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A complex affair

Attraction and repulsion make occludin and ZO-1 function!

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Tight junctions (TJs) are protein complexes comprised of claudins, which anchor them in the membrane and numerous cytosolic scaffolding proteins including MAGI, MUPP1, cingulin and members of the zonula occludens (ZO) family. Originally, their main function was thought to be as a paracellular barrier. More recently, however, additional roles in signal transduction, differentiation and proliferation have been reported. Dysregulation is associated with a wide range of disease states, including diabetic retinopathy, irritable bowel disease and some cancers. ZO proteins and occludin form a protein complex that appears to act as a master regulator of TJ assembly/disassembly. Recent studies have highlighted the structural character of the primary ZO-1:occludin interaction and identified regions on occludin that control association and disassociation of TJ in a phosphorylation-dependent manner. We hypothesize that regions within ZO-1 in the so-called U5 and U6 regions behave in a similar manner.

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Tight junctions (TJs) are mega-protein complexes in cells that bring the membranes of adjacent cells into close apposition mediating cell-cell adhesion and regulating the passive diffusion of fluid, ions and molecules through the paracellular space. They are visualized as a branching network of protein strands that encircle the apical end of each cell in epithelial and endothelial sheets. The strands are primarily composed of the transmembrane claudins although a number of transmembrane, membrane-associated scaffolding, cytoskeletal and signal transduction proteins also colocalize to these strands. To date, more than 50 proteins have been assigned to the TJ complex and so it is perhaps not surprising that components are being implicated in additional functions, such as controlling cell differentiation, proliferation and polarity. Furthermore, because epithelial and endothelial cells are constantly renewing, TJs must balance the dual tasks of maintaining the structural integrity of tissues while allowing for constant remodeling. How this remodeling is regulated has become the subject of intense study and there are many recent excellent reviews on TJ proteins.[1-4]

This commentary focuses on the cytosolic protein Zonula Occludens-1 (ZO-1) and its interaction with the integral membrane protein, occludin. ZO-1 was the first TJ component to be identified and is essential for development, as ZO-1 knockout mice are embryonic lethal. Occludin was identified as the first transmembrane component of TJs. However, the apparently normal formation and physiology of TJs in homozygous occludin knockout mice and the discovery of the claudin superfamily as key structural TJ proteins left the role of occludin in TJ function an open question.[5] Increasingly, the idea that occludin, in concert with ZO proteins, are important regulators of TJ assembly/disassembly and properties is gaining traction.[1] The following sections summarize what is currently known about the primary, secondary and tertiary structures of occludin and ZO proteins and how they relate to their function and regulation. Subsequent sections briefly

