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Research Article

Loosening Tight Junctions

Lessons from the Intestine

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In health, the two major charges of the intestinal epithelium are to absorb the vast quantity of water, ions, and nutrients presented it and to prevent the free mixing of lumenal content with underlying interstitial and vascular fluids. This latter responsibility is often generically referred to as intestinal epithelial barrier function. Here I present a perspective on one component of this barrier, the intercellular tight junction. The thrust of this perspective is that not only are tight junctions crucial to baseline intestinal barrier function, but that these tight junction barriers may be regulated and, as a result of this manipulation, the junctions also play a substantive role in the uptake of nutrients. Although my focus will be on the intestinal epithelium, links will be drawn to work done in other epithelia relating to intercellular tight junctions. Lastly, emerging evidence that tight junctions may be functionally altered in model disease states, even when the epithelium remains confluent, is considered. This latter concept reenforces the notion of the high degree of plasticity expressed by these important barriers.

Tight junctions (ZO) as barriers. As shown schematically in the figure, there exist only two potential pathways for passive permeation across intestinal epithelia: molecules can traverse cells (transcellular pathway) or can pass between cells (paracellular pathway). The paracellular pathway can be divided into two components: the area of the tight junction, more properly termed the zonula occludens (ZO),[1] and the underlying intercellular space. As recently reviewed by Gumbiner (1), and first described by Farquhar and Palade (2), the ZO consists of a narrow belt that wraps epithelial cells at the apical pole (Fig. 1). Within this ZO belt the lateral membranes of adjacent cells are closely apposed and focally appear to be fused. These fusion points or "kisses" course around the cell in an anastomosing fashion. Such kisses appear on replicas of freeze fractured membranes as an interconnected series of strands and grooves, the composition of which is controversial (3–5). It is now clear that the ZO is not the impermeable gasket it was assumed to be when first described. Rather, the ZO displays substantial permeability to small molecules (although under baseline conditions it is a relatively effective seal to molecules 11.5 Å and greater in Stokes radius [6]). Epithelia vary in resistance to passive ion permeation by over two orders of magnitude and it appears that variation in ZO resistance is largely the basis for this variability (for review see reference 7). The logic behind this conclusion is highlighted by considering potential routes for ion flow in the intestine: to passively cross the transcellular pathway a molecule encounters two biomembranes in series, apical and basolateral, each of which is very restrictive to leaks of hydrophilic solutes. In fact, the resistance to passive ion flow across this transcellular pathway is on the order of 1,000–10,000 Ω cm^2 (7). In contrast, the resistance of mammalian small intestinal epithelium is $\sim$ 50–90 Ω cm^2 (8–10). Such observations lead one to suspect that the paracellular pathway is the major site for passive transepithelial permeation, even when considering molecules as small as ions. This expectation is realized by the work of Frizzell and Shultz (11) who showed that at least 85% of passive ion flow across mammalian small intestine takes this paracellular route. Since the intercellular space below the ZO cannot even restrict free diffusion of macromolecules within it (12), it is assumed that under most conditions the major barrier in the paracellular pathway is the ZO. Thus, the importance of the ZO as an intestinal barrier is emphasized by the fact that it appears to be the rate limiting barrier in the pathway across which the majority of transepithelial passive permeation by hydrophilic solutes occurs.

Potential for ZO regulation. In the intestine, as well as in other organs, it has been suggested that ZO barrier function is integrated into specific functional roles of the epithelium. For example, ZO structure often varies in a cell type specific fashion (13–15). In the small intestine, ZOs of crypt cells are structurally irregular and, due to the narrow apex of crypt cells, ZO density in the crypt is very high (80 m/cm^2 of crypt luminal surface area) (16). In contrast, villus absorptive cells in fasted animals have ZOs with more structural subunits and with junctional density only a quarter of that of the crypt (16). Given the notion that intestinal secretion of ions originates from the crypt and consists of the net electrogenic transfer of Cl^- across cells followed by subsequent passive paracellular movement of Na^+ (17), it was suggested that these structural modifications, by facilitating paracellular Na^+ movement in the crypt, assisted in secretion (16). Similar examples of how cell or site specific variations in ZO structure could be integrated into transport processes in other epithelia have also been described (18). What was less clear at this time was the possibility that ZO barrier function could be integrated into epithelial transport events by dynamic, reversible alterations in the ZO structure and function.

