Epithelial barrier assembly requires coordinated activity of multiple domains of the tight junction protein ZO-1

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Summary

Tight junctions (TJs) regulate the paracellular movement of ions, macromolecules and immune cells across epithelia. Zonula occludens (ZO)-1 is a multi-domain polypeptide required for the assembly of TJs. MDCK II cells lacking ZO-1, and its homolog ZO-2, have three distinct phenotypes: reduced localization of occludin and some claudins to the TJs, increased epithelial permeability, and expansion of the apical actomyosin contractile array found at the apical junction complex (AJC). However, it is unclear exactly which ZO-1 binding domains are required to coordinate these activities. We addressed this question by examining the ability of ZO-1 domain-deletion transgenes to reverse the effects of ZO depletion. We found that the SH3 domain and the U5 motif are required to recruit ZO-1 to the AJC and that localization is a prerequisite for normal TJ and cytoskeletal organization. The PDZ2 domain is not required for localization of ZO-1 to the AJC, but is necessary to establish the characteristic continuous circumferential band of ZO-1, occludin and claudin-2. PDZ2 is also required to establish normal permeability, but is not required for normal cytoskeletal organization. Finally, our results demonstrate that PDZ1 is crucial for the normal organization of both the TJ and the AJC cytoskeleton. Our results establish that ZO-1 acts as a true scaffolding protein and that the coordinated activity of multiple domains is required for normal TJ structure and function.

Key words: Tight junction, Zonula occludens, Paracellular permeability, Actin cytoskeleton, MAGUK, PDZ

Introduction

The tight junction (TJ) is a membrane-associated complex at the apico-lateral margin of epithelial cells that regulates the paracellular movement of ions, macromolecules and immune cells. The TJ barrier is composed of three families of transmembrane proteins: claudins (Furuse et al., 1998a), the junctional adhesion molecule (JAM) proteins (Bazzoni et al., 2000), and the TJ-associated Marvel domain proteins (TAMPs) occludin, tricellulin and Marvel D3 (Raleigh et al., 2010). Claudins form adhesive contacts between cells that create a charge and size-selective barrier (Colegio et al., 2002; Furuse et al., 1998b; Morita et al., 1999; Van Itallie et al., 2008), while the TAMPs and JAM proteins appear to play a role in stabilizing the TJ and regulating epithelial permeability in response to pathogens and/or physiological stimuli (Balda et al., 2000; Chen et al., 1997; Raleigh et al., 2011; Van Itallie et al., 2010) (Ikenouchi et al., 2005) (Martìn-Padura et al., 1998). These transmembrane barrier proteins are associated with a cytosolic plaque of proteins that regulate their assembly into a circumferential barrier and association with the cortical cytoskeleton (reviewed by Furuse, 2010). The coordinated interactions of these different components are critical for the assembly and maintenance of an effective epithelial barrier.

The cytosolic proteins ZO-1 and ZO-2 are multi-domain scaffolding proteins within the MAGUK family required for the formation of the TJ. These two proteins are likely to play a redundant role during TJ assembly. The depletion of either ZO-1 or ZO-2 from epithelial cells leads to delayed formation of TJs and a mild increase in permeability, whereas depletion of both ZO-1 and ZO-2 disrupts the localization of the transmembrane proteins at the tight junction and causes a dramatic alteration of TJ barrier function (Hernandez et al., 2007; McNeil et al., 2006; Umeda et al., 2006). The N-terminus of ZO proteins, like other MAGUKs, contains a stereotypically conserved core of protein-binding motifs, including three PDZ domains, an SH3 domain and a GUK domain (reviewed by González-Mariscal et al., 2000). The unique C-terminal domain contains an actin-binding region (ABR) and interacts with many other cytoskeletal structural and regulatory proteins (Fanning and Anderson, 2009; Fanning et al., 1998; Wittchen et al., 1999). The transmembrane proteins claudin, occludin and JAM all bind to distinct domains within the N-terminal half of ZO-1 (Bazzoni et al., 2000; Fanning et al., 1998; Furuse et al., 1994; Itoh et al., 1999; Riazuddin et al., 2006; Umeda et al., 2006). ZO proteins also dimerize with each other through the PDZ2 domain and dimerization of ZO-1 has been hypothesized to be required for the organization of claudins into barrier-forming strands (Fanning et al., 2007b; Utepbergenov et al., 2006; Wu et al., 2007). Finally, ZO proteins regulate cytoskeletal organization within the AJC (Fanning et al., 1998; Fanning et al., 2012; Van Itallie et al., 2009). The N-terminal domains are sufficient to recruit claudins and other TJ proteins to cell contacts at the apical/lateral membrane, but whether this region is sufficient for functional barrier assembly is still unknown (Ikenouchi et al., 2007; Umeda et al., 2006).

The complex molecular structure of ZO-1 and the great diversity in binding partners make dissection of the functional/mechanistic role of this critical component challenging. It is unclear which protein–protein interactions are necessary for epithelial-specific functions, or how the activity of so many binding partners is coordinated by ZO-1. The goal of this study, as the first step in a long term analysis of ZO function, was to identify the ZO-1 binding domains within the N-terminus that are necessary for TJ barrier assembly and cytoskeletal regulation. We hypothesize that if ZO-1 is truly a scaffolding protein, multiple domains will be necessary for ZO-1 to properly regulate TJ assembly, contractile array formation and the establishment of an epithelial barrier. Our laboratory has previously demonstrated that MDCK cells depleted of ZO-1 and ZO-2 have three major phenotypes (Fanning et al., 2012). First, the localization of some, but not all, TJ proteins is significantly attenuated, yet there is little alteration in the localization of the adherens junction proteins. Second, these cells have a significantly expanded apical contractile array, highlighted by an increase in actin and myosin accumulation at the adherens junction. Finally, the depletion of both ZO-1 and ZO-2 leads to an increase in the permeability of large solutes. To better understand how ZO proteins coordinate TJ maintenance, we created recombinant ZO-1 proteins lacking individual binding domains and tested their ability to rescue the three phenotypes observed in the ZO-depleted cells. Our results establish that cytoskeletal organization and TJ structure and function are regulated by a distinct, but overlapping, subset of conserved domains within ZO-1, and suggest that complex interactions between different domains and/or their binding partners is critical for normal barrier assembly.

Results

N-terminal half of ZO-1 is sufficient to rescue TJ protein localization and cytoskeletal organization in ZO-depleted cells

We previously reported that in ZO-depleted MDCK II cells the localization of some, but not all, TJ transmembrane proteins to the AJC is reduced. For example, accumulation of occludin, claudin-1 and claudin-2 at the AJC is significantly diminished relative to control cells, while tricellulin, JAM, claudin-3 and -4 appear normal. In addition, assembly of actomyosin filaments is greatly increased relative to control cells (Fanning et al., 2012) (Fig. 1). These defects are reversed by expression of a full length ZO-1 transgene in these cells (Fanning et al., 2012). To determine to what extent the N-terminal binding domains are sufficient to regulate TJ and cytoskeletal assembly at the AJC, we used a tetracycline (Tet)-inducible system to express the N-terminal portion of ZO-1 (cZNA) extending from the N-terminus through the U6, or acidic domain (amino acids 1–887). As seen in Fig. 1A, cZNA effectively localizes to the tight junction, restores claudin-2, claudin-1 and occludin at the TJ (Fig. 1A; supplementary material Fig. S1) and restores the normal distribution of F-actin and myosin IIB at the AJC (Fig. 1B). These, and subsequent results, are summarized in Table 1. cZNA localization does not exactly mimic full length ZO-1 expression, nor does it restore the phenotypic, wavy junctions of wild type MDCK cells or dKD cells expressing full length ZO-1 (Fig. 1C) (Fanning et al., 2012), therein confirming that the C-terminal region (aa 888–1746) has a role in the orchestrating the typical architecture of the AJC in these cells (Yu et al., 2010). However, we did not investigate these differences further because they did not correlate with changes in the permeability of MDCK cells (Fig. 1D).

