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Aggregating Phenotype in *Lactobacillus crispatus* Determines Intestinal Colonization and TLR2 and TLR4 Modulation in Murine Colonic Mucosa

S. Voltan, I. Castagliuolo, M. Elli, S. Longo, P. Brun, R. D'Inca, A. Porzionato, V. Macchi, G. Palu, G. C. Sturniolo, L. Morelli and D. Martines

Clin. Vaccine Immunol., September 1, 2007; 14 (9): 1138-1148.

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Role of Epithelial Cells in Initiation and Propagation of Intestinal Inflammation. Eliminating the static: tight junction dynamics exposed

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Shen, Le, and Jerrold R. Turner. Role of Epithelial cells in Initiation and Propagation of Intestinal Inflammation. Eliminating the static: tight junction dynamics exposed. *Am J Physiol Gastrointest Liver Physiol* 290: G577–G582, 2006; doi:10.1152/ajpgi.00439.2005.—Like all mucosal surfaces, the intestine forms a barrier that separates the external environment, i.e., the gut lumen, from the protected internal milieu. The intestinal barrier is formed by the epithelial cells that line the luminal surface. Plasma membranes of these cells prevent free passage of hydrophilic molecules across this barrier but do not seal the space between cells. This function is provided by the tight junction. Each cell is encircled at the apicolateral boundary by the tight junction, which seals the paracellular space. The tight junction does not form a completely impermeant seal, however, because that would prevent paracellular absorption of essential nutrients and ions; intestinal tight junctions are “leaky” and allow solutes to be transported paracellularly according to size and charge. Abundant data are available to demonstrate that barrier properties of tight junctions can be modulated in response to physiological, pharmacological, and pathophysiological stimuli, but the structural modifications responsible for these responses are poorly defined. Recent advances in understanding the role of tight junction dynamics in response to such stimuli are the focus of this review.

barrier; cytoskeleton; cytokine; nutrient transport; myosin

TIGHT JUNCTION FUNCTION AND STRUCTURE CAN BE REGULATED

The tight junction barrier is not static, but can be regulated by multiple external factors. For example, physiological stimuli, such as initiation of Na⁺-glucose cotransport, can effect rapid and reversible increases in tight junction permeability (22). This regulation is characterized by small increases in permeability that are thought to fine-tune paracellular transport of nutrients; permeability to small molecules is increased without increased passage of larger molecules. Both morphological and mechanistic studies have suggested that tight junction regulation following initiation of Na⁺-glucose cotransport depends on modulation of the actin cytoskeleton, which is intimately associated with the tight junction (12). Consistent with an essential role for the actin cytoskeleton in tight junction function, it has long been known that pharmacological disruption of actin, such as that induced by actin-depolymerizing drugs, causes extensive tight junction disruption (2). In contrast to regulation triggered by Na⁺-glucose cotransport, the effects of actin depolymerization are drastic, resulting in marked loss of barrier function. Thus this represents an exaggerated form of physiological and pathophysiological responses, making it a useful model for mechanistic studies of tight junction regulation. Many pathophysiological effectors, including proinflammatory cytokines and noninvasive bacteria, also appear to

modify epithelial tight junction barrier function via the actin cytoskeleton (24, 25). In general, the magnitude of these permeability increases is intermediate relative to the small changes that follow Na⁺-glucose cotransport and the larger effects of actin depolymerization. Thus the actin cytoskeleton appears to represent a site of convergence for a diverse array of stimuli that acutely modify the tight junction barrier (Table 1).

