The Tight Junction Protein ZO-1 Establishes a Link between the Transmembrane Protein Occludin and the Actin Cytoskeleton

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The tight junction protein ZO-1 belongs to a family of multidomain proteins known as the membrane-associated guanylate kinase homologs (MAGUKs). ZO-1 has been demonstrated to interact with the transmembrane protein occludin, a second tight junction-specific MAGUK, ZO-2, and F-actin, although the nature and functional significance of these interactions is poorly understood. To further elucidate the role of ZO-1 within the epithelial tight junction, we have introduced epitope-tagged fragments of ZO-1 into cultured MDCK cells and identified domains critical for the interaction with ZO-2, occludin, and F-actin. A combination of in vitro and in vivo binding assays indicate that both ZO-2 and occludin interact with specific domains within the N-terminal (MAGUK-like) half of ZO-1, whereas the unique proline-rich C-terminal half of ZO-1 cosediments with F-actin. Consistent with these observations, we found that a construct encoding the N-terminal half of ZO-1 is specifically associated with tight junctions, whereas the unique C-terminal half of ZO-1 is distributed over the entire lateral surface of the plasma membrane and other actin-rich structures. In addition, we have identified a 244-amino acid domain within the N-terminal half of ZO-1, which is required for the stable incorporation of ZO-1 into the junctional complex of polarized MDCK cells. These observations suggest that one functional role of ZO-1 is to organize components of the tight junction and link them to the cortical actin cytoskeleton.

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The tight junction forms the apical barrier to the paracellular movement of water, solutes and immune cells in polarized epithelia (1). Electron micrographs reveal that the paracellular seal is composed of a series of highly ordered membrane contact sites (2), which in freeze-fracture micrographs can be visualized as a series of interconnected fibrils within the plasma membrane (3). Actin filaments terminate at these membrane contact sites (4, 5), suggesting that the cytoskeleton is involved in the structural and functional organization of the tight junction. Although many of the molecular components of the tight junction have now been identified, little is known about how they interact to regulate the assembly and permeability of the paracellular barrier.

At least one transmembrane component of the tight junction, occludin (6), has been demonstrated to contribute to the paracellular seal in cultured cells (7–10). More recently, investigators have identified two novel transmembrane proteins, claudin-1 and claudin-2, which are also presumably components of the paracellular seal (11). Both occludin and claudins localize to the discrete membrane contact sites, or kisses, within the freeze-fracture fibrils (6). These kisses are in turn intimately associated with the cytosolic plaque proteins ZO-1 (12), ZO-2 (13), and ZO-3 (14). These proteins can be co-immunoprecipitated as a complex from cultured epithelial cells (14, 15), and have been demonstrated to bind directly to the C-terminal 146 amino acids (aa) of occludin (16). Other components of the cytosolic plaque include cingulin (17), TH6 (18), AF-6 (19), and symplekin (20).

The plaque proteins ZO-1, ZO-2, and ZO-3 probably play a unique role in the organization/ regulation of tight junctions. These proteins are members of a family of membrane-associated signaling proteins known as the membrane-associated guanylate kinase homologs (MAGUKs), which include the Drosophila tumor suppressor dlg (21), the Caenorhabditis elegans signaling protein LIN2 (22), synaptic proteins hDlg and PSD95 (23–25), and the erythrocyte membrane protein p55 (26). Members of this family are distinguished by a core cassette of protein-binding domains, which include one or more PSD95/dlg/ZO-1 (PDZ) domains, an SH3 domain, and a region of homology to guanylate kinase (GuK) (27). All of these proteins are associated with cell-cell contact sites such as synapses, intercalated disks, and epithelial tight junctions. Mutations in the genes for Dlg and LIN2 cause severe alteration in cell growth and differentiation (21, 22), suggesting that MAGUKs are involved in signal transduction pathways controlling growth and differentiation (27–29).

The role of MAGUKs in signal transduction may be due in large part to the demonstrated ability of these proteins to organize protein complexes at the plasma membrane. There are several examples in which expression of a given MAGUK with its transmembrane binding partner results in clustering of both proteins within the plasma membrane (30, 31). This property appears to be due to the presence of multiple protein binding motifs within these proteins, as well as their ability to form heterodimers with other MAGUKs. This organizational capacity may also be enhanced by interactions with the cytoskeleton since hdlg, p55, and the human LIN2 homolog all bind to the actin-binding protein 4.1 via a conserved motif...
located between the SH3 and GUK domains (23, 32, 33). Many members of this family have been demonstrated to interact directly with ion channels, transmembrane receptors, and known cytosolic signal transduction proteins (34–36). These observations suggest that MAGUKs can act as a scaffold for signal transduction complexes, organizing cytosolic signaling molecules at the plasma membrane with transmembrane receptors and ion channels.