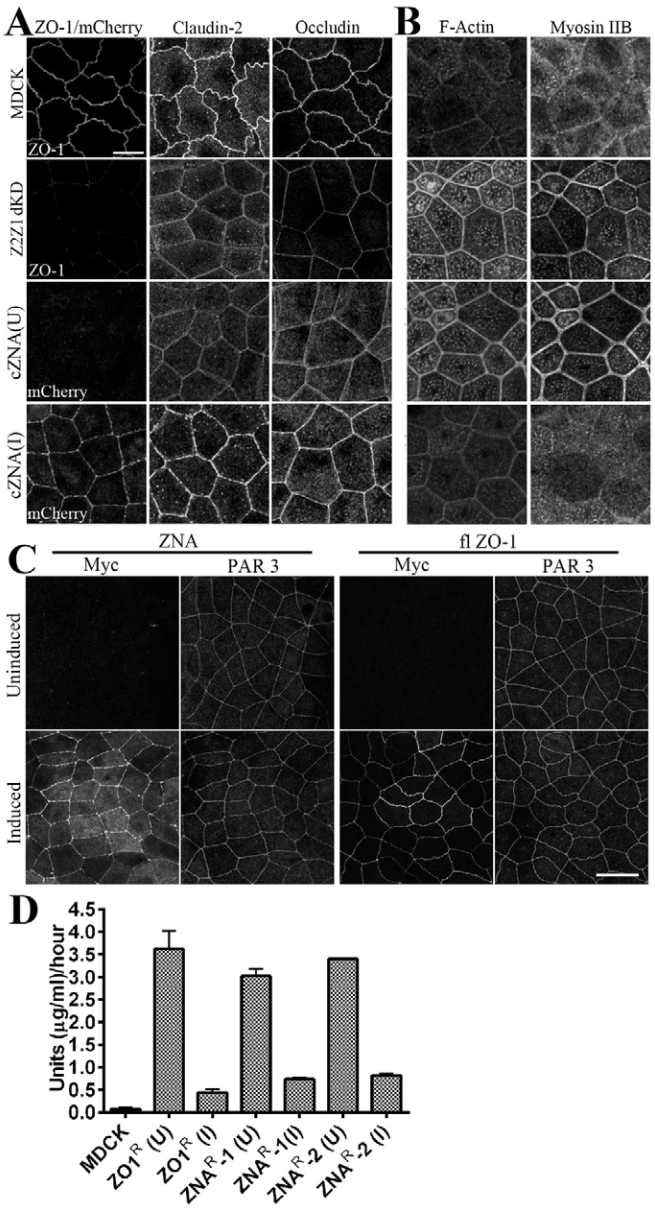

Fig. 1. cZNA is sufficient to reverse most phenotypic alterations observed in ZO-depleted MDCK cells. (**A**) MDCK and ZO-depleted MDCK cells (Z2Z1 dKD) uninduced (U) and induced (I) to express the N-terminal half of ZO-1 (cZNA) were fixed and stained with antibodies against ZO-1 (MDCK and Z2Z1 dKD) or mCherry tag (cZNA), claudin-2, and occludin. (**B**) Cells were stained with antibodies against myosin IIB and TRITC–phalloidin (F-actin). (**C**) Cells expressing a ZNA construct or a full length ZO-1 (fl ZO-1) construct were stained with antibodies against a myc tag to indicate the location of the expressed protein and PAR3 to outline the AJC location. (**D**) Analysis of the paracellular flux of 3.0 kDa fluorescein–dextran in Z2Z1 dKD cells uninduced or induced to express ZNA or full length ZO-1 transgenes. Immunofluorescence images are 1.3 µm maximum density projections of Z-stacks taken through the apical-most aspect of the cells. Scale bars: 10 µm.

The PDZ2, SH3 domains and U5 motif are necessary for the normal localization of ZO-1

Next, we systematically deleted individual binding domains within cZNA in order to determine which ones were responsible for ZO-1 localization, TJ structure and organization of the AJC

Table 1. Effect of domain deletion on ZO-1 localization and function in MDCK cells

	Restoration of normal phenotype				
	Localized to cell–cell contacts	Restores normal contractile array	Claudin 2 localization	Occludin localization	Paracellular permeability
cZNAΔP1	Yes*	No	No	No	No
cZNAΔP2	Yes†	Yes	No†	No	No
cZNAΔP3	Yes	Partial	Yes	Yes	Yes
cZNAΔSH3	No	No	No	No	No
cZNAΔU5	No	No	No	No	No
cZNAΔGUK	Yes	Yes	Yes	Yes	Yes
cZNAΔU6	Yes‡	Yes	Yes‡	Yes‡	Yes
cZNA	Yes	Yes	Yes	Yes	Yes

*Excluded from tricellular junctions.
†Punctate, not a continuous band.
‡Localization also visible on lateral membrane.

cytoskeleton. Fig. 2A outlines each construct and the deletion boundaries. All constructs contained an mCherry tag at the N-terminus to distinguish the expressed protein from residual, endogenous ZO-1. Western blot analysis confirmed that each deletion transgene was expressed at levels comparable to cZNA, and about a tenfold increase over levels of endogenous ZO-1 seen in control cells (supplementary material Fig. S2). All cell lines had comparable levels of occludin, myosin IIB and actin when expression was induced or uninduced. Steady state levels of ZO-3, which are notably reduced in ZO-depleted cells (Fanning et al., 2012), were restored by the expression of all transgenes except cZNAΔP2. This observation suggests that dimerization stabilizes steady state levels of ZO-3. Claudin-2 expression was reduced in some transgene-expressing cells (ZNA, cZNAΔP3 and cZNAΔP2), as was the higher molecular weight species of occludin sometimes associated with phosphorylation (Sakakibara et al., 1997). Neither of these changes in steady state expression pattern had an obvious correlation with changes in TJ permeability or cytoskeletal organization, and was not pursued further.

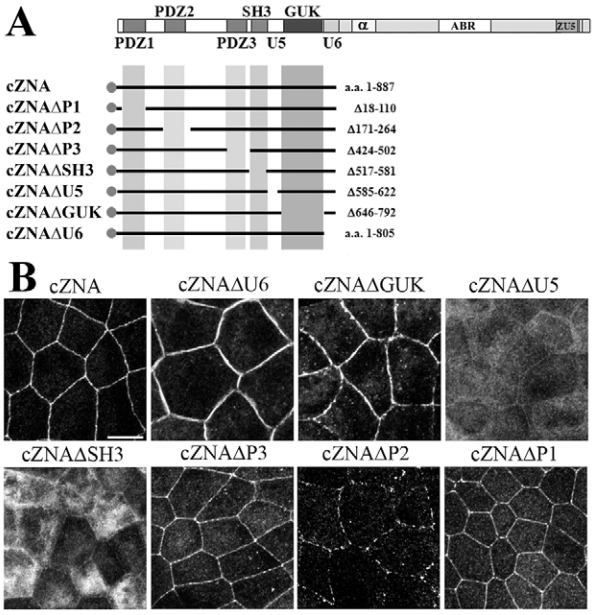

Fig. 2. The SH3, U5 and PDZ2 domains are all necessary for the localization of ZO-1 into a continuous band at cell–cell contacts. Stable Z2Z1 dKD cells were transfected with Tet-inducible cZNA rescue constructs lacking individual binding domains. (**A**) Schematic diagram of rescue constructs indicating the amino acid deletion boundaries. (**B**) Distribution of mCherry-tagged transgenes in induced ZO-depleted MDCK cells. Images are 1.3 μm maximum density projections of Z-stacks taken through the apical-most aspect of the cells. Scale bar: 10 μm.

