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Review

The epithelial tight junction: Structure, function and preliminary biochemical characterization

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Summary

The tight junction, or *zonula occludens* (ZO), forms a semi-permeable barrier in the paracellular pathway in most vertebrate epithelia. The ZO is the apical-most member of a series of intercellular junctions, collectively known as the junctional complex, found at the interface of the apical and lateral cell surface. This structure not only restricts movement of substances around the cells, but may also serve as a 'fence' acting to maintain the cell surface compositional polarity characteristic of epithelial cells. The morphology and physiology of the ZO have been well documented and are briefly reviewed here. The biochemistry of this important intercellular junction remains largely unknown, although a tight junction-specific polypeptide called 'ZO-1' has recently been identified. Preliminary observations regarding the role of this peripheral phosphoprotein in the biology of the ZO are presented.

Introduction

An epithelium is a continuous sheet of cells standing at the interface of, and acting to separate, two biologically distinct compartments. The cells which make up an epithelium come in a variety of shapes, are arranged in a number of configurations, and are classified according to these characteristics.

One of the most striking attributes of epithelial cells is that they are polarized. The apical cell surface faces the lumen, usually the external environment, and is sometimes specialized for interacting with this compartment. The basal cell surface faces the serosal or internal compartment and sits on a specialized layer of extracellular matrix proteins collectively known as the basal lamina. Finally, epithelial cells have lateral cell surfaces which are specialized for interacting with adjacent cells.

As a result of their location, epithelial cells mediate the vectorial exchange of substances between the apical and basal compartments. There are two pathways through which this exchange can take place. Materials can traverse the cell plasma membranes and cytoplasm through the processes of passive or active transport, or substances can go around the cells through the paracellular pathway. There obviously is a need for a permeability barrier in this pathway; otherwise, there would be a free exchange of elements between the two compartments, defeating the purpose of the epithelium.

At the apical end of the lateral epithelial cells surface is a series of intercellular junctions collectively

known as the junctional complex [1] (Fig. 1). The basal-most member of this complex is usually the desmosome, a macular structure resembling a 'spot weld' or point of adhesion between adjacent epithelial cells. More apically is the *zonula adherens,* or intermediate junction, a circumferential adhesive structure. At the apical-most aspect of the lateral cell surface is the tight junction, or *zonula occludens* (ZO). The ZO surrounds each epithelial cell forming a gasket-like seal which restricts the movement of substances through the paracellular pathway.

This article presents a brief review of what is known about the structure and function of the ZO, along with a description of recent progress in the determination of its biochemical composition. This is by no means an exhaustive survey of the literature which deals with the ZO, but rather is intended to provide a current perspective of an exciting and important area of epithelial cell biology. Additional reviews on this subject have recently been published [2–4].

ZO structure

The thin section appearance of the ZO was originally described by Farquhar and Palade in 1963 [1]. In optimum sections it appears as a series of contacts or 'kisses' [5] between the plasma membranes of adjacent cells. The sites of contact have been postulated to represent actual points of fusion between the two membranes, although recent evidence examining the mobility of lipid molecules in this region suggests there is no continuity of the lipid bilayers [6, 7]. A variable amount of fine filamentous material has also been observed on the cytoplasmic surface of the ZO [1, 8,9]. This material, although ill-defined, will play an important role in the attempts to isolate this

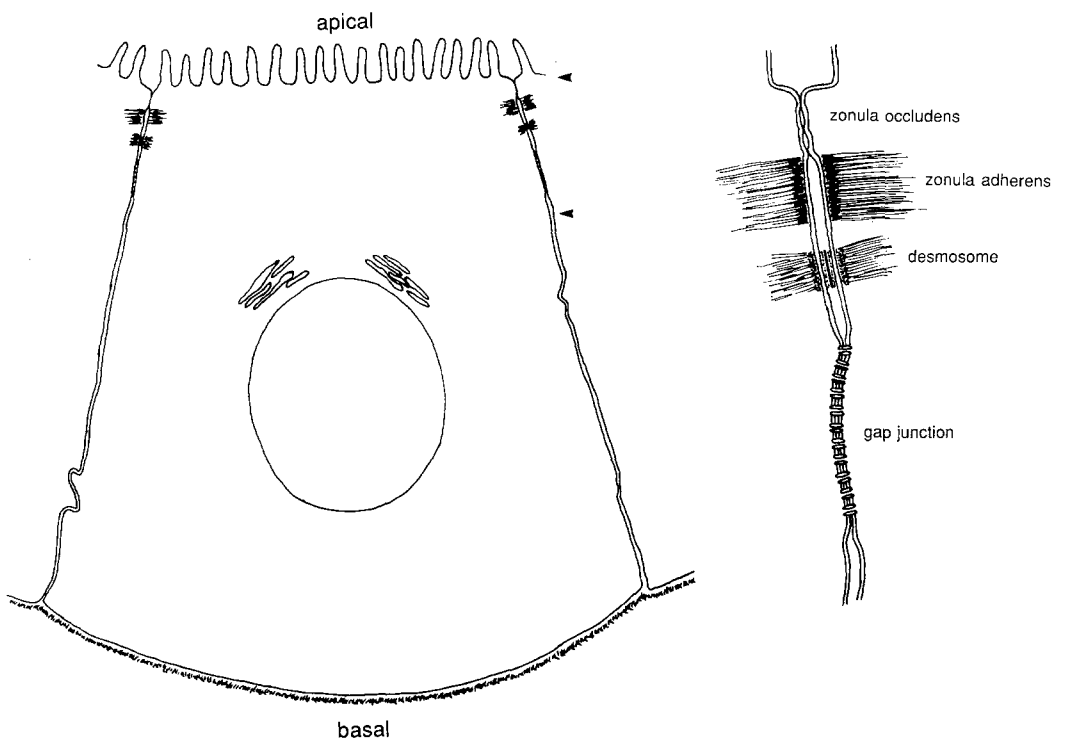

Fig. 1. A schematic cross-sectional view of a typical simple epithelial cell. This polarized cell type displays an apical surface usually facing the external environment, and a basal surface adjacent to the serosal compartment. A portion of the lateral cell surface (between arrowheads) is shown in an expanded view at right. The junctional complex is found at the apical-most aspect of the lateral cell surface and contains the *zonula occludens, zonula adherens,* and desmosome. The gap junction is a macular structure believed to mediate intercellular communication. (Redrawn, with permission, from the Annual Review of Cell Biology, Vol. 1, © 1985 by Annual Reviews Inc.)

structure described below.