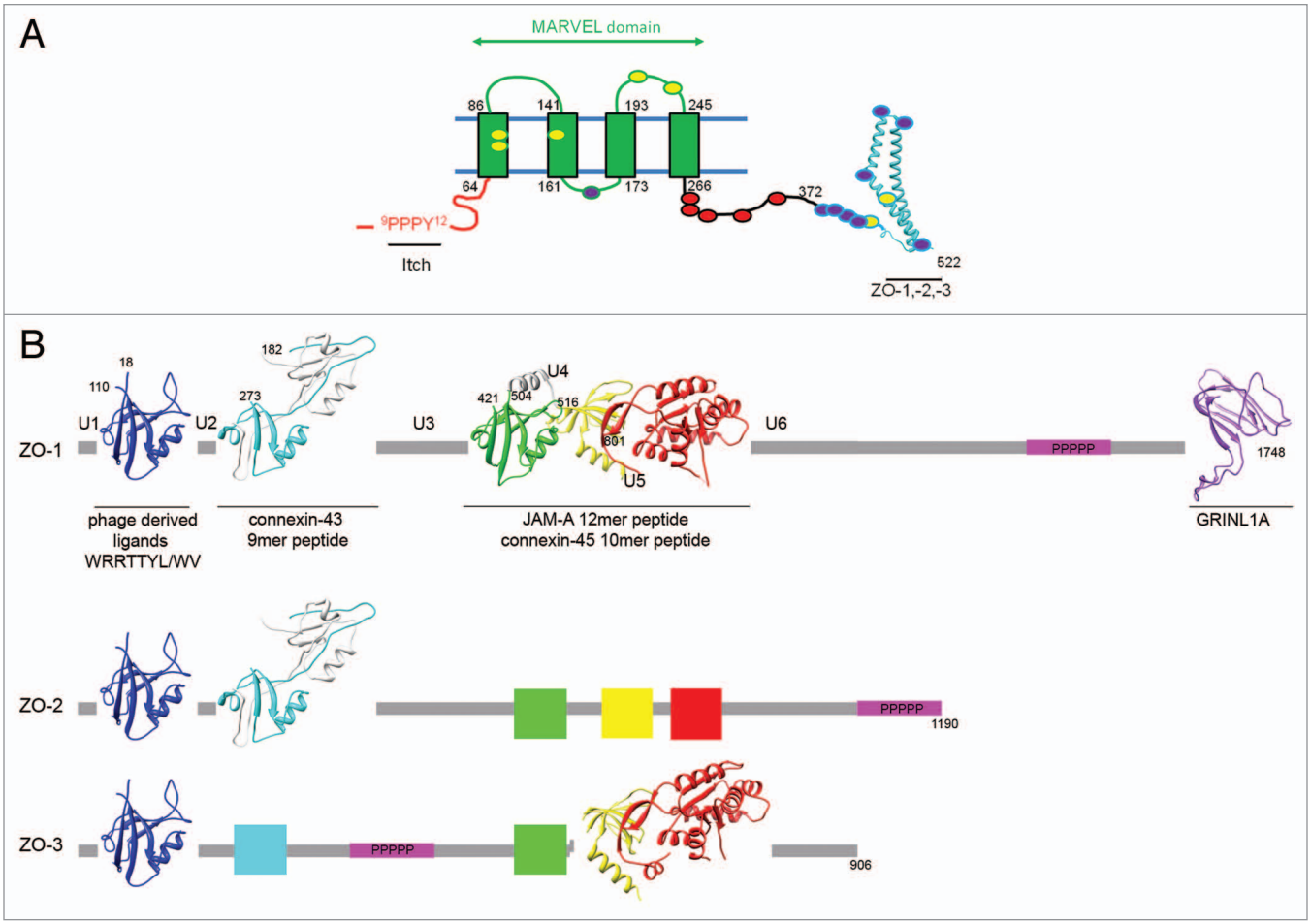

Figure 1. Summary of the structural information for occludin and ZO protein family members. (**A**) Occludin. The N-terminal region, MARVEL domain, proximal C-terminal tail and distal C-terminal tail are colored red, green, black and cyan, respectively. A portion of the distal region is a coiled coil (1WPA.PDB). Ubiquitylation sites identified > 5 times in proteome-wide studies (residues 276, 283, 299, 330 and 343) and phosphorylation sites from site-directed mutagenesis (400, 402, 404, 408, 471, 474, 490 and 508) are drawn as red and purple ovals, respectively. The six cysteine residues at positions 76, 82, 148, 216, 237, 409 and 500 are drawn as yellow ovals. Arrows mark binding partner interaction regions. (**B**) Zonula occludens (ZO). Ribbon drawing of ZO protein domains for which structures are available, linked by gray blocks. PDZ-1, -2 and -3, SH3, GUK and ZU5 are colored blue, cyan, green, yellow, red and violet, respectively. If there are no structures available, the domains are drawn as blocks. A solid black line denotes domains that have structures in complex with binding partners, with the ligand written below.

describe the occludin:ZO-1 complex and speculate about aspects of allosteric regulation. Due to space considerations, where appropriate, reviews replace multiple individual references and in such cases readers are directed to references therein.

Occludin

Structure. Human occludin is a 522 amino acid integral plasma membrane phosphoprotein and is the founding member of the tight junction associated-MARVEL protein (TAMP) family.[1] The 2 other members are tricellulin (MARVELD2), which occurs in tricellular junctions and MARVELD3, which is the newest and least well studied member. They are characterized by the helical MAL and related proteins for vesicle trafficking and membrane link (MARVEL) motifs and are thought to serve distinct but overlapping cellular functions.[6]

Occludin is comprised of four regions: N-terminal, MARVEL, proximal cytoplasmic and distal cytoplasmic domains, respectively (**Fig. 1A**). The N-terminal domain (residues 1–64) is most likely disordered, based on sequence, and contains the ^{9}PPYP^{12} motif required for binding of the E3 ubiquitin ligase, Itch, which is necessary for endosomal recycling.[7] The MARVEL domain is a transmembrane (TM) 4-helix bundle (TM 1–4) containing 2 extracellular loops (EL) between TM 1/2 (EL1, residues 86–141) and 3/4 (EL2, residues 193–245), respectively, and a short intracellular loop between TM 2/3 (IL1, residues 161–173). EL1 is rich in glycine and tyrosine residues, while EL2 is largely hydrophobic and contains a pair of cysteine residues proposed to form intra- or intermolecular disulfide bonds.[8] Both extracellular loops have been implicated in self-association and binding to other proteins. The molecular nature of these interactions, however, is not yet clear. IL1 is very short and its function, if any, is not clear.

The C-terminal cytoplasmic domain is divided into the proximal (residues 266–372) and distal (residues 373–522) regions that participate in a number of protein-protein interaction networks (**Fig. 1B**), including direct interactions with ZO proteins. The *E. coli* expressed recombinant proximal C-terminal region is largely unstructured in solution, alone or with the structured distal region, when characterized by small angle X-ray scattering (SAXS), circular dichroism spectroscopy and NMR spectroscopy (BRT, MCB, FT, JMF unpublished results). However, it is possible that the proximal region adopts a structured or partially structured conformation in the context of intact occludin in biological membranes or when bound to one or more of its binding partners. This idea is supported by the fact that regions of the membrane proximal domain share sequence similarity with ordered regions of other proteins, for example 50% identity with the second half (residues 42–67) of the SH3 domain of Hck and 41% similarity to a ~40 amino acid sequence in thymidylate kinase. While these similarities do not suggest an independently stable domain structure, they lend weight to the idea that, in the correct context, the C-terminal proximal domain could form regions of ordered structure. The C-terminal distal domain is currently the only region of occludin for which there is a three dimensional structure.[9,10] It adopts a coiled-coil structure (occCC, residues 413–522); residues 426–466 form a continuous α-helix (α-1) followed by a short acidic turn (residues 467–471) that connects α-1 to α-2 (residues 472–488). A short, two residue, kink (residues 489–490) links α-2 to α-3 (residues 491–522). The surface of the structure distributes charge in a polarized manner; an acidic surface at one end of the protein and a basic surface at the other. This polarized surface is critical for complex formation and possibly involved in phosphorylation-mediated endocytosis, as will be discussed below.