It is likely that the tight junction MAGUKs like ZO-1 have organizational/functional roles analogous to other MAGUKs. ZO-1 binds to several other tight junction components (13, 16, 37) and has also been demonstrated to interact with several known signaling proteins, such as the Ras substrate AF-6 (19), heterotrimeric G-proteins (38), an unidentified serine kinase (39), and connexin 43 (40). ZO-1 has also been shown to bind directly to F-actin in vitro (41). However, the mechanism of these interactions and their relevance to tight junction assembly is poorly understood. For example, it is not known whether the binding sites for other tight junction proteins reside within the MAGUK-like N-terminal half or within the C-terminal domain, which is unique to tight junction MAGUKs. In addition, although ZO-1 interacts with the cytoskeleton like other MAGUKs, it lacks the band 4.1 binding site found in other members of this family. To better understand the role of MAGUK proteins like ZO-1 in the tight junction, we have used epitope-tagged deletion constructs to map the binding sites for ZO-2, occludin, and F-actin on ZO-1. Furthermore, we have examined how these domains are involved in the assembly of ZO-1 into the tight junction complex. Our results suggest that ZO-1 may serve as a link between the proteins of the tight junction and the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Expression Constructs—A diagram of the expression constructs used in this study is shown in Fig. 1. To insert an epitope tag on the C terminus of the full-length human ZO-1 (ZO1myc), a 203-base pair fragment of the C terminus was amplified from a ZO-1 cDNA using the sense primer 5′-GAAGATGGTCAATAAGGACAG-3′, which is complimentary to a sequence 5′ of a unique MscI site in ZO-1, and the antisense primer 5′-GGACTAGTTTACAAGTCCTCTTCAGATATCAGCTTTTGC-3′, containing ZO-1, which had been digested with MscI and SpeI to remove the 203-base pair sequence encoding the native C terminus. All C-terminal deletion constructs were produced using a variation of this technique. N-terminal deletion constructs were produced by digesting ZO1myc with restriction enzymes that removed specific DNA sequences (for example amino acids 2–156 for del 2–156, and replacing those sequences with an annealed pair of complimentary oligonucleotides that reestablish the appropriate reading frame. Exact details of plasmid construction are available from the authors by request. The pSK (for example amino acids 2–156 for del 2–156), and replacing those

ZO-1 and ZO-2, two 60-mm dishes of transfected MDCK cells were plated into 100-mm dishes at confluent density, grown for 2 days, and incubated for 20 h with 5.0 mM sodium butyrate. For each construct, two dishes of cells were washed twice with PBS− and scraped on ice into 1.0 ml of a hypotonic carbonate buffer consisting of 10 mM sodium carbonate (pH 11.0), 1.0 mM MgCl2, 0.2 mM dithiothreitol, and protease inhibitors (a mixture of leupeptin, antipain, and aprotinin, each at 1.0 mM). The pH of the resulting supernatant was adjusted to 7.0 by the addition of 9.0 μl of 1.0 M HCl and 20 μl of 1.0 M Tris (pH 7.0) before being used in co-sedimentation assays.

To polymerize F-actin, F-actin stocks (Cytokeleton Inc., Denver, CO) were diluted to 2.5 mg/ml in binding buffer (10 mM imidazole, pH 7.0, 150 mM NaCl, 5.0 mM MgCl2, 0.2 mM dithiothreitol, and protease inhibitors described above) and incubated for 30 min on ice, and stabilized by adjusting to 25 μg/ml phallolidin (Molecular Probes, Eugene, OR). F-actin, at a final concentration of 22 μM, was mixed with 60 μl of the cell lysate in binding buffer in a final volume of 200 μl and incubated for 20 min at room temperature. Samples were subsequently spun at 100,000 × g for 20 min. Binding was tested in the presence or absence of 2.0 mM ATP (Boehringer Mannheim) and/or 5.0 μM myosin subfragment-I (S1) (Sigma) as indicated in the legend of Fig. 7. Gel samples were prepared as described previously (45), and examined by Western blotting with the 9E10 antisera.

