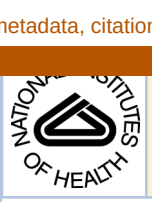

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Zonula Occludens-1 and -2 Are Cytosolic Scaffolds That Regulate the Assembly of Cellular Junctions

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Abstract

The integrity of the tight junction barrier in epithelial and endothelial cells is critical to human health, but we still lack a detailed mechanistic knowledge of how the barrier is formed during development or responds to pathological and pharmacological insults. This limits our understanding of barrier dysfunction in disease and slows the development of therapeutic strategies. Recent studies confirm the long-maintained but previously unsupported view that the zonula occludens (ZO) proteins ZO-1 and ZO-2 are critical determinants of barrier formation. However, ZO proteins can also be components of adherens junctions, and recent studies suggest that ZO proteins may also promote the assembly and function of these junctions during epithelial morphogenesis. We review these studies and outline several recent observations that suggest that one role of ZO proteins is to regulate cytoskeletal dynamics at cell junctions. Finally, we propose a model by which the functional activities of ZO proteins in the adherens junction and tight junction are differentiated by a novel regulatory motif known as the U6 or acidic motif.

Keywords

tight junction; adherens junction; zonula occludens; ZO-1; ZO-2; E-cadherin; cytoskeleton; MAGUK; PDZ; scaffold; permeability; epithelia; morphogenesis

Assembly of Cell–Cell Junctions Is a Multistep Process

Polarized epithelial cells are circumscribed at their apical-lateral margin by two morphologically distinct intercellular junctions that together form what is known as the apical junctional complex (AJC). The adherens junction (AJ) mediates cell–cell adhesion and signaling pathways that control growth, cell morphology, and differentiation.[1] The tight junction (TJ) regulates the movement of ions, macromolecules, and immune cells through the paracellular space and is critical for ion transport.[2,3]

The assembly of the TJ barrier during morphogenesis or tissue repair is a multistep process that involves the initial establishment of adhesive cell–cell contacts, the extension of these contacts laterally along the cell–cell contact zone, and the subsequent (perhaps concurrent) recruitment of transmembrane proteins, such as claudins, junction adhesion molecule (JAM), and occludin/tricellulin that form the paracellular barrier.[4,5] The initial contacts are isolated punctate structures formed by the adhesive activities of the cadherin–catenin

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Conflicts of Interest

The authors declare no conflicts of interest.

complex and can occur within filopodial structures or at discrete sites within a larger contact zone between two cellular membranes (reviewed in Ref. 6). These adhesive contacts are subsequently extended into a circumferential structure known as the zonula adherens (ZA) that circumscribes the apical-lateral boundary of polarized epithelial cells. This latter process is poorly understood, but current evidence supports the hypothesis it involves the coordinated actions of multiple signaling pathways[7,8] with the cortical actin cytoskeleton.

Zonula Occludens Proteins Regulate Tight Junction Assembly

Recent observations from our group and others strongly suggest that the proteins zonula occludens (ZO)-1 and ZO-2, which are members of the membrane-associated guanylate kinase (MAGUK) homologue family of proteins, are critical regulators of TJ assembly. The TJ barrier is composed of transmembrane proteins, such as the claudins, occludin, and tricellulin, that are organized into continuous adhesive strands that circumscribe the apicallateral margin of polarized epithelia.[9] Significantly, many of these transmembrane proteins are also distributed throughout the entire lateral plasma membrane but they are only organized into barrier strands at the apical junctional complex—suggesting there are spatially and temporally regulated steps in the assembly of these proteins into strands.

Recently, the Tsukita group demonstrated that depletion of both ZO-1 and ZO-2 in the EpH4 mammary epithelial cell line completely abrogated TJ assembly.[10] Using a different approach, we have demonstrated that expression of ZO proteins that contain mutations in a highly conserved region, the U6 domain, in MDCK cells can trigger the formation of ectopically positioned TJ transmembrane protein strands on the lateral plasma membrane.[11] These observations, taken together, indicate that the ZO proteins are required for the assembly of TJ transmembrane proteins, such as claudin, into barrier strands and direct the incorporation of these strands into the AJC.

More recently it has been demonstrated that targeted disruption of either ZO-1 or ZO-2 in mice results in embryonic lethality that is correlated with disruption of the paracellular barrier and the structure of cell junctions.[12,13] These observations suggest that the ZO proteins have nonredundant functions during development, unlike their role in strand assembly. However, the mechanistic basis for the early embryonic lethality in these mice is still under investigation.