Function. Occludin serves as an interface between TJs and other proteins involved in a wide range of cellular processes. Although occludin knockout mice produce TJs, there are a number of phenotypic abnormalities in a wide range of tissues, including gastric mucosal hyperplasia, calcification in the brain, testicular atrophy, loss of cytoplasmic granules in striated duct cells of the salivary gland and thinning of the compact bone, suggesting a regulatory, rather than structural, role. These phenotypes cannot be explained by barrier dysfunction of TJs and Furuse and colleagues have suggested that occludin may be involved in epithelial cell differentiation perhaps even coordinating the breakdown of TJs with mitosis.[5] Other studies have demonstrated that when occludin is present, it can control the composition and properties of TJ,[3] suggesting an active role in regulation that is absent when occludin is knocked out. The claudin composition appears to be dependent on the interaction of occludin with ZO proteins and hence permeability of the TJs. Occludin and ZO proteins appear to regulate claudin levels by altering their intracellular trafficking.[11] Thus, occludin may be important in regulating TJ composition, assembly and disassembly.

Zonula Occludens-1 (ZO-1)

Structure. Human ZO-1 is a 1748 amino acid protein and, like the other two ZO protein family members (ZO-2, ZO-3), belongs to the membrane associated guanylate kinase (MAGUK) superfamily that includes post synaptic density protein 95 (PSD-95) and Disc large (Dlg).[12] Many members of this superfamily, including ZO, do not have kinase activity and only share a common fold with functional guanylate kinases. A recent report suggested that the defunct guanosine-5'-monophosphate (GMP) binding pocket in Dlg GUK has evolved into a phospho-Ser/phospho-Thr-binding domain, which may turn out to be a common characteristic and has implications for the regulation of all occludin:ZO protein interactions and TJ dynamics.[13] In this commentary, unless specifically named, functions that apply to all ZO family members (ZO-1, ZO-2 and ZO-3) will be denoted ZO proteins.

ZO and other MAGUK-containing proteins share five domains; 3 tandem PDZ domains (PDZ1, PDZ2, PDZ3), an SH3 domain and a guanylate kinase-like domain (GUK). All are protein-binding modules and the nature of their binding partners defines the scaffolding role(s) played by each family member (**Fig. 1B**). In ZO proteins, the MAGUK region is located within the N-terminal half of ZO-1 and ZO-2 and spans the majority of ZO-3. The remaining half of ZO-1 and -2 is less conserved, and, for the most part, predicted to be disordered. This region, which has within it a proline rich segment, contains the actin-binding module linking ZO-1 and -2 to the cytoskeleton and a small structured domain, ZU5 (ZO-1 and Unc5-like netrin receptors), near its C-terminus. The core binding module of ZO-1 to occludin is comprised of the PDZ3, SH3 and GUK domains (PSG) and it is this region on which we focus family structural discussion. The structure of ZO-1 PSG (3TSW.PDB) did not allow a binding region for occCC to be readily identified because the surface charge distribution was not as striking as that seen in occCC, with positive and negative charge being evenly distributed over the surface of the molecule. This may be due, in part, to a number of residues that were disordered in the crystal structures.

The U or "unique" regions 1–6, are highly variable in sequence within MAGUK containing proteins.[14] Their boundaries are ill-defined and little is currently known about their structures; in general, they are predicted to be disordered or transiently ordered under specific conditions. However, this is not always the case. For example, in the structure of ZO-1 PSG, U4 forms an ordered loop between the C-terminal α-helix of PDZ3 and the first β-strand of SH3,[15] perhaps allowing movement between the domains, as discussed later. U5 is a ~37 amino acid region between the SH3 and GUK domains. It is disordered in the crystal structures of ZO-1 SG and PSG, perhaps a result of using bacterially expressed protein which is not post-translationally modified, or a consequence of the absence of a binding partner. U5, together with U6, a ~85 residue sequence enriched in acidic and S/T residues following the GUK domain, participate in stabilization and regulation of its interaction with occludin in vitro and in TJ localization in vivo.[14]