Immunofluorescence—To analyze the distribution of the epitope-tagged proteins in immature and mature cell-cell contacts, clonal cell lines expressing these constructs were plated at 80% confluence (106 cells/ml) onto acid-washed 12-mm circular coverslips and incubated for 12 h with 5.0 mM sodium butyrate starting 24–72 h after plating, as indicated in figure legend. Alternatively, 106 cells were plated onto 12-mm Transwell filter inserts (Corning Costar Corp., Cambridge, MA), incubated for 10 days, and induced for 24 h with 5.0 mM butyrate. Coverslips or filter inserts were then washed briefly with PBS− and fixed in freshly prepared 1.0% p-formaldehyde for 20 min. Subsequently, coverslips were permeabilized for 30 min with a solution containing 0.2% (w/v) Triton X-100, 2.0% donkey serum in PBS, incubated with 2.0% donkey serum (Life Technologies, Inc.) in PBS for 60 min, and finally incubated for 2 h with an undiluted cell supernatant from the 9E10 hybridoma supplemented with Texas Red-conjugated phallolidin (Molecular Probes, 1:40 dilution), a rat monoclonal antibody against ZO-1 (RA40/76 cell supernatant, 1:10 dilution), or a rabbit polyclonal
against human occludin (Zymed Laboratories Inc. Laboratories, South San Francisco, CA). Following incubation with the appropriate secondary antibody (Jackson Immunoresearch, West Grove, PA), coverslips were mounted on glass slides (Corning) in Vectashield antifade solution (Vector Laboratories, Burlingame, CA). Slides were viewed on a Nikon Microphot FX microscope using a 60× PlanApo lens and photographed using Kodak TMAX 400 film. Alternatively, slides were viewed using a Bio-Rad MRC1024 confocal microscope on a Zeiss Axiovert using a 63× PlanApo lens. Film images were digitized using a Sprintscan slide scanner (Polaroid, Cambridge, MA). Figures were assembled using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

RESULTS

ZO-1 can be divided into an N-terminal half, which constitutes the region of homology to other members of the MAGUK family, and a unique proline-rich C-terminal half. Each of these halves contains several previously identified protein domains whose specific roles are unknown in ZO-1 (46). To address the functional role of these domains in protein binding and tight junction assembly, we created a panel of myc epitope-tagged expression constructs which encode overlapping fragments of the ZO-1 isoform (Fig. 1; see "Experimental Procedures") and introduced these constructs into cultured MDCK epithelial cells.

The Second PDZ Domain of ZO-1 Mediates the Interaction with ZO-2—One of the first molecular interactions to be defined at the tight junction was that between ZO-1 and ZO-2. These proteins can be communoprecipitated from epithelial cell extracts (Fig. 2) under relatively stringent conditions, suggesting a tight and possibly direct interaction (13, 15). However, if ZO-1 does bind directly to ZO-2, then the striking homology between these two proteins (53% identity between human ZO-1 and ZO-2) suggests that these proteins might also form homodimers in vivo. To address these questions and further elucidate the interaction between ZO-1 and ZO-2, the epitope-tagged fusion proteins were communoprecipitated from lysates of transfected MDCK cells. The immunoprecipitates were then resolved by SDS-PAGE and analyzed by Western blotting with antibodies recognizing the endogenous canine ZO-1 and ZO-2 to identify which constructs coprecipitated either of these proteins (Fig. 2). We found that all of the constructs used expressed a polypeptide of the appropriate apparent molecular weight (Fig. 2, myc). In addition, the full-length epitope-tagged construct, ZO1myc, was targeted to the tight junction (discussed below), indicating that the myc epitope does not interfere with these properties of ZO-1.

Analysis of the epitope tag immunoprecipitates with an antibody (R40.76) that recognizes only the endogenous canine ZO-1 isoform (Fig. 2, ZO-1), and not the transfected human polypeptide, indicates that the endogenous protein does not communoprecipitate with the full-length epitope-tagged ZO-1 construct under these conditions, nor does it interact with any of the deletion constructs. This result strongly suggests that ZO-1 does not form homodimers in vivo, but instead forms a simple “αβ” heterodimer with ZO-2. When the same blots were probed with a polyclonal antiserum against ZO-2, we found that only deletion constructs which still contained the second PDZ domain of ZO-1 (PDZ2) could communoprecipitate the endogenous ZO-2 polypeptide (Fig. 2, ZO-2). Furthermore, a construct in which only PDZ2 was specifically deleted (del 159–252) also fails to coprecipitate ZO-2. These results, summarized in Fig. 1, indicate that the PDZ2 domain mediates the interaction with ZO-2.

