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The Unique-5 and -6 Motifs of ZO-1 Regulate Tight Junction Strand Localization and Scaffolding Properties[D]

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The proper cellular location and sealing of tight junctions is assumed to depend on scaffolding properties of ZO-1, a member of the MAGUK protein family. ZO-1 contains a conserved SH3-GUK module that is separated by a variable region (unique-5), which in other MAGUKs has proven regulatory functions. To identify motifs in ZO-1 critical for its putative scaffolding functions, we focused on the SH3-GUK module including unique-5 (U5) and unique-6 (U6), a motif immediately C-terminal of the GUK domain. In vitro binding studies reveal U5 is sufficient for occludin binding; U6 reduces the affinity of this binding. In cultured cells, U5 is required for targeting ZO-1 to tight junctions and removal of U6 results in ectopically displaced junction strands containing the modified ZO-1, occludin, and claudin on the lateral cell membrane. These results provide evidence that ZO-1 can control the location of tight junction transmembrane proteins and reveals complex protein binding and targeting signals within its SH3-U5-GUK-U6 region. We review these findings in the context of regulated scaffolding functions of other MAGUK proteins.

INTRODUCTION

Tight junctions (TJ) are a hallmark of polarized epithelial cells, providing the paracellular barrier required to separate tissue spaces (Van Itallie and Anderson, 2004), contributing to maintenance of apical-basolateral cell polarity and providing a site for cell–cell signaling (Schneeberger and Lynch, 2004; Matter *et al.,* 2005; Shin *et al.,* 2006). At the ultrastructural level, tight junctions appear as highly organized strands that encircle the apical-lateral boundary. These strands are composed of transmembrane proteins such as occludin, tricellulin, and one or more members of the claudin family, which create the paracellular barrier. These proteins are, in turn, associated with a cytosolic plaque of proteins that is closely associated with the cortical cytoskeleton. The sequence of events that bring about the proper continuous apical localization of TJ proteins remains unknown. One approach to this problem has been to define components in upstream pathways required to induce cell polarity (Shin *et al.,* 2006). Another approach has been to elaborate the biochemical interactions among the more than 40 transmembrane and cytosolic components of the TJ and to define their functional role in somatic cells using gene ablation, transcriptional inhibition, or dominant negative strategies (Schneeberger *et al.,* 2004). Despite considerable efforts by many groups, the precise mechanism of junction assembly is poorly understood.

The cytoplasmic protein ZO-1 is proposed to be one of the key regulators of TJ assembly (reviewed in Fanning, 2006). ZO-1 is member of a large family of membrane-associated scaffolding and signaling molecules known as the membrane-associated guanylate kinase homologues (MAGUKs). These proteins are characterized by a core motif of conserved protein-binding domains including one or more PDZ domains, an SH3 domain and a GUK domain (Funke *et al.,* 2005). By analogy to other MAGUKs, ZO-1 is assumed to function as a multidomain scaffold that coordinates the assembly of transmembrane and cytosolic proteins into the TJ and/or regulates the activity of these proteins once assembled. Consistent with this idea, ZO-1 binds all three classes of TJ transmembrane proteins including occludin, claudins, and the CTX (Ig) superfamily (reviewed in Schneeberger *et al.,* 2004). It also binds at least 10 cytoplasmic proteins and various components of the cortical cytoskeleton. However, despite being the first TJ protein identified, the precise role of ZO-1 remains unknown.

Interestingly, experimental evidence so far suggests that ZO-1 alone is not required for cells to form TJs in culture (Umeda *et al.,* 2004; McNeil *et al.,* 2006). For example, removal of the ZO-1–binding domains from occludin or claudins does not affect localization of these proteins to the tight junction (reviewed in Fanning, 2006). In addition, when ZO-1 is deleted from cultured epithelial cells by either homologous recombination (Umeda *et al.,* 2004) or siRNA (McNeil *et al.,* 2006), TJ assembly is markedly slowed, but normal barrier properties are ultimately achieved. These observations suggest that ZO-1 is required for the normal kinetics of TJ assembly. Thus, it is notable that in *Drosophila* the single ZO-1 homolog, Polychaetoid, is required for junc-

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tion remodeling during both tracheal development (Jung *et al.*, 2006) and dorsal closure (Wei and Ellis, 2001), suggesting some undefined role for ZO proteins during dynamic processes of junction remodeling. The critical role of these proteins in tight junction assembly was confirmed by the recent studies of Umeda *et al.* (2006), who found that EpH4 cells were unable to assemble tight junctions when both ZO-1 and its homolog ZO-2 were down-regulated.

Despite the critical role of these proteins for junctional assembly, their precise molecular role is poorly understood. Research into other MAGUK proteins has suggested that there is a underlying structural motif formed by an intramolecular interaction between the SH3 and GUK domains and that this *cis*-interaction regulates properties as diverse as multimerization (Masuko *et al.*, 1999; Nix *et al.*, 2000), transmembrane channel/receptor clustering (Shin *et al.*, 2000), and cytoskeletal interactions (Tsukita and Furuse, 2000a). Positioned between the conserved domains of MAGUKs are highly variable sequences we refer to as "unique" (U) motifs; starting from the N-terminus these are Unique-1 (U1), followed by Unique-2 between the first and second PDZ domains, etc. (see Figure 1A). In other MAGUKs the unique sequences provide regulatory functions through phosphorylation (Sabio *et al.*, 2005), alternative splicing (Tsukita and Furuse, 2000a; Hanada *et al.*, 2003), and inter- or intramolecular protein interactions (e.g., SAP97/hDlg; Wu *et al.*, 2000). These possibilities have not been explored for ZO-1, and we propose that the Unique motifs in ZO-1 regulate the tight junction specific localization and unique organization of transmembrane proteins that are crucial for formation of the paracellular barrier.

In this study we sought to define functions for the SH3-GUK (SG) module and the Unique motifs of ZO-1. We find that, as in other MAGUKs, there is an intramolecular interaction between the SH3 and GUK domains in ZO-1 and speculate that this intramolecular hairpin positions the intervening U5 and adjacent U6 motifs. The U5 motif is required for occludin binding in vitro and for targeting ZO-1 to the TJ in vivo, although we do not know whether these functions are causally linked. The U6 motif inhibits occludin binding in vitro, and when U6 is deleted the expression of this modified ZO-1 induces ectopic strands of occludin and claudins that are displaced onto the lateral cell surface. We speculate that this results from unregulated binding to occludin or another protein. With the exception of cingulin, we find none of the cytoplasmic TJ plaque proteins or adherens junction proteins in the ectopic strands, suggesting ZO-1 is not their primary cue for localization. Our results provide direct evidence that ZO-1 can scaffold other TJ proteins such as occludin or claudins and influence their location on the cell surface. Further, our results indicate that regulated binding activities around the SG module influence TJ assembly and strand localization.

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins

Glutathione *S*-transferase (GST) and maltose-binding protein fusion proteins were induced in BL21 GOLD bacteria cells (Stratagene, La Jolla, CA) and bound to affinity matrices as previously described (Fanning *et al.*, 1998). The concentration of proteins on beads was determined by measuring the A_{280} of the eluate from a predetermined volume of beads and confirmed by SDS-PAGE. MBP fusion proteins were eluted from amylose resin (New England Biolabs, Beverly, MA) in a buffer containing 10.0 mM maltose, 20.0 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1.0 mM EDTA. The expression and purification of the His-tagged ZNG, ZNU5, and ZNU6 from High-5 insect cells has been described (Utepbergenov *et al.*, 2006). All soluble fusion proteins were clarified at 100,000 × *g* for 60 min immediately before use.