The deletion of individual domains had a surprisingly varied effect on the localization of cZNA deletion constructs (Fig. 2B). ZNA protein lacking the U5 (cZNAΔU5) or the SH3 (cZNAΔSH3) domains showed little or no localization to cell–cell contacts. ZNA constructs lacking the GUK (cZNAΔGUK) and PDZ3 (cZNAΔP3) domains localized to the tight junction in a continuous circumferential pattern similar to cZNA. The ZNA construct lacking the U6 domain (cZNAΔU6) localized to the tight junction, but was also organized ectopically along the lateral plasma membrane (Fig. 2C; supplementary material Fig. S3). This ectopic localization pattern has been previously documented by our lab (Fanning et al., 2007a). Interestingly, ZNA lacking the PDZ2 domain (cZNAΔP2), which is responsible for dimerization of ZO1 proteins (Utepbergenov et al., 2006; confirmed in Fig. 3B), localized to the junction, but was unable to form a continuous band around the cells. Instead, it was organized in a distinct, discontinuous pattern along cell boundaries (Fig. 2B; supplementary material Fig. S3). In the current study, we confirmed that the cZNAΔP2 was unable to co-immunoprecipitate with cZNA in transiently transfected HEK cells (Fig. 3B). Furthermore, we demonstrated that the PDZ1, SH3 and GUK domains were not required for cZNA dimerization. These observations suggest that PDZ2-mediated dimerization is required for a continuous circumferential distribution of ZNA at the tight junction. Finally, ZNA lacking the PDZ1 domain (cZNAΔP1) localized to the bi-cellular junctions, but was occasionally absent from the tri-cellular junctions. The observations summarized above suggest that the localization of ZO-1 does not result from a simple binary interaction between the SH3/U5 domains and a single binding partner, but is instead modulated by the coordinated action of several conserved protein-binding domains.

PDZ1 and PDZ2 regulate claudin-2 localization and organization at the AJC

Our previous report established that the subcellular localization of some, but not all, TJ proteins is altered in Z2Z1 dKD MDCK

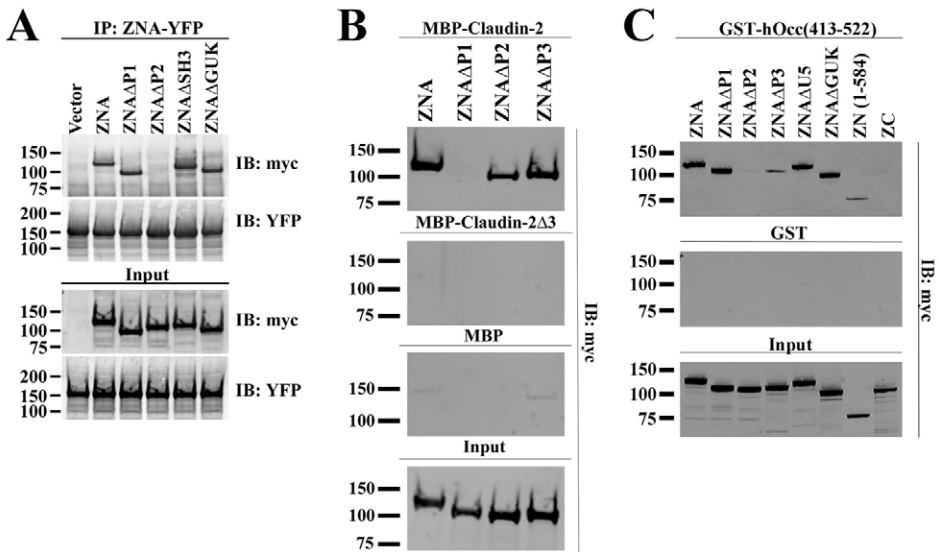

Fig. 3. Conserved domains in ZNA make distinct, and sometimes overlapping, contributions to protein binding *in vitro*. (**A**) The PDZ2 domain is necessary for ZO-1 dimerization. Human embryonic kidney (HEK) cells were co-transfected with YFP-tagged ZNA and the indicated myc-tagged deletion transgene. Complexes were precipitated with an antibody against YFP, resolved by SDS-PAGE and analyzed by immunoblotting (IB) with antibodies against myc and YFP. Note that deletion of the SH3 and GUK domains do not interfere with the co-precipitation of these transgenes with ZNA. (**B**) PDZ1 is required for Claudin-2 binding. HEK cells transfected with the indicated myc-tagged ZNA transgenes were lysed and mixed with MBP, MBP–claudin-2 or a claudin-2 fusion protein lacking the last three amino acids (MBP–claudin-2Δ3). Bound proteins were analyzed by IB with anti-myc antisera, as above. (**C**) PDZ2, PDZ3 and U5/GUK are required for interaction with occludin *in vitro*. ZNA deletion transgenes were incubated with GST or a GST–occludin (aa413–522) fusion protein. Bound proteins were analyzed by IB with anti-myc antisera. Note that multiple domains are required for the interaction of ZNA with occludin. Input protein was 10% of the total.

cells (Fanning et al., 2012). Decreased localization of occludin, claudin-1 and claudin-2 to the AJC are particularly evident, and correlate with changes in permeability, so we used these three proteins as markers of TJ structural integrity. Claudins are well characterized PDZ domain binding proteins (Itoh et al., 1999), and using pull-down assays we confirmed previous observations that PDZ1, but not PDZ2 or PDZ3, were required for interaction of cZNA with a recombinant claudin-2 peptide (Fig. 3A). Consistent with this observation, we found the ZNA construct lacking the PDZ1 (cZNAΔP1) domain, which directly binds to claudin, was unable to rescue localization of claudin-2 (Fig. 4) or claudin-1 (supplementary material Fig. S1) to the AJC. In fact, the small residual amount of claudin-2 normally observed in the tight junction of Z2Z1 dKD cells was notably absent when cZNAΔP1 is expressed in Z2Z1 dKD cells. These observations indicate that direct binding of cZNA to claudin-2 is required for localization to the AJC.

Expression of the cZNAΔP2 construct in ZO-depleted cells was also able to promote the localization of claudin-2 to cell–cell contacts (Fig. 4). However, this population of claudin-2 did not form a smooth, continuous band around the cell boundaries; instead it is distributed in a discontinuous pattern overlapping with the recombinant cZNAΔP2 protein (supplementary material Fig. S4). This observation suggests that PDZ-mediated dimerization of ZO-1 is also required for a continuous circumferential distribution of claudins at the tight junction. Interestingly, cells expressing cZNAΔP3 effectively recruited claudin-2 to the AJC, but claudin-1 localization was not rescued by this construct. These observations reinforce that idea that claudin localization to the AJC may not be regulated solely by binary interactions with ZO proteins.

ZO-1 PDZ domains are required for occludin localization to the AJC

The cytosolic tail of occludin binds to distinct structural elements within the U5 motif and GUK domain *in vitro* (Fanning et al., 1998; Furuse et al., 1994; Schmidt et al., 2004), but whether direct interaction with either of these domains is necessary or sufficient for incorporation of occludin into the AJC has not been directly addressed. To address this issue, we first examined the interaction of the cZNA transgenes with a GST occludin fusion protein (Fig. 3C). cZNA binds robustly to a GST–occludin fusion protein, whereas a fragment encoding the C-terminal 703 aa of ZO-1 showed no detectable interaction. Interestingly, we found that the presence of either the U5 motif or the GUK domain were sufficient for occludin binding, whereas loss of both domains (ZN 1–584) significantly reduced binding to GST–occludin. We also detected a previously unappreciated role for PDZ domains in the interaction of ZO-1 with occludin. Although deletion of PDZ1 had no apparent effect on the interaction of cZNA with GST–occludin, deletion of PDZ2 and PDZ3 both seriously compromised this interaction.

