Mini-Review

The Molecular Organization of Tight Junctions

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It is seven years since the identification of ZO-1, the first protein localized at tight junctions (TJs)[1] (60). Two years later, a second TJ-associated protein, cingulin, was characterized (9). More recently, two additional proteins, ZO-2 (26) and 7H6 (68), have been added to the list. All these proteins belong to the cytoplasmic "plaque" domain of TJs, based on morphological and/or biochemical criteria. In epithelial polarized cells TJs (*zonulae occludentes*) (for reviews see references 23, 56) represent the apical element of a tripartite junctional complex, including the *zonula adhaerens* and the desmosome (16).

In this issue, Tsukitas's group (32) reports the cloning of a 220-kD protein, originally identified at the undercoat of cadherin-containing cell–cell contact sites, in nonepithelial cells (31) and its characterization as the mouse homologue of ZO-1. The occurrence of ZO-1/220-kD protein in nonepithelial tissues (see also reference 29) casts doubt on its specificity as a TJ-exclusive marker and emphasizes the importance of classifying junctions based on their functional properties, composition and morphology. The interaction of ZO-1/220-kD protein with spectrin (31) and its colocalization with cadherins (31, 32) raises new questions about the roles of these proteins in TJ assembly and function. This mini-review will discuss these new perspectives, in the context of past efforts and recent progress in understanding the organization of epithelial TJs.

Tight Junctions and Epithelia: A Structure–Function Relationship

Why are TJs considered one of the most characteristic structural markers of the polarized epithelial phenotype? TJs provide a continuous seal around the apical region of the lateral membranes of adjoining epithelial cells, preventing the free passage of molecules and ions across the "paracellular" pathway, e.g., the extracellular space between the lateral membranes of neighboring cells. This "barrier" function of TJs distinguishes them from other types of junctions, and defines two distinct compartments of the extracellular space, the "luminal" (apical) and the "serosal" (basolateral) compartment, the latter in continuity with the interstitial fluids and, ultimately, the blood. The ability of TJs to act as permeability barrier is shown by using electron dense tracers and measuring fluxes of radiolabeled markers and ions across epithelial monolayers (7, 14).

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1. *Abbreviation used in this paper*: TJ, tight junction.

A second function attributed to TJs in epithelia is to separate apical and basolateral plasma membrane domains, which differ in protein and lipid composition and carry out specialized functions (reviewed in reference 54). TJs are thought to represent the "fence," preventing the free diffusion of lipids in the exoplasmic membrane leaflets and the intermixing of specific apical or basolateral membrane proteins (reviewed in reference 52). However, the precise role of TJs in establishing lipid and protein polarity of the plasma membrane is not clear, as shown by studies on epithelial cells (54) and by the observation that neurons, which lack intercellular TJs, display a functional "fence" to the free diffusion of lipids (35).

The Molecular Composition of TJs: the ZO-1/220-kD Protein

TJs were originally described in epithelial cells by transmission EM, as sites of apparent "fusion" between the exoplasmic leaflets of the plasma membrane (16). Freeze-fracture EM revealed that these sites correspond to a network of intramembranous fibrils on the P face of the fractured membrane and complementary grooves on the E face (21, 58). To account for the appearance of TJ fibrils and their sensitivity to protein fixatives, several models were debated, according to which the fibrils resulted from specific configurations of membrane lipids, proteins, or a combination thereof (27, 33, 50, 58, 63). However, the observation that lipids could not diffuse from one cell to the next via TJs (64) was not consistent with the hypothesis that TJ fibrils were made of lipids. In addition, protein synthesis inhibitors prevent TJ assembly in freshly trypsinized cells (22, 28, 51), and TJ fibrils are resistant to detergent extraction (59).

A breakthrough in the biochemical characterization of TJs was provided by the work of Stevenson and Goodenough, who used a mouse liver TJ-enriched membrane fraction (59) to obtain a specific mAb (60). This antibody was shown to stain the junctional complex of various rat and mouse epithelia and cultured MDCK (canine kidney) cells by immunofluorescence, and to label the TJ-enriched fraction by immuno EM (60). The antigen, denoted as ZO-1, showed a SDS-PAGE mobility corresponding to a polypeptide of 225 or 210 kD in mouse tissues and canine MDCK cells, respectively (1, 60).