In Vitro Binding Studies and Yeast Two-Hybrid Analysis

To measure the interaction between bacterially produced proteins in vitro, GST fusion proteins encoding various ZO-1 polypeptides or the C-terminal amino acids 383-522 of occludin were immobilized on glutathione-agarose. The agarose-bound peptides were washed with binding buffer 1 (PBS supplemented with 1.0 mM EDTA, 0.2% TX-100, and Complete protease inhibitor (Roche Diagnostics, Alameda, CA), resuspended at a final concentration of 3.2 μM, and mixed with either 10.0 μM of a purified MBP fusion protein (see Figures 1B and 2, A and B) or with a range of concentrations from 0.05 to 200 μM (see Figure 2C) in a final volume of 300 μl. Samples were incubated overnight at 4°C with agitation, washed four times in binding buffer 1, and eluted with a solution of 10.0 mM glutathione in Tris-HCL (pH 8.0), 150 mM NaCl. Samples were diluted fourfold in sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Bound proteins were detected by Western blotting with an antisera against MBP (1:10,000 dilution; New England Biolabs), a secondary anti-rabbit conjugated to horseradish peroxidase (1:5000; Amersham/GE Healthcare, Waukesha, WI), and enhanced chemiluminescence (ECL; Amersham/GE Healthcare). Each purified MBP fusion protein was also used to generate a standard curve to confirm the linearity of staining and to extrapolate the amount of protein cosedimenting with each GST fusion protein. X-ray films were digitized and the amount bound was determined as previously described (Fanning *et al.*, 2002).

To measure the interaction of His-tagged ZNG, ZNU5, and ZNU6 with occludin, GST fusion protein encoding occludin C-terminal amino acids 413-522 was immobilized on glutathione-agarose and was resuspended at 0.8 μM in binding buffer 2 (PBS with 1.0% TX-100, 10.0% glycerol and 5.0 mM β-mercaptoethanol) and mixed with 0.025–1.6 μM purified ZNG, ZNU5, or ZNU6 in a final volume 0.5 ml. As a negative control, 0.8 μM of each ZO-1 polypeptide was incubated with 0.8 μM of a GST-occludin construct containing a point mutation that abrogates ZO-1 binding (hDoc 433; Li *et al.*, 2005). All samples were incubated at 4°C for 3.0 h, washed three times in binding buffer 2 and once in PBS and were resuspended in 100 μl of gel sample buffer. Samples were resolved by SDS-PAGE, stained with Coomassie brilliant blue, and scanned with the Odyssey Infrared Imagining System (Licor Biosciences, Lincoln, NE). In saturation binding assays 0.16, 0.8, and 4.0 μg of ZNG and ZNU6 were resolved on the same gel to provide internal standards. Note that the assay was repeated three times with essentially the same results; this includes one assay in which ZNG/ZNU6 were immobilized on glutathione-agarose, and GST-hDoc was in the soluble fraction.

Immunolocalization and Image Acquisition

To image the subcellular localization of GFP transgenes, cells were plated on glass coverslips at a low density (~1.0×10^4 cells/ml) and cultured for 2 d before processing or until cells had just reached confluence. Cells were washed twice in PBS+ (supplemented with 1.8 mM calcium chloride), fixed in freshly prepared 4.0% paraformaldehyde diluted in PBS for 25 min at room temperature followed by two washes with PBS. After the PBS wash, cells were permeabilized in a solution of 0.2% TX-100 in PBS for 15 min, washed three times in PBS, and incubated with a solution of 1.0% bovine serum albumin, 5.0% of normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS to block nonspecific binding. Cells were then incubated for 2 h with a 1:100 dilution of the rat anti-ZO1 hybridoma supernatant (R40.76) followed by four 10-min washes in PBS. This was repeated with a 1:1000 dilution of a Cy3-conjugated donkey anti-rat secondary antibody, and cells were mounted in Mowiol (Calbiochem, San Diego, CA) with 1.0% *n*-propyl gallate.

To image the subcellular localization of myc-tagged transgenes, MDCK II or MDCK II tet off cells (obtained from Keith Mostov, UCSF) were plated on either glass coverslips (see Figures 5 and 8) or 12-mm diameter Transwell-Clear filter inserts (Costar, Cambridge, MA; see Figure 6) at a density of 1.0×10^4 cells/ml and incubated in the presence or absence of doxycycline for the indicated number of days. Cells were then either processed as above, using 1.0% paraformaldehyde (Figures 5 and 6), or fixed for 30 min in ethanol on ice. After fixation, cells were incubated with an antibody against the c-myc epitope and another relevant antibody indicated in the figure legends. Antibody dilutions used are listed above. In rare cases, an anti-ZO-1 serum was substituted for the anti-myc antibody to better visualize ectopic strands.

Wide-field images were acquired on a Nikon E800 microscope (Melville, NY) using 60× or 100× Plan Apo lenses and an Orca ER cooled CCD camera controlled with the Metamorph Imaging software package (version 6.0; Universal Imaging, West Chester, PA). Filter sets and dye combinations have been previously described (Fanning *et al.*, 2002). Confocal images were acquired on a Zeiss LSM510 Meta using a 100× Plan Apo lens (Thornwood, NY). Confocal Stacks and image projections were generated with Zeiss LSM Image Browser version 3.2. Contrast adjustment and montages were generated using Adobe Photoshop (version 6.0; San Jose, CA).

Freeze Fracture Electron Microscopy

Freeze fracture electron microscopy was performed as previously described (Medina *et al.*, 2000).

RESULTS

The SH3 and GUK Domains of ZO-1 Form an Intramolecular Complex

All MAGUK proteins contain a core motif of three conserved protein-binding domains that include a PDZ, SH3, and GUK domain, which are linked together by a series of unique regions (Figure 1A). ZO proteins are distinct because in addition to the unique region that links the SH3 and GUK domains, which we designate as the unique-5 (U5) domain, they also contain a C-terminal region that varies from 145 to 944 amino acids. Although most of this region is highly divergent among the ZO MAGUKs, there is a short region of homology immediately adjacent to the GUK domain, which we designate the unique-6 (U6) motif (Supplementary Figure 2). Despite the presence of these unique sequence elements relative to other MAGUKs, structural modeling of ZO-1 onto the known structure of PSD-95 (McGee *et al.*, 2001; Tavares *et al.*, 2001) suggests that intramolecular interaction between the SH3 and GUK domains might also be conserved in the ZO MAGUKS (data not shown). We tested this interaction directly using yeast two-hybrid analysis and fusion protein binding assays.

Surprisingly, our initial yeast two-hybrid analysis indicated that neither the GUK domain alone (ZG4) or a combination of the U5 + GUK domain (ZUG) were able to bind to the SH3 domain (ZS1) in a yeast two-hybrid assay (Figure 1B). Instead, we found that both the GUK domain and flanking C-terminal amino acid residues (ZUGC) were required for binding to the SH3 domain. Further analysis indicated that amino acids 627-890 (ZGU) encoded the minimal region that could bind to ZS1. This region includes both the GUK domain (aa 642-801) and the adjacent C-terminal residues between aa 804-888, which we refer to as U6. This domain is conserved in ZO-1, 2, and 3 and *Drosophila* Polychaetoid (Supplementary Figure 2). A construct lacking the first third of the GUK domain (ZΔGC) also failed to bind to ZS1, indicating that an intact GUK domain is required for binding to the SH3 and that the U6 motif alone is incapable of mediating this interaction.

We attempted to confirm these binding interactions in vitro using recombinant proteins purified from *Escherichia coli* (Figure 1C). In these assays, however, we found that the U6 motif was not required for binding to the SH3 domain; both the U5+GUK domain (ZUG) and the U5+GUK+U6 domain (ZUGC) were able to bind the SH3 (ZS1) domain. We conclude that the U6 motif is not always required for GUK binding to the SH3 domain. Furthermore, in both the yeast two-hybrid analysis and fusion protein binding assays neither the SH3 domain construct ZS1 (Figure 1, B and C) nor the GUK domain constructs ZUG and ZUGC (data not shown) were able to bind to a construct encoding the entire SG module (SH3+U5+GUK+U6 domain, also known as ZSGC). These results suggest that, as in PSD-95, the intramolecular (*cis*) interaction between the SH3 and the GUK domain is favored over an intermolecular (*trans*) interaction between different domains. Together, these observations support the hypothesis that ZO-1, like other MAGUKS, is capable of forming an intramolecular hairpin and that U6, which is unique to the ZO-MAGUKs, may also be part of this interacting module of domains.