These observations raise several critical questions. First, how do ZO proteins bring about the coordinated assembly of barrier proteins, such as the claudins, into a highly organized circumferential barrier? Second, at what step in epithelial barrier morphogenesis do ZO proteins act? Third, how is the assembly of the barrier linked to the assembly of other junctional complexes, such as the AJ? Based on the results reviewed below, we speculate that ZO proteins act at several stages of assembly and that their role is based on three very important properties of the ZO MAGUKs: (1) their ability to act as a temporally and spatially regulated scaffold for other TJ proteins, such as occludin/tricellulin and claudin; (2) their ability to bind to and regulate components of the cortical actin cytoskeleton; and (3) their ability to promote cadherin-mediated cell–cell adhesion.

ZO Proteins Are Multidomain Scaffolding Molecules

How do the ZO proteins act as scaffolds? The three ZO proteins, ZO-1, -2, and -3, are multidomain polypeptides (Fig. 1). They have an N-terminal half with a domain structure similar to other MAGUK proteins that contains 3 PSD-95/discs-large/Zonula occludens-1 (PDZ) domains,an Src homologty-3 (SH3) domain, and a region of homology to guanylate kinase (for review, see Refs. 9,14). Interspersed between these conserved protein-binding domains are so-called unique (U) motifs. The protein-binding motifs within the N terminus

mediate direct interactions with all of the knownTJ transmembrane proteins and many of the cytosolic proteins that localize to TJ. For example, the first PDZ domain (PDZ1) binds to claudins, PDZ3 binds to JAM proteins, and the U5+GUK domain binds to occludin and tricellulin.[15–18] In ZO-1, like other MAGUKs, the U5 domain (also known as the HOOK domain), located between the SH3 and GUK domains, is required for junction localization, whereas the U6 domain regulates the activity of the U5 and GUK domains in a fashion not yet fully understood (discussed below).[11] Interestingly, the AJ proteins α catenin, AF-6/afadin, and ARVCF have also been reported to bind within the N-terminal half of ZO-1 and ZO-2.[19–22] The possible functional relevance of this is considered below.

The C-terminal regions are unique to the three ZO MAGUKs and not present in proteins such as PSD95, CASK, and Dlg. Although highly divergent at the amino acid level, the C-terminal regions all bind directly to F-actin or with other actin-binding proteins, such as protein 4.1 and cortactin.[17,23,24] Both AF-6/afadin and cingulin also appear to have binding sites in the C terminus.[11,25]

Importantly, the second PDZ domains of ZO MAGUKs mediate homodimerization and heterodimerization between these three molecules.[26,27] Although the functional role of PDZ dimerization is poorly understood, one recent study suggested that cross-linking of ZO proteins within the SH3/GUK regions is critical for strand assembly.[10] This is not without precedent; in the synaptic MAGUKS PSD-95 and SAP102, “domain swapping” between SH3 and GUK domains of these two proteins results in the formation of heterodimers.[28] Thus, we predict that the ability of ZO proteins to promote the assembly of TJ proteins into the paracellular barrier not only depends upon their ability to bind directly to many different junctional proteins but also on an intrinsic ability to cross-link these proteins into larger arrays. The critical questions that arise include: What protein–protein interactions are necessary for assembly of TJ transmembrane proteins into strands and the incorporation of these strands into the AJC? Does multimerization play a role in the assembly of barrier strands? How is the temporal and spatial assembly of TJ proteins into strands controlled?

Very little is known about how binding of proteins to ZO-1 is regulated or how cell signaling pathways control TJ assembly and barrier function, although many different pathways have been implicated (for review, see Ref. 29). Interestingly, it was recently demonstrated that Ca-calmodulin binds to the GUK domain of ZO-1,[30] suggesting the novel hypothesis that intracellular calcium levels control some ZO-1 functions. Again, such a mechanism is not without precedent. Ca-calmodulin binds within the U5 region of other MAGUKs, and this interaction regulates multimerization *in vitro*.[28,30] Another regulator of TJ assembly, Gα12, binds to the SH3 domain and appears to regulate the interactions of ZO-1 with several TJ ligands.[31] Thus, there are several lines of evidence that suggest that regulated interactions with the region encoding the SH3, U5, and GUK domains are critical for TJ assembly.

Do ZO Proteins Regulate Adherens Junction Assembly and/or Cell–Cell Adhesion?