Additional information is gained by visualizing the ZO with freeze-fracture electron microscopy, a technique which splits membrane bilayers and exposes structures contained within the hydrophobic interior. The classic image of the ZO is one of a network of branching and anastomosing fibrils lying within the plane of the membrane at the interface of the lateral and apical membrane domains [10, 11] (Fig. 2). These fibrils correspond to the points of the membrane contact seen in thin section [8, 12, 13]. There is also evidence that along the lines of cell-cell contact there are pairs of fibrils, offset with respect to each other, with one being contributed by each cell [14, 15].

It is known that the ZO is a highly dynamic membrane specialization whose fibrils are capable of substantial structural reorganization. Mechanical stress exerted on toad large intestine epithelium [16] and cultured monolayers of mouse mammary epithelial cells [17] cause the ZO fibril branching pattern to appear more elongated or horizontally arranged, much as one would expect the elements of a chain-link fence to appear when the ends are pulled. This evidence suggests that physical tension can produce movement of the junctional fibrils.

A more dramatic example of the dynamic nature of the ZO can be found in the seminiferous epithelium, where the process of spermatogenesis takes place. The ZOs in this unique epithelium are formed by the Sertoli cells and act to separate the basal from adlumenal spermatogenic compartments. During the process of maturation, spermatocytes start in the basal compartment and periodically migrate up through the junction into the apical compartment where they are eventually released in the lumen of the

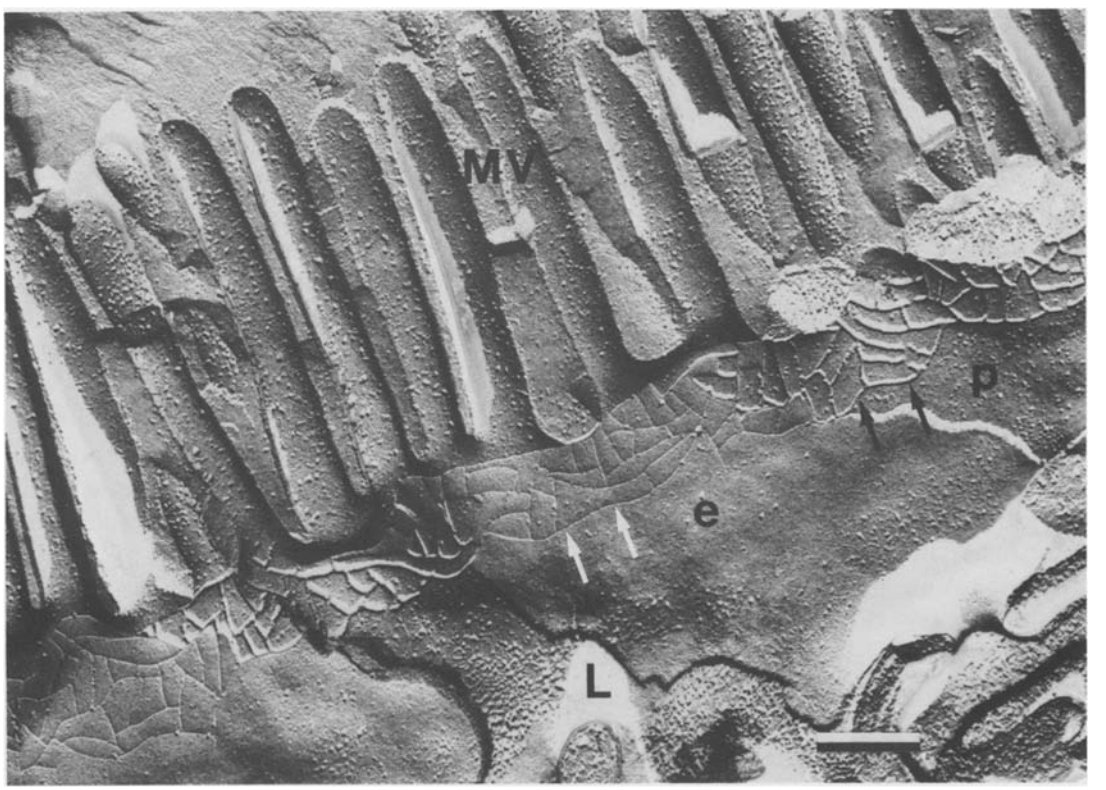

Fig. 2. A freeze-fracture electron micrograph of rat small intestine absorptive epithelial cells fixed with glutaraldehyde. The characteristic image of the ZO is visible, showing branching and anastomosing fibrils (black arrows) in the fracture face adjacent to the cytoplasm (p) and corresponding grooves (white arrows) in the external half of the membrane bilayer (e). The ZO stands between the apical surface, here seen in the form of microvilli (MV), and lateral cell membrane (L). Bar = 200 nm.

seminiferous tubule as spermatozoa. Interestingly, these cells migrate through the ZO without compromising the permeability barrier [18], indicating a highly labile relationship between the fibrils and the passing spermatocytes.

The complexity of the ZO fibril branching pattern seen in freeze-fracture is thought to be related to the permeability of the ZO. Based on a comparison of data from a variety of epithelia, a hypothesis was made that an inverse logarithmic relationship exists between the number of fibrils encountered along the apical-lateral axis and junctional permeability [19, 20]. Exceptions to this rule have been cited in developing tissues [21], in systems perturbed experimentally [22–24], and in a heterogeneous epithelium [22], leaving the general validity of the hypothesis in dispute. This situation has been examined in detail in intestinal epithelium [25, 26] and in cultured epithelial cells [27, 28]. It is clear that an accurate analysis of the structure-function relationship must take into account the fact that ZOs from different cell types within an epithelium, as well as from different areas of the same cell, are heterogeneous with respect to both freeze-fracture morphology and permeability measurements. When this heterogeneity is viewed as a circuit of parallel resistors of different values occurring at different frequencies, most epithelia conform to the Claude and Goodenough hypothesis [19, 20]. Current work cited below, however, adds new information to this interesting controversy.