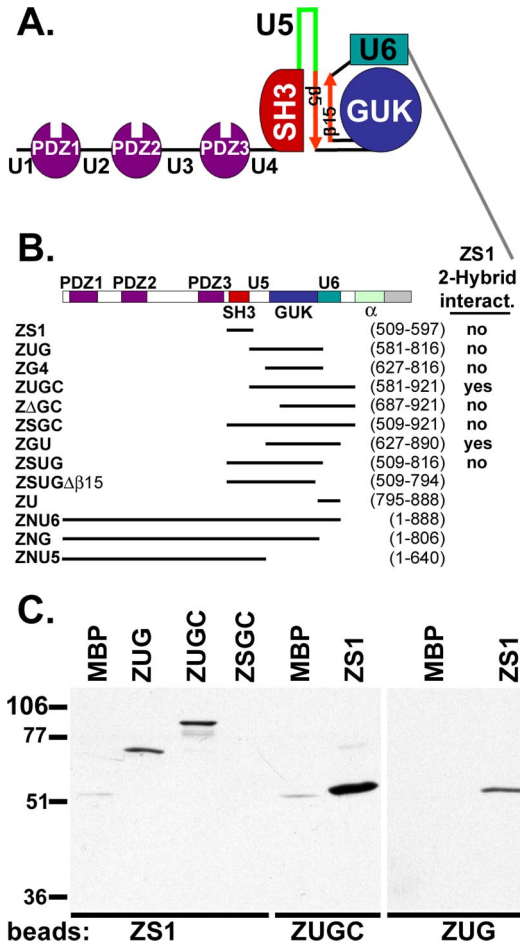

Figure 1. The SH3 and GUK domain interaction is conserved in ZO MAGUKs. (A) Model of ZO-1 structure. The SH3 domain packs against the GUK domain and is stabilized, in part, by an interaction between beta strands β5 and β15 that flank the GUK domain. The PDZ, SH3, and GUK domains are separated by Unique (U) regions that have no discernable sequence homology to other MAGUK proteins. The U5 and U6 regions pack closely against the SH3 and GUK domains and constitute functional motifs that are conserved among ZO MAGUKs. This model is based on the crystal structure of PSD-95 core motif, and the diagram is adapted from McGee *et al.* (2001). (B) Diagram of ZO-1 inserts used for yeast two-hybrid and fusion protein–binding assays. Construct names are acronyms for the domains included (S, SH3; U, U5 or U6; G, GUK; C, C-terminal aa 816-921; N, N-terminal aa 1-597; ΔG, deletion of aa 643-687 in GUK domain; Δβ15, deletion of aa 794-816 in GUK domain) and are listed in linear order from N- to C- terminus of the recombinant polypeptide. The amino acid residues incorporated into each construct are indicated in parentheses. The results of yeast two-hybrid assays between these constructs and the SH3 domain of ZO-1 (ZS1) are indicated in the right-hand column; "yes" indicates a robust interaction, whereas "no" indicates a failure to interact as determined by colony growth on selective media as described in Supplementary Methods. (C) Fusion protein binding assays between the SH3 domain (ZS1) and fusion proteins encoding the U5+GUK (ZUG), U5+GUK+U6 (ZUGC), or SH3+U5+GUK+U6 (ZSGC) domains of ZO-1. GST fusion proteins were immobilized on glutathione-agarose and mixed with bacterial cell lysates containing the indicated MBP fusion proteins. Binding was assessed by Western blotting with an antisera against MBP.

The U6 Domain Regulates the Binding of Occludin to ZO-1

It is known that the formation of a stable intramolecular complex between the SH3 and the GUK domains inhibits multimerization and/or ligand binding to the GUK domain in PSD-95 and SAP-97 (Fujita *et al.*, 2000; Schneeberger *et al.*, 2004). Because multimerization between ZO MAGUKs is regulated by interactions between the second PDZ domains (Fanning *et al.*, 1998; Utepbergenov *et al.*, 2006), we focused

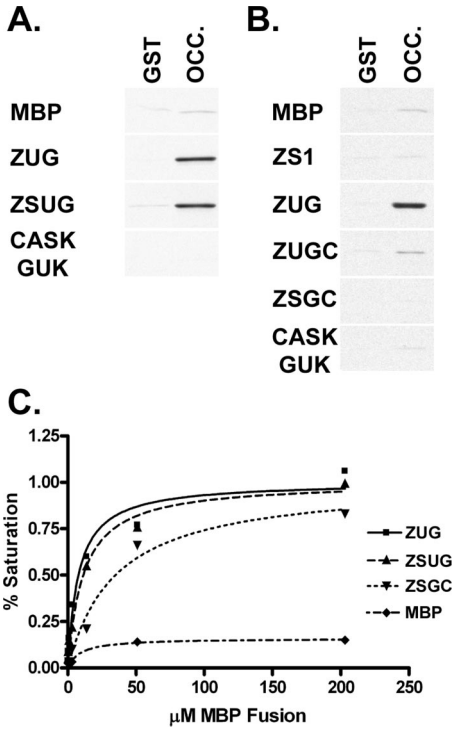

Figure 2. The U6 motif inhibits the binding of occludin to the SH3-GUK motif in ZO-1. (A and B) GST and GST-occludin (aa 377-521) were purified from bacteria, immobilized on glutathione-agarose, and mixed with purified MBP fusion proteins at a 1:1 M ratio. Binding of MBP fusion proteins to GST occludin was detected by Western blotting with an anti-MBP antisera. (C) Saturation binding assays were performed by incubating increasing concentrations (0.1–200.0 μM) of purified MBP fusion proteins encoding the U5+GUK (ZUG), SH3+U5+GUK (ZSUG), and SH3+U5+GUK+U6 (ZSGC) domains and GST-occludin (3.0 μM) immobilized on agarose. Binding was detected by Western blotting as above, and curves were plotted by nonlinear regression as described in *Materials and Methods.* Note that ZSGC, containing the U6 domain, has a 3–5-fold lower affinity for occludin (K_d = 34.7 ± 5.7) than ZUG or ZSUG (K_d = 11.1 ± 2.9 and 7.7 ± 2.5 μM, respectively).

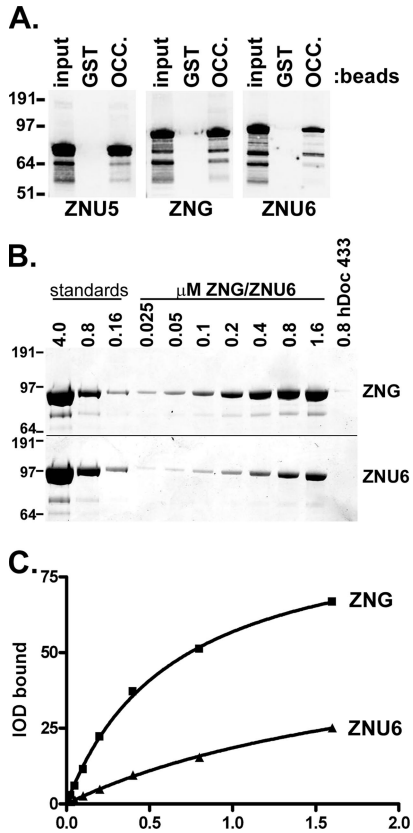

Figure 3. The U6 motif inhibits binding of occludin to the U5 motif of ZO-1. (A) Occludin binds to the U5 motif of ZO-1. GST and GST-occludin (0.8 μM) were immobilized on glutathione-agarose and mixed with equimolar concentration of purified ZO-1 polypeptides ZNU5 (aa 1-640), ZNG (aa 1-806), and ZNU6 (aa 1-888). The bound polypeptides were resolved by SDS-PAGE, transferred to nitrocellulose, and stained with an antibody against ZO-1. A stoichiometric amount of ZO-1 polypeptide (input) was resolved to determine efficacy of binding. (B) Saturation binding of GST-occludin with the His-tagged ZO-1 polypeptides ZNG and ZNU6. The purified ZO-1 polypeptides (0.025–1.6 μM) were incubated with 0.8 μM GST-occludin immobilized on agarose. As a negative control, both ZO-1 polypeptides (0.8 μM) were mixed with a GST fusion encoding a mutated occludin polypeptide (hDoc 433) that does not bind to ZO-1. The bound polypeptides were analyzed as above. Known concentrations of ZNG and ZNU6 (0.16, 0.8, and 4.0 standards) were loaded on the same gel to confirm the linearity of Coomassie binding. (C) Binding curves from B (above) were plotted using nonlinear regression analysis of the scanned Coomassie gel and are representative of three different experiments. Note again that binding of ZNU6 to occludin (K_d = 2.25 ± 0.19 μM) is ~3.5-fold lower than that of ZNG (K_d = 0.65 ± 0.04 μM).

