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Myosin light chain kinase: pulling the strings of epithelial tight junction function

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Abstract

Dynamic regulation of paracellular permeability is essential for physiological epithelial function, while dysregulated permeability is common in disease. The recent elucidation of the molecular composition of the epithelial tight junction complex has been accompanied by characterization of diverse intracellular mediators of paracellular permeabiltiy. Myosin light chain kinase, which induces contraction of the perijunctional actomyosin ring through myosin II regulatory light chain phosphorylation, has emerged as a key regulator of tight junction permeability. Examination of the regulation and role of MLCK in tight junction dysfunction has helped to define pathological processes, characterize the role of barrier loss in disease pathogenesis, and may provide future therapeutic targets to treat intestinal disease.

Keywords

tight junction; myosin light chain kinase; TNF-α; IBD

Introduction

Epithelial cells serve an essential role in homeostasis by providing and regulating the physical barrier between tissue compartments.[1] In the intestine, the epithelium forms a barrier between the sterile tissues and the harsh environment of the lumen. This barrier must be selectively permeable to allow the absorption and secretion of large volumes of solutes and fluids while maintaining a barrier to pathogens and xenobiotic materials. In addition, it is now clear that the barrier function is dynamic, and can be regulated by a variety of physiological and pathophysiological stimuli.[2] The apical and basal plasma membranes of mechanically-linked epithelial cells form the majority of the barrier surface, and constitute the transcellular barrier, but the space between adjacent cells, that is, the paracellular space, must also be sealed to maintain the continuity of the barrier. Throughout the gastrointestinal tract, the *transcellular* and *paracellular* routes are used for transport of solutes and fluids through and between epithelial cells. Specific pumps, transporters, and channels on the apical and basolateral plasma membranes facilitate transcellular transport. In contrast, paracellular transport is passive, occurring down electrochemical gradients established either by the activity of the transcellular transporters or by external events, such as ingestion and luminal digestion of nutrients.

Paracellular transport is regulated by an intercellular seal, formed by two main protein complexes located at the apical-most part of the lateral membrane, collectively termed the apical junctional complex (Fig. 1).[3] The adherens junction, which contains cadherin and catenin proteins, is linked to the dense ring of perijunctional actin and myosin that underlies

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the apical junction complex. While critical for structural integrity, the adherens junction does not contribute to sealing of the paracellular space. The tight junction, which is located just apical to the adherens junction, is the essential determinant of paracellular flux.[4, 5] Tight junction-associated proteins, including the claudin family and occludin,[6] and the specialized lipid composition of local membranes[7] form a seal that limits paracellular flux. As with the adherens junction, the tight junction protein complex is attached to perijunctional actomyosin ring[8] by direct and indirect protein–protein interactions.[9, 10] As discussed below, these interactions are critical to tight junction structure and function.[11–13]

Myosin light chain kinase

Original studies assumed that the tight junction was a static barrier. However, demonstration of rapid modulation of structure and barrier function by plant cytokinins, compounds derived from purines, suggested the possibility of physiological tight junction regulation. This was subsequently described as a consequence of Na^+-nutrient cotransport in mammalian small intestine.[14–19] However, the contribution of the paracellular pathway to overall nutrient and water absorption was considered controversial.[20–25] Many reasons exist for the failure of some studies to demonstrate paracellular water and nutrient absorption, most of which reflect technical issues and, potentially, the species studied.[24, 25] The clearest *in vivo* example of the role of physiologic tight junction regulation in paracellular glucose transport is provided by a perfusion study of rat jejunum.[26] This work showed that D-glucose absorption over a wide range of perfusate concentrations could only be explained by the sum of active (i.e., transcellular) and passive (i.e., paracellular) transport.[26] As discussed below, *in vivo* perfusion studies of mouse jejunum have confirmed the critical contribution of tight junction regulation and paracellular transport to overall nutrient and water absorption in health and disease.