Identification of an Occludin-binding Site within Amino Acids 633–876 of ZO-1—Pan et al. (16) have identified a ZO-1 binding site within the C-terminal aa 358–504 of chicken occludin, and have established that this domain binds directly to the full-length ZO-1 polypeptide. To identify the reciprocal occludin binding site on ZO-1, we performed binding assays between the epitope-tagged ZO-1 constructs and a GST fusion protein encoding this 146-aa domain. Total lysates from MDCK cells transfected with the epitope-tagged cDNA constructs (Fig. 3A, total) were incubated with the immobilized GST-occludin (GST-occ) fusion protein, and the myc-tagged proteins bound to

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The GST fusion protein were identified by Western blotting with the anti-myc antisera (Fig. 3A, bound). The relative amount of protein binding to GST-occ was assessed by comparing the amount of myc-tagged protein in the bound fraction to that in the total lysate or unbound fraction. In all cases, the expression of deletion constructs was identical to or greater than that of the full-length construct ZO1myc.

An initial comparison of tagged constructs encoding the N- and C-terminal halves of ZO-1 (z1–876 and del 67–1033, respectively) indicates that only the N-terminal half of ZO-1 (z1–876), the C-terminal half of ZO-1 (del 67–1033), or transfected with the vector alone (pCB6) are compared with the fraction of the lysate that is bound to GST-occ (bound). Note that the full-length and N-terminal half of ZO-1 interact with GST-occ, but that the C-terminal half fails to bind. Panel B shows the bound proteins from a survey of all of the epitope-tagged ZO-1 constructs. In each case expression was confirmed either by examination of the total cell lysate or by Western blotting of the unbound fractions and determined to be equivalent to that seen with the full-length protein.

The GST fusion protein were identified by Western blotting with the anti-myc antisera (Fig. 3A, bound). The relative amount of protein binding to GST-occ was assessed by comparing the amount of myc-tagged protein in the bound fraction to that in the total lysate or unbound fraction. In all cases, the expression of deletion constructs was identical to or greater than that of the full-length construct ZO1myc.

An initial comparison of tagged constructs encoding the N- and C-terminal halves of ZO-1 (z1–876 and del 67–1033, respectively) indicates that only the N-terminal half of ZO-1 associates with the GST-occ fusion protein (Fig. 3A). Progressive C-terminal deletions beyond aa 876 severely diminish the ability to associate with GST-occ (Fig. 3B). For example, no binding is detected with the constructs z1–412, z1–549, and z1–572. These observations suggest that sequences within the 305-aa domain between aa 572 and 876 mediate a strong interaction with occludin (Fig. 1). The binding site can be further refined from the observation that the construct del 294–633 also binds to the GST-occ fusion protein. Interactions with PDZ1 (del 2–156), PDZ2 (del 159–252, del 1–245), PDZ3 (del 294–633), and the SH3 domain (del 550–603) do not appear to be required for binding to occludin (Fig. 3). These results suggest that the primary occludin binding site resides within the 244-aa domain between aa 633 and 876 (Fig. 1).

The N-terminal MAGUK-like Half of ZO-1 Associates with Tight Junctions When Expressed in MDCK Cells—To determine the contribution of the N-terminal half, which interacts with ZO-2 and occludin, and the unique C-terminal half of ZO-1 to tight junction localization of ZO-1 in MDCK cells, we examined the subcellular localization of the epitope-tagged constructs encoding these domains by indirect immunofluorescence. Cells were transiently transfected with the full-length epitope-tagged ZO-1 (ZO1myc), a construct encoding the N-terminal half of ZO-1 (z1–876), or a construct encoding the C-terminal half of ZO-1 (del 67–1033) and were double-labeled with antibodies against occludin and the myc epitope (myc). A scanning laser confocal microscope was used to collect sequential 0.36-μm-thick face-on (top) or vertical (bottom) sections. Note that both the full-length and N-terminal constructs colocalize with occludin at the tight junction, whereas the C-terminal construct is distributed uniformly along the lateral plasma membrane. The horizontal field size is 87.5 μm.
terminal construct appeared less focused at the membrane, and was often observed to accumulate in other actin-rich structures such as apical microvilli and within discrete puncta on the basal surface (data not shown).