on whether SH3-GUK interactions might regulate binding of ZO-1 to the transmembrane protein occludin, a known ligand for the ZO-1 GUK region (Fanning *et al.*, 1998). Using in vitro binding assays, we found that a construct encoding either the U5+GUK domains (ZUG) or the SH3+U5+GUK (ZSUG) domains were both capable of binding to a purified GST-occludin fusion protein encoding amino acids 377-522 of the cytosolic tail (Figure 2A). Saturation binding analysis indicted that the affinities of ZUG (K_d = 11.1 ± 2.9 × 10^{-6} M) and ZSUG (K_d = 7.7 ± 2.5 × 10^{-6} M) for occludin were essentially identical (Figure 2C). In contrast, neither the SH3 domain (ZS1) nor the GUK domain from another MAGUK, CASK, demonstrated significant binding to occludin (Figure 2B). These observations indicate that, unlike several other MAGUKs, the formation of a stable intramolecular complex between the SH3 and GUK domains does not necessarily inhibit binding of other proteins to this region.

However, the U6 motif had a dramatic inhibitory effect on the binding of occludin to the GUK region of ZO-1. Constructs that contain the U6 motif, such as ZUGC and ZSGC, were unable to bind effectively to occludin (Figure 2B). This is reflected in a 3–5-fold decrease in the affinity of ZSGC for GST-occludin calculated by saturation binding analysis (K_d = 34.7 ± 5.7 × 10^{-6} M; Figure 2C). These results indicate that although SH3-GUK interactions do not inhibit binding of occludin to this region, the presence of the U6 motif does either directly or indirectly decrease binding of occludin to the isolated U5-GUK elements of the SG module.

The results described above suggest that occludin binds to either the U5 (aa 581-529) or the GUK domain (aa 642-801). To resolve which of these domains is involved in occludin binding, we measured the in vitro affinity of GST-occludin for various HIS-tagged ZO-1 polypeptides purified from insect cells (Figure 3). We found that polypeptides encompassing the entire N-terminus up to and including the U5 motif (ZNU6: aa 1-640) or the GUK domain (ZNG; aa 1-801) bound effectively to GST-occludin. In contrast, a longer fragment that included the U6 motif (ZNU6; aa 1-888) bound

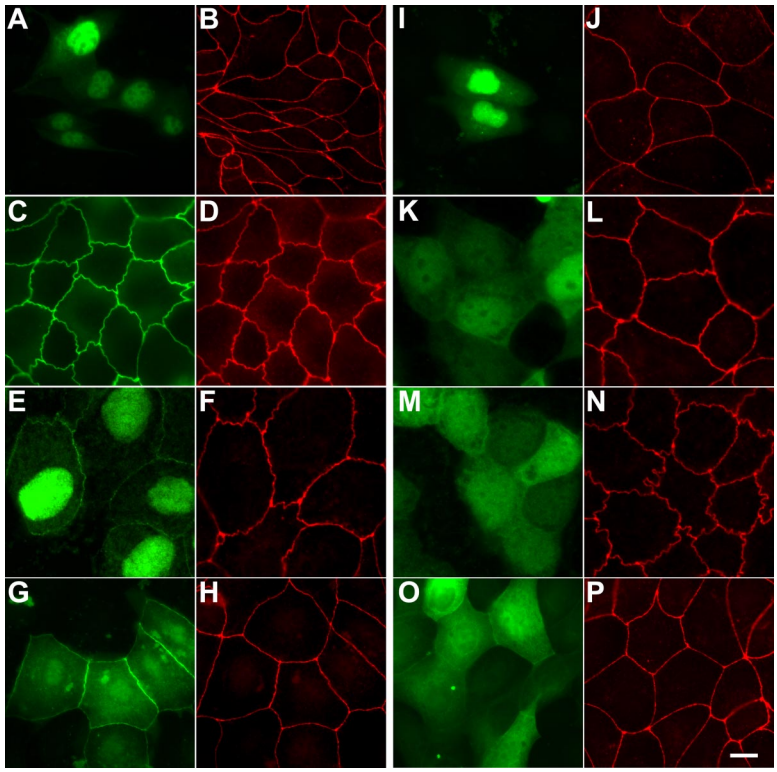

Figure 4. The U6 motif inhibits tight junction localization of GFP fusion proteins containing the SH3-GUK module. Stable MDCK II cell lines expressing GFP fusion proteins encoding GFP alone (A and B), ZO1 FL (full-length ZO-1; C and D), ZUG (E and F), ZSUG (G and H), ZSUGΔβ15 (I and J), ZU (K and L), ZUGC (M and N), and ZSGC (O and P) were plated on glass coverslips and grown to confluence. Cells were fixed and stained with an antisera specific to the endogenous ZO-1. The distribution of the GFP fusion (green) and ZO-1 (red) were observed by wide-field microscopy. Bar, 10 μm.

with lower affinity, consistent with the inhibitory role observed in binding studies using fusion proteins (Figure 3A). These results, in conjunction with previous studies (Fanning *et al.*, 1998), indicate that the U5 motif alone is sufficient for binding to occludin in the context of the larger N-terminus and that this interaction is antagonized by the presence of the U6 motif.

We further examined the effect of the U6 motif on occludin binding by directly measuring the affinity of ZNG and ZNU6 for occludin by saturation binding assays (Figure 3B). The ZNG polypeptide, which lacks the U6 motif, had a noticeably higher affinity for occludin (K_d = 0.65 ± 0.04 × 10^{-6} M) than did ZNU6 (K_d = 2.25 ± 0.17 × 10^{-6} M). Neither protein interacted with a GST-occludin protein containing a point mutation in the ZO-1 binding domain (hDOC433; Li *et al.*, 2005). Furthermore, although the affinities of the insect cell–derived proteins for occludin are about 10-fold higher than the GST constructs, perhaps reflecting more efficient folding or processing of the insect cell produced protein, the magnitude of the inhibition (3–4-fold) by the U6 motif is almost identical. These observations suggest that at least one function of the U6 motif may be to regulate the interaction of ZO-1 with other TJ proteins.

The U6 Motif Can Inhibit TJ Localization of the SH3-GUK Module In Vivo

Our previous examination of the localization of C-terminal deletion constructs indicated that a region of ZO-1 encoding the U5+GUK+U6 domains (aa 584-888) was necessary for localization to the TJ (Fanning *et al.*, 1998). To determine which domain is sufficient to direct localization to the TJ and to determine if this activity might be regulated by the U6 motif, we created a panel of GFP-tagged constructs that mirrored those used in the binding assays above (Figure 1B). These constructs were introduced into MDCK II cells and their subcellular distribution was monitored relative to the endogenous ZO-1 (Figure 4). Although GFP alone is concentrated in the cytoplasm and nucleus (Figure 4, A and B), the GFP-U5+GUK construct (ZUG) was incorporated into points of cell–cell contact where it colocalized with endogenous ZO-1 (Figure 4, E and F). This localization was similar to that of a full-length GFP-tagged ZO-1 transgene (Figure 4, C and D), but of lower relative intensity. The ZSUG construct, encoding the SH3+U5+GUK, was incorporated in ZO-1 positive cell–cell contacts even more robustly than ZUG (Figure 4, G and H), suggesting perhaps that SH3-GUK hairpin formation facilitates localization to intercellular junctions. Consistent with this hypothesis, ZSUGΔβ15, which contains a GUK domain mutation that inhibits hairpin formation (Shin *et al.*, 2000; McGee *et al.*, 2001) failed to localize to cell–cell contacts (Figure 4, I and J), and neither the GUK nor the U5 motif alone localized to the TJ (data not shown). Taken together, these observations suggest that the combination of the U5 and GUK domains is the smallest structural unit that can direct localization to cell–cell contacts.