While study of intact tissue, either *in vivo* or *ex vivo*, was critical to the initial demonstration of tight junction regulation, it was not suitable for investigation of the underlying mechanisms. However, study of intact tissue did provide one essential clue. Ultrastructural studies showed that Na^+-glucose cotransport-induced tight junction regulation was associated with perijunctional actomyosin condensation.[15, 18, 27] This led to the hypothesis that perijunctional actomyosin contraction might regulate the tight junction barrier function. Development of a model for study of Na^+-glucose cotransport-induced tight junction regulation in cultured epithelial monolayers demonstrated that permeability was affected in a size-selective manner, that is, permeability to small, but not large, molecules was increased.[28] This model also allowed biochemical analysis, which revealed increased myosin II regulatory light chain (MLC) phosphorylation, a biochemical marker of actomyosin contraction, following Na^+-glucose cotransport activation.[28] Further, pharmacological inhibition of myosin light chain kinase (MLCK) prevented Na^+-glucose cotransport-induced tight junction regulation, both in cultured monolayers and isolated rodent mucosae.[28] Subsequent *ex vivo* analyses confirmed Na^+-glucose cotransport-induced tight junction regulation in human jejunum, and quantitative fluorescence microscopy demonstrated MLC phosphorylation within the perijunctional actomyosin ring of absorptive enterocytes within these tissues. Thus, MLCK-dependent MLC phosphorylation is an essential intermediate in physiological tight junction regulation.

MLCK is necessary for TNF-α–induced barrier loss

A role for the cytoskeleton in the pathophysiological tight junction regulation was first suggested by the observation that MLC phosphorylation is markedly increased following infection with enteropathogenic *E. coli*.[29] Shortly thereafter, a role for MLCK in tight junction dysfunction induced by tumor necrosis factor (TNF-α) was examined.[30] Analyses

of cultured monolayers demonstrated TNF-α–induced barrier loss could be corrected acutely using a highly-specific pseudosubstrate peptide MLCK inhibitor.[31] Further study demonstrated that TNF-α activated MLCK by at least two separate mechanisms: increased transcription and increased enzymatic function.[32]

While some details of TNF-α–induced barrier loss are best identified using highly manipulable *in vitro* models, determination of the effect on transepithelial transport requires use of *ex vivo* or *in vivo* models. Thus, an *in vivo* perfusion system was developed to allow quantitative analysis of barrier function and fluid transport in neurovascularly intact mouse jejunum.[33] Mice were treated with anti-CD3 to activate T cells systemically.[34] This induced jejunal barrier loss and reversal of net fluid movement, from absorption to secretion, in a TNF-α–dependent manner.[33, 35] Ultrastructural examination revealed that this was associated with perijunctional actomyosin contraction (Fig. 2) similar to that induced by Na^+-glucose cotransport, and phosphorylation of perijunctional MLC.[33] Furthermore, mice lacking long MLCK, the intestinal epithelial MLCK isoform,[36] or mice treated with the peptide inhibitor of MLCK failed to phosphorylate intestinal epithelial MLCK and were protected from both barrier loss and fluid secretion.[33] Thus, MLCK is critical effector of pathological barrier dysfunction *in vivo*.

MLCK regulation in the gastrointestinal tract

The emergence of MLCK as a critical regulator of epithelial paracellular permeability has provided an opportunity to prevent intestinal barrier dysfunction in experimental models and examine the potential therapeutic benefit of this intervention. As noted above, TNF regulates MLCK transcription and enzymatic activity.[31, 32, 37] Moreover, MLCK is expressed in intestinal epithelia as two splice variants. Short, or smooth muscle, MLCK[38] is not expressed in intestinal epithelia.[36] Long MLCK is derived from the same gene as short MLCK, but uses an upstream promoter that gives rise to 5′ transcriptional and translational start sites and additional amino terminal protein sequence.[38] Two long MLCK isoforms, MLCK1, or full-length long MLCK, and MLCK2, which lacks a single exon within the unique, long MLCK upstream sequences, are expressed in intestinal epithelia. These splice variants have distinct subcellular localizations and functions,[36] and their expression is differentially regulated during epithelial differentiation. MLCK1 is predominantly expressed in villous epithelium, while MLCK2 is expressed throughout the crypt villus axis. Moreover, MLCK1 is concentrated at the perijunctional actomyosin ring and specific MLCK1 knockdown increases barrier function.[36, 39] Finally, the ability of cultured intestinal epithelial monolayers to regulate barrier function after initiation of Na^+-glucose cotransport, which develops during enterocyte differentiation, coincides with the onset of MLCK1 expression.[36] Given that MLCK participates in multiple cellular processes, MLCK1 may be the preferred molecular target for therapeutic MLCK inhibition.