The role of these different conserved domains in occludin localization was similarly complex. Consistent with the binding assays, the induction of cZNAΔP2 was unable to restore occludin localization and the induction of cZNAΔP3, which demonstrated attenuated binding, showed a much weaker rescue of occludin localization than that seen with cZNA. Similarly, cZNAΔGUK, which binds well to GST–occludin, was able to restore occludin localization to the AJC (Fig. 5). However, induction of cZNAΔP1, which interacts quite effectively with GST–occludin in pull-down assays, was unable to rescue localization of occludin. These observations, taken together, would suggest

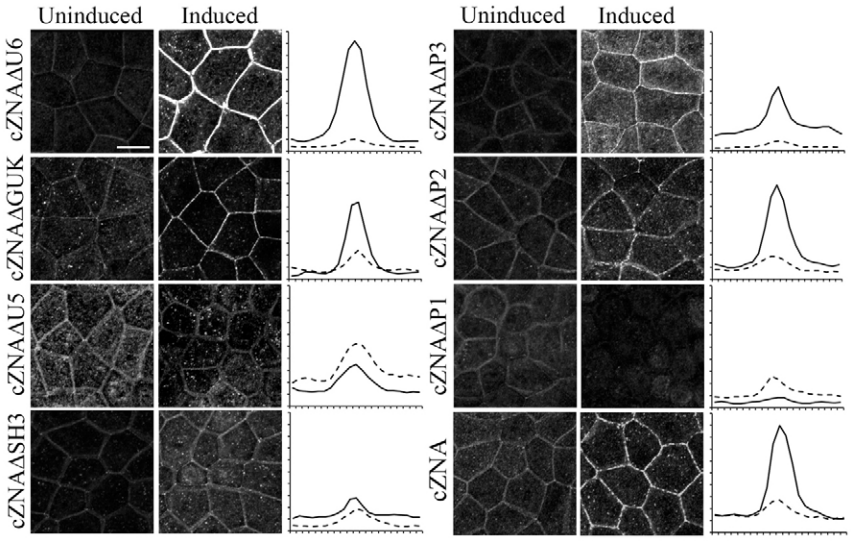

Fig. 4. The PDZ1, PDZ2 and SH3/U5 domains are necessary for claudin-2 localization to cell–cell contacts. Induced and uninduced Z2Z1dKD cells were fixed and stained with antibodies against claudin-2. cZNA images from Fig. 1 have been added for direct comparison and line scans. Images are 1.3 µm maximum density projections of Z-stacks taken through the apical-most aspect of the cells. Line scans represent the intensity of claudin staining at the cell–cell junction. Each line on the graph represents an average of ten line scans taken across the cell–cell junction of a different cell within the image shown in the figure. The *x*-axis represents distance along the 3 µm line scan and the *y*-axis represents arbitrary intensity units (each mark=20 units). Solid lines show data from cells induced to express the transgene and dotted lines are from uninduced cells. The axes for each line scan graph are the same scale. Line scans of claudin-2 in cZNAΔP2 cells were taken specifically at areas of the junction where claudin was localized. Scale bar: 10 µm.

that the localization of occludin to the TJ is not a simple matter of direct interactions with binding motifs in the U5 and GUK domains. Instead, occludin interaction with ZNA and localization to the TJ is dependent on the activity of other conserved domains, such as PDZ1, PDZ2 and PDZ3.

Localization of ZO-1 to the AJC is required for TJ assembly

Induction of either the cZNAΔSH3 or the cZNAΔU5 constructs, which are unable to localize to the membrane, was unable to restore normal localization of claudin-2 or occludin, indicating that ZO-1 localization to the AJC is required for normal localization of these proteins. Expression of the cZNAΔU6 construct effectively promoted localization of both occludin and claudin to cell–cell contacts. However, claudin and occludin were not restricted to the AJC in these cells, but instead colocalized with cZNAΔU6 in the lateral plasma membrane in the strand-like arrays characteristic of this mutation (supplementary material Fig. S2) (Fanning et al., 2007a). Taken together, our observations suggest that the localization of TJ proteins like occludin and claudin is regulated by a complex interaction between the activities of multiple domains.

The PDZ1 domain is necessary for normal cytoskeletal organization at the AJC

ZO-depleted MDCK cells have an expanded contractile array, as demonstrated by the increased localization of both F-actin and myosin IIB at the AJC (Fanning et al., 2012). Neither cZNAΔSH3 nor cZNAΔU5, which do not localize to the junctions, were able to restore a normal actin and myosin IIB distribution, indicating that normal cytoskeletal assembly requires localization of ZO-1 to the AJC (Fig. 6). Expression of the cZNAΔP1 construct was also unable to restore a normal contractile array within Z2Z1 dKD cells (Fig. 6). Most other constructs tested were able to effectively restore the normal distribution of F-actin and myosin IIB to the AJC (compare Fig. 6 and Fig. 1B). However, cells expressing cZNAΔP3 displayed an intermediate phenotype in which the accumulation of actin and myosin IIB within the AJC of these cells was

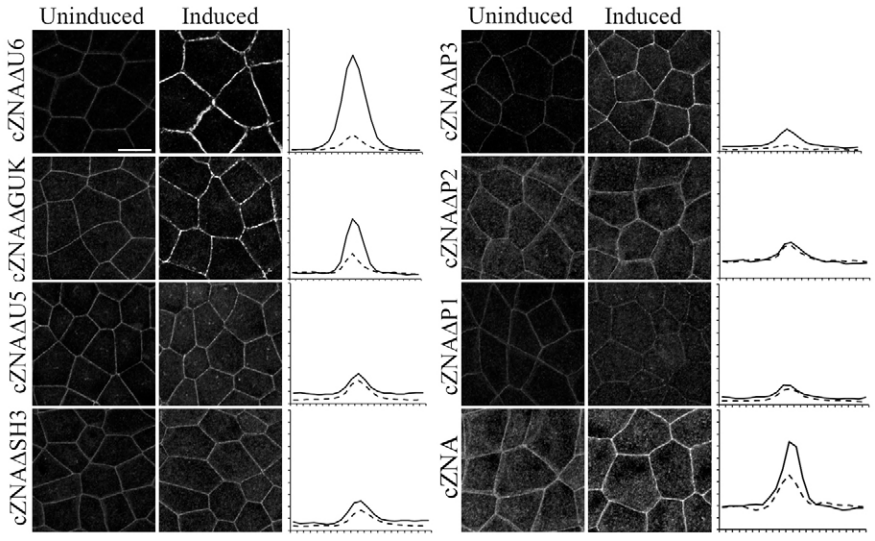

Fig. 5. The PDZ1, PDZ2 and SH3/U5 domains are necessary for occludin localization to cell–cell contacts. Z2Z1dKD cells with uninduced and induced expression of rescue constructs were labeled for occludin. cZNA images from Fig. 1 have been added for direct comparison and line scans. Images are 1.3 µm maximum density projections of Z-stacks taken through the apical-most portions of the cells. Line scans represent the intensity of occludin staining at the cell–cell junction. Each line on the graph represents an average of five line scans, each taken across the cell–cell junction of a different cell within the image shown in the figure. Solid lines show data from cells induced to express the transgene and dotted lines are from cells not expressing the transgene. The *x*-axis represents distance and the *y*-axis represents arbitrary intensity units (each hash mark=20 units). The axes for each line scan graph are the same scale. Scale bar: 10 µm.