Function and regulation. Functional analyses of binding partners highlight

roles for ZO proteins in TJ, AJ and gap junction structure and regulation, in signal transduction pathways and in transcriptional regulation.[16] Some interactions have been localized to specific domains (reviewed in ref. 17), such as ZO-1 PDZ1, which interacts with the C-termini of claudins and connexin 36 or ZO-1 SG which binds calmodulin and α-catenin, whereas in other cases, such as Coxsackie and Adenovirus receptor (CAR) binding, the specific interacting domains have yet to be mapped. The fact that multiple proteins appear to interact with the same binding sites on ZO proteins suggest that there must be some mechanism for regulating access. One obvious regulatory mechanism would be the multiple phosphorylation sites in U2–6. In other MAGUK proteins, such as PSD-95, regulation by sequences located within the U-regions control either inter-domain interactions between adjacent modules or allosteric regulation of binding partners.[18-20] Thus, phosphorylation of U-regions could alter these interactions however, none of these phosphorylation sites have been characterized in detail for any ZO proteins. In addition, the possibility of allostery in the binding of individual domains to their protein partners has recently been suggested. Thus, understanding the role(s) of domain:domain and domain:U region interactions may be critical to defining individual functions for ZO-1, -2 and -3 and the interplay between them.

The structure of the occludin:ZO-1 complex: Primary interaction interface. Since occludin is a membrane protein, structure determination of the entire protein, with or without its binding partners, is a massive undertaking, although we are working toward this goal. Furthermore, ZO-1 presents challenges because it is predicted to contain large regions that are only transiently ordered. In such cases, the general approach to gain structural information is to work with the minimal interacting domains of each: in this case the occCC domain and ZO-1 PSG.[15]

The structure of the complex was determined using small angle X-ray scattering (SAXS) by fitting crystal structures of occCC and ZO-1 PSG into the SAXS envelope.[21] The specific residues that comprise the primary binding interface in occCC, identified by NMR chemical shift perturbation and cross saturation measurements, consist of the acidic head formed by the turn between α-1 and α-2. It has the sequence ^{468}REESEEYM^{475} and contains two characterized in vivo phosphorylation sites.[2] In addition, the predominantly basic face of helix V in the GUK domain was identified as part of the occludin binding site in ZO-1. For occludin binding, helix V has also been implicated biochemically and structurally[21,22] in calmodulin binding and α-catenin binding,[23] suggesting that binding of each is competitive. This may account, in part, for the migration of ZO-1 from adherens junctions and TJs during the formation of the later junctions. This work answered a long standing question about the exact nature of the interaction, and using in vitro binding experiments, suggested a previously undocumented role for ^{471}S phosphorylation. It further suggested possible additional allosteric controls for the occludin:ZO protein complexes.

Is a Secondary Occludin-Binding Interface Generated by a Concerted Movement of Multiple Elements in ZO-1?

The interaction of the acidic head of occCC and a basic surface of helix V in ZO-1 GUK may not comprise the entire interface, as the buried surface area on each molecule is unlikely to be sufficient to fully stabilize the complex. Indeed, other regions in occCC and ZO-1 PSG have also been implicated in the complex stability. In occludin, specific lysine residues on one face of the basic surface of occCC also contribute to stability[9] and in ZO-1, the U5 region is also an indispensable element of complex stability as its deletion abrogates binding of occCC and localization of ZO-1 at the junctional complex.[14]

In order to try and gain a more complete model of the occCC:ZO-1 PSG complex, we superimposed the structure of ZO-3 SG (3KFV.PDB) onto ZO-1 PSG (3TSW.PDB) as the ZO-3 structure contains an 9 additional ordered residues (66% identical) of the U5 region (**Fig. 2A**). These residues form part of a helix that extends in the general direction of the primary interface. In superimposing the structures, however, we were surprised to find that the GUK domain of ZO-3 was rotated ~20° relative to the equivalent regions in the structure of ZO-1. To determine whether other ZO-1 SG or PSG structures contained similar displacements, we compared the available ZO protein structures, using 3TSW.PDB as the reference. We found that the ZO-3 GUK domain is rotated by ~35° relative to its equivalent position in the structure of ZO-1 SG (3LH5.PDB). A similar but smaller rotation was observed when comparing 3TSW.PDB with ZO-1 PSG in complex with a 12mer peptide of JAM-A (3TSZ.PDB) or ZO-1 SG, whereas a ~90° rotation was seen for SG of the related protein MAGUK Discs large 1(Dlg1; 3UAT.PDB). The differences are unlikely to be artifacts of crystallization, as suggested,[15] because ZO-1 PSG (3TSZ.PDB) superimposes well onto ZO-1 SG (3LH5.PDB) in its equivalent domains despite their different packing environments. Thus, these domain movements may be relevant to understanding protein binding and regulation.

