 at its cytoplasmic tail can regulate trafficking and retention of claudin-2 to the tight junction [59*].

Mechanisms of leak pathway flux

Much less is known about the mechanisms of the tight junction leak pathway. The leak pathway is often associated with reduced expression or internalization of tight junction proteins such as occludin, ZO-1, or tricellulin [25,36,60,61].

There are several models to account for increased leak pathway, and they are not mutually exclusive. One possibility is that increased large molecule flux is due to transient strand separations allowing large molecules to cross the tight junction in a stepwise manner. Another possible explanation is that leak may be due to the physical geometry of tricellular contacts. Overexpression of tricellulin, a member of the TAMP family, which localizes to tricellular tight junctions, appears to diminish this type of leak [61]. Finally, loss of occludin is associated with a ~62.5 Å barrier defect suggests a model of leak associated with large paracellular channels [58]. The structural basis of these channels remains unclear.

Tight junction dynamics are also an important part of leak pathway regulation. TNF-α treatment increases the diffusion rate of occludin within the plasma membrane and promotes occludin endocytosis. These effects can be blocked through specific inhibition of MLCK [16,25] or by blocking occludin/ZO-1 interactions at the occludin OCEL domain [36].

Summary

Tight junctions form the essential paracellular barrier in all epithelia. While these structures were once thought to be static and largely impermeant, we now understand that tight junctions are dynamic structures. Further, paracellular flux is regulated via at least two distinct mechanisms: pore and leak. The pore pathway selectively regulates transepithelial permeability to small ions, whereas the leak pathway is a charge non-selective pathway which allows much larger macromolecules to pass. Improved understanding of the molecular mechanisms and differential roles of these pathways in disease will lead to novel therapeutic approaches to treat barrier dysfunction.

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Highlights

1. Paracellular flux is regulated by dynamic tight junction protein interactions.
2. Claudin interactions define small molecule flux across tight junction pores.
3. Macromolecular flux occurs via the non-selective tight junction leak pathway.
4. Pore and leak pathways are differentially regulated in health and disease.

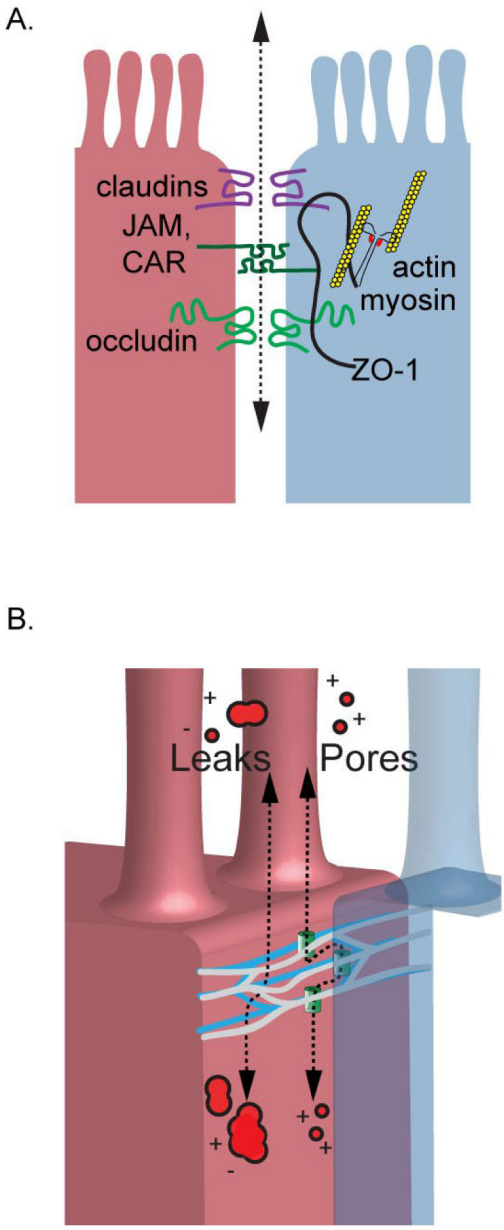

Figure 1.

Tight junctions establish the rate limiting step for paracellular flux between epithelial cells. (**a**) The barrier is established by multiple interactions between transmembrane proteins situated on adjacent cells. Transmembrane proteins are linked to the actin cytoskeleton providing one means of barrier regulation. (b) Ultrastructurally, the tight junction appears and a meshwork of anastomosing strands that encircle the epithelial cells. Current models of barrier function suggest at least two distinct pathways of trans-tight junction flux. A high capacity pore pathway regulates paracellular flux of small ions and molecules but does not pass macromolecules. A low capacity leak pathway passes ions and macromolecules in a charge and relatively size non-selective manner.