Precisely how the anatomically defined subunits of the ZO relate to ZO barrier function is not yet certain. However, cur-

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1. *Abbreviations used in this paper:* ZO, zonula occludens.

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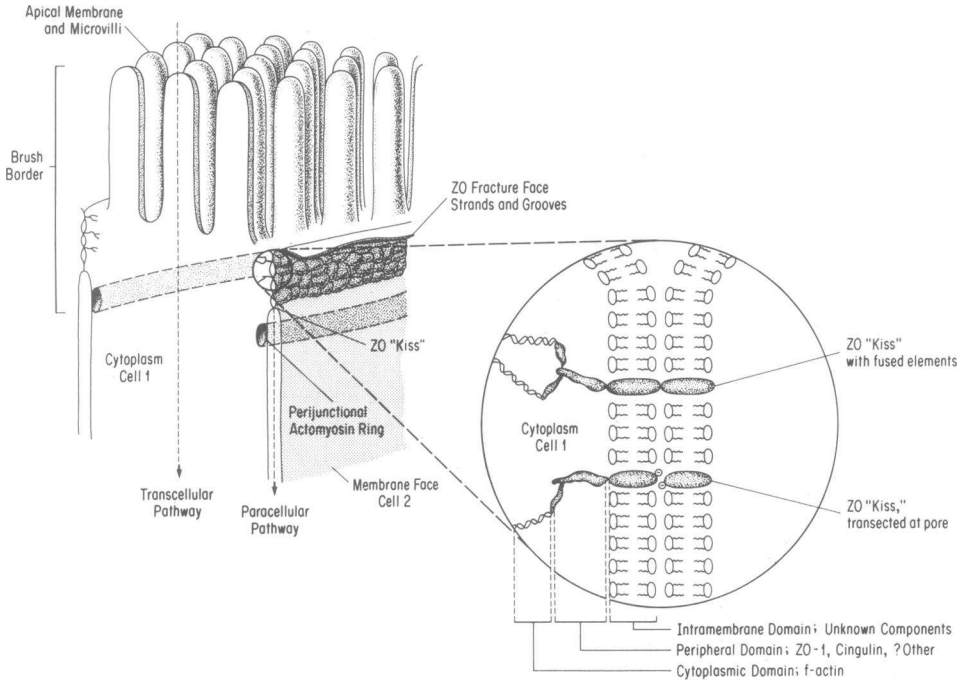

Figure 1. Schematic illustrations depicting tight junction (ZO) location and structure in intestinal epithelial cells. On the left is shown the brush border region of a transected cell. Fragments of the lateral membranes of flanking, neighbor cells are shown. The membrane face of the neighboring cell in the foreground is shown to highlight the freeze fracture appearance of the ZO. The inset displays a speculative model of the molecular substructure of the ZO (see text). Note that not only might the cytoskeleton modulate the ZO indirectly by tensile forces within a perijunctional actomyosin ring which circumferentially wraps the cell and inserts on the lateral membrane just below the ZO (*left*), but direct cytoskeletal-ZO interactions also appear to occur (*inset*).

rent knowledge in this area allows one to deduce a tentative model that aids in understanding how the function of the ZO could be altered. Taking into consideration a variety of indirect data, such as ZO structure-function correlations (6, 19, 20), ZO ion selectivity sequences (7, 21, 22), ZO sieving characteristics (6), and ZO charge selectivity (23, 24), one can formulate the following speculative model of how the ZO might function: ZO kisses/strands could be viewed as relatively impermeable structures in which discontinuities, "channels" or pores, reside (Fig. 1). As with channels of biomembranes, it is proposed that these channels may open and close (20). The interior of the channel would appear to be highly hydrated and contain fixed negative charges. Assuming this model is correct, it is evident that there exist numerous potential ways to modify ZO barrier function: the number of kisses/strands (i.e., ZO subunits) could be changed, the probability of channels being in the open state could be altered, or physical characteristics of the pore interior might be changed.