Despite the unique role of MLCK1 in tight junction regulation, MLCK1 and MLCK2 transcription appear to be activated similarly by TNF-α.[37] There has been debate regarding the signaling events that lead to increased MLCK transcription, The first study examining this found that inhibitors of NF-κB could block TNF-α–induced MLCK upregulation at extremely low concentrations.[32] In contrast, NF-κB inhibition required use of these agents at significantly greater doses that actually enhanced TNF-α–induced MLCK upregulation.[32] A subsequent study suggested that NF-κB was critical to TNF-α–induced MLCK upregulation.[40, 41] Although both of these studies used the Caco-2 intestinal epithelial cell line, which is derived from a human colonic adenocarcinoma, the first used the well-differentiated, absorptive (surface) enterocyte-like BBe subclone,[31, 32, 42] while the second study used the less well-differentiated parent line.[40, 41] A detailed analysis of the human long MLCK promoter showed that this discrepancy likely explains the difference in

mechanism of transcriptional regulation.[37] While the promoter was responsive to both AP-1 and NF-κB, the data show that TNF preferentially activates NF-κB in poorly-differentiated monolayers and AP-1 in well-differentiated monolayers.[37] While activation of MLCK transcription by TNF has been demonstrated *in vivo*,[37, 39] the mechanism of transcriptional regulation has not been defined. However, transgenic mice expressing an epithelial-specific IκBα mutant, which functions as an NF-κB super repressor, were protected from anti-CD3–induced tight junction regulation.[43]

A plethora of studies have identified distinct mechanisms to control MLCK expression and activity, which appear to be altered in disease states. While numerous groups have shown MLCK increases MLC activity by phosphorylation at Ser19, the regulation of MLCK expression is less well defined. Perhaps with greatest relevance to inflammatory bowel disease (IBD), numerous studies have established the ability of inflammatory cytokines TNF-α and interferon (IFN)-γ, which are elevated in Crohn's disease, to induce barrier loss *in vitro* at relatively high doses. Critically, TNF-α and IFN-γ inhibition can reverse barrier loss and substantially reduce inflammation in patients and animal models. At low doses, TNF-α and IFN-γ act synergistically to decrease barrier function *in vitro* [31, 32, 44], which may be more relevant to the pathogenesis of human disease where both cytokines are elevated. In monolayers primed with IFN-γ, TNF-α decreased barrier function and increased MLC phosphorylation. Barrier function was restored by specifically inhibiting MLCK, suggesting that MLCK activity was responsible for the loss of barrier function [31]. The molecular mechanism leading to increased MLCK activity was subsequently studied in this model and it was found that MLCK protein [32] and gene [37] expression was also increased by IFN-γ/TNF-α, and corresponded with increased MLC phosphorylation. Thus, MLCK was found to be inducible by TNF-α, uncovering a novel mechanism of epithelial barrier regulation by the cytokine. Crucially, this observation is supported by patient data. In intestinal resections and biopsies, MLCK expression was slightly increased in ileal epithelia of patients with inactive Crohn's disease, and this increased further in active disease, correlating with histological disease activity [39]. MLC phosphorylation was also increased in the colon tissues of patients with active disease (Fig. 3). Taken together, these studies provide a key insight into the regulation of MLCK by inflammatory cytokines, and the role of MLCK activity in the pathogenesis leading to epithelial barrier loss in IBD.