In contrast, a construct encoding only the U6 motif (GFP-ZU) remained in the cytosol (Figure 4, K and L), indicating that the U6 motif alone is not sufficient to mediate localization to cell–cell contacts. Furthermore, when this domain was added to either the U5+GUK domains (ZUGC; Figure 4, M and N) or the SH3+U5+GUK domains (ZSGC; Figure 4, O and P), these two GFP constructs no longer localized to cell–cell contacts. These observations suggests that the U6 motif may influence ZO-1 localization in vivo in polarized epithelial cells.

Expression of ZO-1 Constructs Lacking the U6 Motif Results in a Novel Pool of Laterally Displaced Tight Junction Strands Lacking Wild-Type ZO-1

To better understand the functional role of different domains within the SG module, we established myc-tagged trans-

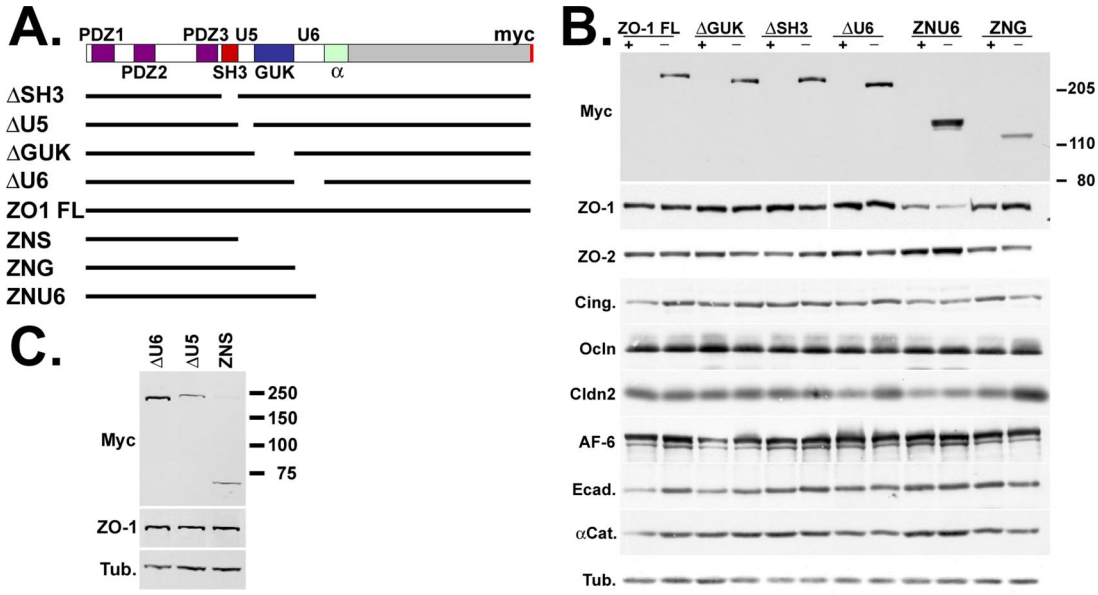

Figure 5. Inducible expression of ZO-1 transgenes in stable MDCK II tet-off cells lines. (A) Schematic diagram of ZO-1 transgenes subcloned into the pTRE expression vector. All transgenes are tagged at the C-terminus with a single myc epitope. The conserved PDZ, SH3, and GUK domains and the unique (U) regions separating these domains (U5 and U6) are indicated to scale. (B) Western blot of whole cell lysates from individual cell lines grown in the presence (+) or absence (−) of 40.0 ng/ml doxycycline and probed with antibodies against various tight junction and adherens junction proteins. Cing, cingulin; Ocln, occludin; Cldn2, claudin-2; Ecad, E-cadherin; αCat, α-catenin; Tub, β-tubulin. (C) Western blot of ΔU5 and ZNS expression in noninducible MDCK II clonal cell lines is compared with ΔU6 from above.

genes lacking the SH3, U5, GUK, or U6 domains (Figure 5A). These constructs were subcloned into either the eukaryotic expression vector pTRE, which is transcriptionally silent in the presence of doxycycline (Gossen *et al.*, 1995), or into the constitutively active expression vector pCB6. At least 3, and as many as 10, stable cell lines were isolated for each transgene, and protein expression was confirmed by immunoblotting (Figure 5, B and C) and immunocytochemistry (see below). Representative cells lines demonstrating roughly equivalent expression levels (±10% by scanning densitometry) were chosen for more extensive analysis, although it should be noted that ZNU6 expression levels were consistently much higher than other clones and that expression from the pCB6 transgenes was consistently low.

An analysis of protein in both induced and uninduced cells indicated that the myc-tagged transgenes were undetectable in cell lines incubated with doxycycline (Figure 5B) and that expression of the myc-tagged transgenes had little, if any, effect on the expression of endogenous ZO-1 or ZO-2. Similarly, there were few obvious changes in the steady state levels of other TJ proteins such as cingulin, occludin, claudin-2 (Figure 5B), and claudin-4 (not shown) or among adherens junctions proteins E-cadherin, AF-6, α-catenin (Figure 5B), and β-catenin (not shown). We also could not detect any changes in the detergent extractability (in 1.0% TX-100 or 1.0% NP-40 + 0.5% sodium deoxycholate) of the myc transgenes relative to endogenous ZO-1 polypeptide or in the extractability of the cadherin/catenin complex (data not shown). We did not examine these parameters in the ΔU5 and ZNS lines.

However, there were dramatic differences in the subcellular localization among several of the myc-tagged proteins. As in previous studies, we found that both a full-length ZO-1 (ZO-FL) and a construct composed of amino acids 1-888 (ZNU6) were effectively incorporated into the TJ in polarized MDCK II tet-off cells (Figure 6, A, B and C, D, respectively), whereas an N-terminal construct composed of amino acids 1-584 (ZNS) was restricted to the cytosol (Figure 6, E and F). These observations imply that the information required for ZO-1 localization to the TJ resides within the U5, GUK, and/or U6 domain(s). Deletion of the SH3 domain alone (Figure 6, I and J) or the GUK domain alone (Figure 6, K and L) has no apparent effect on TJ localization. In contrast, deletion of the U5 motif (Figure 6, G and H) eliminates localization to the TJ.

The distribution of constructs lacking the U6 motif was strikingly different from those lacking the other conserved domains. Full-length ZO-1 lacking the U6 motif (ΔU6; Figure 6, M and N) and an N-terminal construct consisting of amino acids 1-806 (ZNG; Figure 6, O and P) were distributed much more diffusely at the cell cortex relative to the endogenous ZO-1. At low magnification, this manifested as a thickened band of ZO-1 staining at the apex of the lateral cell borders, with occasional punctuate staining outside of the apical ring. At higher magnification (Figure 6, Q–T), these thickenings resolved into linear strands that looped and wandered several micrometers from the apical junctional complex. Some of these strands were also clearly disconnected from the apical junctional complex.