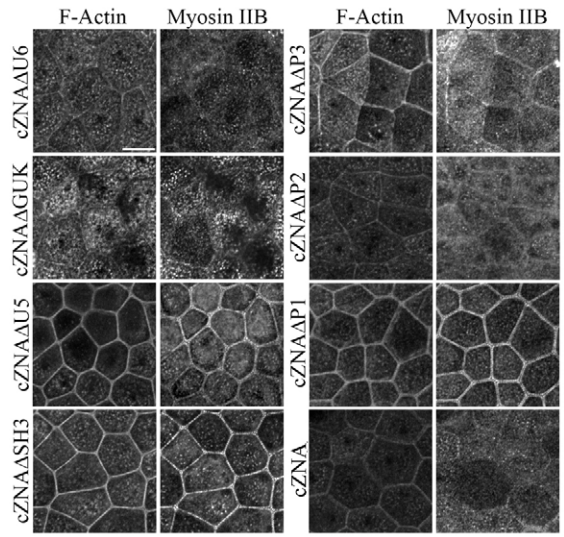

Fig. 6. The PDZ1, and to a lesser extent PDZ3, are required for normal contractile array assembly. Z2Z1dKD cells expressing rescue constructs were labeled for F-actin and myosin IIB. cZNA images from Fig. 1 are included for comparison. Images are 1.3 μm maximum density projections of Z-stacks taken through the apical-most portions of the cells. Scale bar: 10 μm.

attenuated compared to Z2Z1 cells, but not restored to a completely normal distribution. Thus, PDZ1, and to a lesser extent PDZ3, are involved in the normal regulation of cytoskeletal structure within the AJC.

Ability of rescue constructs to establish a functional barrier

ZO proteins are necessary for normal cell permeability (Fanning et al., 2012; Umeda et al., 2006; Van Itallie et al., 2009). Our previous results demonstrated that cells lacking both ZO-1 and ZO-2 maintain normal (or slightly increased) transepithelial electrical resistance (TER), but have a significantly increased permeability for larger molecules through the 'leak pathway' (Fanning et al., 2012). Re-expression of either ZO-1 or ZO-2 in ZO-depleted cells reduced permeability to normal or near normal levels (Fig. 1). Thus, to identify domains involved in the regulation of the leak pathway, we examined the flux of 3 kDa fluorescent dextrans across Z2Z1 dKD cell monolayers induced to express the transgene. The results, shown in Fig. 7, are presented as the fold increase over wild type MDCK cells culture at the same time as the experiment cells.

Rescue constructs had one of three effects on the increased permeability of ZO-depleted cells: they reduced, increased or had no effect on permeability relative to uninduced cells (Fig. 7). Expression of cZNA, cZNAΔGUK, cZNAΔU6 and cZNAΔP3 constructs in Z2Z1 dKD cells all reduced permeability to levels characteristic of control MDCK cells. Induction of the cZNAΔP2 construct within Z2Z1 dKD cells had no effect on permeability; these cells continued to maintain an increased permeability to dextrans similar to uninduced cells. Finally, we found that cells expressing cZNAΔP1, cZNAΔSH3 or cZNAΔU5 constructs further increased epithelial permeability above levels seen in uninduced Z2Z1 dKD cells. This effect was most dramatic in cells expressing cZNAΔP1, where we saw a nearly tenfold increase in permeability to 3 kDa dextran compared to uninduced cells. Interestingly, cells expressing cZNAΔP1 and cZNAΔU5,

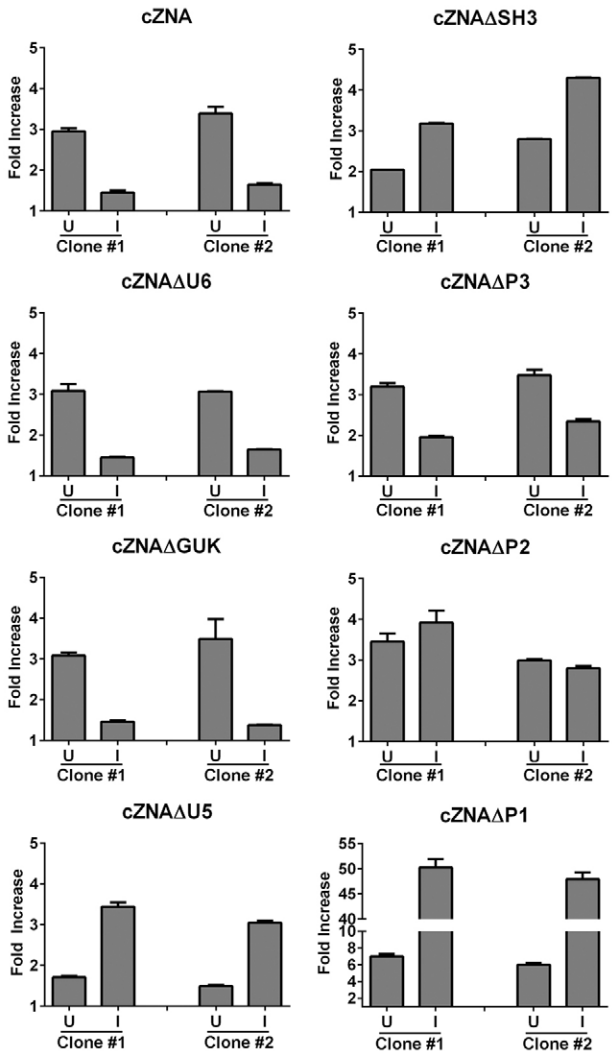

Fig. 7. PDZ1, PDZ2 and SH3/U5 domains contribute to epithelial barrier formation. Analysis of the paracellular flux of 3.0 kDa fluorescein–dextran in ZO-depleted cells induced (I) and uninduced (U) to express rescue constructs. The *y*-axis is the fold increase in dextran flux relative to control MDCK cells cultured at the same time. All values are the mean ± standard deviation from quadruplicate readings and are representative of at least three experiments.

also had a significant decrease in TER compared to wild type MDCK cells (supplementary material Fig. S5). Permeability of larger dextrans was not assessed, because we found no significant difference in the permeability of 40 kDa dextrans in ZO-depleted cells relative to control cells (data not shown).

The U2 and U3 domains are not required for normal ZO-1 function

The N-terminal portion of ZO-1 contains two additional large unique regions, the U2 and U3 domains. ZNA constructs lacking these domains (ZNAΔU2 and ZNAΔU3) localize to the AJC and their expression within Z2Z1 cells restores epithelial permeability to near normal levels (supplementary material Fig. S6). ZNAΔU2 and ZNAΔU3 expression also restores normal claudin, actin and myosin localization to the AJC (supplementary material Figs S7, S8). Thus, the U2 and U3 domains, which have no known binding

partners, are not necessary for normal ZO-1 function within MDCK cells.

Discussion

Many factors contribute to the formation of epithelial TJs and the regulation of epithelial permeability. Our results indicate that ZO-1 behaves as a multi-domain scaffold, and that coordinated activity of multiple conserved protein-binding motifs is required for normal organization and function of the steady state junction (Table 1). This includes regions like SH3 and U5, which promote ZO-1 localization, the PDZ2 domain, which mediates dimerization, and the PDZ1 domain, which binds directly to claudins. Our results define a core set of functional activities that are critical to TJ structure and maintenance of epithelial permeability.