It has been appreciated for several years that the assembly and integrity of TJs are intertwined with that of AJs. Disruption of E-cadherin-mediated cell–cell adhesion with extracellular antibodies to E-cadherin[32] or small interfering (si)RNA[33] delays TJ assembly. Furthermore, ZO-1 and ZO-2 localize to AJ with cadherin and catenins at the earliest punctuate cell–cell contacts prior to proteins such as occludin or claudin and bind directly to AJ proteins, such as α catenin, AVRCF, and AF-6/afadin.[20,22,34,35] Thus, the prevalent hypothesis is that ZO proteins use direct interactions with AJ proteins as a positional signal

early during AJ assembly and subsequently recruit TJ proteins into an apical complex that is ultimately segregated from the AJ.

While this appears to be one role for the ZO proteins, the evidence suggests they are actually active components in AJ formation or cell adhesion. ZO proteins are components of the AJ in nonepithelial cells, such as fibroblasts and cardiomyocytes, and are primarily localized to AJs in nonvertebrates, such as *Drosophila* and *Caenorhabditis elegans*.[25,36–38] In addition, several groups have identified defects in AJ assembly in cells lacking ZO proteins. For example, the extension of AJ plaques into a circumferential belt was delayed by more than 24 h in ZO-deficient EpH4 cells, and disruption of the ZA was observed in fully polarized cells.[39,40] Similarly, Hernandez *et al*. found that knockdown of ZO-2 alone was sufficient to briefly delay AJ assembly and disrupt E-cadherin localization in MDCK cells.[41] Finally, the combination of weak mutations in the single *Drosophila* ZO protein PYD and the AF-6/afadin homologue Canoe, which on their own have no AJ defect, have severe AJ defects when combined.[25] Stronger Pyd alleles appear to have more dramatic alterations in adhesion that lead to defects in dorsal closure and eye morphogenesis.[42,43] Significantly, exogenous PYD transgenes can only rescue AJ defects when they localize to the AJ, and this requires the U6 motif discussed below.[42] These observations suggest that ZO proteins not only serve to recruit TJ proteins to the circumferential belt but they also directly regulate the activity of AJs.

How ZO proteins might regulate AJ assembly is a matter of speculation. ZO proteins do not appear to be required for the *de novo* assembly of cadherin or catenins into primordial AJ in cultured epithelial cells[39,40] or for their localization to AJ in *Drosophila* embryos.[25,43,44] Instead, they appear to regulate the extension of AJ proteins into a circumferential complex[39] and morphogenetic processes, such as tubulogenesis[44] and dorsal closure[42] and tissue morphogenesis in the pupal eye.[43] One hypothesis that might explain these observations is that ZO proteins can directly promote adhesion of the cadherin–catenin complex during dynamic reorganization of cell–cell junctions. This hypothesis is at least partly supported by the observation that E-cadherin/α catenin chimeras lacking the ZO-1 binding domain in α catenin cannot promote cell–cell adhesion in L cell fibroblasts lacking endogenous E-cadherin, although E-cadherin/α catenin chimeras can do so.[45]

The U6 Motif Regulates the Assembly of Tight Junction Strands

In epithelial cells, ZO proteins are necessary for the assembly of TJ proteins, such as claudin, into TJ strands. We hypothesize that the spatial, and perhaps temporal, positioning of these strands is dependent upon the U6 motif. This conserved motif, which is unique to ZO MAGUKs, is immediately C terminal to the GUK domains in all ZO proteins (Fig. 1). We have demonstrated that this motif negatively regulates incorporation of ZO-1 into the TJ, that it inhibits the binding of occludin to ZO-1, and that deletion of this motif results in assembly of ectopically positioned strands.[11] These results were confirmed by Ikenouchi *et al*.[39] who demonstrated that this motif is required for the localization of TJ strands to the AJC in ZO-1/ZO-2-depleted cells. ZO-1 transgenes that lack the U6 domain can initiate the polymerization of claudins on the lateral surface of ZO1/ZO2-depleted cells but they are unable to incorporate claudins into a circumferential barrier.[39] Thus, the U6 motif regulates the positioning of TJ strands in the AJC as well as the incorporation of transmembrane proteins, such as occludin or tricellulin, into these strands.