Mechanism of MLCK-dependent barrier regulation

While the data above demonstrate that MLCK is a critical mediator of tight junction barrier function, the downstream events activated by MLCK are only beginning to be defined. First, while Na^+-glucose cotransport and TNF-α both regulate tight junctions by MLCK-dependent processes, the impact each has a distinct impact on barrier function. Na^+-glucose cotransport induces a size-selective increase in permeability that is limited to small molecules.[25, 27, 28] In contrast, TNF-α–induced increases in paracellular flux of small and large molecules.[32, 33, 45]

Inducible expression of constitutively-active MLCK in cultured intestinal epithelia caused a size-selective increase in permeability similar to that following initiation of Na^+-glucose cotransport.[46] Detailed analysis of tight junction structure in these monolayers demonstrated MLCK-dependent reorganization of perijunctional actin, occludin, and ZO-1.[46] The normally smooth, arc-like tight junction profiles viewed *en face* were modified to irregularly undulating profiles after MLCK activation.[46] This resulted in a nearly 20% increase in tight junction length which, by virtue of an increase in potential paracellular channels, could partially explain the MLCK-induced increase in paracellular flux. This may have also been associated with a change in lipid composition of tight junction membrane microdomains, as occludin was redistributed to a higher density population of glycoplipid- and cholesterol-rich

membranes.[46] However, given the critical role of claudins in defining paracellular permeability,[47–51] it is notable that neither the *en face* profiles nor density of membranes containing claudin-1 and claudin-2 were affected by MLCK activation.

To better understand the mechanism of physiological MLCK-dependent barrier regulation, the dynamic behaviors of occludin, ZO-1, claudin-1, and actin were examined in monolayers with active Na^+-glucose cotransport.[52, 53] MLCK inhibition markedly reduced ZO-1 exchange between tight junction and cytosolic pools, but did not affect dynamic behavior of other tight junction proteins or perijunctional actin.[53] Exchange of a ZO-1 mutant lacking the actin binding region was not affected by MLCK inhibition, thereby demonstrating that this domain mediated the observed increased in ZO-1 anchoring.[53] Further, either ZO-1 knockdown or expression of the free actin binding region, as a dominant negative inhibitor, prevented MLCK-dependent barrier regulation.[53] Thus, physiological MLCK-dependent barrier regulation occurs via a ZO-1–dependent process. The role of occludin in this form of tight junction barrier regulation has not been established.

Similar to physiological tight junction regulation, pathophysiological MLCK-dependent barrier loss is associated with increased undulation of *en face* ZO-1 profiles.[33] However, *in vivo* TNF-α–induced tight junction regulation is also accompanied by occludin internalization.[33] This caveolin-1–dependent endocytosis is prevented by MLCK inhibition.[33, 54] Further, *in vivo* occludin overexpression reduced TNF-α–induced barrier dysfunction by ~50%.[54] Similar data have demonstrated a critical role for occludin during *in vitro* TNF-α–induced barrier loss.[55] Thus, occludin endocytosis is a critical intermediate in pathophysiological tight junction regulation, both *in vitro* and *in vivo*. While not reported, it may be that this occludin endocytosis is responsible for the lack of size selectivity in pathophysiological, relative to physiological, MLCK-dependent barrier loss.

The role of MLCK-dependent barrier regulation in disease initiation and progression

Identification of MLCK as a central mediator of intestinal epithelial tight junctions has enabled further characterization of its role in disease pathogenesis as well as the wider role of barrier loss in disease initiation and progression. Patient data supporting the critical role of barrier function include the increased risk of relapse from remission in Crohn's disease patients with increased intestinal permeability.[56] The observed permeability increases in a subset of healthy first degree relatives of Crohn's disease patients[57, 58] also suggests a role for tight junction dysregulation in disease initiation, while, simultaneously, demonstrating that barrier dysfunction alone is insufficient to cause disease.

To determine the contribution of MLCK to initiation and development of disease, transgenic mice that express constitutively-active MLCK within the intestinal epithelium were developed.[59] As expected, these mice displayed increased intestinal epithelial MLC phosphorylation and paracellular permeability. While mucosal immune activation, including increased TNF-α, IFN-γ, IL-10, and IL-13, as well as increased numbers of lamina propria T cells were observed,[47, 59] these mice did not develop spontaneous disease. Thus, the mice may be similar to healthy first degree relatives of Crohn's disease patients. However, when immunodeficient mice expressing constitutively-active MLCK were challenged with adoptive transfer of naive T cells, they developed colitis more rapidly than non-transgenic littermates.[59] In addition, disease in the transgenic mice was more severe in terms of cytokine production, histopathology, and overall survival.[59] Thus, while insufficient to initiate disease, epithelial tight junction dysregulation can accelerate disease progression and enhance barrier function. Conversely, delayed disease onset and reduced severity have been

reported after adoptive transfer of naive T cells into long MLCK knockout mice.[60] Thus, targeted MLCK inhibition could be of therapeutic benefit.