In 1991 Itoh et al. (32) reported the characterization of a 220-kD protein at the undercoat of cadherin-based cell–cell adhesion sites, and now this protein is identified as the mouse homologue of ZO-1 (32). Cadherins are transmembrane proteins which were first identified in developing tissues (30,

34), and which mediate Ca^{2+}-dependent cell–cell adhesion in epithelial and nonepithelial cells (for reviews see 19, 62). In some cell types, cadherins are found concentrated at specialized junctions such as epithelial *zonulae adhaerentes* (5) and cardiac intercalated discs (20, 65). Other members of the cadherin family are found at desmosomes (13, 36, 42, 66). However, no cadherins have been detected in TJs so far.

Are the TJ-associated protein ZO-1 and the cadherin-associated 220-kD protein purified by Itoh et al. (31) the same protein? Itoh et al. showed by rotary-shadowing EM that the 220-kD antigen purified from rat brain appears like a spherical particle (diameter 25–50 nm) (31). On the other hand, Anderson et al. reported that ZO-1 is a monomer with an asymmetric shape, based on hydrodynamic analysis of ^{35}S-labeled ZO-1, immunoaffinity isolated from MDCK cells (1). This discrepancy may not be significant, since it may be due to the different techniques used, or to the aggregation of ZO-1 or its interaction with elongated proteins, in the samples analyzed by Anderson et al. (1). It is also possible that ZO-1 may exist in different forms of association (31). Probes for ZO-1 and 220-kD protein hybridize to transcripts of similar, if not identical size in epithelial and nonepithelial cells (2, 29, 32). The sequence identity between the partial amino acid sequence of rat ZO-1 (67) and the corresponding region in the mouse 220-kD protein is rather low (77.5%), considering that the two clones were obtained from related species. On the other hand, human and rat ZO-1, at least in the short region reported by Willott et al., are 78% identical (67).

In summary, the evidence from molecular studies, and the crossreactivity of anti-ZO-1 mAbs with the 220-kD fusion protein (32), strongly favors the idea that ZO-1 and the 220-kD protein are very closely related, if not identical. Determination of the complete sequence of ZO-1/220-kD protein cDNAs from human, rat, and other sources will probably confirm this idea, and will be useful to identify functional and conserved domains across species.

Other TJ Proteins in Nonepithelial Cells?

The notion that ZO-1/220-kD protein is not TJ exclusive is demonstrated by the observation that it also occurs in nonepithelial tissues, such as cardiac myocytes, fibroblasts (31), in astrocytes, Schwann cells, and nonepithelial tumors (29), and in epithelial structures devoid of typical TJs, such as the slit diaphragms of the glomerular podocytes (55). However, the quality of the antibody probes, the expression and/or availability of specific epitopes in different cell types, and the occurrence of isoforms may complicate the interpretation of the results obtained. For example, only one of the two spliced isoforms of ZO-1/220-kD protein is expressed in slit diaphragms (37).

Are other TJ proteins also localized in nonepithelial cells and tissues, or at least in cells lacking "classical" TJs?

Cingulin (140 kD) was localized in epithelial TJs, by immuno EM of intestinal cells and immunofluorescence of cultured cells and frozen tissue sections (9, 10). The biophysical properties of purified cingulin, its shape in the EM (9, 10) and its partial cDNA sequence (Citi, S., J. Kendrick-Jones, and D. Shore. 1990. *J. Cell Biol.* 111:409a) show that cingulin is a coiled-coil dimer. Cingulin is present in the junctional regions of epithelial cells, from chicken intestine, kidney, liver, pancreas, retina (10), human normal and neoplastic colon (12), guinea pig cochlea (53), and rat epididymis (6). Cingulin immunofluorescent labeling was detected in the nonkeratinizing, stratified epithelia of the chicken esophagus and cornea (10), but immunoblotting analysis failed to reveal cingulin in human epidermis and metastatic squamous carcinomas (12). Notably, these tissues are not known to contain continuous TJs. Cingulin staining is absent from chicken kidney glomeruli (10), and anti-cingulin antibodies do not stain the slit diaphragms of the glomerular epithelium (M. Farquhar, personal communication). Therefore ZO-1, but not cingulin is present in this type of epithelium. During mouse pre-implantation development cingulin reactivity is detected throughout oogenesis and early embryogenesis, unlike ZO-1, but similarly to uvomorulin/E-cadherin (17). Embryonic cingulin is assembled at junctional sites at a later stage with respect to ZO-1 (17, 18), probably reflecting sequential roles of the two proteins in the de novo formation of the TJ. Thus, direct comparison of cingulin and ZO-1/220-kD protein distribution during development and in glomerular podocytes has revealed some differences.