As seen from the perspective outlined above, the ZO pore essentially represents a site between cells in which the volume of extracellular fluid is severely restricted. Displacement of the conductive extracellular solution results in a substantial paracellular resistance. Thus, it would not be surprising if the resistance of extracellular fluid within the intestinal ZOs approximated the resistance of the epithelium. In other words, the ZO imparts resistance on the epithelium by squeezing out, at this one site, the ion-containing and therefore conductive solution of the paracellular space. This view of ZO behavior highlights yet another way in which ZO resistance could be manipulated. Since the specific resistance of ion containing solutions depends both on the specific ionic composition and concentration within the solution, relatively modest alterations in the relative concentrations of specific species of ions within the minuscule volume of the ZO pores could result in substantial alterations in ZO resistance. This speculative possibility could be accomplished in several ways. For example, if the lateral membranes of adjacent cells contained pumps within the area of the ZO, such as Na^+-K^+-ATPase, ions of substantially different conductivity would be exchanged (not to mention the fact that, if the stoichiometry of exchange varied from 1:1, changes in ionic strength and, therefore, conductivity within the ZO pore might also occur). Such alterations in fluid composition within the pore should result in substantial changes in ZO resistance, as measured with biophysical techniques, even though no structural alterations in the ZO (even at the molecular level) occurred.

Above are highlighted a few of the events that might potentially lead to modification of ZO function. Recent evidence suggests that at least some of these events may indeed occur. Examples include experimentally induced alteration in ZO subunit number (number of strands/kisses) (8, 24–27), and alteration in surface charge within the pores of the ZO as suggested by altered ZO charge selectivity (28). Recent work using two strains of MDCK cells which have either high ($\sim$ 3,000 Ω cm^2) or low ($\sim$ 100 Ω cm^2) resistance suggests that in this case the basis of the difference in resistance observed is not due to strain-related differences in ZO subunit number (29). Rather, in this case, it appears that variation in the density, probability of being in the open state, or other physical characteristic of the ZO pores determines the strain-related variation in resistance (29).

The suggestion that ZOs could potentially be regulated as a consequence of intracellular events came initially from a host of observations. First, a variety of intracellular mediators were shown to alter ZO function and/or structure in at least some epithelia. Using microelectrode impalement techniques Duffey et al. (25) showed that the ZOs of amphibian gallbladder epithelium display enhanced resistance to passive ion flow as intracellular cAMP is elevated (25). Concurrently, ZOs gain additional, structurally defined, subunits and ZO charge selectivity is altered. Similarly, cAMP appeared to substantially alter ZO function in goldfish (30) and in flounder (28) intestine. Also, exposure of amphibian gallbladder epithelium to Ca^{2+} ionophore appears to enhance ZO resistance and induce

alterations in ZO charge selectivity and structure (27) (whether these latter effects are specifically due to elevated intracellular free Ca^{2+} is clouded by the fact that large concentrations of ionophore were utilized). Lastly, using a kidney epithelial cell line it was shown that activation of protein kinase C with phorbol esters diminished ZO resistance (31, 32).

Given that a variety of intracellular activation signals can influence ZOs in at least some epithelia, the question arises as to how these signals are transmitted to the area of the ZO. There are little direct data bearing on this issue. However, a host of indirect data suggest that the cytoskeleton is anatomically and functionally tied to the ZO. Thus, it is possible that ZOs may be manipulated by the cytoskeleton as are many other components of the cell surface which have cytoskeletal links (33). This hypothesis, which links functional alterations in ZOs to cytoskeletal rearrangement, is supported by the observation that structural changes occur in the cytoskeleton adjacent to the ZO during the above described response to cAMP (25).