**N-terminal Amino Acids 633–896 Are Required for Stable Association with the Tight Junction**—To better understand how different domains within the N-terminal half of ZO-1 direct the assembly of ZO-1 into the tight junction, we looked at the distribution of epitope-tagged constructs at different stages during the assembly of tight junctions. MDCK cells stably transfected with each construct were dissociated by treatment in PBS-EDTA, trypsinized, and replated on glass coverslips at a 80% confluent density. The expression of the epitope-tagged construct was then induced either 24 or 72 h after plating by the addition of sodium butyrate into the culture media. At 24 h, cells are still subconfluent, relatively flat, and have not yet developed a barrier to ions. In contrast, by 72 h cells appear columnar, densely packed, and have developed a transepithelial electrical resistance characteristic of this line of MDCK cells (110–130 ohms × cm²). Thus, we define junctions or cell-cell contacts at the 24- and 72-h time points as functionally immature and mature, respectively. We have observed that the myc-tagged constructs used here are fortuitously not expressed in stable lines in the absence of sodium butyrate (data not shown). Thus, the expression of a construct can be induced at different points during the assembly of the tight junction.

There is a dramatic difference in the distribution of different myc-tagged constructs in immature (24 h) and mature (72 h) cell-cell contacts. In both immature and mature cell-cell contacts, the distribution of the full-length ZO-1 and z1–876 are indistinguishable. Both are tightly associated with the plasma membrane at sites of cell-cell contact (Fig. 5) and colocalize with the endogenous occludin (Fig. 4). The constructs z1–412 and z1–572, which are progressively truncated from the C terminus, are also targeted to cell-cell contacts in subconfluent cells (Fig. 5), although the pattern of staining was often more discontinuous and less intense than with the full-length construct. However, neither of these proteins were incorporated to any significant extent into mature tight junctions. These results suggest that aa 573–876 are required for stable incorporation of the N-terminal half of ZO-1 into the tight junction. Significantly, this region contains a membrane-associated signaling proteins, binds to the transmembrane protein occludin. The construct del 159–252 is associated with both immature and mature cell-cell contacts, suggesting that interactions with ZO-2 mediated by the PDZ2 domain are not required for targeting to cell-cell contacts.

The construct z1–193, which includes the PDZ1 domain, is not incorporated into either immature or mature cell-cell contacts, but instead has a distinct nuclear distribution. A faint but distinct nuclear localization of the full-length and N-terminal construct z1–876 was also detected in immature cell-cell contacts (Fig. 5). The physiological relevance of nuclear localization is unclear, although nuclear localization of the endogenous ZO-1 has been previously reported under similar conditions of low cell density (47).

**The Unique C-terminal Half of ZO-1 Associates with F-actin Structures at the Plasma Membrane**—The distribution of the C-terminal construct is markedly different from that of z1–876 or the full-length construct. As previously noted, in mature cell-cell contacts the C-terminal construct is uniformly distributed along the lateral plasma membrane (Fig. 4), and does not concentrate within the tight junction. Del 67–1035 is also concentrated in lamellar structures that penetrate beneath adjacent cells (Fig. 5), and within punctate or linear strands that colocalize with the focal adhesion protein FAK (data not shown). The distinction between N- and C-terminal constructs is even more dramatic at earlier times during the assembly of tight junctions. After only 12 h in culture, the full-length and N-terminal construct z1–876 are only found in a continuous band at points of cell-cell contact (Fig. 6). The distribution of these two constructs at cell-cell contacts was much more punctate and diffuse than that found in more mature monolayers (Fig. 5) and was indistinguishable from that of adherens junction protein α-catenin (data not shown). In contrast, the C-terminal construct was distributed along actin stress fibers at points where they terminated at the basal plasma membrane, and appeared to be only weakly associated with the plasma membrane at cell-cell contacts (Fig. 6).

In rare instances of very high level expression, the full-length construct Z01myc could also be found in basal lamellae, focal contacts, and stress fibers, as well as sites of cell-cell contacts. Such a pattern was never observed with z1–876. These results suggest that the C-terminal half of ZO-1 mediates interactions with the actin cytoskeleton at the plasma membrane while the N-terminal half, which binds to both ZO-2 and occludin, mediates the specific localization of ZO-1 to tight junctions.