Allosteric regulation of ligand binding has been hypothesized for various MAGUK superfamily members.[18-20] Modeling studies of rat PSD-95 PSG based on the existing structures of SG and PDZ3 suggest that the PDZ3 domain interacts with either the SH3 or GUK domains, to produce different functional forms,[24] which could be a general property of all family members. Some experimental support for this idea can be seen in the overlay of ^{1}H-^{15}N correlation spectra of ZO-1 PDZ3 and ZO-1 PSG from unpublished studies in our lab (**Fig. 2B**). We have assigned the spectra of the isolated ZO-1 PDZ3 domain[25] (residues 409–517) and compared it with an equivalent unassigned spectrum for ZO-1 PSG. Typically, any differences in chemical shift of the PDZ3 resonances should localize to the interface with SG. As expected, a significant number of peaks of the isolated PDZ3 domain show minimal changes in the context of PSG, indicating that there is no gross conformational change in PDZ3 within the context of the PSG construct. However, a number of resonances corresponding to residues that are near the

PDZ3:SH3 interface showed large chemical shifts (> 0.5 ppm) suggesting intimate contacts between these domains that could alter their functional properties and perhaps account for the increased affinity for the JAM-A peptide in this case.[15] In a separate experiment, the ^{1}H-^{15}N correlation spectrum of SG was compared with that of PSG (data not shown). It contained a larger number of shifted resonances than expected if the only differences occur at the PDZ3:SH3 interface. Together, these data support the hypothesis that the PSG module is conformationally labile and interactions between PDZ3 and SH3 have structural effects that propagate throughout the PSG structure. This may potentially be a mechanism to regulate the coordinated binding activities of individual domains in PSG allowing the assembly of complexes with specific compositions.

Further support for this idea comes from our structural studies of the occCC:ZO-1 PSG complex using fully deuterated occCC and random fractionally ^{2}H,^{13}C,^{15}N-labeled ZO-1 PSG. In the absence of occCC, the ZO-1 PSG TROSY-HSQC spectrum was reasonably well dispersed.[21] However, analysis revealed that it did not account for the amide protons of ~50 amino acids, presumably because they are labile in the structure. Binding of occCC resulted in the appearance of ~16 peaks in the ZO-1 PSG relative to its unbound state. Although the entire ZO-1 PSG spectrum has not yet been assigned, based upon the crystal structure of ZO-1 PSG, we expect that many of the residues that order upon occCC binding are in U5. Together these results are consistent with a model in which the rotation of the GUK domain relative to SH3 could act in concert with the ordering and disordering of the U5 region to regulate occCC binding.

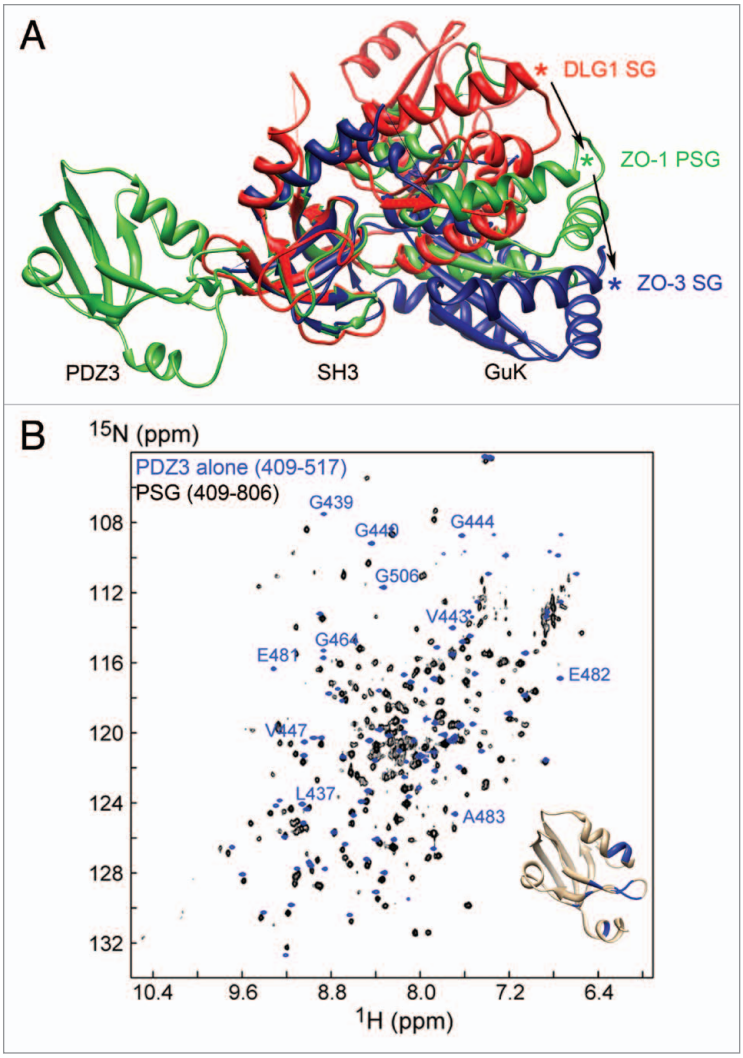

Figure 2. Flexibility of the ZO-1 PSG module in crystal structures and NMR spectra. (**A**) Superposition of the crystal structures of ZO-1 PSG (3TSZ.PDB), ZO-3 SG (3KFV.PDB) and DLG1 SG (3UAT.PDB) showing the different positions of GUK. The molecules are colored the same as the labels. (**B**) Overlay of the ^{1}H-^{15}N correlation spectra for ^{15}N, ^{2}H-PSG and ^{15}N-PDZ3. PDZ3 resonances with the largest shifts labeled. Inset: Ribbon drawing of the structure of PDZ3 (3TSV.PDB) in the same orientation as (**A**) with the residues labeled in the spectra colored blue.

