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Regulation of Epithelial Permeability by the Actin Cytoskeleton

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Abstract

The actin cytoskeleton is a dynamic structure necessary for cell and tissue organization, including the maintenance of epithelial barriers. The epithelial barrier regulates the movement of ions, macromolecules, immune cells and pathogens, and is thus essential for normal organ function. Disruption in the epithelial barrier has been shown to coincide with alterations of the actin cytoskeleton in several disease states. These disruptions primarily manifest as increased movement through the paracellular space, which is normally regulated by tight junctions. Despite extensive research demonstrating a direct link between the actin cytoskeleton and epithelial permeability, our understanding of the physiological mechanisms that link permeability and tight junction structure are still limited. In this review we explore the role of the actin cytoskeleton at tight junctions and present several areas for future study.

Keywords

tight junction; ZO-1; paracellular diffusion; cell dynamics

Introduction

Epithelial sheets are necessary to maintain normal organ operation by establishing boundaries to the movement of ions and molecules. This allows the formation of distinct tissue compartments and ion gradients that drive transport across the epithelium. Examples include kidney tubules, ducts within the liver, and the lining of the gastrointestinal tract and lungs. In many of these examples, the epithelium is also a barrier between the organ tissue and the external environment, representing the first layer of defense against pathogens. Epithelial cells form sheets by binding to each other through apically located adherens junctions (AJ) and more basally located desmosomes (Hartsock and Nelson 2008). Movement of pathogens and large macromolecules through the space between two cells is restricted by the tight junctions (TJ); a complex molecular structure located just above the AJ (Shen et al. 2011). Disruption of the junctions, and the barrier they establish, is a common feature of disease states and is associated with the establishment of infections, increased inflammation, and malabsorption (Berkes et al. 2003; Hollander 1999). The actin cytoskeleton is directly connected to cell junctions and plays an important role in assembly and maintenance of these structures (Fanning 2001). The purpose of this review is to explore previous research investigating the role of the actin cytoskeleton in regulating TJ dynamics and, consequently, epithelial permeability. We conclude by proposing areas in need of further exploration to better understand how these processes interact in both healthy and diseased tissues.

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Tight Junction Structure

The molecular components of TJs have been extensively reviewed and thus will only be briefly discussed in this manuscript (Balda and Matter 2008; Ballard et al. 1995; Furuse 2010; Van Itallie and Anderson 2004). There are three large families of transmembrane proteins that are found at the TJ: the occludin/tricellulin family of MARVEL domain proteins, the Junction Adhesion Molecule (JAM) family of immunoglobulin-like cell adhesion molecules, and the claudins (Furuse et al. 1998a; Furuse et al. 1993; Martin-Padura et al. 1998). Claudins are necessary for barrier formation, while occludin and JAM family proteins seem to be required for regulation of barrier permeability and signaling (Citi et al. 2009; Furuse 2010; Severson and Parkos 2009). Claudins create dynamic, interconnecting strands within the lateral membrane that form homo and heterotypic contacts with claudins of adjacent cells (Furuse et al. 1998b; Morita et al. 1999; Sasaki et al. 2003; Tsukita et al. 2001). Recent studies have demonstrated that the assembly of claudin-based contacts creates 4Å radius, charge-selective ion pores within the paracellular space (Colegio et al. 2002; Van Itallie et al. 2008).

These transmembrane proteins are associated with a cytosolic plaque of proteins; more than thirty have been described to date (Fanning and Anderson 2009; Shen et al. 2011). These include scaffolding, cytoskeletal, signaling, and polarity proteins. Although the role of many of these proteins in TJ assembly and permeability is poorly understood, several are either directly or indirectly associated with the actin cytoskeleton (Reviewed in (Fanning 2001; Fanning and Anderson 2009)). Of particular note is the Zonula Occludens (ZO) family of tight junction proteins. These multidomain proteins bind directly to F-actin and the barrier-forming claudins and are necessary for assembly of the TJ (Fanning et al. 1998; Umeda et al. 2006). These interactions are discussed further below.

Epithelial Permeability in Health and Disease

Epithelial permeability is defined as the movement of solutes and ions across the epithelium. Permeability varies greatly between organ systems. For example, the gall bladder, intestinal epithelium and the proximal tubules of the kidney are all considered to be leaky to ions and water. The skin, bladder and stomach have low permeability and restrict water and solute flow (Ballard et al. 1995). Variation can also be found within different tissues that compose an organ. For example, there is a 100-fold difference between the permeability of the proximal tubule and the collecting duct of the kidney (Denker and Sabath 2011). Epithelial permeability is the sum of two different pathways. The first, transcellular permeability, represents the transport of molecules and ions across the cell membrane, generally via transporters and channels. The second, paracellular permeability, is the passive diffusion of solutes and ions between cells. For the purpose of this review we will focus on the regulation of paracellular permeability by the cytoskeleton.