ZO physiology

The barrier function of the ZO has been demonstrated by ultrastructural examination of epithelia exposed to electron dense tracer molecules. In 1960, Miller [29] demonstrated that hemoglobin was unable to pass the ZO region in mouse kidney epithelium, an observation later confirmed by numerous investigators in other epithelia using protein markers [1, 30, 31] or ionic lanthanum salts [8, 12, 32, 33]. In general, tracers applied to one surface of an epithelium migrated up to the ZO, but were unable to pass to the other side. Unfortunately, the image of tracer stopped at the ZO is misleading in that it implies that this structure is absolutely impermeable. In fact, careful electrophysiological analysis has shown that the ZO is permeable to certain molecules, that this permeability varies from epithelium to epithelium, and that ZO permeability within a given epithelium is dependent on the extracellular environment.

A common and convenient way to measure the permeability of the ZO is to place electrodes on either side of an epithelium and to measure the transepithelial resistance. Because plasma membrane resistances are, in most situations, relatively so high, transepithelial resistance values reflect the resistance to current flow through the ZO, and hence its ionic permeability [19, 34–36]. Membrane conductance can contribute to overall transepithelial resistance under certain conditions (e.g. during hormonal stimulation), but can be neglected for the purposes described here. Transepithelial resistance values vary over a wide range, from a low of 6 ohm-cm^2 in the mammalian kidney proximal convoluted tubule [37] to over 2000 ohm-cm^2 in the amphibian urinary bladder [38]. Careful physiological analysis of many epithelia suggests that fixed paracellular channels are responsible for paracellular ion permeation, and that these channels are, in most cases, more permeable to cations than anions [35, 39–42].

Electrical measurements only provide information regarding the movement of ionic species across the epithelium. Paracellular permeability has also been characterized by the transepithelial flux of radiolabeled non-transported solutes such as sucrose and inulin [28, 43–46]. In two different epithelia the putative ZO channels limit the permeation of molecules with hydrodynamic radii greater than 3.6 Å and are impermeable to those with radii greater than 15 Å [28, 46].

ZO permeability varies in response to a wide variety of external stimuli. Increases in transepithelial flux of radioactive sucrose occurs in mammary gland during lactation [47], and a similar increase in junctional permeability was observed in liver following bile duct occlusion [48]. Osmotic gradients across an epithelium also act to alter both the permeability and morphology of the ZO [42, 49–52]. cAMP increases transepithelial resistance in rabbit ileum [53] and in *Necturus* gallbladder [54]. In addi-

tion, junctional permeability is hormonally controlled in the canine-derived MDCK epithelial cell line [55]. Changes in ZO freeze-fracture morphology, indicative of possible change in permeability, have been documented in dividing cells [56, 57] and in thyroid epithelium in response to changes in thyroid activity [58].

Recent exciting work from the laboratories of Pappenheimer and Madara indicates that absorption of nutrients from the intestinal lumen proceeds primarily through the ZO and paracellular pathway, rather than by active transport through the cells [59–61]. According to their hypothesis, elevated lumenal concentrations of glucose or amino acids act in a still undefined manner through their Na-coupled active transport systems to cause contraction of the actin-myosin based cytoskeleton in the cytoplasm adjacent to the junctional complex. This coincides with morphological changes in the thin section and freeze-fracture appearance of the ZO and an increase in paracellular permeability. Nutrients then move through the paracellular pathway in the lumenal to serosal direction by solvent drag (water flow), driven by the higher osmolarity in the paracellular space created by the Na-coupled transport. This represents an entirely new concept of intestinal absorption and provides interesting new information on the control and function of the ZO and paracellular pathway.

The structure and function of the ZO is also dependent on the concentration of Ca^{2+} in the environment surrounding the epithelium [41, 62–65]. Typically, when exposed to either calcium-free media or to solutions containing the calcium chelator EGTA, an immediate and dramatic decrease in transepithelial resistance occurs. The effect is reversible, however, in that physiologic concentrations of calcium return resistance value to normal. The decrease in transepithelial resistance caused by calcium-free conditions is paralleled in some cases by an alteration in thin section [41] and/or freeze-fracture appearance of the ZO [24, 65, 66]. This work has lead to the general belief that the calcium ion is directly involved in the maintenance of ZO structural integrity. However, more recent work described below on isolated ZO-enriched preparations from liver indicates that the junction is structurally stable in the absence of Ca^{2+} [67].

The ZO and cell polarity

One manifestation of epithelial cell polarity is the compositional asymmetry of the apical and basolateral plasma membranes (see ref. 68 for a review). A large body of indirect evidence suggests that the ZO plays a role in the maintenance of this membrane asymmetry. This concept is based, in part, upon freeze-fracture observations. DeCamilli *et al.* [69] observed that in pancreatic acinar cells the density of intramembranous particles (IMPs), thought to correspond to integral membrane protein molecules, was much greater in the lateral membrane than in the apical membrane. When the cells were treated with EGTA, large discontinuities appeared in the freeze-fractured ZOs, and the apical-basolateral IMP density differential disappeared [65, 66]. Similar results were obtained using cell surface labeling in frog urinary bladder epithelial cells [70] and in isolated mouse intestinal cells [71]. Alternatively, these results could be explained by a possible equalization of surface protein composition by altered synthetic and/or degradative mechanisms or by cytoskeletal perturbations induced by the calcium-free conditions.

Ultrastructural localization of cell surface antigens demonstrates that the boundary between apical and basolateral domains occurs precisely at the ZO [72–76]. This body of evidence does not rule out an additional role for other nonjunctional mechanisms in the maintenance of cell membrane protein polarity. In fact, recent work from the laboratory of Nelson indicates that submembranous cytoskeletal elements, including fodrin and ankyrin, may be involved in anchoring key integral membrane proteins of the basolateral surface in cultured epithelial cells [77–79].