Functional links between the cytoskeleton and the ZO were first described in cultured renal epithelium (34) and in gallbladder epithelium (26). These seminal studies took the approach of pharmacologically manipulating the cytoskeleton and subsequently assessing the alterations that occur in ZO function. The intestinal epithelium has provided a useful model in which to further examine this putative ZO-cytoskeletal link since the cytoskeleton of these cells has been so extensively characterized. As shown in the diagram, the apex or "brush-border" of intestinal absorptive cells consists of a carpet of microvilli. The cytoskeletal underpinning of the brush border is so extensive that the brush border can be isolated from the cell and purified thus permitting biochemical characterization and parallel morphological studies of this specific cell region. Such studies have now reasonably defined the molecular topography of the brush border cytoskeleton (see reference 35 for recent review). One characteristic feature that has been noted is a circumferential ring of actin and myosin, which wraps each absorptive cell brush border (36–38). This perijunctional actomyosin ring appears to associate with the lateral plasma membrane just below the ZO (Fig. 1). This ring is also termed a contractile ring since studies of isolated brush borders show that, using divalent cations and ATP, morphologic alterations suggestive of ring contraction can be elicited (36, 38) and, in parallel, myosin becomes phosphorylated (37). What was unclear was whether ring contraction could be induced to occur in an intact epithelial sheet and, if so, whether such contraction would alter the ZO as some had hypothesized (35). This suggestion was certainly plausible since it had been shown that mechanically applied lateral tension (39), which presumably would be the result if rings contracted, was capable of altering ZO structure. Using cytochalasin D, an agent that affects actin microfilaments, we were able to demonstrate that intestinal absorptive cell ZOs became perturbed in structure and that ZO charge selectivity and resistance became markedly diminished (9). In parallel, the perijunctional ring became segmented and condensed and the brush borders became rounded, all features suggesting that, in analogy to isolated brush borders, ring contraction had occurred. Supporting this view was the subsequent finding that the effects of cytochalasin D on ZO structure, ZO function, and ring condensation were energy dependent and appeared to be interrelated (40). Similar data regarding pharmacologically stimulated ring contraction and enhanced ZO permeability have been generated using a model intestinal epithelium (41), monolayers composed of the human intestinal epithelial cell line T_{84}. Thus, not only do macroscopic sphincters reside along the axis of the alimentary canal, but these perijunctional actomyosin rings which amount to intracellular "sphincters" abound in the intestinal epithelium!

Recently it has been recognized that subtle but direct anatomical associations appear to exist between the cytoskeleton and the ZO (42, 43). Detergent extracted preparations of intestinal absorptive cells display plaque-like condensations of electron-dense material immediately adjacent to the cytoplasmic face of the ZO (42). This material often specifically localizes at the sites of kiss/strands within the ZO. It is possible that this material in part represents ZO-1 (5, 44), a ZO specific peripheral membrane phosphoprotein, which is a candidate molecule for linking the cytoskeleton with the ZO. As determined using both immunoelectron microscopic (43) and detergent extraction (42) techniques, actin microfilaments intimately associate with these plaque-like condensations that flank the ZO. These data raise the speculative possibility that not only may the ZO be indirectly affected by tension within the perijunctional actomyosin ring, but perhaps elements of the ZO could be directly manipulated through cytoskeletal interactions mediated by ZO specific proteins such as ZO-1. Recently another ZO specific protein, termed cingulin, has been identified. Western blot analysis of epithelial cell homogenates reveals two polypeptides of molecular mass 140 kD and 105 kD which label with anticingulin antibodies (44). Preliminary studies have indicated that ZO-1 and cingulin are antigenically distinct molecules (29), thus at least two ZO specific proteins exist in intimate association with the ZO. The functional significance of ZO-specific proteins is unclear but their existence provides additional clues as to how cytoplasmic signals specifically influence ZO structure and function. On the basis of such data, a tentative working model of the direct structural relationships between the cytoskeleton and the ZO is presented as an enlargement in Fig. 1.

Intestinal ZO as a regulated transport pathway in health. To this point we have discussed ZOs predominantly in relation to barrier function, a topic which, on the surface, largely appears to be distinct from that of vectorial transport across epithelia. What follows will indicate that absorptive cell ZOs constitute a major absorptive transport pathway in the intestinal epithelium and will show how intimately intertwined absorptive and barrier function of epithelia can be.

As expressed in major reviews and texts (45, 46), the predominate view of how uptake of hydrophilic nutrients such as glucose and amino acids occurs can be summarized as follows: glucose is cotransported across the apical membrane with Na^+, and, via the Na^+-K^+-ATPase pump and by basolateral glucose facilitated transport, these solutes are subsequently deposited into the paracellular space. Absorption of additional water across the ZO driven by the deposition of these osmotically active solutes in the paracellular space (or by the creation of a hypertonic subepithelial compartment by other means such as the putative countercurrent exchanger in the villus core [47]) occurs subsequently. In summary, this view would hold that nutrient uptake is largely a transcellular event. It now appears that activation of Na^+ coupled cotransporters on the apical membrane of absorptive cells by substrates such as glucose or amino acids also results in alteration of the ZO (48, 49) and