The paracellular barrier has two distinct, measurable characteristics: charge selectivity and size selectivity. Three different assays can be used to measure permeability depending on the characteristic being studied: transepithelial resistance (TER), the flux assay, and the polyethylene glycol (PEG) assay (Van Itallie and Anderson 2011). TER is an instantaneous measurement of ion movement across the epithelial barrier. The flux assay measures the movement of large molecules, such as mannitol (4.2Å), inulin (15Å), or fluorescent dextrans over an extended period of time, typically one to three hours. The PEG assay is a variant of the flux assay used to measure the diffusion of a graded series of uncharged polyethylene glycol oligomers of different sizes across the epithelial barrier. This assay allows for the simultaneous assessment of the movement of solutes of various sizes and thus allows the researcher to determine the effective ‘pore size’ of the paracellular pathway.

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In normal tissues, paracellular permeability is typically limited to the diffusion of select ions and macromolecules with a molecular radius of less than 4.0Å. Alterations in the TJ, especially those associated with disease states, can lead to the paracellular diffusion of larger molecules, microbes and the movement of immune cells between tissue spaces (Reviewed in (Berkes et al. 2003; Shen et al. 2009)). Many patients with inflammatory bowel diseases (IBD) have leaky intestinal epithelial barriers (Ivanov et al. 2010). In one example, Crohn's disease, the TJ strands of the intestinal epithelium are incorrectly arranged (Zeissig et al. 2007). This disruption is associated with a decrease in epithelial resistance, the increase in large molecule flux, and the presentation of leak-flux diarrhea (John et al. 2011). Asthma is another inflammatory disease known to have a disruption in epithelial permeability. The TJs of the bronchial airway epithelia are significantly disrupted in asthma patients compared to healthy individuals (Elia et al. 1988). The increased permeability of lung epithelium results in an increased incidence of damage from air particulates and an increased ability of pathogens to transverse the epithelial barrier, both of which further contribute to a hyperactive inflammatory response (Holgate 2007). The disruption of TJ structure and function often associated with disease states is frequently correlated with the misregulation of cytoskeletal dynamics.

Relationship between the Cytoskeleton and Tight Junction Structure

Microscopy Evidence

Some of the very earliest ultrastructure analyses of epithelial cells demonstrated that TJs are closely, perhaps even directly, associated with the perijunctional actomyosin ring (PAMR) adjacent to AJs. Studies using quick-freeze, deep-etch methods to visualize the cytoskeleton revealed the presence of actin filaments adjacent to TJs, just inside of the plasma membrane (Hirokawa et al. 1983; Hirokawa and Tilney 1982). These and subsequent TEM studies by Madara and colleagues (1987; 1988) demonstrated that many of these filaments appear to directly contact points of close cell-cell contact associated with tight junctions. Immuno-gold labeling and myosin S1 fragment decoration of epithelia confirmed that the microfilaments observed in association with TJs are predominantly made up of actin filaments. (Drenckhahn and Dermietzel 1988; Madara 1987)

Pharmacological Evidence

The linkage between the actin cytoskeleton and TJ structure has also been demonstrated exhaustively by pharmacological studies. Disruption of actin filament dynamics in tissue explants by plant cytokinins, cytochalasin D, or phalloidin leads to a significant alteration in TJ strand arrangement (Bentzel et al. 1980; Madara et al. 1986). Such treatments triggered the formation of "stray" strands, visible by freeze fracture electron microscopy, dipping along the lateral membrane well below the typical tight meshwork found in the apical portion of the cell(Bentzel et al. 1980) or a decrease in the number and cross-linking of strands (Madara et al. 1986). Cytochalasin D treatment in cultured cells also causes an alteration in ZO-1 localization from a continuous band at the apical junction to a discontinuous series of puncta (Stevenson and Begg 1994). Similar effects have been observed following Cytochalasin B treatment (Ma et al. 1995). These studies demonstrate that the structure of tight junctions is linked, either directly or indirectly, to the integrity of the actin cytoskeleton. How these changes in cytoskeletal organization are transmitted to the tight junction is poorly understood, but one hypothesis is that they are a result of direct contacts between molecular components of the tight junction and cytoskeleton.