In nonepithelial tissues such as chicken smooth muscle and heart, clear cingulin labeling was detected only along capillary endothelial junctions (10). Cingulin labeling was absent from chicken lens cells, which are rich in gap junctions, and in vinculin- and A-CAM/N-cadherin–containing adherens junctions (10). Cingulin was not detected by immunoblot in human nonepithelial tumors (12). In conclusion, the present evidence indicates that in adult polarized epithelial cells both cingulin and ZO-1/220-kD protein are good TJ markers, but unlike ZO-1/220-kD protein, cingulin has not so far been detected in adherens junctions of fibroblasts and cardiac myocytes.

The available data for ZO-2 and the 7H6 antigen are not sufficient to draw conclusions on their exclusive association with TJs. ZO-2 was identified as a 160-kD protein found in ZO-1 immunoprecipitates (26). Recently antibodies have been developed against ZO-2, and immuno EM reveals that the protein is associated with TJs of isolated MDCK plasma membranes (L. Jesaitis and D. Goodenough, personal communication). 7H6 is a 155-kD protein which was recently localized by immuno EM in the cytoplasmic domain of rat liver TJs and by immunofluorescence in the epithelial junctions of liver, intestine, lung, and kidney (68). The distributions of ZO-2 and 7H6 in nonepithelial cells and tissues have not been determined.

A Model for the Architecture of Epithelial TJs

The diagram shown in Fig. 1 presents a hypothetical model for the organization of TJs. The nature of the intramembrane particles seen in freeze-fractured TJs in unknown, although it can be speculated that they are proteinaceous, and might form a selective channel for the passage of molecules through the paracellular pathway.

Measurements of the distance of the immunogold label from the plasma membrane in EM micrographs indicates that ZO-1/220-kD protein is the closest to the TJ membrane (15–25 nm) (61; see also reference 31), followed by 7H6 (40 nm) (68) and cingulin (40–60 nm) (9, 10, 61). Apart from the association of ZO-1 with ZO-2 (26), no other protein–protein interaction between TJ proteins has been demonstrated. Similarly, it is not known whether any TJ protein interacts with F-actin or with actin-binding proteins, al-

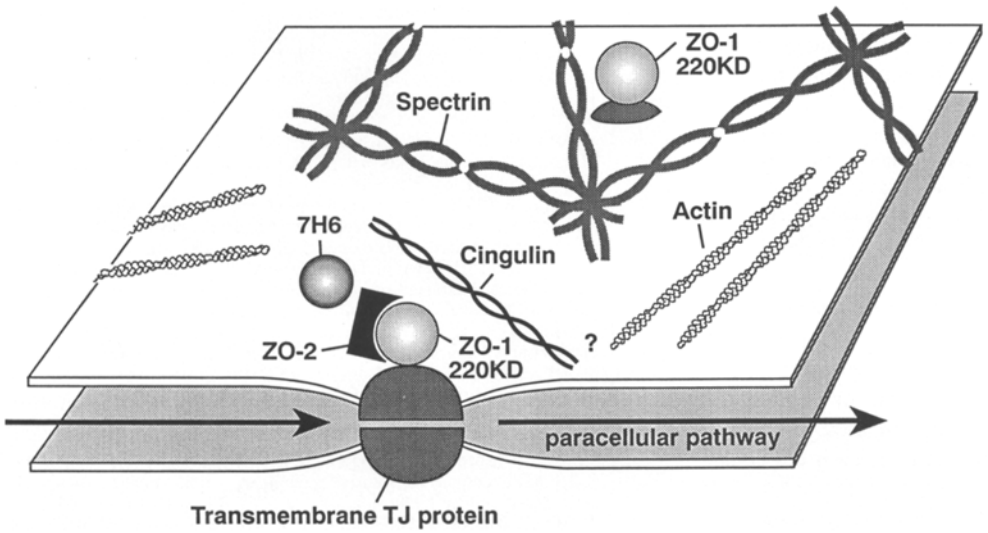

Figure 1. A schematic illustration of the organization of TJs in epithelial cells. The apical regions of the lateral membranes of adjoining cells are represented, with a putative transmembrane TJ protein located at the points of fusion. ZO-1 is depicted as a sphere, interacting with the cytoplasmic domain of the putative membrane TJ protein and ZO-2 (*bottom*), and with spectrin (*top*). Cingulin is depicted as a helical dimer, possibly interacting with actin-containing microfilaments (*?*). The shapes of ZO-2 and 7H6 are purely speculative, and the scale is approximate. Other membrane and cytoskeletal proteins are omitted for clarity. See text also.

though cingulin is structurally similar to the rod portions of myosins, and it was speculated that it may interact with the actin cytoskeleton (10). F-actin is localized within the filamentous material underlying the TJ membrane (15, 38), and its functional role in the maintenance of TJs is shown by several studies with microfilament-active drugs, such as cytochalasins (reviewed in reference 39).