Apical and basolateral plasma membranes of epithelial cells also differ in lipid composition [80]. Lipid probes inserted into the external half of the apical plasma membrane bilayer of MDCK cells stopped at the level of the ZO and did not diffuse into the basolateral domain. Restriction of movement past the tight junction was abolished when external Ca^{2+} levels were reduced. Conversely, if these probes were integrated into the internal half of the bilayer, free diffusion into the basolateral membrane occurred even at normal calcium concentrations

[81]. Similar results were obtained in other epithelial cell lines [6]. These experiments indicate that the barrier to lipids found in the region of the ZO acts only in the external half of the membrane, while in the internal half free diffusion between the two membrane domains take place.

The role of the ZO in the development of epithelial cell polarity is more controversial. A temporal correlation between the appearance of the ZO and the polarization of cell surface proteins in cultured epithelial cells has been demonstrated [82, 83]. A strong argument can be made, however, against the ZO having a role in the development of cell polarity in mouse embryos. In this well characterized system, single unpolarized cells isolated from a 8-cell embryo can be induced to polarize, as determined by fluoresceinated con-A binding, by contact with individual cells isolated from 2-, 4-, 8-, or 16-cell embryos [84, 85]. Obviously, the normal zonular ZO architecture can not exist between two cells, although specific junctional components could be acting to influence the polarization process.

The polarized budding of enveloped viruses has also been used to assess the role of the ZO in the development of cell polarity. In the normal situation certain viruses bud only from the apical surface, while others bud exclusively from the basolateral surface. It has been shown that budding can occur in a polarized manner in single MDCK cells attached to a substrate [86]. However, it has also been found that other non-viral cell surface proteins are incompletely polarized in single MDCK cells in the absence of intercellular junctions [87].

In summary, epithelial cell polarization is a complex process which involves many different factors acting in concert, including attachment to substrate, organization of the cell cytoplasm, assembly of the junctional complex, and arrangement of the cell surface. The exact role of the ZO in polarization will be more easily ascertained through the use of specific probes for ZO components.

The relationship of the ZO to the cytoskeleton

The precise mechanisms through which the epithelial cell acts to control the paracellular permeability at the ZO remain undefined. The work of Pappenheimer and Madara described above on the paracellular pathway-mediated absorption of nutrients in the intestine provides evidence that the cytoskeleton acts to control ZO permeability *in vivo* [59–61]. Additional evidence is based on the treatment of epithelial cells with drugs known predominantly for their interactions with cytoskeletal components. Phalloidin, a mushroom toxin which stabilizes the filamentous form of actin, disrupts hepatocyte ZOs and induces large arrays of ZO-like fibrils in freeze-fracture images of the lateral plasma membrane [88]. Similar treatment was also shown to increase the permeability of liver ZOs to sucrose, inulin, and lanthanum [89]. Conversely, phalloidin and cytokinins, plant hormones which also disrupt microfilaments, cause a decrease in junctional permeability in *Necturus* gallbladder [90, 91].

Additional investigations have examined the effects of the actin-disrupting drugs cytochalasin B and D on ZO structure and function [46, 92–96]. Madara and his colleagues [46, 96] have carefully documented the effects of cytochalasin D on the ZOs of intestinal absorptive cells and noted increases in ZO permeability, condensation of filamentous elements in the cytoplasm immediately adjacent to the ZO, and disruption of the freeze-fracture appearance of the ZO in drug treated tissue. Similar results were obtained in intestinal epithelia subjected to varied osmotic loads [42].

Observations in different systems provide morphological evidence of a spatial relationship between defined filamentous cytoplasmic elements and the ZO [1, 8]. S_1-decorated actin filaments have been observed adjacent to the ZO in quick-frozen, deep etched, rotary shadowed hair cells of the chick ear [9], and in direct end-on association with the points of membrane contact at the ZO in detergent-extracted intestinal absorptive cells [97]. These observations represent the first physical evidence of a relationship of a known cytoskeletal protein and the ZO. Vinculin and alpha-actinin, two proteins believed to mediate microfilament-membrane interactions, have been localized to the junctional complex region by immunocytochemistry [98–100]. Although both were localized near the *zonula adherens,* no significant association with the ZO was found. The *zonula adherens* is also the site of a cir-

cumferential ring of actin and myosin which exhibits contractile activity [16, 101–104]. Taken together, these observations have led to the speculation that epithelial cells regulate paracellular permeability through tensile forces generated through the adjacent cytoskeleton [46, 59, 96, 97, 102]. The molecular details of cytoskeleton-ZO interactions remain to be elucidated.

Biochemistry of the ZO

While a great deal of evidence exists characterizing the ZO from both the morphological and physiological viewpoints, little information exists on its biochemical composition. Numerous investigations have indirectly approached this question, but only recently has progress been made in the dissection of the ZO at the molecular level.

Originally it was thought that the fibrils seen in freeze-fracture images of the ZO were composed of protein. In tissue fixed with the protein crosslinker glutaraldehyde, the fibrils appeared as continuous cylinders lying within the plane of the membrane. In the absence of chemical fixation, the fibrils appeared instead as a linear series of individual IMPs [13, 105, 106], leading to the belief that the fixative acted to cross-link adjacent fibril protein molecules.

It has been clearly shown that proteins participate in ZO formation. When freshly trypsinized MDCK cells are plated on a permeable substrate they develop a characteristic transepithelial resistance. If, however, they are plated in the presence of either cyclohexamide or puromycin, two protein synthesis inhibitors, no resistance develops [93, 107, 108]. Gumbiner and Simons [109] demonstrated that antibodies to the cell adhesion molecule uvomorulin (L-CAM), an integral membrane protein, inhibit the formation of ZOs in MDCK cells. More recently it has been shown that this antibody inhibits the formation of desmosomes and *zonulae adherentes* as well, indicating that the normal interaction of cell adhesion molecules on adjacent cells is necessary for the assembly of the entire junctional complex [110]. These results suggest that proteins play a key role in ZO assembly, but do not offer direct information on the composition of the ZO.