REGULATORS OF THE ACTIN CYTOSKELETON MODIFY EPITHELIAL BARRIER FUNCTION

Although proximal signaling pathways remain an area of active investigation, a relatively small number of distal signaling pathways have been implicated in physiological and pathophysiological tight junction regulation. As noted above, many of these involve modulation of actin cytoskeleton structure or function. This is supported by data showing that the apical junctional complex, composed of the tight junction and adherens junction, is located adjacent to a dense band of actin and myosin, the perijunctional actomyosin ring that encircles each cell. One important class of molecules that regulate actin includes the small GTPases Rac, Rho, and Cdc42. These regulate tight junction barrier properties in part through actin cytoskeletal reorganization. Expression of either constitutively active or dominant negative small GTPases disrupts barrier function (10), suggesting that the role of small GTPases in tight junction regulation is not as simple as “on” or “off,” but requires active cycling between these states. Consistent with this, both positive and negative GTPase regulators, including GEF-H1 and Smurf1, modify epithelial barrier function. The importance of the small GTPases to pathological barrier disruption is demonstrated by the effects of bacterial toxins that covalently modify small GTPases. For example, *C. difficile* toxins glucosylate and, thereby, inactivate Rho. This results in disruption of the perijunctional actomyosin ring and massive loss of barrier function that is similar to that induced by actin-depolymerizing drugs (7). In contrast, the *E. coli* toxin cytotoxic necrotizing factor-1 activates Rho GTPases via deamidation. This also causes loss of barrier function (8) but does not grossly disrupt the perijunctional actomyosin ring (8). Phosphorylated myosin II regulatory light chain (MLC), which is normally associated with the tight junction, is, however, displaced after cytotoxic necrotizing factor-1 treatment (8).

The role of MLC phosphorylation has also been extensively studied with regard to tight junction regulation. In fact, this may be one mechanism of Rho action, because the effector Rho-associated kinase can enhance MLC phosphorylation by inhibiting MLC phosphatase and may also directly phosphorylate MLC (11). However, in intestinal epithelial cells, MLC phosphorylation is primarily accomplished by the long isoform of MLC kinase (MLCK) (5). Activation of this kinase is required for the increased paracellular permeability induced by

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Table 1. Mechanisms of cytoskeletally mediated tight junction regulation

Stimulus	Cellular Effectors	Cytoskeletal Effector	Actin Architecture	Tight Junction Protein Internalization	Tight Junction Protein Complex Dispersal	Endocytic Route
Physiological Na ⁺ -glucose cotransport	Akt2, ezrin, NHE3, MLCK	MLC phosphorylation	Actomyosin ring condensation	No		
Pathophysiological Interferon- γ		Myosin ATPase activation	Actin-coated vesicles	Yes	No	Macropinocytosis
TNF Transforming growth factor- β	Par6, Smurf1	MLCK activation RhoA degradation	Actomyosin contraction Loss of actomyosin ring, increased stress fibers	Yes	Yes	
Enteropathogenic <i>E. coli</i> infection	MLCK	MLC phosphorylation	Actomyosin ring alteration	Yes	Yes	
<i>E. coli</i> CNF-1 toxin	Activation of Rac, Rho, and Cdc42	Phosphorylated MLC displacement	Actomyosin ring condensation, actin cable assembly	Yes	Yes	Caveolae
<i>Clostridium difficile</i> toxins	Rho GTPase inactivation		Loss of perijunctional actin	Yes	Yes	Caveolae
Experimental Cytochalasin/latrunculin		Actin depolymerization	Actomyosin ring fragmentation	Yes	Yes	Caveolae
MLCK catalytic domain expression	MLCK	MLC phosphorylation	Actomyosin ring condensation	No	Yes	
Ca ²⁺ chelation		Myosin ATPase activation	Actomyosin ring collapse	Yes	No	Clathrin

MLC, myosin regulatory light chain; MLCK, MLC kinase; NHE, Na⁺/H⁺ exchanger.

Na⁺-glucose cotransport, and MLCK inhibition prevents this physiological tight junction regulation in vitro and in vivo (22).

THE ACTIN CYTOSKELETON IS A PRIMARY TARGET OF IMMUNE-MEDIATED EPITHELIAL BARRIER DYSFUNCTION

In addition to a role in physiological tight junction regulation, MLCK has also been implicated in pathological barrier disruption in response to infectious and inflammatory stimuli. These include the barrier dysfunction induced by enteropathogenic *E. coli*, *Giardia*, and TNF (16, 24, 25). Most of this work has been performed in vitro, but several recent studies have extended these observations to in vivo models. For example, we have recently shown that MLCK is absolutely required for both the barrier dysfunction and diarrhea that are induced after in vivo immune activation (4). In this model, the principal cytokine mediator of both barrier dysfunction and diarrhea was TNF, because TNF-neutralizing antibodies prevented the barrier dysfunction and diarrhea associated with in vivo immune activation (4). A role for MLCK was demonstrated by the observations that either genetic knockout of the long-MLCK isoform expressed in enterocytes or mucosal application of a highly specific peptide MLCK inhibitor each prevented barrier dysfunction and diarrhea without interfering with the immune response (4). This is consistent with in vitro studies (23) demonstrating that MLCK transcription is activated by TNF, suggesting that control of MLC phosphorylation is critical to the epithelial cell response to TNF. TNF is also implicated in the sepsislike clinical syndrome, which includes increased colonic paracellular permeability, bacterial translocation, and inflammation, that is induced by lipopolysaccharide administration (14). In this case, systemic administration of a general kinase inhibitor that preferentially targets MLCK prevented increases in colonic paracellular permeability, bacterial trans-

location, and inflammation, supporting a central role for MLCK in the response to lipopolysaccharide (14).