ZO-1 must localize to the junction in order to rescue permeability, tight junction assembly and regulation of contractile array

This report reveals that localization of ZO-1 to the apical junction complex is necessary for proper regulation of the tight junction structure, permeability and organization of the Zonula adherens (ZA) cytoskeleton. The larger SH3–U5–GUK region has been previously implicated in junction localization (Fanning et al., 2007a; Umeda et al., 2006) of ZO-1 and other MAGUKs (reviewed by Fanning et al., 2007a). Our results indicate that the SH3 domain and the U5 motif are required for localization of ZO-1 to cell–cell contacts, but that the PDZ2 domain and the U6 motif appear to 'fine-tune' the distribution of ZO-1 within the lateral domain (Fig. 2) (Fanning et al., 2007a). Our recent structural analysis of ZO-1 suggests that the U5 domain is, in fact, a flexible loop extending from the core SH3 domain (Lye et al., 2010). This report would thus confirm that they are acting as a single functional unit.

The simplest model is that the SH3/U5 unit regulates TJ localization through interactions with an as yet unidentified 'anchor protein'. The U5 motif has no known binding partners besides occludin, and occludin and its orthologs are not necessary for TJ assembly (Raleigh et al., 2010; Saitou et al., 2000; Yu et al., 2005). Similarly, although the SH3 domain of ZO-1 binds to at least five different proteins, most of these proteins do not appear necessary for tight junction assembly (Balda and Matter, 2000; Meyer et al., 2002; Tsapara et al., 2006). The one exception is the AJ protein AF-6/afadin. The formation of both AJ and TJ are compromised in AF6/afadin-depleted epithelia (Ikeda et al., 1999; Komura et al., 2008; Lorger and Moelling, 2006; Ooshio et al., 2010; Zhadanov et al., 1999). However, AF-6/afadin does not localize to the TJ in polarized cells, and co-immunoprecipitation studies suggest that AF-6/afadin and ZO-1 only interact prior to TJ formation (Ooshio et al., 2010). Thus, we believe any role for AF-6/afadin in TJ assembly is likely an indirect effect of its role in adherens junctions. The identity of the factor(s) that recruit ZO proteins to the AJC is still a matter of great speculation.

The PDZ1 domain is necessary for claudin-1, -2 and occludin localization and for formation of the TJ barrier

The seminal studies by Umeda et al. (Umeda et al., 2006) demonstrated that ZO proteins are necessary for the assembly of claudins and occludin into barrier strands and incorporation of these strands into the apical circumferential complex. Unlike the cells used by Umeda et al., our Z2Z1 dKD cells still incorporate many TJ proteins, such as claudin 3, claudin 4, JAM and tricellulin, into the AJC (Fanning et al., 2012). However, there are several proteins that are not effectively incorporated into the AJC in ZO-depleted MDCK cells, including claudin-1, claudin-2, occludin and cingulin. We did not examine the distribution of cingulin in cZNA rescue cells, because cingulin binds to the C-terminus of ZO-1 and does not appear to play a major role in the regulation of TJ structure or the epithelial barrier (Guillemot et al., 2012).

Our examination of claudin-1, claudin-2 and occludin revealed a complex interaction between these proteins and ZO-1. As predicted, our results confirm that PDZ1 is required for direct binding to claudin-2 *in vitro* (Itoh et al., 1999), and is necessary for claudin-2 localization to cell–cell contacts. However, we found that claudin-1 localization depended not only on PDZ1 for AJC localization, but also required PDZ3. The reason for this is currently unclear. Claudin-1 binds exclusively to PDZ1 in all of the ZO proteins (Furuse et al., 1998a), so our observations suggest that some PDZ3 ligand might stabilize claudin-1 at the AJC. Taken together, our studies suggest that different claudins are recruited to or stabilized at the AJC by diverse mechanisms.

Another surprising result of these studies was that the PDZ1 domain is not just required for the localization of claudin-2, but also for the localization of occludin to the TJ. The occludin-binding site has been previously mapped to structural elements within the U5 motif and the GUK domain (Fanning et al., 1998; Schmidt et al., 2004). Our results demonstrate that either domain is sufficient for binding to ZO-1, but the absence of both significantly attenuates the interactions between ZO-1 and occludin. However, we found that cZNAΔP1, which associates well with occludin in our binding assays, is unable to recruit occludin to the AJC, indicating that direct binding of ZO-1 to occludin is not sufficient to recruit occludin to junctions. A previous study by Furuse et al. demonstrated that claudins could markedly potentiate the assembly of occludin into cell–cell contacts in L-cells transfected with claudins (Furuse et al., 1998b). These observations lead us to hypothesize that the maintenance of occludin at tight junctions is mediated by direct or indirect interactions with organized claudin strands.

Our studies also strongly suggest that the PDZ1 domain is necessary for the establishment of a physiological barrier. Expression of cZNAΔP1 was not only unable to restore normal localization of claudin-1 and -2 to the AJC, but it also further enhanced the barrier disruption seen in ZO-depleted cells. This included a 10-fold increase in flux through the leak pathway and a 5-fold decrease in TER relative to the parental dKD cells. We hypothesize that cZNAΔP1 acts as a dominant negative, and that cZNAΔP1 displaces endogenous ZO-1 from junctions and establishes an underlying scaffold that cannot directly interact with barrier forming claudins. We interpret our observations to suggest that direct interaction between ZO proteins and at least some claudin polypeptides, like claudin-1 and -2, are required for steady state structure and/or function of the barrier. While this seems like the most likely hypothesis, we cannot rule out that the involvement of PDZ1 in cytoskeletal assembly at the AJC (discussed below) may also be involved in the regulation of the barrier. Future studies will be directed at resolving this important issue.

The PDZ2 domain, a ZO dimerization motif, is required for normal physiological barrier formation

The PDZ2 domain of ZO-1 is necessary for the dimerization of ZO proteins (Fig. 3) and (Fanning et al., 2007b; Utepbergenov

et al., 2006) and binding to connexins (Giepmans, 2004). While it is theoretically possible that interaction of ZO-1 with connexins promotes TJ barrier assembly, we believe it to be unlikely. MDCK cells do not appear to have gap junctions or to be dye coupled under normal conditions (Armitage and Juss, 2003; Jin et al., 2004). Furthermore, the loss of TJ structure is not obvious in most mouse connexin knockout models (Dobrowolski and Willecke, 2009; Eckert and Fleming, 2008). Instead, our interpretation is that dimerization mediated by PDZ2 is necessary to recruit at least some TJ proteins, like ZO-1, claudin-1, -2 and occludin, into a fully functional barrier. This is based on our observation that cZNAΔP2 does not form a continuous band around the apical portion of the cell, but is instead co-distributed in a punctate pattern at the tight junction. A similar punctate pattern is observed for claudin-1 and -2. Our interpretation of these observations is that cZNAΔP2 polypeptide is still able to recruit claudins into the AJC, but is unable to 'cross-link' these proteins into a continuous array.

Interestingly, the cZNAΔP2 polypeptide is also unable to bind to occludin or restore its normal distribution at the TJ. The implication of these observations is that dimerization of ZO-1 is required for biochemical interaction with occludin, and that this is somehow linked to occludin incorporation into the AJC. Unlike claudin, the distribution of occludin in cells expressing cZNAΔP2 is indistinguishable from that observed in uninduced or ZO-depleted cells. These observations imply that occludin and claudin-2 are stabilized at junctions by distinct mechanisms.