**The C-terminal Half of ZO-1 Associates with F-actin in Vitro**—The lateral plasma membrane, basal lamellae, and cell-cell contacts sites are rich in F-actin. The co-distribution of the C-terminal construct with stress fibers and several different compartments within the actin cytoskeleton suggests that ZO-1 may cross-link components of the tight junction like occludin and ZO-2 with the cytoskeleton via its C-terminal domain. This would be consistent with the recent observations of Itoh et al. (41), which demonstrated that a baculovirus-produced fragment of the C-terminal half bound directly to F-actin. We confirmed and extended these results. Cell lysates from transfected cells were incubated with F-actin, and the ability of the myc-tagged constructs to cosediment with actin was examined by immunoblotting (Fig. 7A). In the absence of F-actin, little if any of the myc-tagged protein is found in the pellet. However, addition of F-actin results in a significant shift of both the full-length ZO-1 and the C-terminal construct into the pellet fraction. In contrast, there is no detectable sedimentation of the N-terminal construct. These results confirm that ZO-1 interacts with F-actin through its C-terminal domain. Interestingly, addition of skeletal muscle myosin S1 at equimolar concentrations with actin results in a loss of ZO-1 from the pellet fraction. The addition of ATP, which effectively removes myosin from F-actin, results in the reattachment of ZO-1 to F-actin. ATP alone has no effect on the binding of ZO-1 to F-actin, and the addition of myosin S1, ATP, and cell lysate have no effect on the polymerization of F-actin (Fig. 7B). These results suggest that ZO-1 binds specifically to F-actin in an ATP-independent manner, and suggest that the binding site on F-actin might overlap with that of myosins.

**DISCUSSION**

In the present study, we have identified domains in the tight junction protein ZO-1 that mediate interactions with ZO-2, occludin, and F-actin. The N-terminal half of ZO-1, which is structurally similar to other members of the MAGUK family of membrane-associated signaling proteins, binds to the tight junction proteins occludin and ZO-2, while the interaction with the actin cytoskeleton is mediated by the unique proline-rich C-terminal half. Furthermore, we have identified a 244-aa domain within the N-terminal half of ZO-1, which is required for the stable association of ZO-1 with the tight junction and which includes the occludin-binding site. Thus, the N-terminal half of ZO-1 has a functional role analogous to other MAGUK proteins organizing transmembrane and cytosolic components at specialized cell-cell contact sites. Unlike other MAGUKs,
ZO-1 has a unique C-terminal domain that mediates direct interaction with actin. We suggest that ZO-1 forms a functional link between the paracellular seal and the actin cytoskeleton.

The N-terminal MAGUK-like Half of ZO-1 Mediates the Interactions with ZO-2 and Occludin—The nature of the interaction of ZO-1 with ZO-2 and occludin suggests that ZO-1 has a role in the organization of protein complexes analogous to that of other MAGUKs. Using coprecipitation assays, we found that ZO-2 specifically interacts with the second PDZ domain of ZO-1. PDZ domains are a conserved motif found in a diverse array of proteins, and have been shown to mediate protein-protein interactions at the plasma membrane (48, 49). The majority of the PDZ domains examined to date bind to consensus motifs located at the C terminus of transmembrane proteins (50, 51), although several PDZ domains also interact directly with other PDZ domains (31, 36). Since ZO-2 does not have a C-terminal sequence resembling any of the known consensus motifs, we predict that ZO-1 might bind directly to one of the three PDZ domains in ZO-2. The ability to coimmunoprecipitate ZO-1 and ZO-2 under harsh conditions without stoichiometric coprecipitation of another protein supports the idea of a direct interaction between these two proteins (13, 15), and several other MAGUKs have been demonstrated to form heterodimers by direct interaction between PDZ domains (31).

However, we have been unable to confirm the interaction between ZO-1 and any of the three PDZ domains in ZO-2 using...
yeast two-hybrid, blot overlay, or fusion protein binding assays
(data not shown). These in vitro experiments are difficult to interpret because of an inability to confirm proper folding and post translational processing of the polypeptides used. Nevertheless, they raise the possibility that the interaction between ZO-1 and ZO-2 is mediated by a third protein.