Analysis of projections reconstructed from confocal image stacks confirmed that these ectopic strands were not present in uninduced cells (Figure 7, A–C) and that the strands themselves were restricted within the lateral membrane domain of polarized cells expressing ΔU6 (Figure 7, D–F) or ZNG (Figure 7, G–I). The ectopic strands were seen in both stably and transiently transfected cells and were apparent as early as 5 h after plating of the cells (data not shown). They were also visible at very low levels of induction, whereas ZO1-FL and ZNU6 failed to generate ectopic strands at levels of expression at least 10-fold greater than ΔU6 or ZNG. Finally, identical ectopic strands were generated in transfected Caco-2, MCF-7, and NRK epithelial cells (data

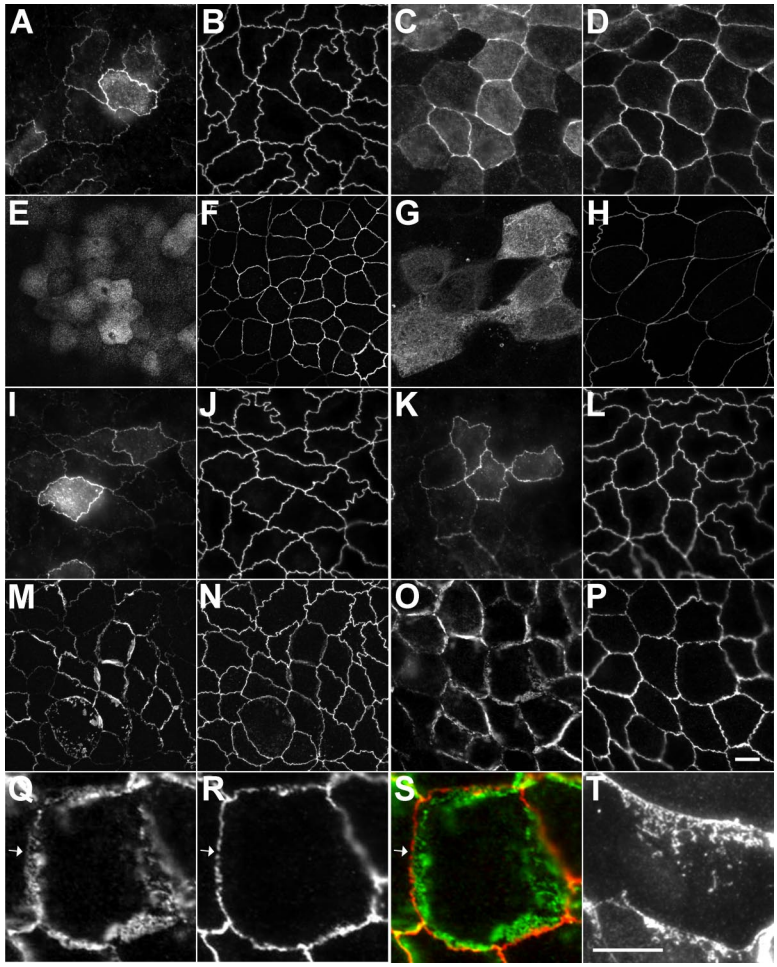

Figure 6. The U5 and U6 motifs are required for localization of ZO-1 to the apical tight junction. Cell lines expressing the myc-tagged transgenes ZO1-FL (A and B), ZNU6 (C and D), ZNS (E and F), ΔU5 (G and H), ΔSH3 (I and J), ΔGUK (K and L), ΔU6 (M and N), and ZN (O and P) were plated on glass coverslips and incubated in the absence of doxycycline for 4 d. Cells were then fixed and stained with an antisera against c-myc (A, C, E, G, I, K, M, O, Q, and T) and antibodies specific for the endogenous ZO-1 (B, D, F, H, J, L, N, P, and R). The center field in O and P is magnified 2.6-fold in Q and R, respectively, and are merged in S (green, myc; red, endogenous ZO-1). (T) Another high magnification image of the ectopic strands observed in ΔU6 cells. All images obtained by wide-field microscopy except E, F, G, H, M, and N, which were reconstructed from 5.0–8.0-μm-thick confocal stacks. Bar, 10.0 μm in A–P, and 4.0 μm in Q–T.

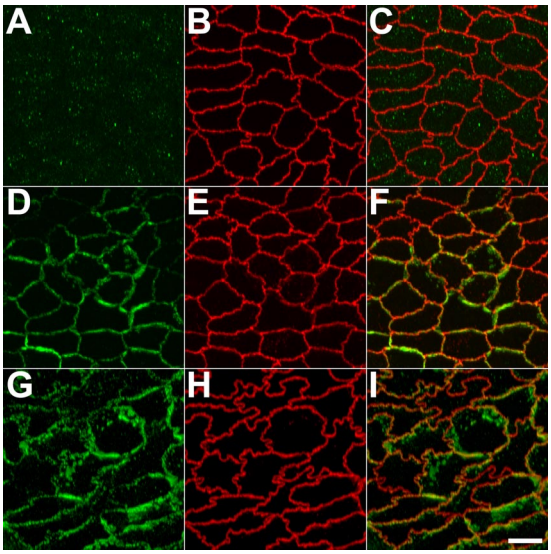

Figure 7. ZO-1 polypeptides lacking the U6 motif are concentrated within disorganized strands on the lateral surface of MDCK cells. MDCK II cells transfected with ΔU6 (A–F) or ZNG (G–H) were plated on filter inserts and grown in the presence (A–C) or absence (D–F and G–H) of doxycycline for 10 d. Cell were then fixed and stained with an antibody against c-myc (A, D, and G) and a sera specific for the endogenous ZO-1 (B, E, and H). The images shown are projections of 5.0 μm confocal stacks that have been rotated by 48°. Bar, 10 μm.

not shown), suggesting that this dominant phenotype is not a unique characteristic of a specific epithelial cell type.

Further analysis of the staining patterns reveals other interesting features. For example, in cells that express ΔU6 or ZNG there are often discontinuities in the normally linear pattern of staining of endogenous ZO-1 (see arrow, Figure 6, Q and R). These breaks are not observed in cells expressing ZO1-FL, ΔSH3, or ΔGUK. Furthermore, there is no apparent colocalization of the endogenous ZO-1 with the ectopic strands containing ZNG (Figure 6, Q–S), suggesting that interaction between the transgene and the endogenous ZO-1 is not required for the formation of the ectopic strands.

The alterations induced by ΔU6 and ZNG are also observed at the ultrastructural level by freeze-fracture electron microscopy. In mock-transfected (Figure 8A) and ΔU6-expressing MDCK cells grown in the presence of doxycycline (i.e., inhibited; Figure 8B) there is a characteristic distribution of continuous orthotopic strands. These usually have a depth of 1-3 strands with regular cross-links, as previously described. The same distribution was observed in cells expressing ZNU6 (Figure 8D) and ZO-1 FL (not shown), indicating that the presence of excess ZO-1 alone does not induce formation of additional strands. In contrast, cells expressing ΔU6 (Figure 8C) and ZNG (Figure 8, E and F) have a markedly expanded array of TJ strands. These appear as focal expansions of cross-linked strands that extend from the TJ, which can extend down the entire lateral membrane, but are most often less than a depth of 10–15 strands. We

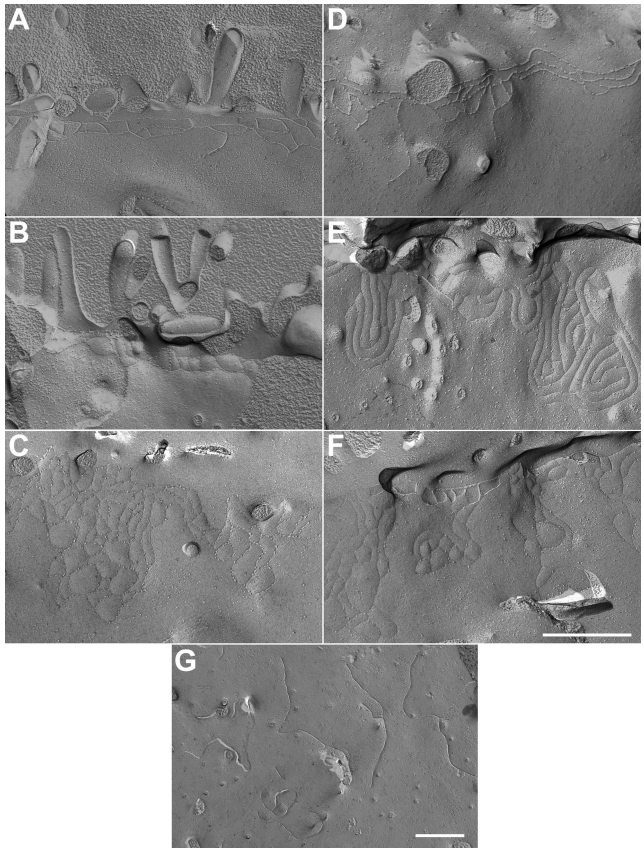

Figure 8. Cell expressing ΔU6 constructs show novel freeze fracture strands extending onto the lateral membrane. Stable MDCK cell lines were fixed and analyzed by freeze fracture electron microscopy. Cell lines expressing ΔU6 (C) or ZNG (E and F) show bizarre collections of tight junction fibrils that extend from the orthotopic junctional strands or that can be free-floating on the lateral surface (G), which were not present in cells transfected with vector alone (A), ZNU6 (D), or in ΔU6-transfected cells grown in the presence of DOX. Replicas A–F were imaged at 38,000× magnification and replica G at 21,000× magnification. Bar, 500 nm.