Potential of MLCK inhibition as a therapeutic approach

The prospect of preventing initial development of Crohn's disease, maintaining remission, and reducing severity of active flares by inhibition of epithelial long MLCK is compelling. The safety of such an approach is supported by the observation that long MLCK knockout mice are healthy[61] and are at least partially protected from many stressors.[33, 61–64] However, established pharmacological inhibitors such as ML-7 and ML-9, are not useful, as they inhibit many kinases at concentrations necessary to block MLCK.[65] Although inhibitors with greater specificity are available,[61, 66, 67] these are also unsuitable, as they cannot distinguish between long and short MLCK, whose catalytic domains are derived from a single gene and are, therefore, identical.[38] Thus, a pharmacological approach targeting epithelial long MLCK enzymatic activity will also inhibit the short MLCK expressed in smooth muscle. Toxicities that would follow are demonstrated by the perinatal death of genetically-modified mice lacking the MLCK catalytic domain,[68] and the hypotension, gut dysmotility, and viscus obstruction of mice with smooth muscle specific MLCK deletion.[69, 70] Thus, targeting of MLCK enzymatic activity may not be a viable approach to therapy when the risk of adverse effects is considered. One possible future direction may involve direct targeting of specific MLCK isoforms. Due to its expression within well-differentiated intestinal epithelia, direct targeting of long MLCK1 might prevent tight junction dysfunction without systemic toxicities. The presence of a unique IgCAM domain that contains src phosphorylation sites could represent an alternative targets to inhibit MLCK1.[71] However, these studies are limited and further investigation is needed.

Conclusions

In summary, understanding the contribution of epithelial paracellular permeability to physiological processes in the intestine, and its dysregulation in disease, has provided invaluable insight into disease mechanisms. While not yet practical, isoform-specific long MLCK inhibition may ultimately provide a viable approach to restoring tight junction barrier function and preventing or treating intestinal disease.

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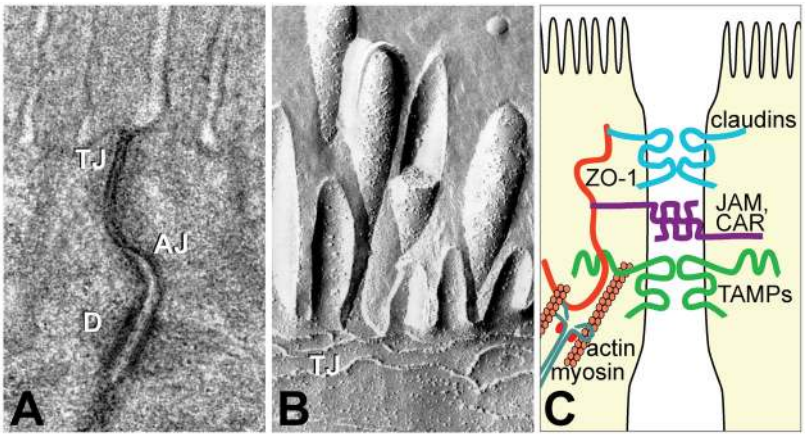

Figure 1.

The apical junctional complex. (A) Transmission electron micrograph shows the tight junction, TJ, located most apically, the adherens junction, AJ, located below, and desmosomes, D, located basolaterally. (B) Freeze-fracture electron microscopy shows that the TJ is composed of particulate intramembranous strands. Diagrammatic representation of the apical junctional complex. Myosin and actin interact with the tight junction through plaque proteins, such as ZO-1. Integral membrane proteins, including junctional adhesion molecules (JAM), Coxsackie adenovirus receptor (CAR), and tight junction-associated MARVEL proteins (TAMPs), such as occludin, bridge the intercellular space. Figure reproduced from Shen *et al.*[72] with permission.