In the case of enteropathogenic *E. coli* infection, some data suggest that barrier disruption may occur in two phases (20). In the initial stages of infection, bacterial factors that enter the intestinal epithelial via the type III secretion system appeared to be required for loss of barrier function and altered tight junction structure (20). In contrast, the type III secretion system was not required for barrier dysfunction and tight junction disruption after more prolonged enteropathogenic *E. coli* infection (20). Although the reason for this difference is not clear, it is interesting that mucosal TNF was elevated after prolonged infection, suggesting that, at later times, the enteropathogenic *E. coli*-induced inflammatory response, rather than the organism itself, is responsible for the observed barrier loss. The data emphasize the complexity of these stimuli that activate multiple signaling cascades and make it difficult to determine which changes are due to MLC phosphorylation and which require additional effectors.

MLCK ALONE IS SUFFICIENT TO CHANGE TIGHT JUNCTION STRUCTURE AND FUNCTION

To define the contribution of isolated MLCK activation to tight junction assembly, Hecht et al. expressed a constitutively active MLCK in Madin-Darby canine kidney epithelial cells (6). These cells failed to develop significant barrier function, suggesting that tight junction assembly was disrupted, although tight junction structure was not assessed (6). To evaluate MLCK activation in mature monolayers with assembled tight junctions we developed an inducible system to express the same constitutively active MLCK in Caco-2 intestinal epithelial cells (19). Constitutively active MLCK expression was initiated only after monolayer assembly was complete (19).

This resulted in MLC phosphorylation, decreased barrier function, and size-selective increases in paracellular permeability (19). Both the magnitude of barrier loss and the size specificity of the permeability increases were similar to tight junction regulation induced by Na⁺-glucose cotransport (19). Moreover, the increased permeability associated with constitutively active MLCK expression was rapidly reversible on MLCK enzymatic inhibition (19). Thus MLCK activation alone is sufficient to recapitulate physiological Na⁺-glucose cotransport-dependent tight junction regulation.

This model of barrier regulation by isolated MLCK activation has allowed us to assess the effects of MLC phosphorylation on tight junction structure without interference from the complex web of signaling events that follow other stimuli. We found that MLC phosphorylation alone is sufficient to drive downstream reorganization of tight junction structure. The most dramatic example of this is illustrated by ZO-1, a peripheral membrane protein associated with the tight junction. MLC phosphorylation caused ZO-1 to be reorganized from generally smooth arclike profiles into an irregular series of fine and course undulations (19). Like increases in tight junction permeability, ZO-1 redistribution is reversible on MLCK inhibition. Occludin, a transmembrane protein whose role in the tight junction is incompletely understood, was redistributed with ZO-1 (19). In contrast, claudin-1, a member of the claudin transmembrane protein family that defines tight junction ion permeability, was reorganized at the tight junction and also internalized into membrane vesicles after MLC phosphorylation (19). This difference in redistribution after MLC phosphorylation between claudin-1, which does not directly interact with actin, and ZO-1 and occludin, which do bind to actin, suggests that these direct interactions between tight junction proteins and actin may be involved in MLCK-dependent tight junction regulation.

CAVEOLAE-MEDIATED ENDOCYTOSIS IS REQUIRED FOR ACTIN DEPOLYMERIZATION-INDUCED TIGHT JUNCTION DISRUPTION

Although MLC phosphorylation can regulate tight junction structure and function, the complex interactions between the actin cytoskeleton and the tight junction remain incompletely understood. To address this problem, we chose to study the tight junction disruption that follows actin depolymerization (2). This model stimulus induces an acute and dramatic decrease in barrier function. Our first goal was to identify the morphological changes that accompanied loss of barrier function. Treatment of epithelial monolayers with latrunculin A, which binds to actin monomers and prevents them from polymerizing into filaments, caused barrier function and F-actin content to decrease significantly within 5 min (17). Despite this, no changes in tight junction protein distribution were detected by immunofluorescent analysis of monolayers fixed 5 min after latrunculin A addition (17). This suggests that either the changes occurring are not detectable by light microscopy or that the kinetic resolution of the approach is insufficient to recognize these changes (17). We therefore developed a novel approach to allow time-lapse imaging of tight junctions while simultaneously measuring barrier function (17).