perturbation of ZO structure (49). Since apparent condensation of the perijunctional actomyosin ring accompanies these changes, it is suggested that, in analogy to the data previously described, enhanced ring tension underlies this ZO response. Furthermore, activation of these cotransporters alters the sieving characteristics of ZOs such that there is enhanced clearance of nutrient sized molecules (50). Lastly, it appears that this nutrient-induced change in ZO resistance is maximal at nutrient concentrations that saturate the transcellular uptake pathway. The following alternative theory of nutrient absorption arises from these observations and is summarized as follows: exposure of the intestinal epithelium to lumenal glucose (or amino acids) results in the same events outlined above under the existing dogma of transcellular nutrient absorption but, in addition, ZOs become leaky to nutrient-sized molecules due to enhanced cytoskeletal tension. Thus, as water flows across the ZO, substantial nutrient absorption occurs by solvent drag. This view suggests that as luminal nutrient concentration rises above that at which the capacity of the transcellular uptake mechanism is saturated, an increasingly large percentage of nutrient will be harvested via solvent drag across the ZO. Since the cotransporters of the apical membrane are saturated at low nutrient concentrations (below 25 mM for glucose) but, after a meal, nutrient concentrations in the proximal intestine often exceed 200 mM (51, 52), it follows that the ZO may be a, if not the, major pathway of nutrient uptake. The amount of nutrient absorbed by solvent drag would necessarily depend on the concentration of nutrient within the lumen. Thus, expressed another way, one should expect increasing nutrient absorption from the intestine as lumenal solute concentration increases, even when the transcellular transport pathway is saturated. Also predicted by this view is that, at high concentrations of lumenal nutrients, the precise composition (glucose vs. amino acids) of the lumenal solute would not substantially influence net absorption as long as one of the cotransporters was saturated. These predictions, based on the above trans-ZO solvent drag theory of absorption, have been or are being realized. For example, it is clear that glucose uptake by the intestinal epithelium (in the presence of an intact vasculature) continues to increase with escalating luminal nutrient concentration well past the point of saturation of the transcellular pathway (53, 54). Further, it now appears that oral rehydration solutions combining glucose with glycine (attempting to enhance absorption by using two transcellular absorption pathways instead of one) are no more efficacious in providing volume absorption in patients with secretory diarrhea than are solutions containing high concentrations of glucose alone (55).

ZO dynamics in intestinal disease. It comes as no surprise that ZO barrier function can be severely altered in many intestinal diseases. Such diseases are often characterized histologically by small erosions or even macroscopic ulcers. It is intuitively obvious that when epithelial cells are separated by such huge distances, the site at which the "ZO" resides, the ulcer, will not represent a significant barrier to diffusion. However, there do exist diseases in which the intestinal epithelium remains confluent while ZOs leak relatively large molecules, in the extreme, even macromolecules such as albumin. Celiac-sprue is a good example of such a disorder (56). Given the plasticity of ZOs in health it is only natural that these crucial barriers might also be affected in disease. Recent studies of modulation of intestinal ZOs in disease states have been carried out in hopes not only of gaining insights into what may go wrong with these barriers, but also in hopes of gaining further insights into the mechanisms whereby individual intestinal epithelial cells regulate ZOs. Given the uncertainties and complexity of many of the animal models of intestinal disease, these studies have relied heavily on a model intestinal epithelium consisting of T_{84} cells (57). T_{84} cells grow as confluent monolayers, display high baseline resistance, have ZOs with subunit structure-function correlates not dissimilar to those seen in native intestinal epithelium (6), and harbor actin-rich perijunctional rings (41) which, like those of natural intestinal epithelial cells, segment and condense upon exposure to cytochalasin D, an event accompanied by enhanced ZO permeability (41).

T_{84} cells have been used as a model for studies of ZO barrier function in the state of acute inflammation. Acute inflammation of the intestine is characterized by movement of polymorphonuclear leukocytes out of subepithelial microvasculature and into and across the epithelium (58). This process may be modeled by the placement of isolated PMN on one side of T_{84}, or other (59, 60), monolayers and of a chemotactic factor such as an *n*-formylated-peptide on the opposite side. Under such conditions PMN move across T_{84} monolayers by crossing the ZO (61). With large numbers of transmigrating PMN, ZO function is dramatically impaired. In T_{84} monolayers, impairment takes the form of diminished transepithelial resistance, enhanced transepithelial flux of inert tracers such as mannitol and inulin and, during the phase in which ZOs are actively being impaled by PMN, leaks to macromolecules (61). Upon ablation of chemotactic conditions, these barrier alterations are readily reversed. It does not appear that products released by the PMN are responsible for these defects in ZO permeability since (*a*) when PMN are densely layered onto monolayers and stimulated with the chemotactic agent in the absence of a chemotactic gradient, no change in barrier function occurs and (*b*) selected inhibitors of products released by PMN under chemotactic conditions do not prevent altered barrier function (62). It does appear that an adhesion plaque that forms before transmigration between the PMN and epithelial cells may be the "foothold" from which the PMN is able to generate the force required to open the ZO (62). Thus, we speculated that the opening of the ZO which occurs during PMN transmigration is produced by mechanical force (62) just as mechanical force may underlie the ZO perturbation induced by the above outlined pharmacologic (8, 40) and physiologic (49) manipulations; the difference being that, in these latter instances, the mechanical force putatively is generated by the cytoskeleton within the epithelial cell, whereas with PMN transmigration, the mechanical force is generated externally by the PMN pseudopod.