Molecular interactions between the TJ and actin cytoskeleton

Although there are several cytoskeletal proteins that localize to the tight junction, there are only four TJ proteins known to directly bind to actin: ZO-1, ZO-2, ZO-3, and Cingulin.

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Cingulin is a cytosolic protein that localizes to the TJ and binds directly to F-actin and nonmuscle myosin II (Citi et al. 1988; Cordenonsi et al. 1999; D'Atri and Citi 2001). However, loss or depletion of cingulin does not dramatically disrupt the assembly or permeability of TJs (Guillemot and Citi 2006; Guillemot et al. 2004). Instead, it appears to regulate aspects of cell proliferation, signaling, and epithelial morphogenesis (Aijaz et al. 2005; Citi et al. 2009; Terry et al. 2011). In contrast, the ZO proteins are potentially critical links between the TJ barrier and the cortical cytoskeleton. They bind directly to claudin, occludin, and JAM; all three of the transmembrane proteins found within the TJ (Bazzoni et al. 2000; Fanning et al. 1998; Furuse et al. 1994; Itoh et al. 1999; Umeda et al. 2006). They all also bind directly to F-actin (Fanning et al. 1998; Itoh et al. 1997; Wittchen et al. 1999), as well as to regulators of cytoskeletal structure and dynamics. ZO-1 is a particularly prodigious cytoskeletal scaffold. It binds to the cytoskeletal proteins cingulin, protein 4.1, AF-6/afadin, cortactin, alpha-actinin4, and myosin 1C (Chen et al. 2006; Cordenonsi et al. 1999; Goldblum et al. 2011; Kremerskothen et al. 2011; Mattagajasingh et al. 2000; Ooshio et al. 2010). Additionally, ZO-1 has been shown to interact with regulators of myosin II activity like Shroom2 and MRCKβ (Etournay et al. 2007; Huo et al. 2011) and regulators of RhoGTPase signaling such as the cdc42GEF, Tuba and Paracingulin (Otani et al. 2006; Pulimeno et al. 2011). Both ZO-1 and ZO-3 have been implicated in the assembly of F-actin at cell-cell contacts (Ikenouchi et al. 2007; Wittchen et al. 2003; Yamazaki et al. 2008). However, to date the hypothesis that cytoskeletal interactions with ZO proteins are required for barrier assembly or regulation has not been directly tested. Furthermore, despite having a long list of actin binding and regulatory proteins that localize to the TJ, it is still unclear as to which, if any, are critical for stabilizing the connection between the TJ transmembrane proteins and the cytoskeleton.

Mechanisms of Actin Cytoskeletal Regulation of Epithelial Permeability

Pharmacological Disruption

In addition to providing evidence for a linkage between the TJ and the actin cytoskeleton, early pharmacological studies also provided evidence for a potential link between actin dynamics and epithelial permeability. Early investigations found that cytochalasin D and B treatments lead to increased permeability within epithelial cells, as measured by both TER and Flux assays (Ma et al. 1995; Madara et al. 1986; Madara et al. 1988; Meza et al. 1980). These effects are dose dependent and can be reversed upon removal of the treatment (Bentzel et al. 1980; Ma et al. 1995; Stevenson and Begg 1994). Other actin altering treatments, such as cytokinins, lead to a decrease in cellular permeability (Bentzel et al. 1976; Bentzel et al. 1980). These early studies led investigators to question whether the cytoskeleton plays a role in the etiology of barrier dysfunction associated with disease, or perhaps even if it has a role in absorption within healthy tissues.

Regulation of Permeability by Apical Contraction of the PAMR

Many inquiries into the role of the cytoskeleton in barrier dysfunction and normal absorption have focused on the perijunctional actomyosin ring, mostly because of its close association with epithelial cell contacts. A series of early studies simulating luminal conditions after a meal demonstrated that glucose diffuses through the paracellular spaces between cells after PAMR contraction (Madara and Pappenheimer 1987; Pappenheimer 1987; Pappenheimer and Reiss 1987). It was hypothesized that contraction of this ring may alter paracellular permeability by creating small gaps between cells.