We developed fluorescent fusion constructs of the three representative tight junction proteins as well as β -actin (17).

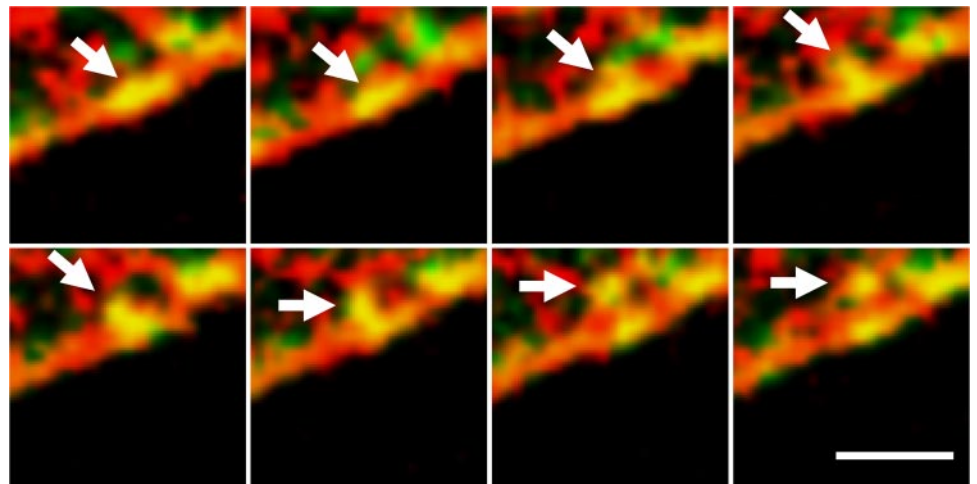
We selected ZO-1, the first identified tight junction plaque protein; occludin, the first identified tight junction transmembrane protein; and claudin-1, one of the first two identified members of the claudin family of proteins. The constructs were carefully validated to assure that they were correctly targeted to tight junctions and did not disrupt tight junction assembly, response to latrunculin A, or targeting of endogenous proteins. In contrast to the results with fixed preparations, the live cell imaging approach allowed us to recognize redistribution of occludin at early time points after latrunculin A addition. Within minutes, occludin distribution along the tight junction became uneven, with alternating sites enriched in and depleted of occludin (17). As barrier function was lost, occludin was internalized into cytoplasmic vesicles, primarily from the sites of enrichment. In contrast, ZO-1 and claudin-1 distributions were not appreciably altered until well after barrier function was lost (17). We therefore focused on occludin internalization and, using the live-cell imaging results as a guide, were able to develop a new fixation procedure that preserved occludin-containing vesicles induced by latrunculin A treatment.

It is striking that a number of studies have reported the appearance of tight junction proteins in cytoplasmic vesicles after exposure of epithelial monolayers to a variety of chemical and pathophysiological stimuli. For example, disruption of tight junctions by calcium chelation (9), enteropathogenic *E. coli* infection (20), or TNF all cause tight junction protein internalization in vitro and in vivo (3, 4, 23). However, in each case, tight junction protein internalization was defined at time points associated with or following maximal barrier loss, making it impossible to determine whether internalization is directly related to loss of barrier function or is a secondary event.

To determine whether the occludin internalization we noted was required for loss of tight junction barrier function, we asked whether membrane traffic inhibitors could prevent latrunculin A-induced barrier loss (17). Two treatments that inhibit membrane traffic, cooling to 14°C and incubation in hypertonic media, each prevented barrier loss and occludin internalization despite latrunculin A-induced actin depolymerization. Thus the endocytic events illustrated by occludin internalization appear to be required for latrunculin A-induced barrier loss (17).