An alternative model has been proposed which envisions the ZO fibril as being composed of an inverted cylindrical lipid micelle lying within a linear fusion of the external leaflets of the plasma membrane [111]. This model was based on the observation that ZO-like fibrils accumulated in the lateral membranes of prostate epithelial cells in tissue slices incubated at 37 °C in the presence of cyclohexamide [112] and that these fibrils appeared continuous in optimally frozen and freeze-fractured unfixed samples. A second model suggests that proteins may be present at the ZO fibrils to stabilize the lipid micelles [113].

It has been shown, however, that ZO fibrils are structurally resistant to treatment with detergents. Goodenough and Revel [8] observed a branching fibril pattern similar in appearance to freeze-fractured ZOs in negatively stained isolated liver plasma membranes briefly washed with the detergent sodium deoxycholate (DOC) and in whole liver following acetone extraction. Similarly, Hirokawa and Tilney [9] observed the ZO fibril network in quick-frozen deep etched rotary shadowed hair cell of the chick ear following extraction with 1% Triton X-100. It was later found that extensive washing of liver plasma membranes in the presence of DOC and EGTA generated a preparation which contained the ribbon-like remnants of the hepatocyte junctional complex [67]. When viewed in negative stain, these junctional ribbons contained a pattern of fibrils believed to be elements of the ZO. Freeze-fracture of this preparation revealed that the ZO fibrils remain structurally stable embedded within the detergent-treated ribbon matrix (Fig. 3). While not impossible, it is unlikely that lipid domains would remain intact following exposure to these detergents [114].

Isolation and preliminary characterization of ZO-enriched preparations

In an attempt to get a better handle on the molecular nature of the ZO, efforts were made to isolate subcellular fractions containing this structure, as had been done for the desmosome [115–117] and gap junction [118, 119]. Using liver as a starting material and taking advantage of earlier observations on the isolation of bile canaliculus-enriched membrane

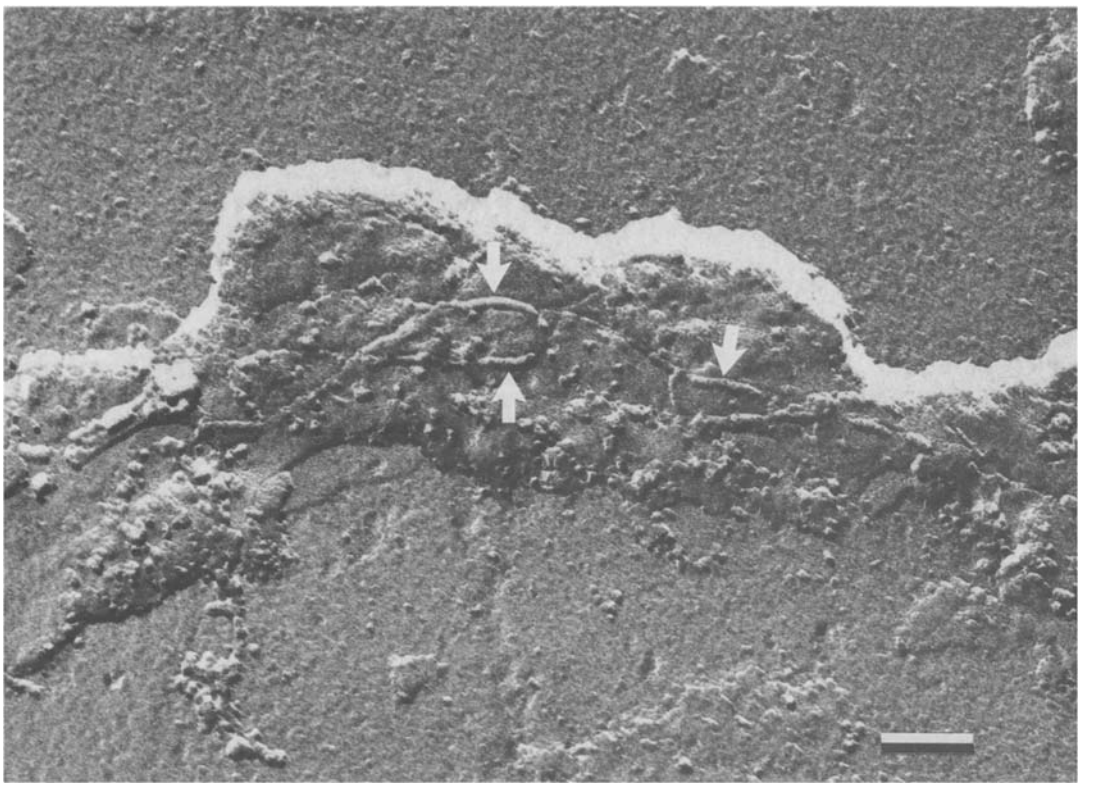

Fig. 3. Freeze-fracture replica of a junctional complex-derived ribbon structure generated by treatment of isolated mouse liver plasma membranes with the detergent sodium deoxycholate. ZO fibrils (arrows) can be seen embedded within the ribbon. Bar = 100 nm.

fractions [8, 120], a detergent- and EGTA-resistant preparations enriched for the ZO regions was obtained [67]. This isolation was made possible by the insolubility of a dense layer of 'fuzzy' material applied to the cytoplasmic surface of the junctional complex membranes. Although this preparation contained elements of the ZO embedded within the fuzzy material, it also contained other components of the junctional complex as well as nonjunctional material. Efforts to further enrich the preparation for the ZO were fruitless, largely due to the fact that the only assay for this structure was morphology. Attempts to find a treatment which selectively removed the non-ZO contamination had to be assessed by the somewhat tedious thin section, negative stain, or freeze-fracture techniques. Protocols that removed the contaminating materials also destroyed the morphological integrity of the ZO (B. Stevenson, unpublished data).