also observe patches of strands on the lateral membrane that are disconnected from the apical junctional complex (Figure 8G). Thus, expression of constructs lacking the U6 motif has a dramatic effect on the organization of TJ strands within the plasma membrane. Of interest, however, we note that although the region occupied by strands may be expanded, we rarely, if ever, see breaks in the most apical orthotopic strands. There is always at least one strand observed between the apical and lateral domains. Correlating the light and ultrastructural data suggests that the endogenous ZO-1 remains in the intact orthotopic TJ strands and the ectopic strands are associated with ZO-1 lacking U6.

ZO-1 Directs the Subcellular Localization of Tight Junction Transmembrane Proteins

To better understand the nature of the ectopic strands formed by the ΔU6 constructs, we used immunocytochemistry to determine whether other junction components are recruited into these novel structures. Interestingly, we found that many of the transmembrane proteins normally associated with TJs, such as occludin (Figure 9, A and B), claudin-2 (Figure 9, C and D), and JAM-1 (Figure 9, E and F) colocalized with the ZNG-myc transgene in ectopic strands. In contrast, all of the cytosolic TJ proteins examined were absent from the ectopic st4 rands. These include cingulin (Figure 9, G and H), ZO-2 (Figure 9, I and J), and α-catenin (Figure 9, K and L) as well as ZO-3, β-catenin, AF-6, PAR3 (data not shown) and E-cadherin (Supplementary Figure 3). Similar results were obtained in cells expressing the ΔU6 construct with the notable exception that in these cells cingulin was now recruited into the ectopic strands (Supplementary Figure 4). These observations suggest that the ZO-1 polypeptides displaced into the lateral plasma membrane are able to recruit their normal transmembrane ligands into this ectopic structure, but that with the exception of cingulin they are not able to recruit their normal cytosolic binding partners.

It is notable that we did not detect any reorganization of adherens junction proteins, either transmembrane or cytosolic, into these ectopic structures or outside of their normal apical location. Further, we saw no changes in the polarized distribution of other apical and lateral markers, such as gp135, Na-K ATPase, and ezrin (Supplementary Figure 3).

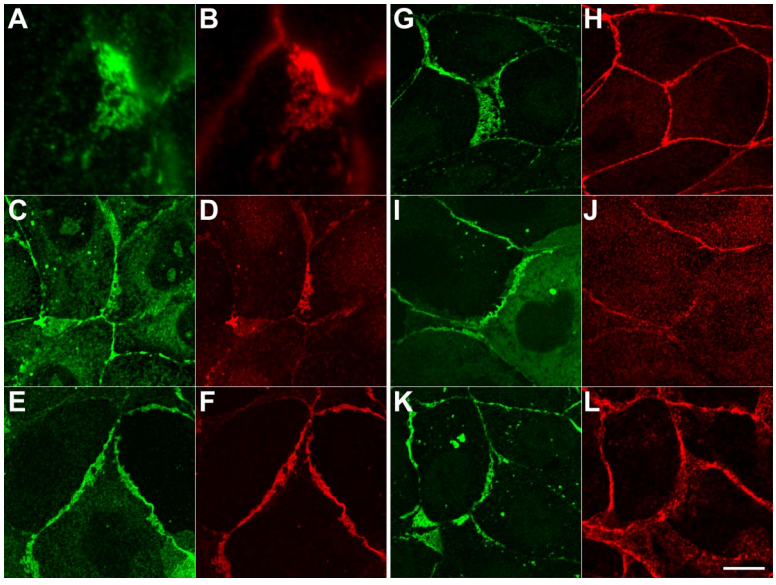

Figure 9. Transmembrane proteins of the tight junction, but not cytosolic proteins, are incorporated into the ectopic strands formed by ΔU6 constructs. MDCK cells expressing ΔU6 or ZNG were plated at low density in DOX-free media, fixed after 4 d and stained with antibodies against c-myc (B, D, F, H, J, and L) and antisera recognizing the transmembrane proteins occludin (A), claudin-2 (C), and JAM-A (E) or the cytosolic proteins cingulin (G), ZO-2 (I), and α-catenin (K). The images shown are maximum projections of 2.5–5.0-μm confocal stacks. Bar, 10 μm.

These observations indicate that the changes in TJ structure and transmembrane protein localization observed in ΔU6 cell lines do not result from a global change in cell polarity or membrane trafficking. There was also no observable effect on the physiological properties of MDCK II cells expressing any of these reported transgenes. Steady state transepithelial electrical resistance (TER; Supplementary Figure 5) and the flux of [^{3}H]mannitol were identical in cell lines grown in the presence or absence of doxycycline. Furthermore, we did not see any significant change in the redevelopment of TER after calcium depletion-repletion (also known as "calcium-switch" assay; Supplementary Figure 5). Thus, the dominant effect of U6 motif deletion on the distribution of TJ proteins is not translated into changes in overall cell physiology.

DISCUSSION

Extending the MAGUK Paradigm

Although there is considerable diversity in the molecular and cellular functions of MAGUK proteins, there is strong conservation of certain structural and functional paradigms. Most MAGUKs are associated with highly organized plasma membrane domains, such as synapses or cell–cell junctions, and most seem to serve as molecular scaffolds that regulate the assembly, location, and signaling functions of protein complexes. In addition, all MAGUKs contain the SH3-GUK (SG) module. The crystal structure of PSD-95 shows that in this module the SH3 and GUK are not independent domains, as generally supposed, but instead fold into an intramolecular hairpin in which individual structural elements are shared between the two domains (McGee *et al.,* 2001; Tavares *et al.,* 2001). This characteristic interfolded SG module appears to be a key regulatory region. For example, in some MAGUKS the "domain swapping" that results from a regulated exchange of intramolecular SH3-GUK interactions for intermolecular interactions promotes multimerization (Masuko *et al.,* 1999; Tsukita and Furuse, 2000b). In other MAGUKs, the intramolecular SH3-GUK interaction regulates binding of other proteins to the GUK domain (Wu *et al.,* 2000; Mehta *et al.,* 2001). Notably, in the ZO-1 null cells described by McNeil *et al.* (2006), both the SH3 and the GUK regions are specifically required for the rescue of normal assembly kinetics, supporting a critical role for these conserved domains in ZO-1 function.

Although all MAGUKS contain a U5 motif between the SH3 and GUK domains, most terminate after the GUK domain. The ZO MAGUKs are unique in having a conserved U6 region (Supplementary Figure 2) as well as long C-terminal sequences, which in ZO-1 bind protein 4.1 (Mattagajasingh *et al.,* 2000) and F-actin (Fanning *et al.,* 2002). In the *Drosophila* Polychaetoid protein the U6 motif is alternatively spliced; isoforms containing the U6 domain localize to cell junctions, whereas those without are distributed along the lateral cell membrane (Wei *et al.,* 2001). These observations suggest that the U6 domain has an important regulatory role. We propose that the U5 and U6 motifs are critical elements of the SG module. Although the U5 motif in ZO-1 (the region between the SH3 and GUK domains) shows no sequence homology among MAGUKs, it clearly shares a role in protein binding and subcellular localization that is common to all MAGUKs.