Multiple studies of tight junction protein internalization have reported that, depending on the stimulus, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis can each mediate tight junction protein internalization (Table 1) (3, 8, 9). However, the contribution of each different endocytic mechanism to barrier loss has not been assessed. We therefore sought to determine the pathway by which the observed latrunculin A-induced occludin internalization occurred and whether blockade of this pathway could prevent barrier loss. We first sought to separate dynamin-dependent processes, clathrin- and caveolae-mediated endocytosis, from the dynamin-independent process of macropinocytosis. Expression of dominant negative dynamin II prevented latrunculin A-induced occludin internalization, suggesting that either clathrin- or caveolae-mediated endocytosis was involved (17). To further dissect this process, a variety of approaches were used. First, latrunculin A-induced occludin endocytosis was monitored in cells expressing fluorescent clathrin light chain or caveolin-1 fusion proteins. Occludin colocalized with caveo-

Fig. 1. Occludin cointernalizes with caveolin-1 after actin depolymerization. Madin-Darby canine kidney monolayers expressing mRFP1-occludin (red) and caveolin-1-EGFP (green) were treated with latrunculin A and imaged at 15-s intervals (17). A focus of caveolin-1 and occludin concentration forms within the tight junction (arrow, bright yellow region) and is then internalized (arrows). In contrast to other intracellular occludin stores, which are red, the internalized structure remains yellow, indicating the colocalization of occludin and caveolin-1. Bar = 2 μ m.



lin-1, but not clathrin light chain, during internalization (Fig. 1). Consistent with this morphological result, pharmacological inhibition of caveolae-mediated, but not clathrin-mediated, endocytosis prevented latrunculin A-induced occludin internalization. Finally, pharmacological inhibition of caveolae-mediated, but not clathrin-mediated, endocytosis prevented latrunculin A-induced barrier loss. Thus caveolae-mediated endocytosis of tight junction components such as occludin is necessary for the barrier loss that accompanies actin depolymerization (17). These data represent the first direct link between tight junction barrier function and specific pathways of tight junction protein endocytosis.

Although the described occludin endocytosis was induced by a nonphysiological stimulus, the observation does have important implications for tight junction protein internalization in response to pathophysiological stimuli. For example, the occludin internalization that is induced by TNF *in vitro* (23) and *in vivo* (4) (Fig. 2) requires MLC phosphorylation. Moreover, we have recently shown that inhibition of MLCK, either pharmacologically or by genetic knockout, prevents TNF-dependent occludin endocytosis, barrier dysfunction, and diarrhea *in vivo* (4). Thus the mechanistic insight provided by the studies of latrunculin A-induced barrier loss may direct future analyses of pathophysiologically relevant barrier dysfunction.

TIGHT JUNCTIONS ARE DYNAMIC IN STEADY-STATE EPITHELIAL MONOLAYERS

Although studies of barrier loss provide insight into the dynamic processes activated during tight junction disruption, they do not address steady-state tight junction structure. One recent report demonstrated that the strands formed by a fluorescent claudin-1, claudin-1-EGFP, are dynamic structures when the fusion protein is expressed in fibroblasts (15). Although these strands do not represent actual tight junctions, because the latter are absent in fibroblasts, this dynamic behavior may explain the apparent flexibility of the tight junction in epithelia. For example, barrier function is maintained, both *in vitro* and *in vivo*, despite ongoing cell migration within and even extrusion from the monolayer. Thus one may conclude that the tight junction has some structural flexibility. In other cases, tight junction deformability is associated with removal of proteins from the tight junction (13), an observation consistent with local tight junction disassembly.

Although the strands may be dynamic, the molecular structure of the strands has not been explored. Fluorescence recovery after photobleaching (FRAP) analyses showed that claudin-1 is not mobile within the strands formed in transfected fibroblasts (15), suggesting that tight junction strand structure is static at a molecular level. This concept is supported by studies describing a plethora of interactions among tight junction proteins, including claudins, occludin, cingulin, ZO-1, and other PDZ domain-containing proteins, and between tight junction proteins and cytoskeletal proteins, such as actin and myosin (1, 21). The available data therefore support the prevailing view of the tight junction as a large, stable multiprotein