Direct interactions between ZO-1 and F-actin are not required for barrier assembly or regulation of actomyosin contractile arrays at the AJC

Tight junction structure and function are dependent on cytoskeletal integrity, and can be acutely regulated by changes in cytoskeletal dynamics (Shen and Turner, 2005). ZO-1 binds directly to F-actin through a well-defined ABR region within the C-terminus (Fanning et al., 1998; Itoh et al., 1997; Wittchen et al., 1999) and it has been proposed that this direct interaction transduces cytoskeletal signals that regulate the assembly and function of the TJ barrier (Shen et al., 2006; Van Itallie et al., 2009; Yu et al., 2010). In one notable example, Yu et al. demonstrated that the ABR was required for the MLCK-dependent changes in TJ dynamics and paracellular permeability to ions in CACO2 cells (Yu et al., 2010). Tight junctions also show acute changes in structure and function in response to physiological and pathological stimuli that are dependent on the cytoskeleton (reviewed by Cunningham and Turner, 2012). Our results suggest that the mechanistic aspects of cytoskeletal regulation within the AJC are quite complex. We have confirmed the previous observation (Ikenouchi et al., 2007) that the ZO-1 ABR is not required to recruit TJ proteins to the apical junction complex. Furthermore, we demonstrate for the first time that the ABR, and presumably direct interaction with F-actin, is not required for maintenance of steady state barrier to solutes (also known as the leak pathway) in MDCK cells. Whether the ABR or other C-terminal binding domains are required for acute barrier response to physiological stimuli, particularly those that activate MLCK, will be the subject of future studies.

We found that the ABR is also not required for the normal regulation of contractile actomyosin arrays at the AJC. These arrays, normally associated with the adherens junction, are required for morphogenetic changes in cell shape that drive tissue formation. However, we note that the change in junction topology in Z2Z1 dKD cells from a relaxed/wavy configuration to a straight configuration is not rescued by cZNA. These observations have two important implications. First, they confirm that the steady state architecture of the AJC is controlled by elements within the C-terminus of ZO-1. One prediction, given previous results (Yu et al., 2010; Shen et al., 2006), is that regulation of junction topology involves direct interaction of the ABR with F-actin. However, we note that similar changes in junction topology in ZO-1 single KD cells are at least partially rescued by a ZO-1transgene lacking only the ABR, suggesting C-terminal domains outside of ABR may also be involved in regulating junction topology (Van Itallie et al., 2009). Second, our observations imply that the assembly of large contractile arrays in Z2Z1 dKD cells is not directly responsible for the change in junction profiles from wavy to straight. Thus, we propose that changes in junction topology and the formation of contractile arrays at the AJC are regulated by distinct cytoskeletal mechanisms.

Much to our surprise, we found that it was PDZ1, and to a lesser extent PDZ3, that are the critical domains for regulation of contractile array formation at the cell junctions. There is no known F-actin-binding domain within PDZ1 or PDZ3 of ZO-1 (Fanning et al., 1998; Itoh et al., 1997), therefore regulation of the contractile array is likely through the binding of ZO-1 to cytoskeletal regulatory proteins and/or other cytoskeletal components. For example, PDZ1 is known to bind to alpha-actinin 4 and ARVCF (Chen et al., 2006; Kausalya et al., 2004). Future studies will address the role of PDZ1 ligands in regulating cytoskeletal structure.

Closing remarks

Our results suggest that PDZ1 interactions, ZO protein dimerization and localization to the apical junctional complex are critical elements of ZO-protein-mediated TJ organization. They also point to a previously unappreciated complexity in how different proteins are recruited and stabilized at junctions. In three examples discussed here, ZO-1-dependent localization and binding appears to be mediated by interactions between multiple domains and/or their binding partners. This observation suggests several intriguing possibilities. For example, it suggests that the interaction between different ZO protein ligands may stabilize their distribution at the tight junction, or that steric and allosteric regulation plays a role in ZO protein scaffolding. This study also highlights the importance of identifying the factor(s) that bind to the SH3/U5 motifs and recruit ZO proteins to the AJC. Understanding which proteins are necessary to assemble and maintain a functioning TJ and elucidating how these proteins are regulated is critical for the development of therapeutic approaches for disorders associated with aberrant permeability.

Materials and Methods

Cell lines and expression vectors

MDCK Tet-Off cells (clone T23; Clontech, Mountainview, CA) were maintained at 37℃ and 5% CO_2 in standard growth medium: DMEM (high glucose, Mediatech, Manassas, VA) medium supplemented with 10% tetracycline-free fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO), penicillin, and streptomycin. Cells for immunocytochemistry and permeability assays were maintained on 12 mm diameter Transwell filter inserts with a 0.4 μm pore size (Corning, Corning, NY). Cells were maintained for 7 days with fresh media changes every 2–3 days.

The Z2Z1 dKD cells were constructed as previously described by Fanning et al. (Fanning et al., 2012). C-myc-tagged ZO-1 transgenes lacking the PDZ2, PDZ3, SH3, GUK, and U5 regions, or encoding the ZNA and ZNAΔU6 transgenes, were previously created in the pCB6 expression vector (Fanning et al., 2007a). The ZNA and ZNAΔU6 constructs were transferred from pCB6 vectors to pTRE vectors. To create the ZNAΔP2, ZNAΔP3 and ZNAΔSH3 constructs, *Eco*RI–*Nhe*I fragments encompassing the deletion were subcloned from the pCB6 vectors and inserted into the corresponding *Eco*RI–*Nhe*I site within the pTRE ZNA vector. The ZNAΔU5 construct was created by transferring a *Stu*I fragment from pCB6 ZO1ΔU5 into the corresponding *Stu*I site in pTRE ZNA. To create the ZNAΔGUK construct, an *Acc*I fragment from the pCB6 ZO1ΔGUK was inserted into the corresponding *Acc*I site in the pTRE ZNA. To construct ZNAΔP1, PCR was used to amplify an *Age*I–*Nhe*I fragment encoding amino acids (aa) 110–582 (primer sequences: 5′-GCACCGGTAAAGTTCAAATACCAGTAAGTCG-3′ and 5′-CTGCGAAGACCTCTGAATCT-3′). Site-directed mutagenesis was then used to insert an Age I site at aa16 in pTRE ZNA (primer sequence: 5′-CACAGCAATGGAGGAAACCGGTATATGGGAACAACATACA-3′), and the PCR fragment encoding aa 110–582 was subcloned into the corresponding *Age*I–*Nhe*I site of this modified pTRE ZNA. To insert an mCherry tag and *Eco*RI site was inserted in the 5′ untranslated region of each pTRE construct using site-directed mutagenesis, and an *Eco*RI–*Not*I fragment encoding mCherry was subcloned into the *Eco*RI–*Not*I sites of the appropriate pTRE vector. To create pTRE ZNAΔU2, we synthesized a gene fragment (GeneArt/Life Technologies) encompassing an 847 bp *Eco*RI–*Bsp*EI fragment of human ZO-1 encoding aa 1–339, absent sequence encoding aa 123–185. This fragment was subcloned into the *Eco*RI–*Bsp*EI sites of pTRE ZNA. Similarly, to create pTRE ZNAΔU3, we synthesized a gene fragment encompassing a 940 bp *Eco*RI–*Xba*I fragment of ZO-1 encoding aa 1–450, absent sequences encoding aa 277–419. This fragment was subcloned into the *Eco*RI–*Xba*I sites of pTRE ZNA.

The resulting pTRE vectors were co-transfected with a pTK-hyg plasmid into Z2Z1 dKD cells. Stable clones were selected in 200 µg/ml hygromycin B (Sigma-Aldrich). To inhibit transgene expression, cell lines were maintained in the standard growth medium supplemented with 15 µl/l doxycycline. Transgene expression was induced by maintaining cells in the standard growth media.