Does the U6 region in ZO-1 Mimic the Acidic Loop of the occCC to Regulate Occludin Binding?

The U6 motif (amino acids 795-888), which immediately follows the GUK domain in the amino acid sequence of ZO-1, has also been identified as a key regulator of occludin binding.[14] Little is known about its structure since it is disordered in the crystal structure of the PSG:U6 protein (3SHW.PDB). Moreover, in preliminary NMR studies in our lab with this construct, the additional resonances corresponded to a disordered conformation, while SAXS analysis suggested the addition of a mass near the occludin binding region of ZO-1 (BRT, MCB, FT and JMF, unpublished). This latter observation is consistent with a model in which U6 competes with occludin for binding to GUK, but it poses a different question of what triggers the conformational change? Fanning and his colleagues have reported that occCC has an ~3-fold reduction in binding in vitro to a construct of baculovirus expressed ZO-1 (NZO-1, residues 1–888) that includes U6 relative to that of the shorter ZO-1 fragment (residues 1–806) without U6.[14] Because U6 is enriched in serine and threonine residues, we wondered whether it might be a disordered hyper-phosphorylatable region. We noticed that sequences such as ^{809}T-S-D-D-L-D ^{840}D-S-D-Y-E-D-T-D-T-E in U6, are very similar the sequence of the acidic loop of the occCC (^{469}E-E-pS-E-E-pY) that is part of the interface with ZO-1. It is possible that U6 mimics regions of occCC under specific conditions, perhaps in concert with a more extended ZO-1 PSG structure, to compete with binding of occCC to the ZO-1 GUK domain. In

the next section we extend this discussion to include phosphorylation, glycosylation and ubiquitylation sites as major modifications that regulate complex stability.

Regulation of the occludin:ZO-1 Complex by Phosphorylation, Glycosylation and Ubiquitylation

To date, a number of occludin phosphosites have been identified in targeted studies and characterized in vitro with a smaller subset being characterized in cells, or in vivo; they have been the subject of many excellent reviews summarizing their location and effects and will not be described in detail here.[1,2,4] In occludin, sites that are primarily in the proximal C-terminal domain have been found in studies investigating particular modifications within the whole proteome. These data are stored in an interrogatable database available online (www.phosphosite.org). For occludin, the residues ^{287}Y, ^{313}S, ^{315}Y, ^{325}Y, ^{342}Y, ^{368}Y, ^{370}S, ^{376}T, ^{400}T, ^{402}Y, ^{404}T and ^{408}S are listed as phosphorylated (n > 5). This data should be used with caution as they were obtained from a range of cells, with or without TJs, and disease states. However, ^{408}S phosphorylation is functionally relevant, resulting in altered TJ permeability and somewhat reduced affinity for ZO-1.[1] The distal C-terminal region also contains phosphorylation sites located between the helices at ^{471}S, ^{474}Y and ^{490}S that affect TJ integrity.[2,10,21] These studies established a direct link between occludin phosphorylation in the coiled coil domain and complex formation with ZO-1 and TJ properties.

Not all phosphorylation events reduce the stability of the occludin:ZO-1 complex and result in disassembly of TJs; some enhance the occludin:ZO-1 interaction and may decrease TJ permeability.[2] For example, in occCC, phosphorylation of ^{490}S results in a decrease in occludin:ZO-1 complex stability while phosphorylation of ^{471}S enhances its stability in vitro and perhaps in vivo.[10,21] In addition to phosphorylation, serine/threonine residues may also be modified by O-β-glycosylation, contributing to the regulatory diversity of these residues. This modification appends an uncharged bulky group compared with phosphorylation. In this regard, Butt and coworkers recently suggested that occludin contains so-called Yin-Yang sites, which are either phosphorylated or O-β-glycosylated, at ^{408}S and ^{490}S. They propose that the crosstalk between these two modification systems may contribute to a functional switch[26] and speculate that it might be used to prevent Hepatitis C virus (HCV) infection, which exploits some TJ proteins, such as claudins and occludin, as host factors to gain entry to the cell.[27] These studies and a host of others demonstrate the importance of post-translational modifications involving serine, threonine and tyrosine in occludin and highlight the need to fully evaluate how the different phosphorylation states of occludin contribute to its in vivo function.[1,4]

At present, little is known about phosphorylation in ZO proteins and its effect on complex stability. It has been reported that ZO-1 is phosphorylated, but only at the whole protein resolution. Balda and colleagues used a series of deletion constructs to more accurately define the serine phosphorylation region.[28] They identified a region of U5 between residues 589–616 that bound a serine kinase and was phosphorylated in MDCK cell extracts. It contains two serine residues (609 and 610) and our preliminary studies indicate that S609D and S610D phosphomimetic single site variants of ZO-1 PSG do not significantly inhibit the capture of purified GST-occCC (BRT, JMF unpublished results). Although they have not been observed in a curated database that collates the results of phosphoproteomic studies (www.phosphosite.org), others in the U5 region have, including ^{588}Y (observed 126 times) and ^{617}S (observed 25 times). These were found most often in control samples rather than in cell models of disease and so it remains possible that phosphorylation of one of these residues may play a role in U5 structure or the affinity of ZO proteins for occludin. The absence of information about the phosphorylation state of ZO protein, their role in its affinity for binding partners and in TJ properties represents a key bottleneck in our understanding of the roles of ZO.