In light of these difficulties, monoclonal antibody techniques were utilized in an attempt to generate probes specific for ZO components, using the ZO-enriched preparation from liver described above as immunogen. Antibody-producing hybridomas were screened first with a modified ELISA using SDS-denatured protein. This allowed selection of a clone which identified antigen on SDS-PAGE/immunoblots. Positives from the ELISA were then used to immunofluorescently stain liver frozen sections. The unique histology of this tissue permitted the unequivocal identification of antibodies reacting with elements of the junctional complex. To date, one hybridoma line (R26.4) has been fully described which satisfies these criteria [121].

Liver stained with ZO-specific antibodies produces a distinct pattern, showing pairs of bright dots on either side of the lumen of the bile canaliculus in cross section, and parallel lines running along the lateral hepatocyte surface in longitudinal view, in both cases exactly where the tight junction is located

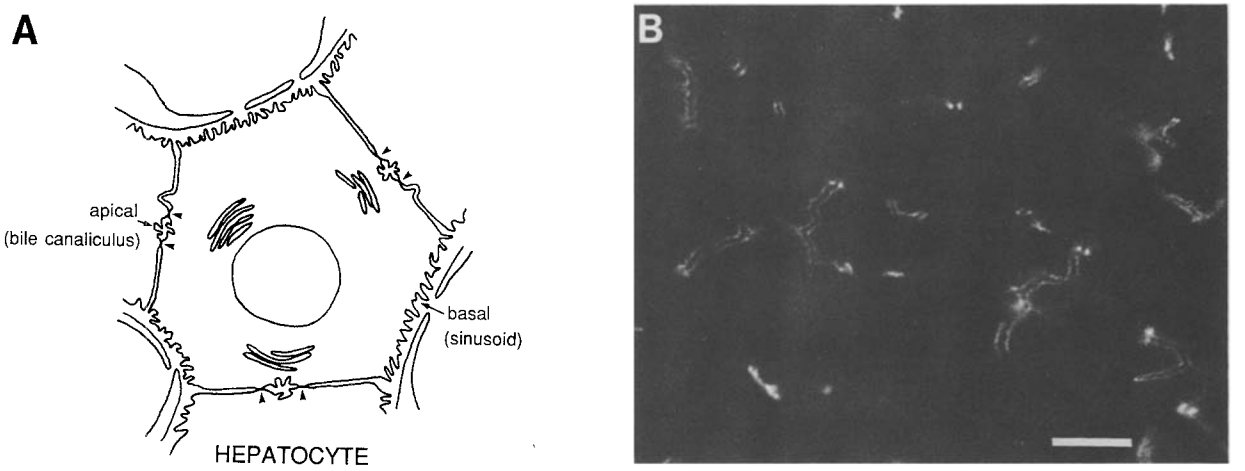

Fig. 4. (A) Schematic diagram showing the unique histology of the liver epithelial cell (hepatocyte). The apical surface surrounds the lumen of the bile canaliculus, a tubular structure which forms a network running throughout the liver. As a result of this complex network, any cross-sectional view of a single hepatocyte will show multiple apical surfaces. ZOs are located immediately adjacent to the apical domain (arrowheads). The basal cell surface abuts the blood sinusoid. (Redrawn, with permission, from the Annual Review of Cell Biology, Vol. 1. c 1985 by Annual Reviews Inc.) (B) Immunofluorescent localization of the ZO-specific polypeptide ZO-1 in cryostat sections of rat liver. ZO-1 staining appears in cross-section as pairs of bright dots on either side of the lumen of the bile canaliculus and in longitudinal section as pairs of parallel lines running along the lateral hepatocyte surface. Bar = 10 μm.

(Fig. 4). Ultrastructural localization performed on isolated liver plasma membranes demonstrated labeling exclusively associated with the cytoplasmic surface of the ZO, seemingly clustered around the points of membrane contact. Immunoblots showed that these antibodies reacted with a polypeptide of 225,000 Daltons (225 kD) that was enriched in the junctional ribbon isolation protocol used to generate the immunogen. This polypeptide was named 'ZO-1' to indicate that it is the first protein shown to be found solely at the ZO [121].

ZO-1 has been found in a variety of other rodent epithelia. Immunofluorescent staining of kidney, colon, and testis demonstrates activity in the ZO region; arterial endothelial ZOs are also stained with the R26.4 antibody [121]. Additional mouse or rat tissues, including lung and brain, also show ZO-1 activity (D. Goodenough, R. Dermietzel, unpublished data). When a coverslip of MDCK cells (derived from dog kidney) is exposed to this antibody, a discreet network of staining is seen surrounding each one of the cells at the level of the ZO, indicating that this epitope is found in the ZOs of other species as well. Immunoblots of either whole MDCK cells or MDCK plasma membrane fractions demonstrate the presence of the ZO-1 polypeptide at approximately the same molecular weight as that from liver (see below). No ZO-1 activity has yet been convincingly demonstrated in invertebrate tissue (C. Green, N. Lane, unpublished data).

The physical characteristics of ZO-1 have been described in detail [122]. ZO-1 can be extracted from mouse liver or MDCK plasma membranes with urea or elevated pH conditions, but not by nonionic detergents, indicating that it is peripherally associated with the junctional membrane. Experiments using MDCK cells labeled with (^{32}P)orthophosphate provide evidence that ZO-1 is a phosphoprotein phosphorylated exclusively at serine residues. Gel filtration and rate-zonal sedimentation analysis of ZO-1 from liver and MDCK sources suggest that this protein behaves as an asymmetric, monomeric entity, and that ZO-1 from liver has a higher molecular weight than that from MDCK cells. This molecular weight difference is verified by SDS-PAGE, which shows that ZO-1 has an apparent size of 225 kD in mouse tissues and 210 kD in the canine-derived MDCK cell line. There are approximately 30,000 ZO-1 molecules in a MDCK cell, as determined by Scatchard binding analysis of radioactively labeled

monoclonal antibodies to total cell protein. This is roughly similar to the amount of fibril IMPs in an average MDCK cell ZO based on calculations made from published freeze-fracture images [123], suggesting there is close to a one-to-one relationship between ZO-1 and the fibril elements.