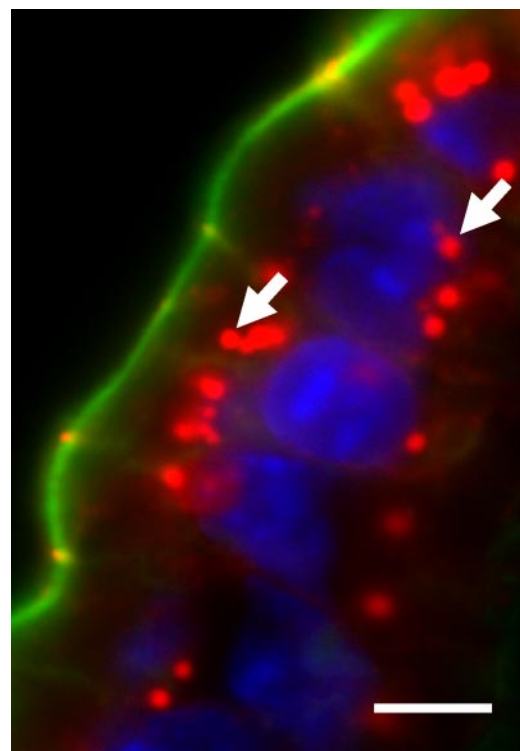


Fig. 2. Occludin is internalized *in vivo* after immune activation. T cells were activated *in vivo* by intraperitoneal injection of anti-CD3 antibody (4). This resulted in TNF-dependent occludin (red) internalization (arrows). F-actin (green) and nuclei (blue) are shown for reference. Bar = 5 μ m.

complex maintained by numerous protein-protein interactions (1, 21).

Despite this concept of tight junction structure, our initial time-lapse movies suggested that occludin expressed in resting epithelial monolayers might flow within the tight junction (17). This caused us to ask whether occludin could diffuse within the tight junction. Moreover, if such diffusion did occur, we wanted to know whether this represented movement of a protein complex, including occludin, claudin, and ZO-1. To answer these questions, we performed FRAP studies on polarized epithelial monolayers expressing fluorescent-tagged tight junction proteins. Our data showed that occludin fluorescence did recover in photobleached areas of the tight junction (18). Further analyses suggested that this fluorescent recovery occurred by diffusion of occludin molecules within the tight junction. Similar to occludin, ZO-1 recovered significantly following photobleaching (18). However, it did so with kinetics significantly more rapid than those of occludin. This raises the possibility that ZO-1 moves by a mechanism distinct from that employed by occludin. Recovery of claudin-1 fluorescence was also observed in photobleached regions. However, in contrast to occludin and ZO-1, this recovery was limited (18). This suggests that claudin-1 does not diffuse as a complex with occludin and ZO-1. Because the rates and extents of recovery differ among occludin, ZO-1, and claudin-1, we conclude that these proteins do not diffuse as complexes within the tight junction. Thus these data suggest that our current model of tight junction strands as stable multiprotein complexes must be revised to allow for rapid and extensive movement of some, but not all, components within the strand. We must therefore begin to think of the tight junction as highly dynamic in both function and structure.

A DYNAMIC MODEL OF TIGHT JUNCTION STRUCTURE

It is clear that individual protein components are delivered separately during tight junction biogenesis and may dissociate from one another during tight junction disassembly. It has, however, been assumed that assembled tight junctions in intact epithelia are composed of large, stable protein complexes (1, 20). The FRAP studies summarized above are inconsistent with this accepted static model of tight junction structure. Specifically, the observation that FRAP kinetics are unique to each of the proteins studied indicates that the observed exchange does not represent exchange of macromolecular protein complexes. These results therefore demand that the prevailing model of tight junction structure be revised. A new model must incorporate the ideas that the tight junction itself is a dynamic structure and that interactions between different tight junction proteins may be transient. It follows that constant engagement and release of these interactions must occur within the tight junction. This suggests the possibility that small changes in the dissociation constants between tight junction proteins may alter tight junction structure and function.

UNANSWERED QUESTIONS

To establish a new model of the tight junction, it will be important to further characterize the dynamic behavior of tight junction proteins in living cells. Even better would be to understand these processes in living tissues. Characterization of the properties of each protein that define its unique dynamics

would also enhance our understanding of tight junction organization and aid in developing an accurate molecular model of tight junction structure. This would logically give rise to testable hypotheses. For example, one might ask whether tight junction protein internalization is a consequence of changes in the dissociation constants between individual tight junction proteins. Ultimately, such studies may allow us to understand the role(s) of tight junction protein dynamics in barrier regulation, including the dysregulation that occurs in disease.

In summary, the tight junction is a far more dynamic and complex structure than previously recognized. Although significant progress has been made, greater understanding of tight junction maintenance and regulation in health and disease is needed. Ultimately, such knowledge will provide means to selectively and specifically regulate the tight junction that may allow development of targeted therapies for diverse diseases.

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