Due, in part, to whole proteome studies, other post-translational modifications such as ubiquitylation, are being identified (www.phosphosite.org). While these should be viewed with some caution, as many of these studies use cells that do not contain TJs and may not be involved in the actual functions of TJ proteins, they still contribute to our knowledge. Ubiquitylation as a means of TJ remodeling is emerging as a common theme, but few details of the specific sites in individual proteins are available. Potential candidate sites in the proximal C-terminal domain of occludin have been identified in two whole proteome studies using HEK293, MV4-11, HCT116 and/or 293T cells (**Fig. 1A**). The specific role of ubiquitylation at these residues is not clear since HEK293, MV4-11 and 293T cells do not form TJs, and HCT116 are not differentiated or polarized cells and do not exhibit typical and functional TJs. Nevertheless, TJ-like structures are present under some culture conditions. Thus, ubiquitylation at these residues may reflect housekeeping signals that may or may not involve the Itch ubiquitin ligase or endosomal trafficking of occludin.

How Does occludin:ZO-1 Complex Formation Regulate TJ Properties?

The structure of the occCC:ZO-1 PSG complex provides a basis for understanding how phosphorylation of ^{471}S in occludin could stabilize the complex and suggests a potential model for why phosphorylation at ^{490}S could destabilize it. However, despite the strong correlation between the presence of this complex at TJs and reduced occludin ubiquitylation, endosomal cycling and TJ permeability, its exact role(s) are not yet clear. One possibility is that complex formation directly inhibits endosomal recycling by sterically blocking the ubiquitylation sites. The absence of ubiquitylation would promote retention of occludin and claudin at the junction, resulting in TJs with low permeability. This direct physical model has the advantage that TJ properties are directly coupled to occludin:ZO-1 complex formation, stability and regulation by phosphorylation. Based upon our current SAXS derived model in which the proximal C-terminal domain is largely unfolded and does not contribute to ZO-1 binding, ubiquitylation would likely occur

in the occCC domain, which is in direct contact with ZO-1. There is indeed a cluster of conserved lysine residues on the surface of the occCC domain (residues 433, 444, 485, 488, 504 and 511) and adjacent to ^{490}S, that have been previously implicated in stabilizing its complex with ZO-1 constructs containing the U6 domain.[9] In addition, phosphorylation of ^{490}S upon VEGF treatment of BREC disrupts the occludin:ZO-1 complex and results in increased TJ permeability.[11] Potentially, phosphorylation of ^{490}S could disrupt a largely ionic interaction between the lysine-rich region in occludin and some acidic region on ZO-1, such as the acidic U6 region. Consistent with this interpretation, site directed mutagenesis of lysine pairs (K433D/K444D, K485D/K488D and K504D/K511D) and the phosphomimetic substitution, ^{490}S to glutamic acid, affect complex stability with full-length ZO-1 but not the *E. coli* expressed PSG.[9] U6 could either promote or inhibit the binding of occludin in a conformation dependent manner. A limitation of this model, however, is the lack of data for ubiquitylation of lysine residues in occCC, since all of the sites identified to date lie in the "unstructured" proximal domain, although the functional significance of these is not known.

There are a number of alternate models that account for how occludin:ZO-1 complex formation could regulate endosomal cycling and TJ permeability due to their presence in large multisubunit complexes. For example, in its role as a scaffold, ZO-1 could recruit a protein that sterically blocks access to the lysine residues in the occludin proximal domain, although it is not known whether any of the ZO-1 binding partners have this property. Moreover, due to the wide range of complexes involving ZO-1, this model would require coordinated binding of the hypothetical protein; currently there is no evidence for this type of coordination. Alternatively, the Itch-binding motif in occludin (^{9}PPYP^{12}) might be obscured upon occludin:ZO-1 complex formation and thus be inaccessible to Itch. Disruption of the complex would expose the PPYP sequence, resulting in rapid Itch-dependent ubiquitylation of occludin and its targeting to the ESCRT pathway for endosomal trafficking. A limitation of these proposed models is the lack of a corresponding structural model that accounts for how access to the ^{9}PPYP^{12} motif could be controlled in the complex either by additional binding sites in ZO-1, other unknown binding partners in a tripartite complex or through occludin oligomerization masking this site.

Is There a Functional Role for Occludin and ZO Protein Oligomerization in TJ Regulation?