Support for the regulation of epithelial permeability by actomyosin contraction comes from several studies focused on the activity the F-actin motor protein nonmuscle myosin II. Chemical inhibition of myosin II by blebbistatin treatment leads to a decrease in TER in

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intestinal epithelial cells (Ivanov et al. 2004). Similarly, knockdown of myosin IIA in several intestinal epithelial cell lines also causes a decrease in epithelial permeability (Ivanov et al. 2007). This effect is not observed with myosin IIB or IIC depletion, suggesting a degree of specificity in the function of myosin isoforms that could not be revealed by pharmacological studies. Epithelial permeability is also sensitive to the activation state of myosin light chain (MLC). Both direct and indirect activation of MLC in epithelial cells causes a significant reduction in TER and an increase in mannitol flux (Hecht et al. 1996; Turner et al. 1997). Of interest, the permeability of a larger tracer, inulin, is not affected by activation of MLC, implying that regulation of paracellular permeability by actin contraction is size selective (Turner et al. 1997). In fact, regulation of MLC phosphorylation may be a key step in the regulation of paracellular permeability. Turner et al (1999) showed that a decrease in phosphorylated MLC induced by activation of protein kinase C (PKC) led to a dose dependent increase in TER in caco-2 cells. Similarly, inhibition of myosin light chain kinase with a membrane soluble inhibitor can reverse the pathological barrier defects induced by microbial challenge or inflammatory cytokines (Feighery et al. 2008; Zolotarevsky et al. 2002). Thus, regulation of MLC phosphorylation may be an effective method for treatment of disorders associated with increased epithelial permeability.

This contractile model provides an attractive hypothesis, but the role of MLC and actomyosin contraction is likely more complicated. Activation of MLC not only induces actomyosin contraction within the PAMR, but also triggers a redistribution of ZO-1 and occludin in CACO2 cells (Shen et al. 2006). Therefore, it is still unclear as to whether MLC activation is altering paracellular permeability via the widening of spaces between two cells due to PAMR contraction, as visualized by Madara and Pappenheimer (1987), or by directly regulating the organization of TJ proteins.

Regulation of TJ barrier by Rho GTPases

The Rho GTPases are critical cellular regulators of actomyosin contractility and filament dynamics; and epithelial junctions are highly sensitive to factors that disrupt the activity of these proteins (Jou et al. 1998; Nusrat et al. 1995). For example, both activation and inactivation of Rho A activity leads to disruption of the PAMR and increased permeability; indicating a critical need for precise regulation of Rho activity at the tight junction (Bruewer et al. 2004; Jou et al. 1998; Nusrat et al. 1995). In these early studies the mechanism in which Rho A was regulating permeability was unclear, because both the PAMR and the TJ structure were disrupted, preventing a separation of actin dynamics and TJ dynamics. However, inhibition of a well-known downstream RhoA effector, Rho Kinase (ROCK), in T84 cells disrupted the PAMR without effecting TJ protein localization and, interestingly, led to an increase in permeability (Walsh et al. 2001). This study suggests that Rho A is likely regulating paracellular permeability through a direct effect on cytoskeletal organization or contractility and not by an indirect effect on TJ structure.

What has been less clear until recently is whether the activity of Rho GTPases and their effectors are specifically activated at junctions. There are at least two mediators of Rho activity that localize at the tight junction, GEF-H1 and p114RhoGEF. Depletion of either of these proteins leads to an increase in paracellular permeability at the membrane (Benais-Pont et al. 2003; Terry et al. 2011). p114RhoGEF forms a complex with cingulin, myosin IIA, and ROCKII at the membrane. In the absence of p114RhoGEF, Rho A activity is significantly reduced at the cell membrane and is redistributed through the cytosol, simultaneously with the disruption of ZO1 localization at the membrane (Terry et al. 2011). Based on these studies it is likely that Rho GEFs, particularly p114RhoGEF, are key regulators of Rho activity at the TJ and subsequent regulation of paracellular permeability.

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Endocytosis

Finally, the actin cytoskeleton has been shown to alter paracellular permeability by regulating the endocytosis of TJ proteins. The induction of actin depolymerization by latrunculin A simultaneously reduces TER and induces the caveolin dependent internalization of occludin (Shen and Turner 2005). IFN-γ treatment of epithelial cells also reduces TER as well as the internalization of occludin, JAM, and claudin-1. Unlike latrunculin A treatment, TJ reorganization induced by IFN-γ required RhoA and downstream ROCK activation of the myosin II motor protein and did not require actin filament turnover (Utech et al. 2005). MCLK activity has also been demonstrated to reduce TER and induce caveolin-1 dependent occludin endocytosis after *in vivo* TNF treatment (Marchiando et al. 2010). These studies demonstrate that actin cytoskeletal activity associated with the endocytosis of TJ proteins is closely correlated with a loss of paracellular permeability as measured by TER. However, the pathways which lead to actin cytoskeletal regulation of endocytosis vary depending on the method of induction.