Occludin also interacts with the N-terminal half of ZO-1, but unlike other MAGUKs the interaction with this transmembrane protein is not mediated by a PDZ domain. Instead, our binding assays indicate that there is a strong interaction between GST-occ and the 244-aa domain between aa 633 and 876 of ZO-1. This region contains both the GUK domain (the region of homology to guanylate kinase) and an acidic domain of unknown function. Several proteins have recently been identified that bind to the GUK region of other MAGUKs, although none of these proteins shows any homology to occludin (52, 53). Both domains are also conserved in ZO-2 (63% identity) and ZO-3 (43% identity), suggesting that these proteins may also bind directly to occludin. Indeed, ZO-3 has recently been demonstrated to bind directly to the cytosolic tail of occludin (37), although the nature of the binding site has not been determined. However, the interaction between ZO-1 and occludin does not require ZO-2, since the deletion of the PDZ2 domain does not interfere with the ability of ZO-1 to bind occludin.

**Differential Localization of ZO-1 Constructs Supports a Multistep Assembly of Tight Junctions—**The localization of ZO-1 to the plasma membrane coincides with the formation of cell-cell contacts (54, 55), although development of a measurable paracellular barrier sometimes lags behind this initial association by days. Previous localization studies in MTD-1A cells have demonstrated that ZO-1 is initially assembled into actin-rich cadherin based cell-cell contacts, and subsequently segregated into a distinct apical compartment as cells became polarized (56). Thus, the assembly of tight junctions is presumably a multistep process that may involve distinct protein-protein interactions over time. At the earliest time that we can detect ZO-1 at the plasma membrane (12–24 h; see Figs. 5 and 6), it is diffusely distributed at points of cell-cell contact, sometimes focused within discrete puncta. By 72 h after plating, when an electrically resistive barrier has formed, the distribution appears as a tight band at the apical junction complex (Fig. 5). Although any construct containing aa 194–876 is found in immature cell-cell contacts (z1–412, z1–572, and z1–876; see Fig. 1), only constructs containing 573–876 are localized to the mature tight junction in polarized epithelial cells (z1–876, Fig. 1).

### Figure 7

**A** myosin S1:  
- - - + +  
ATP: - - + - +  
Actin: - - + + +  
ZO1myc: Sup Pel Sup Pel Sup Pel Sup Pel Sup Pel lystate  
z1-876: Sup Pel Sup Pel Sup Pel Sup Pel Sup Pel  
d67-1033: Sup Pel Sup Pel Sup Pel Sup Pel Sup Pel  

**B** myosin S1:  
- - - + +  
ATP: - - - - +  
lysat: - + + + +  
Actin: + + + + +  

The C-terminal half of ZO-1 mediates the interaction with F-actin. A, cell lysates from clonal lines expressing either the full-length (ZO1myc), N-terminal half (z1–876), or C-terminal half (d67–1033) of ZO-1 were mixed with F-actin and tested for their ability to co-sediment in the presence (+) and/or absence (−) of ATP and myosin subfragment-1 (myosin S1) as described in methods. The soluble (Sup) and actin-bound (Pel) fractions were resolved by SDS-PAGE and the epitope-tagged proteins detected by Western blotting with the anti-myc antibody 9E10. B, Coomassie-stained gel of the same assay. Note that the amount of F-actin pelleting is unaffected by the addition of lysate, myosin S1, or ATP.
plasma membrane during different stages of assembly might be mediated by distinct protein-protein interactions.

The specific interactions that mediate association with immature versus mature cell-cell contacts is still unresolved. One model suggests that junction formation is initiated by cadherin engagement, and that ZO-1 is recruited to new cell-cell contacts by interacting with a cadherin-binding protein like α- or β-catenin (41, 56–58). Both of these proteins have the potential to interact with ZO-1, and target to the plasma membrane at sites of cell-cell contact (41, 57). In addition, the distribution of both ZO1myc and z1–876 is indistinguishable from that of α catenin in very early cell-cell contacts (data not shown). Thus, the domain between aa 194 and 572, which is required for incorporation into early cell-cell contacts, may in fact interact with one of the catenins. The sequences that are required for association with mature cell-cell contacts (aa 573–876) include the 244-aa domain that binds to occludin, raising the possibility that association with occludin is required for the stable assembly of ZO-1 into mature tight junctions. However, ZO-1 distribution appears normal in epithelia generated from mice lacking occludin (59), suggesting that interaction with occludin is not absolutely required for assembly of ZO-1 into the tight junction. Understanding assembly will thus ultimately require the further resolution of these two domains and the identification of the proteins which interact with them.