Oligomerization of TJ proteins is required for function. It is well established that some claudins and ZOs can form homo- or hetero-oligomers, and there is accumulating evidence that occludin can also form homo-oligomers.[29,30] However, unlike ZO, the mechanism(s) of this process are not yet clear. EL2 can promote occludin:occludin association, perhaps via an intermolecular disulfide bond between ^{216}C and ^{237}C, or disulfide-independent interactions involving both EL2s.[2] Certainly, the extracellular environment readily supports disulfide formation, although the jury is still out as to whether it happens in vivo. By contrast, oligomerization of occludin via disulfide bond formation involving cysteine residues in TM1 (^{76}C and ^{82}C) and TM2 (^{148}C) is less likely.[8] While the membrane environment contains sufficient oxygen for this process, its low dielectric constant prevents the formation of the obligate thiolate intermediate. For example, cysteine scanning mutagenesis of transmembrane proteins requires the addition of a catalyst, such as Cu(II)-(o-phenanthroline)$_3$ for disulphide bond formation, making it unlikely that the cysteine residues contribute to oligomerization in this way. In support of this, a very recent study has suggested that the MARVEL domain itself mediates oligomerization and correct sorting of occludin in epithelial tissues by a disulfide independent mechanism.[31]

Disulphide bond formation involving ^{409}C in the hinge region of the proximal C-terminal domain has recently been implicated in redox- and/or ^{408}S phosphorylation-sensitive dimerization and alteration of TJ permeability in response to changes in cellular conditions.[8,30] Typically, cysteine residues in cytoplasmic proteins are not involved in disulfide bonds due to high glutathione concentrations that maintain a reducing environment in this compartment. However, oxidative stress can produce conditions that promote disulfide bond formation of some cytoplasmic cysteine residues and this mechanism is exploited by many redox sensor proteins.[32] In stress sensing proteins, cytoplasmic disulfide bond formation is usually coupled to a large conformational change that catalyzes thiolate formation, allows transduction of the redox signal and prevents the rapid reduction of these bonds. In the case of occludin, ^{409}C is likely to be solvent exposed and is located in a highly acidic region adjacent to a conserved phosphorylatable hot-spot.[33] These conditions would be unfavorable for formation and stabilization of a disulfide bond since the negative charge density would hinder thiolate formation (pK_a ~9), even in a more oxidizing environment. Even if it could be made, the resultant solvent exposed disulfide bond would not be particularly stable. Thus, if ^{409}C plays a role in occludin oligomer formation, it is likely to involve either a major conformational change altering the environment of the resultant disulfide bond, or is mediated by additional cellular factors.

The consequences of occludin oligomerization on its binding to ZO proteins would likely depend upon the structure of the resultant dimer. Dimerization via the extracellular domain, and likely the transmembrane domain, might not directly affect ZO protein binding and could have a beneficial effect upon TJ structure by allowing ZO protein mediated crosslinking between adjacent occludin-containing complexes enhancing the stability of the resultant 2:2 complex. The effect of dimer formation via intracellular moieties such as ^{409}C is less clear. Its properties would greatly depend upon whether the coiled-coil structure of the distal region is disrupted, or the binding site of ZO proteins altered by this process. However, it is conceivable that it could destabilize the complex and result in the observed increased permeability. In any case, it is clear that the oligomerization of state of occludin is a fertile area for future studies.

The occludin:ZO-1 Complex and Viral Entry

The regulatory nature of the occludin:ZO-1 complex makes it a potential site of access for various pathogens. It has been established that some viruses exploit the dynamic nature of TJ to gain access to the cells.[27] For example, hepatitis C virus uses claudin-1 and occludin to enter liver cells.[34] In other cases, viral infection correlates with mislocalization or downregulation of tight junction proteins, for example in HIV-1 infected patients with HIV-encephalitis show a disorganization of TJ proteins and altered expression of both occludin and ZO-1 and phosphorylation of ^{508}S in occCC; Hantaan virus infections result in increased cellular permeability and, in the kidney of affected individuals, mislocalization of ZO-1.[35] Some viruses, such as respiratory syncytial virus (RSV) gain entry by other mechanisms, but may exploit TJ proteins to spread infection, as increased expression of occludin, claudin-4 and ZO-1 is observed in RSV-infected primary nasal epithelial cell culture resulting in increased polarity.[36] These are all in vivo or in cell observations and there is little if any structural characterization of the interaction. One exception is the nucleocapsid protein of Hantaan virus, which contains a highly conserved ~75 amino coiled-coil domain (2IC6.PDB) reminiscent of occCC; it contains an acidic tip and a basic face. In a peptide-based binding assay, phosphorylation of ^{471}S in the occCC acidic head increases affinity of ZO-1. If the nucleocapsid domain has evolved as an occludin mimetic, it might compete for ZO-1 binding causing the observed mislocalization in Hantaan virus-infected cells and current studies in our lab will test this hypothesis in vitro.

The Future Promise of Structural Techniques to Augment Our Understanding of TJ Biology

The recent structural papers mark the beginning of an exciting new phase in TJ biology. Additional structure function studies of larger occludin:ZO-1 constructs and potential regulatory complexes are needed to really determine how TJ are regulated and how loss of regulation could occur in disease states.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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