Recombinant phage used to express a fusion protein immunoreactive with anti-ZO-1 monoclonal antibodies have been identified from a rat kidney lambda gt-11 expression library (J. Anderson, C. Van Itallie and B. Stevenson, unpublished data). The 1 kb cDNA insert codes for a polypeptide of approximately 38 kD, so obviously represents only a portion of the full length molecule. A preliminary screen of the sequence of this cDNA shows no homologies with previously sequenced DNAs or proteins, suggesting it codes for a previously undescribed polypeptide. Polyclonal antisera raised against this fusion protein in three different rabbits give identical staining patterns to that produced by the original monoclonal antibodies. Pending further confirmation, such a cDNA clone will be of obvious use for a variety of molecular analyses.

Preliminary analysis of the biological role of ZO-1

Preliminary experiments have been performed on two strains of MDCK cells which differ in their junctional permeability (B. Stevenson, J. Anderson, D. Goodenough and M. Mooseker, unpublished data). Strain I cells have a typical transepithelial resistance of approximately 3000 ohm-cm^2, while strain II cells give values of about 100 ohm-cm^2 [55]. It was found that when these cells are grown on filters they are roughtly the same size and shape, and hence the same linear junctional density, a measure of the linear amount of ZO in a given area of epithelium [20]. They also have approximately the same amount of ZO-1 in each type of cell, expressed in terms of molecules of ZO-1/micron of ZO.

Taken together, these results and the similarity in amounts of freeze-fractured ZO fibril IMPs and ZO-1 determined by Anderson *et al.* [122] imply that there would be little difference in the freeze-fracture images of the ZOs in the two strains of cells. In fact, quantitative freeze-fracture determined that there was no morphological difference in these cells, either in the average number of fibrils encountered in the apical-basolateral axis or in network density [24], a measure of the complexity of the ZO fibril branching pattern. There is also no difference in the frequency of distribution in the fibril number observations in the two strains. Knowing the linear density and frequency of fibril number observations allows the calculation of predicted resistance values for each strain of cell based on the Claude and Goodenough hypothesis [19, 20]. These values are 26.5 ohm-cm^2 for the high resistance strain I and 35.7 ohm-cm^2 for the low resistance strain II. Compared to actual transepithelial resistance values, this prediction is satisfactory for the low resistance strain, but is grossly inaccurate for the high resistance strain I cells.

There are several possible explanations for these observations. These strains of MDCK cells may represent an exception to the Claude and Goodenough structure-function relationship [19, 20]. It may be that the structures responsible for permeability are not revealed by freeze-fracturing since they must be on the outside surface of the membrane, and this is covered by the E fracture face [124]. Another any kind of ultrastructural investigation, such as different ZO channel characteristics, must also be taken into account in a comparison of ZO parameters from different epithelia. More detailed analysis is dependent upon identification of the channel-containing ZO component.

The distribution of ZO-1 in MDCK monolayers has been investigated in a number of physiologically interesting conditions (B. Stevenson, J. Anderson, M. Mooseker, unpublished data). Exposure to calcium-free conditions causes a characteristic sequence of events (Fig. 5). Transepithelial resistance values rapidly fall to zero, and ZO-1 immunofluorescence distribution changes from the typical discreet honeycomb network to one where ZO-1 activity appears dispersed and clumped within the plane of the plasma membrane, diffuse throughout the cell cytoplasm, or in a contracted or 'purse-string' morphology. Cytochalasin D treatment of MDCK monolayers causes the transepithelial resistance to fall to 20% of control levels and ZO-1 to be distributed in a more clumped morphology along the lateral cell surface