Since spectrin tetramers bind to ZO-1/220 kD (31), spectrin could play a role in the architecture of TJs (Fig. 1). Interestingly, ZO-1/220-kD protein shows size and isoelectric focusing properties similar to ankyrin (4), although it is immunologically (31) and structurally distinct (32) from it. The role of spectrin and ankyrin in organizing the submembrane cytoskeleton in polarized epithelial cells (44, 46, 47) and the recent isolation of complexes containing Na^+,K^+-ATPase, ankyrin, fodrin(spectrin), and E-cadherin from MDCK cells (48) raises further questions. Does ZO-1/220 kD define a new class of spectrin-binding proteins, and what is its spatial relationship with spectrin/fodrin in situ? Could ZO-1 behave as ankyrin, and form complexes with E-cadherins, spectrin, and Na^+,K^+-ATPase?

Association of Cadherins with ZO-1/220-kD Protein

A key observation demonstrating the role of E-cadherin in the morphogenesis of epithelia was that transfection of nonepithelial cells with E-cadherin cDNAs induced them to show an epithelial morphology and adhere more tightly (43, 45). Itoh et al. (31) have now added an exciting new layer to this experimental model, by demonstrating that ZO-1/220-kD protein colocalizes with N-, P-, and E-cadherin when cotransfected into fibroblasts.

Cadherin-mediated cell–cell adhesion is believed to be critical in the establishment and maintenance of TJs and epithelial cell polarity. Antibodies against the extracellular domain of E-cadherin prevent the assembly of functional TJs and disrupt preexisting junctions (3, 24, 25). Transfection of fibroblasts with uvomorulin/E-cadherin induces redistribution of surface membrane Na^+-K^+ATPase (41). Removal of extracellular calcium, which disrupts the adhesive functions of cadherins (62), leads to the functional opening of TJs, the separation of ZO-1 and cingulin from the peripheral membrane (2, 8, 57), and the loss of cell polarity (23, 25).

Since ZO-1/220 kD was not localized at adhesion sites in transfected fibroblasts unless cadherins were also present, cadherins must play a role in promoting the assembly of ZO-1 in the adhesion sites. This observation raises intriguing questions.

First, does ZO-1 interact directly with the cytoplasmic domains of cadherins, or indirectly, for example through catenins (49)? Studies with purified proteins in vitro and characterization of immunoprecipitates from these fibroblasts should provide answers to these questions. Second, what is the role of ZO-1 in nonepithelial cells? The expression of ZO-1/220-kD protein in these cells (29, 31) is neither correlated with the presence of TJ intramembranous fibrils, nor with established "barrier" or "fence" functions. However ZO-1/220-kD protein might participate in the organization of the spectrin/fodrin cytoskeleton at cell–cell contacts (see above).

Third, and perhaps most important, why does ZO-1/220-kD protein so specifically associate with TJs in epithelial cells that also have cadherin-based adherens junctions? Itoh et al. suggest that TJs may contain an unidentified type of cadherin, with a higher affinity for ZO-1/220-kD protein than E-cadherin. A TJ cadherin might provide TJs with adhesion properties, and help bring ZO-1/220-kD molecules in register in the regions of cell–cell contact. A putative TJ cadherin may have an unconventional calcium sensitivity, since TJs persist in membrane vesicles in the absence of Ca^{2+} (59) and cell–cell contact areas with strong ZO-1 and cingulin labeling are observed in MDCK cells in the absence of extracellular calcium (2, 8, 57). If ZO-1/220-kD protein and cadherins are indirectly associated, the selective localization of ZO-1/220-kD protein in TJs may be due to its higher affinity for a cadherin-associated protein. A third possibility is that a variable domain in ZO-1/220-kD protein may define multiple isoforms of the protein, with different affinity constants for proteins of TJs and of cadherin-containing

adherens junctions. Finally, other proteins may "trap" ZO-1/220-kD protein in the TJ or competitively prevent the association between cadherin and ZO-1/220-kD protein at the *zonulae adhaerens*.

Clearly, more work is necessary to identify and characterize the transmembrane protein component(s) of TJs.

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