Immunocytochemistry

Filter inserts were processed as previously described by Fanning et al. (Fanning et al., 2012). All inserts were fixed for 30 minutes in ice-cold ethanol. Primary antibodies were used as follows: claudin-2 (Invitrogen; 1:100), occludin (Hycult Plymouth Meeting, PA; 1:100), myosin IIB (Covance, Emeryville, CA; 1:250), ZO-1 (R40.76; (Anderson et al., 1988); 1:25) and mCherry (Clontech; 1:500). The appropriate species-specific affinity-purified Cy2-, Cy3-, or Cy5-conjugated secondary antibodies were purchased from Jackson Immunoresearch and used at a dilution of 1:100. F-actin was labeled using tetramethylrhodamine isothiocyanate (TRITC)–phalloidin (Sigma Aldrich; 1:500). Samples were imaged on a Zeiss LSM710 confocal microscope using a 60× PlanApo lens. Photomultiplier settings in each channel were identical for control, Z2Z1 dKD, and rescue construct cells (both induced and uninduced conditions). Image stacks were acquired through the 1.33 µm apical-most portion of the cell with a step size of 0.33 µm. All volumes were processed in the ZEN image browser (Zeiss, Thornwood, NY) and are presented as maximum-density projections. At least two clones for each rescue transgene were analyzed and a representative image was provided for the figures. To examine cZNAΔP2 localization, transgene expression was attenuated by adding low levels of doxycycline to the culture media. This improved the signal to noise ratio for the tagged transgenes, but had little effect on the overall distribution of the transgene at cell–cell contacts.

Western blots

Western blots were performed as previously described by Fanning et al. (Fanning et al., 2012). Lysates were collected from 7-day cell cultures grown on filter inserts. Primary antibodies were used as follows: ZO-1 (R40.76; (Anderson et al., 1988); 1:2000), mCherry, (Clontech; 1:1000), claudin-2 (Invitrogen; 1:100), occludin (Hycult; 1:500), actin (Millipore; 1:1000), myosin IIB (Covance; 1:1000) and GAPDH (Sigma-Aldrich; 1:2000). The appropriate species-specific secondary antibodies coupled to IRDyes (Rockland, Gilbertsville, MD) were used at 1:10,000. Western blots were imaged with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Epithelial permeability

For both TER and FITC–dextran flux assays cells were plated at subconfluent density and cultured for 7 days on 6.5 mm filter inserts with a 0.4 µm pore size (Corning, Corning, NY). All measurements were performed in quadruplicate. Each experiment was performed a minimum of three times; one representative experiment is shown in the provided figures. TER was measured using an EVOM volt–ohm meter (World Precision Instruments, Sarasota, FL). FLUX assays were performed as follows. Cells were rinsed twice with Hank's buffered saline solution with calcium ($HBSS^+$) (Mediatech, Manassas, VA) and then incubated with fresh $HBSS^+$ (200 µl on top of insert and 800 µl below the insert) for 30 minutes at standard culture conditions. The apical chamber was then replaced with 75 µl of a 0.3 mg/ml solution of 3 kDa fluorescein–dextran in $HBSS^+$ and placed in a receiving chamber containing 600 µl of fresh $HBSS^+$. After an initial 2 hours incubation, which allows cells to stabilize, the inserts were removed and transferred to a new receiving chamber containing 600 µl $HBSS^+$ and incubated for an additional 2 hours. Flux was assayed by measuring the concentration of FITC–dextran present in the basal chamber after the second 2-hour incubation period using a Synergy HT plate reader (BioTek, Winooski, VT).

Co-immunoprecipitation assays

Human embryonic kidney (HEK) 293 Tet-Off cells were transfected with YFP-tagged ZNA and a myc-tagged deletion transgene using Fugene HD according to the manufacturer's suggestions (Promega, Madison, WI). After 2 days, cells were lysed on ice in immunoprecipitation (IP) buffer (20 mM TrisCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS; pH 7.5) supplemented with protease inhibitors (Roche Diagnostics, Mannheim, Germany) and clarified by centrifugation at 10,000 ***g***. The supernatant was pre-cleared with protein-G–Sepharose (GE Biosciences) for 2 hours, incubated overnight at 4℃ with 3.0 µg of mouse anti-GFP serum (Life Technologies, Grand Island, NY) and supplemented with 20 µl Protein G Sepharose for an additional 2 hours at 4℃. The beads were washed three times with IP buffer, resolved by SDS-PAGE and analyzed by western blotting with anti-myc (clone 9B11; Cell Signaling Technology, Beverly, MA) and anti-GFP (A6455; Invitrogen) sera. Western blots were imaged on an Odyssey Imager (LICOR, Lincoln, NE).

Claudin-2 tail binding assay

Recombinant 6×HIS-tagged maltose-binding protein (MBP), MBP claudin-2 tail, and an identical claudin-2 construct lacking the last 3 amino acids (MBP–claudin-2Δ3) were expressed in *E. coli* and purified on Nickel-Sepharose HP (GE Healthcare, Piscataway, NJ) as previously described (Van Itallie and Anderson, 2004). The concentration of protein bound to beads was assessed by UV spectroscopy and confirmed by SDS-PAGE and Coomassie Brilliant Blue staining. Protein-conjugated beads were diluted with Sepharose CL-4B (GE Healthcare) to a final concentration of 0.2 µg/µl resin. For pull-down assays HEK 293 Tet-Off cells were transfected with myc-tagged ZNA transgenes as described above. After 2 days, cells were lysed on ice for 10 min in 1 ml lysis buffer (20 mM Hepes, 150 mM NaCl, 1% v/v NP-40, 20 mM imidazole, protease inhibitors; pH 7.4) and clarified by ultracentrifugation at 100,000 ***g***. The supernatants were pre-cleared overnight with 50 µl of Ni-MBP resin, diluted 1:1 in HEK lysis buffer and incubated with 25 µl of MBP claudin-2 tail or MBP claudin-2Δ3 resin for 2 hours at 4℃. Beads were washed three times with lysis buffer and eluted in 200 µl of 400 mM imidazole (pH 7.4). Protein samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antisera against myc epitope. Westerns were imaged as detailed above.

GST–occludin binding assay

GST and human GST–occludin tail (aa 413–522) were expressed in *E. coli* as previously describe (Fanning et al., 2007a). Cells were harvested by centrifugation at 10,000 ***g***, resuspended in 10.0 ml resuspension buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA; pH 7.2) and incubated with 1.0 mg/ml chicken lysozyme for 30 min on ice (Sigma-Aldrich, St. Louis, MO). The bacterial lysate was adjusted to 1.0% Triton X-100 and 250.0 U/ml Benzonase (Sigma-Aldrich), incubated on ice an additional 10 minutes and clarified by centrifugation at 20,000 ***g*** for 30 minutes. The soluble bacterial lysate was incubated with 0.25 ml of glutathione agarose (Sigma-Aldrich) for 1 hour at 4℃ with gentle mixing and washed three times in resuspension buffer. HEK cells were transfected with myc-tagged ZNA transgenes as described above and, after 2 days, lysed on ice in 1.0 ml of occludin lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 0.05% w/v SDS, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, protease inhibitors, pH 7.5) supplemented with phosphatase inhibitors (Roche Diagnostics) and clarified by ultracentrifugation at 100,000 ***g*** for 30 minutes at 4℃. 500 µl of clarified supernatant was diluted with equal volume lysis buffer and pre-cleared with 40 µg of GST-agarose slurry for 2 hours at 4℃. The cleared lysate was incubated with 40 µg of GST–occludin resin (~1.0 µM) overnight at 4℃. The resin was then washed three times in occludin lysis buffer (w/o protease/phosphatase inhibitors) and once in TrisCl/NaCl pH 8.0. Bound protein was eluted in 50 µl of elution buffer (25 mM TrisCl, 10 mM reduced glutathione; pH 9.0). The eluted protein was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antiserum against the myc epitope.

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Author contributions

L.S.R. and A.S.F. participated in the conception, execution and design of experiments, data interpretation, and manuscript preparation. M.T.B. participated in execution of experiments and manuscript preparation. J.M.A. participated in conception, data interpretation, and preparation of the manuscript.

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