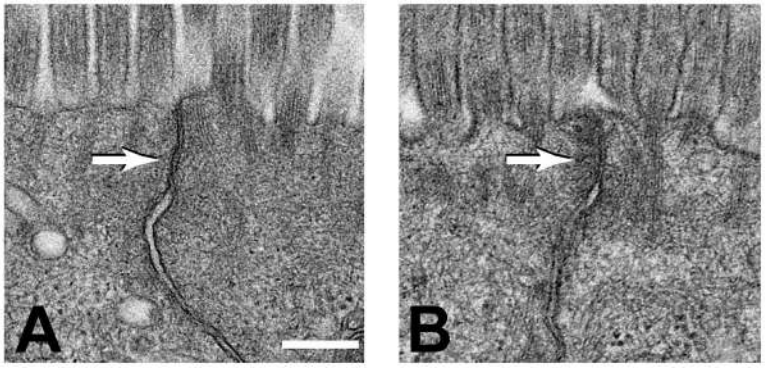

Figure 2. T cell activation induces perijunctional actomyosin condensation

The tight junctions (arrows) of jejunal villous enterocytes within control (A) and anti-CD3–induced T cell activation (B) mice. Note the perijunctional cytoskeletal condensation induced by T cell activation. Figure reproduced from Clayburgh *et al.*[33] with permission.

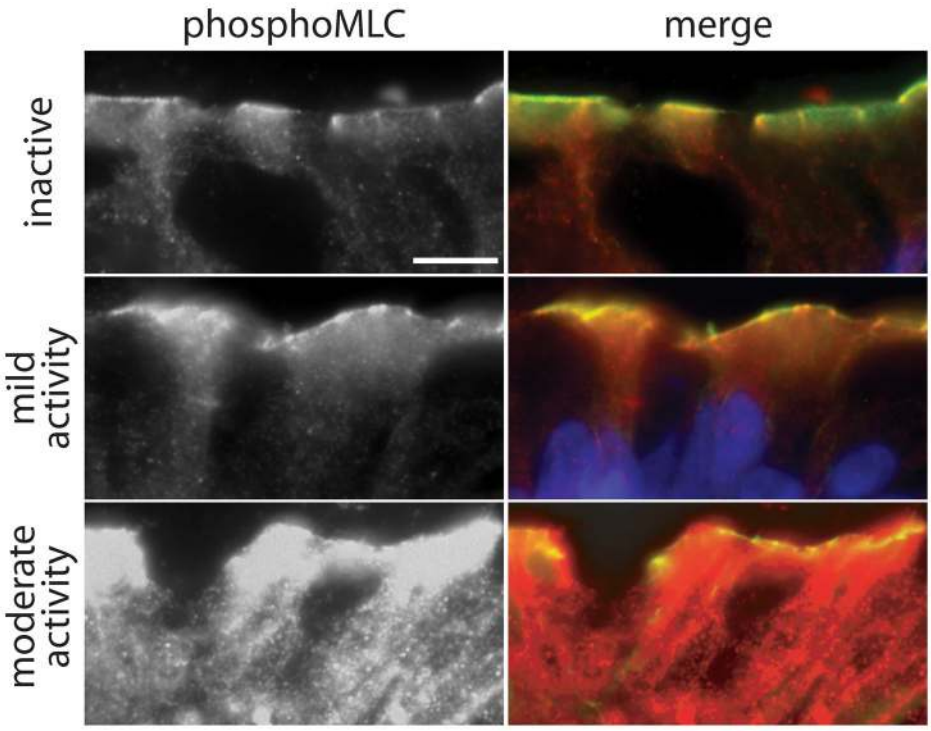

Figure 3.
Myosin light chain (MLC) phosphorylation is correlated with inflammatory activity in inflammatory bowel disease. Phosphorylated MLC (red) is primarily detected at the perijunctional actomyosin ring (green) in biopsies without active disease. While still predominantly within the perijunctional actomyosin ring, the intensity of phosphorylated MLC detection is markedly enhanced with increasing disease activity. Matched exposures are shown. Bar = 5 μm. Figure reproduced from Blair *et al.*[39] with permission.