Unexpectedly, in vitro models of intestinal disease also promise to yield insights into cytoskeletal-ZO relationships. For example, toxin A, a protein exotoxin of *Clostridium difficile,* causes a severe enterocolitis which, in part, may be due to its effects on inflammatory cells (63). In order to determine whether this toxin could also exert direct effects on intestinal epithelial cells, T_{84} monolayers were exposed to this agent. Toxin A disrupted barrier function of T_{84} monolayers such that transepithelial resistance was nearly abolished within 6–8 h (64). The way in which this toxin abolished resistance in this early phase of epithelial perturbation was remarkable. The monolayers remained confluent, cells remained abutted to their neighbors, and no biochemically or morphologically discernable evidence of cytotoxicity was seen (64). Flux data in-

dicated that this toxin-elicited increase in permeability was restricted to molecules less than 5 Å in Stokes radius. Since toxin A did not increase the permeability of T_{84} cell plasma membrane to a hydrophilic solute 3.6 Å in radius, the above alterations in permeability were attributed to the toxin-elicited alterations in intercellular ZOs. Analysis of the cytoskeleton showed that a prominent effect of toxin A was diminution of f-actin staining in the perijunctional ring. Such findings define a useful model to study cytoskeletal-ZO interactions and suggest that ZO-perijunctional ring-interaction, even in the baseline state, may subtly influence the sieving characteristics of ZOs.

Given the apparent plasticity of ZOs and the putative relationships between ZOs and the cytoskeleton, it will not be surprising if various other disease-related challenges substantially alter intestinal epithelial barrier function even if epithelial continuity is maintained. For example, recently it was recognized that the inflammatory mediator interferon-gamma directly effects barrier function of T_{84} monolayers in the absence of cytotoxicity and appears to do so by altering ZO permeability (65). Others have noted that, coincident with the accumulation of envelope protein in the cell surface, ZOs of Madin-Darby Canine Kidney cells infected by vesicular stomatitis virus become greatly diminished in barrier capacity (66). Further analyses of such systems should enhance understanding of the factors involved in eliciting abnormal epithelial permeability in disease states.

Conclusions. As modeled by intestinal epithelia, ZOs behave quite unlike the static gaskets they were assumed to be in the not too distant past. It appears this cell surface structure intimately associates with cytoskeletal components and that such ZO-cytoskeletal associations may be functionally relevant. Not only can ZO structure and function be altered by pharmacologic manipulation of the cytoskeleton, but, in some models, intracellular messengers, such as cAMP, can also affect the ZO, putatively by generating cytoskeletal alterations. More than that, physiologic signals, such as the activation of Na-coupled nutrient transporters on the apical membrane of intestinal villus absorptive cells, can substantially alter ZO structure and function. Indeed, it appears the intestine would be unable to harvest the vast quantity of nutrients presented daily in the absence of this regulated ZO pathway. Evidence suggests that such ZO regulation may ultimately be tied to tensile force emanating from an intracellular sphincter-like actomyosin ring. Lastly, the plasticity of the ZO is also manifest in several model disease states. These latter findings not only serve as a basis for understanding how epithelial permeability can change so greatly with disease, even in the presence of retained epithelial confluency, but also provide models to further dissect ZO-cytoskeletal relationships. It is becoming clear that modulation of intestinal epithelial ZOs is important in health and disease. It is now time to attempt to understand with more precision the nature of the intracellular pathways that modulate this cell surface structure which is so crucial a part of intestinal epithelial barrier function.

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