Our published observations indicate that the U6 motif does not directly target ZO-1 to the AJC. Instead, sequences within the U5 motif appear to be necessary and sufficient for TJ localization, much like the U5 domain (also known as the “HOOK”) in other MAGUK proteins.[11,46,47] Thus, the ability of the U6 motif to inhibit TJ localization suggests that the

U6 domain somehow regulates the activity of the U5 motif. In a similar manner, the inhibition of occludin binding suggests that U6 also inhibits the binding of some proteins to the GUK domain. Interestingly, the U6 domain is also necessary for ZA assembly in ZO-1/ZO-2-depleted cells; ZO-1 transgenes lacking the U6 motif cannot rescue the defect in AJ assembly in these cells.[39,40] Thus, the U6 domain appears to act as a bifunctional switch that inhibits TJ assembly (incorporation into AJC and binding to occludin/tricellulin) but promotes AJ assembly. We propose that the U6 motif distinguishes between the dual activities of ZO proteins in the AJ and TJ (discussed below) and suggest that it regulates the segregation of AJ and TJ proteins that develops within the AJC of polarized epithelia.

ZO-1 Regulates the Cortical Cytoskeleton at Cell–Cell Contacts

A third hypothesis currently being tested in our laboratory is that ZO-1 regulates both AJ and TJ function by coordinating the assembly or dynamics of the cortical cytoskeleton. Both TJs and AJs are intimately associated with the cortical actin cytoskeleton, and there is a considerable body of evidence that indicates that the assembly and functional activity of cell–cell junctions relies on the integrity of the cytoskeletal arrays with which they are physically associated.[7,9] AJ proteins like α catenin, AF-6, and vinculin all bind directly to F-actin and regulate actin dynamics *in vitro*, and disruption of cytoskeletal interactions with these proteins can clearly disrupt AJ assembly and cell–cell adhesion.[21,45,48,49] The assembly of the TJ, like the AJ, is also sensitive to physiological and pharmacological regulators of cytoskeletal dynamics, and ZO proteins bind directly to F-actin and to several proteins that can regulate cytoskeletal dynamics (reviewed in Ref. 50). For example, both ZO-1 and ZO-2 bind to cortactin, which promotes and stabilizes the assembly of branched filaments at the plasma membrane.[24] ZO-1 has also been demonstrated to interact with vasodilatorstimulated phosphoprotein (VASP),[51] protein 4.1,[23] shroom2,[52] and the Cdc42 guanine nucleotide exchange factor (GEF) Tuba.[53]

Disruption of ZO protein expression has a dramatic effect on cytoskeletal dynamics at AJ and TJ. In MDCK cells, depletion of ZO-1 alters the dynamics of F-actin redistribution following calcium switch,[54] and depletion of ZO-2 alone disrupts F-actin structures associated with junctions.[41] More dramatically, depletion of both ZO-1 and ZO-2 in EpH4 cells delays the formation of circumferential actin bundles by 72 h, eliminates the band of F-actin that is normally tightly associated with the ZA, and disrupts the incorporation of myosin II into AJ.[39,40] Finally, loss of the single ZO-1 orthologue in *C. elegans* disrupts the accumulation of F-actin at AJ during embryogenesis.[38] These observations support the hypothesis that ZO proteins, or their binding partners, are regulators of F-actin dynamics at cellular junctions.

The functional/mechanistic relevance of these cytoskeletal interactions with ZO proteins to TJ or AJ biology is still poorly understood. One possibility is that ZO proteins passively link transmembrane proteins, such as claudin, occluding, or the cadherin/catenin complex, to the cortical cytoskeletal and that this has some role in stabilizing junctional complexes in the plasma membrane. An alternative possibility is that ZO proteins directly regulate the assembly or dynamics of F-actin at AJ or TJ and may thus regulate the downstream morphogenetic processes of epithelia, such as tubulogenesis, wound repair, or epithelial–mesenchymal transitions. A recent study demonstrated that ZO-1 was required for three-dimensional cyst formation in MDCK cells,[55] and Wittchen *et al.* found an increased rate of wound closure in cells expressing ZO-3 truncation mutants.[56] It has also been demonstrated that ZO-1 is required for localization of Tuba and myosin II to cell junctions.[40,53] Disruption of Tuba expression delays junction assembly, alters junctional F-actin, and results in changes in cell shape.[53] Finally, Ikenouchi *et al.* found that rac activation, which is critical for cytoskeletal dynamics, was markedly reduced in ZO1/ZO2 knockdown cells.[39] Together

these results support the hypothesis that ZO proteins recruit and/or activate the machinery of actin filament assembly at junctions during critical points in epithelial morphogenesis.