In the current study we find that the SH3-GUK intramolecular fold is conserved in ZO-1 and we begin to define functions for the unique motifs flanking this module. The U5 motif binds occludin and is required for TJ localization. C-terminal sequences flanking the GUK domain, the U6 motif, inhibit occludin binding in vitro, whereas in vivo its removal from full-length ZO-1 results in laterally displaced TJ strands. Our results point to important regulatory properties within the SG module of ZO-1 and its flanking effector motifs. In addition, the ability of truncated ZO-1 to direct ectopic placement of TJ strands provides direct evidence ZO-1 can determine the location of other TJ proteins.

The U5 Motif Mediates Localization of ZO-1 to the Tight Junction

The U5 motif within the SG module plays a role in the localization of several MAGUKs. Consistent with this, we find that the U5 motif is required for localization of ZO-1 to the TJ. Removing U5 from otherwise full-length ZO-1 produces a soluble protein exclusively located in the cytosol. In *Drosophila* DLG this region is referred to as the HOOK domain and is required for localization to neuromuscular junctions and epithelial septate junctions (Thomas *et al.,* 2000; Tsukita *et al.,* 2001), whereas in CASK this region is required for localization to the lateral plasma membrane of polarized epithelia (Lee *et al.,* 2002). In other MAGUKs the U5 motif is not as critical for membrane localization. For example, in PSD-95 a combination of N-terminal palmitoylation, the first two PDZ domains, and a C-terminal motif all contribute to localization (Craven *et al.,* 1999). In hdlg/SAP97 the N-terminus is both necessary and sufficient for localization to synapses and/or cell–cell junctions (Wu *et al.,* 1998; Hanada *et al.,* 2003). Nevertheless, in hdlg/SAP97 complex alternative splicing of the HOOK domain creates isoforms with distinctly different protein associations and subcellular distributions (Lue *et al.,* 1996; Wu *et al.,* 1998; Hanada *et al.,* 2003). Thus, U5/Hook motifs in many MAGUKs are either directly or indirectly involved in subcellular localization.

What Recruits ZO-1 to Tight Junctions?

Consistent with a previous report (Muller *et al.,* 2005), our results demonstrate that the U5 motif binds to the C-terminal tail of occludin. This does not directly prove that occludin recruits ZO-1 to the TJ; there are several possible ligands of the ZO-1 SG module. These include α-catenin (Muller *et al.,* 2005), the transcription factor ZONAB (Balda and Matter, 2000) and a serine-threonine kinase ZAK (Balda *et al.,* 1996), which bind to the SH3 domain. Our preliminary data suggest that cingulin may also bind within the SG module (Fanning, unpublished results). Whether any of these proteins recruits ZO-1 to the TJ is unclear. Deletion of the occludin and cingulin genes in mice has no effect on the localization of ZO-1 to TJs (Saitou *et al.,* 2000; Guillemot *et al.,* 2004), and cell culture studies suggest that ZONAB localization is itself dependent on ZO-1 (Balda and Matter, 2000). One speculation is that components of the polarity complex may be involved in the localization of ZO-1. However, we have to date been unable to detect an interaction of ZO-1 with PAR3, aPKCζ, or Pals-1 by immunoprecipitation (data not shown).

The U6 Motif Is a Negative Regulator: What Regulates the Regulator?

Our results suggest the U6 motif negatively regulates binding and localization functions of the U5 motif. Using in vitro fusion protein–binding assays, we observe that the U6 motif can inhibit binding of the SG module to occludin. This is true of both bacterially expressed proteins, which are limited to the SG module region, as well as much larger fragments expressed in insect cells, which include all three PDZ domains and the N-terminus. Similarly, when fragments of

ZO-1 containing only the SH3-U5-GUK-U6 regions are expressed in MDCK, we find that the U5 motif is required for TJ localization and that inclusion of the U6 motif inhibits localization. Why then do the endogenous ZO-1 and full-length transfected ZO-1, both of which contain the U6 motif, bind occludin and localize to TJs? We speculate the inhibitory activity of the U6 motif is somehow regulated in the cell, and this possibility is currently under investigation. Regulation might occur through binding another protein or phosphorylation. To date, we have been unable to identify proteins that bind to the U6 motif using yeast two-hybrid analyses. Furthermore, specific phosphorylation sites in ZO-1 have not been identified, although both tyrosine and serine phosphorylation of ZO proteins are associated with changes in ZO-1 localization and junction structure. However, the serine-threonine kinase ZAK binds to the SH3 domain and phosphorylates serine residues within the U5 motif (Balda *et al.*, 1996). This finding suggests a specific candidate for further analysis.

A region of homology to the U6 motif is found in all three ZO MAGUKs as well as the *Drosophila* homolog PYD (Supplementary Figure 2). In flies, alternative splicing of the U6 motif (also known as exon 6) determines whether the protein is located at adherens junctions or the lateral membrane (Wei *et al.*, 2001; see Supplementary Figure 2). We have been unable to identify splicing of U6 in mammals either in the databases or by RT-PCR, suggesting U6 function might be regulated posttranslationally. Other MAGUKs lack the U6 domain, and binding to the GUK domain appears to be regulated either by the physical interaction between the SH3 and GUK domains or by N-terminal sequences that bind directly to the SG module (Brenman *et al.*, 1998; Wu *et al.*, 2000; Mehta *et al.*, 2001; Sabio *et al.*, 2005). In our assays, the regulation of occludin binding and ZO-1 localization by the U6 motif does not require physical interaction between the SH3 and GUK domains. The U6 motif can inhibit binding of occludin to the U5 motif and localization of GFP transgenes even in the absence of the SH3 domain. Thus, the U6 motif appears to regulate occludin binding by a mechanism that is independent of SH3-GUK hairpin regulation. It remains to be determined whether this is true for the other ligands of the ZO-1 core motif.

ZO-1 Can Direct the Localization of Other TJ Proteins

ZO-1 is not normally present at the lateral plasma membrane. However, in most polarized epithelia, a significant fraction of the transmembrane proteins like occludin and claudins are normally resident in the lateral plasma membrane, although they are not organized into strands. We demonstrate that when the U6 motif is removed, the modified ZO-1 is found on the lateral plasma membrane where it is associated with strands of occludin, JAM-1, and claudins. Thus, ZO-1 can stabilize the assembly of TJ strands and do this even outside the context of the apical junction complex. Previous studies suggest that ZO-1 plays a role in enhancing the kinetics of junction assembly (Umeda *et al.*, 2004), perhaps via regulation of cytoskeletal dynamics (McNeil *et al.*, 2006), but that ZO-1 alone was not necessary for tight junction assembly. However, Umeda *et al.* (2006) have demonstrated that cultured cells with reduced levels of both ZO-1 and ZO-2 fail to form tight junctions and that reexpression of ZO-1 alone in these cells is sufficient to restore tight junction structure and the paracellular barrier. We believe that combined these observations strongly support the hypothesis that ZO-1 plays a direct scaffolding role in organizing proteins within the TJ.

What Causes Ectopic Stands?

One explanation for the ectopic strands is that without the U6 motif ZO-1 is not stably anchored at the TJ and migrates with associated strand proteins into the lateral plasma membrane. This seems unlikely, because our studies indicate that the U6 domain is not required or sufficient for junction localization. A second possibility that is more consistent with our biochemical results is that removal of the U6 domain creates a ZO-1 molecule in which the protein-binding properties of the SG module are constitutively activated. We speculate that the unregulated interaction of ZO-1 with its pool of ligands on the lateral membrane interferes with the normal incorporation of these proteins into the TJ. Furthermore, this interaction is still capable of stabilizing the assembly of TJ strands. Thus, we speculate that ZO-1 binding to transmembrane proteins must normally be both temporally and/or spatially regulated, or strands would form within any domain that contained these transmembrane proteins (as seen with the ΔU6 mutants). This hypothesis is consistent with the current model that tight junction assembly only occurs following recruitment of ZO-1 to adherens junctions. We speculate that after localization to the apical junctional complex, ZO-1 is activated to bind other tight junction proteins and that this initiates the organization of transmembrane proteins like claudins into the characteristic apical strands.

By drawing analogies with better understood MAGUKs, we have produced new insights into the function of ZO-1 and the key role played by the SG module and its flanking regulatory motifs. Future studies will be directed toward understanding spatial and temporal regulation of this module during junction assembly.

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