Future Directions

Which cytoskeletal proteins are essential for TJ barrier regulation?

Based on the studies presented in this review it is clear that the actin cytoskeleton is physically and functionally connected to the TJ. We also have a list of proteins located at the TJ which can bind to and/or regulate actin dynamics. However, we do not know how or even if many of these proteins affect actin dynamics at the junction, or if they are required for junction assembly. Nor do we know the precise mechanism by which the activity of these proteins is coordinated, although the studies outlined here suggest a critical role for the Rho family of GTPases. While cellular models can address many of these issues, the interpretation of such studies is complicated by the fact that many of the cytoskeletal proteins that localize to the TJ, or bind to TJ proteins, have other cellular roles. Thus, it will be important to identify approaches to specifically disrupt actin dynamics at the TJ.

Does epithelial permeability fluctuate in healthy tissues?

As described above, there is considerable evidence that changes in cytoskeletal structure and dynamics at the tight junction are involved in the etiology of barrier dysfunction in disease states. It is also generally assumed that TJ permeability can be acutely regulated in normal tissues, although there is little evidence to support this hypothesis. Madara and Pappenheimer (1987) have suggested that the intestinal epithelium undergoes cytoskeleton-mediated changes in permeability depending on the presence of a meal, but the relevance of this pathway to glucose absorption in most mammals has proven controversial (Fine et al. 1993). Nevertheless, it is clear from both animal and cell culture studies that regulated actomyosin activity is critical for the maintenance of the barrier (Shen et al. 2011); so it is not inconceivable that passive diffusion of nutrients or water might be regulated in normal tissues such as the intestine or the lung. Thus, further examination of these issues in animal models, although difficult, is warranted.

How is the cytoskeleton regulating epithelial permeability and barrier protein dynamics?

The many studies discussed in this review clearly demonstrate that the cytoskeleton regulates epithelial permeability; however the predominant mechanism for this regulation is still unclear. The current research proposes three different models. In the first, alterations in cytoskeletal dynamics lead to the fragmentation and/or redistribution of TJ protein strands (i.e. claudins) within the lateral membrane (Bentzel et al. 1980; Madara et al. 1986). The second model suggests that the actin cytoskeleton regulates TJ permeability by regulating the endocytosis of TJ proteins (Shen and Turner 2005). Finally, in the third model, contraction of the PAMR induces changes in epithelial permeability without disruption of

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the TJ structure. Unfortunately, only a limited number of studies have been able to disrupt the cytoskeleton without also altering TJ structure (Van Itallie et al. 2009; Walsh et al. 2001). Therefore, it is still unclear as to the frequency in which cytoskeletal dynamics alter epithelial permeability without a significant disruption of TJ structure; both in pathological or healthy states. Finally, one cannot yet rule out that other aspects of cytoskeletal dynamics are involved in the regulation of epithelial permeability, such as actin polymerization (Ivanov et al. 2005). It was recently reported that activation of cofilin, an actin depolymerizing factor, decreases TER in CACO2 cells (Nagumo et al. 2008). These observations suggest that there is a need to more closely examine the effects of actin filament dynamics on the distribution and behavior of barrier proteins.

Assessing cytoskeletal regulation of epithelial permeability

Epithelial permeability is typically measured using TER or Flux. Each type of assay provides data for a different mode of diffusion between cells. Ions diffuse between cells through claudin channels while large molecules are believed to diffuse through larger, transient openings. The nature of these transient openings is unclear, but they are clearly influenced by pharmacological manipulation of myosin activity (Van Itallie et al. 2009; Yu et al. 2010). It has also become increasingly clear that these two modes of diffusion through the paracellular space can be independently regulated (Balda et al. 2000; Van Itallie and Anderson 2011), but the mechanistic basis for this observation is unclear. One issue that has clouded an accurate assessment of cytoskeletal effects on permeability has been that many studies provide data from only one type of permeability assay. In order to have a complete understanding of epithelial permeability in a given system, both ion and large molecule permeability need to be measured.

What about other components of the cytoskeleton?

Finally, our review has focused on the role of the actin cytoskeleton in regulating epithelial permeability. However, there is evidence from one study that microtubules may play a role in regulating the formation of the PAMR and TJ assembly and disassembly (Ivanov et al. 2006). Further studies are needed to elucidate whether microtubule dynamics play a significant role in TJ assembly and stability and thus also play a part in the regulation of paracellular permeability.

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