Conclusion

Recent studies have confirmed the long-held view that ZO proteins are critical regulators of TJ assembly and have presented the novel hypothesis that ZO proteins may also promote AJ assembly. The critical elements of these functions are likely to be the ability of ZO proteins to bind to and cross-link other junction proteins into higher order arrays and to link these arrays to the underlying cortical cytoskeleton. Whether ZO proteins have a more active role in the dynamics of this cytoskeletal array remains to be determined. It is also not clear if or how the various signaling pathways that regulate junction assembly might work through ZO proteins. However, it is clear that the U6 motif is an important regulatory element in ZO proteins that warrants further study in the context of these signaling pathways.

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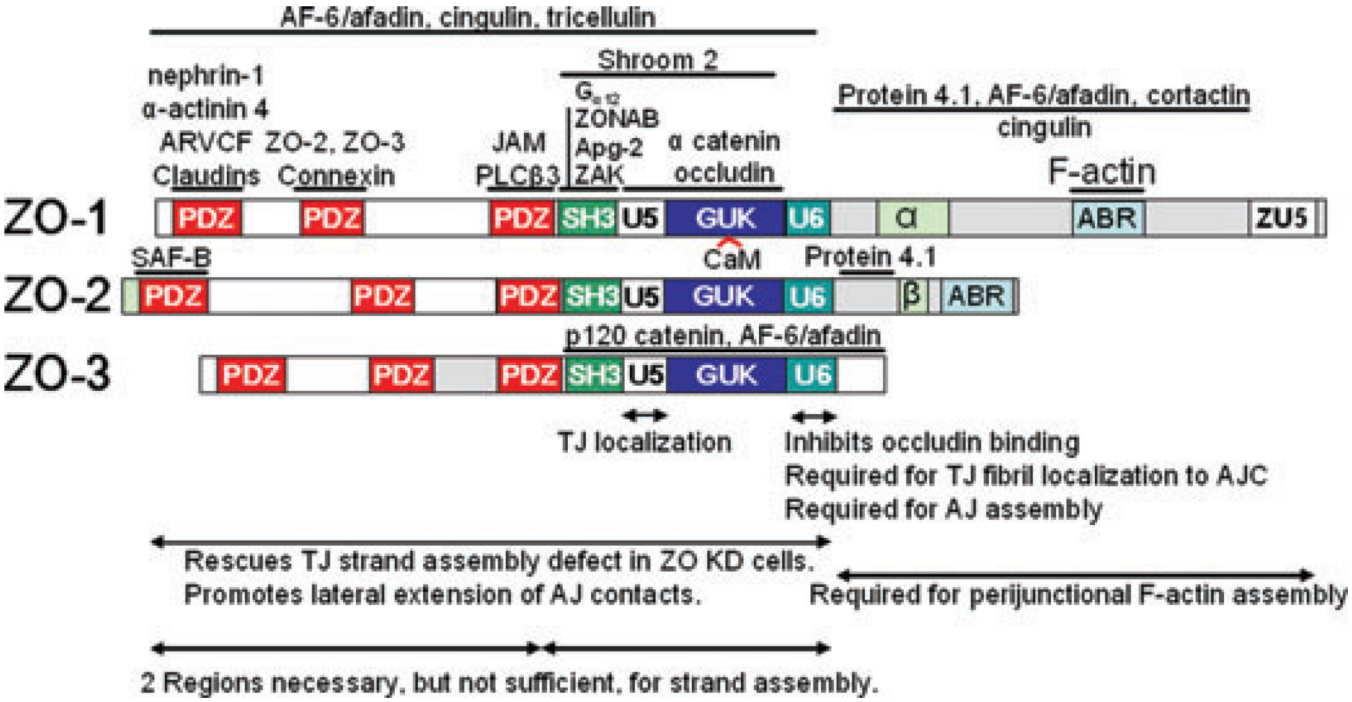

Figure 1.

Zonula occludens (ZO) proteins are multidomain scaffolds required for tight junction (TJ) assembly. Protein ligands and their binding sites on ZO-1 (top), ZO-2, and ZO-3. The functional roles of different regions are indicated (bottom). See text for details. ABR, actin-binding region; ARVCF, armadillo repeat gene deleted in velo-cardiofacial syndrome; CaM, calmodulin; GUK, guanylate kinase-like domain; PDZ, PSD-95/discs-large/zonula occludens-1 domain; PLCB3, phospholipase-C B3; SAF-B, scaffold attachment factor-B; SH3, Src Homology-3 domain; ZAK, zonula occludens kinase; ZONAB, ZO-1-associated nucleic acid binding protein; α, β, and ZU5, alternatively spliced domains of unknown function.