**ZO-1 Links Actin Filaments to Proteins of the Tight Junction.**—The present study demonstrates that ZO-1 not only binds to F-actin in vitro, as previously reported (41), but also associates specifically with actin filaments at the plasma membrane of cultured MDCK cells in vivo. The C-terminal half of ZO-1, when expressed in subconfluent cells, preferentially targets to actin filaments that terminate at the plasma membrane in focal contacts, cell-cell contacts, microvilli, and within lamellae. This is similar to the observations of Howarth et al. (60, 61) and Yonemura et al. (56), who found that ZO-1 localizes with a subset of actin filaments at cell-cell contacts and in lamellae of cultured astrocytes and transformed fibroblasts. In contrast, Itoh et al. (41) have reported that a construct encoding the C-terminal half of ZO-1 is uniformly distributed along stress fibers in cultured fibroblasts. This may be due in part to a difference in cell types, expression levels, or the exact nature of the constructs used. In our own studies in the NRK fibroblast line, we find that our C-terminal construct concentrates primarily within the sites of focal and cell-cell contact (data not shown). These results taken together suggest that ZO-1 may have some mechanism which promotes association with a specific subset of actin filaments at the membrane that is independent of interactions with ZO-2, occludin, or α-catenin. The precise location of the actin-binding motif within the C-terminal half of ZO-1 is currently under investigation.

It is not clear to what extent interactions with F-actin are involved in assembly of ZO-1 into the tight junction, or if the cytoskeleton has a general role in the assembly of the tight junction. The N-terminal construct, which lacks ability to bind to F-actin, is still efficiently incorporated into both immature and mature cell-cell contacts. However, there is considerable evidence suggesting that the perijunctional actin is involved in the maintenance or assembly of the paracellular seal (62, 63). Signaling pathways that affect the organization of perijunctional actin (64–67) often modify tight junction permeability (63). For example, activation of small GTP-binding proteins of the Rho family triggers a marked reorganization of perijunctional actin and increase in paracellular permeability (65, 68). In addition, treatment of cultured epithelial cells with cytotoxic lasins causes a decrease in the size and complexity of tight junction fibrils (69–72). However, as most of these interventions cause widespread alterations of the cytoskeleton, the results are difficult to attribute to specific affects on tight junctions.

Several recent studies suggest that actomyosin contraction is also directly involved in the physiological regulation of paracellular permeability. The tight junction is adjacent to a perijunctional ring of actin that is capable of circumferential contraction both in vitro and in vivo (5, 73). Studies in both isolated tissues and cultured cells have demonstrated that activation of the sodium glucose transporter leads to cytoskeletal contraction and a concurrent increase in paracellular permeability (5, 74, 75). In cultured cells this has been shown to occur in concert with the phosphorylation of myosin light chain, which is known to activate nonmuscle myosin contractility (76). In addition, Hecht et al. (77) have shown that the introduction of a constitutively activated myosin light chain kinase into cultured cells results in a contraction of the cortical cytoskeleton, a decrease in transepithelial electrical resistance, and increased paracellular flux. These observations have led to the idea that paracellular permeability is directly regulated by cortical tension generated by actomyosin contraction.

The results of this study are consistent with the idea that ZO-1 has an organizational role analogous to that of other MAGUKs. For example, ZO-1 binds to both the transmembrane protein occludin and the peripheral membrane protein ZO-2, suggesting that it may be involved in the organization of these proteins at the tight junction. This role is supported by the recent observation that ZO-1 is involved in the targeting and incorporation of occludin into tight junctions.2 Like other MAGUKs, ZO-1 also has the potential to act as a scaffold for the organization of signal transduction proteins (19, 38–40). Finally, ZO-1 binds to F-actin (41), suggesting that it may mediate the anchoring or assembly of actin filaments at the tight junction. To date neither ZO-2, ZO-3, nor occludin have been demonstrated to interact directly with F-actin. The unique C-terminal regions of ZO-2 and ZO-3 show little similarity to ZO-1, and neither protein contains the conserved protein 4.1 binding motif found in other MAGUKs. Thus, it is possible that actin-binding is limited to ZO-1, and that ZO-1 may have the unique potential among tight junction MAGUKs to organize both structural and signaling components of the paracellular seal.

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