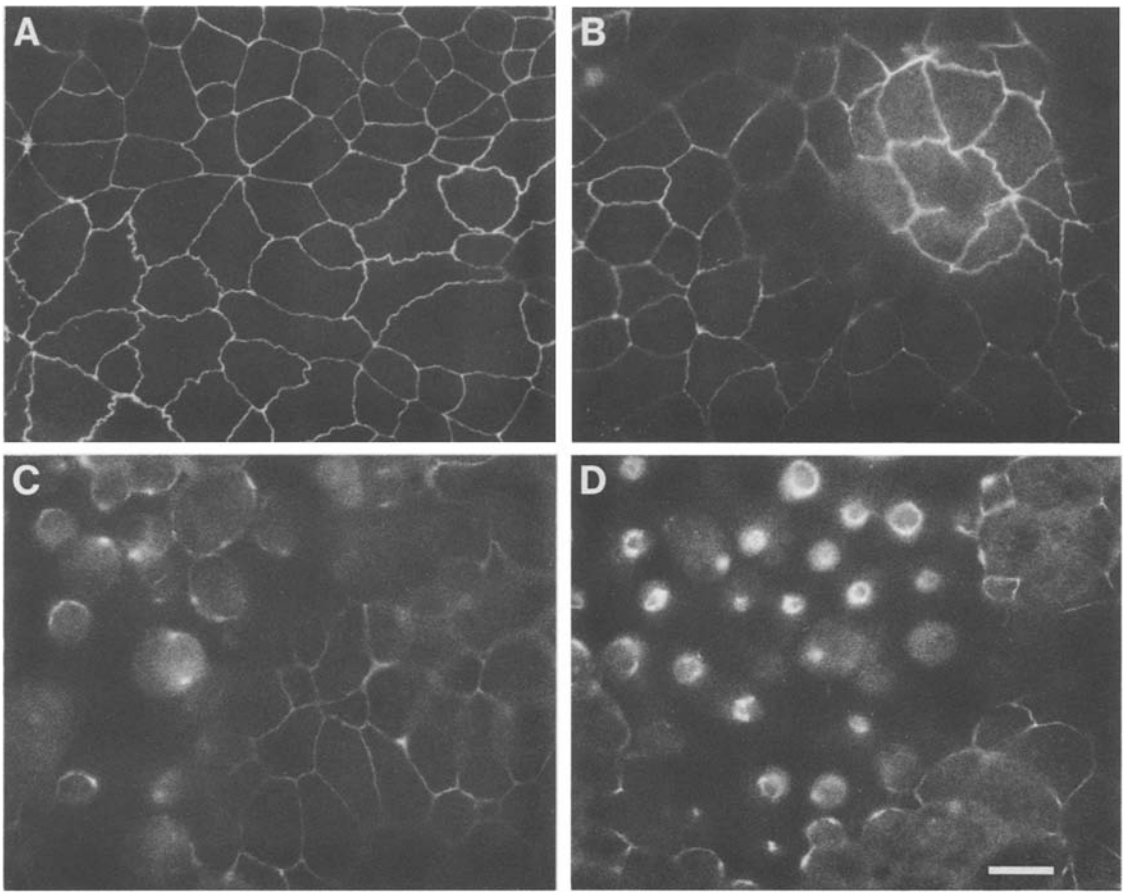

Fig. 5. Immunofluorescent localization of ZO-1 in monolayers of the MDCK I epithelial cell line grown on permeable filters and treated with calcium-free media. (A) Control monolayer prior to experimental treatment. ZO-1 staining appears as a discrete continuous network surrounding each cell at the level of the ZO. (B) 1 min after exposure of the monolayer to calcium-free Spinner media blisters appear within the monolayer. Here cells show a brighter staining of ZO-1 in the periphery and a more diffuse staining in the cytoplasm. The transepithelial resistance of this monolayer is 33% of the control. (C) 10 min following calcium-free conditions the transepithelial resistance has fallen to zero and cells have begun to detach from each other and round up. These cells show diffuse ZO-1 activity throughout the cell cytoplasm and clumped staining in the plane of the plasma membrane. (D) At 20 min the transepithelial resistance remains at zero and most of the cells have rounded up and show a 'purse-string' ZO-1 staining pattern. Bar = 10 μm.

(Fig. 6). This provides further evidence for a relationship between elements of the cytoskeleton and the ZO. Not surprisingly, the cell can also alter the distribution of ZO-1 under normal conditions. When MDCK monolayers are costained for ZO-1 and DNA it appears that cells which are in the process of dividing have a brighter peripheral ZO-1 staining pattern as well as a more diffuse staining in the cell cytoplasm (Fig. 7). Experiments designed to investigate the biochemical properties of ZO-1 under these diverse conditions are in progress.

Conclusions and a model for ZO structure

Since its original description in 1963, many elegant investigations have provided interesting insights into the structure and function of the ZO. ZO biochemistry, on the other hand, is a nascent field which has recently yielded the identity of the first ZO-specific component. Information obtained in the process of this identification leads to a number of conclusions.

The isolation of ZO-enriched preparations from mouse liver utilizes the selective stability of the ZO

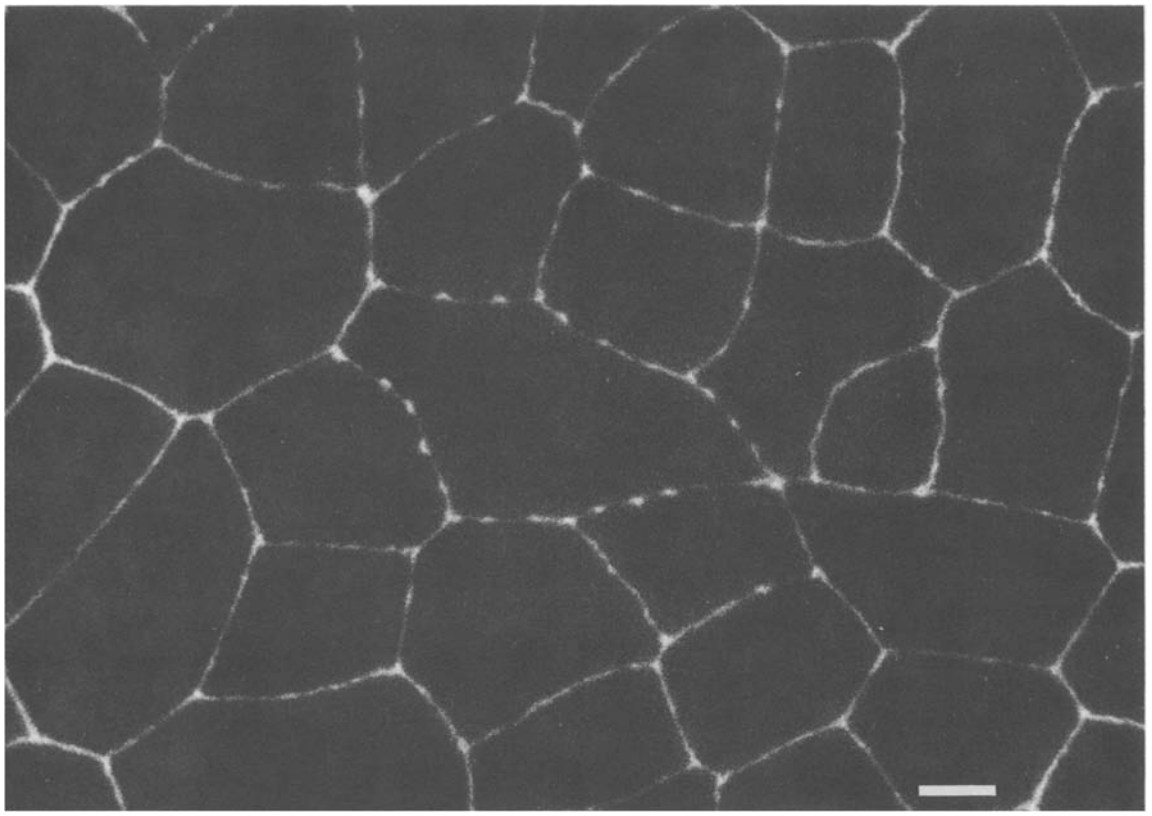

Fig. 6. Immunofluorescent localization of ZO-1 in an MDCK I monolayer grown on a permeable filter and treated with 2 μg/ml cytochalasin D (in DMSO) for 1 h at 37°. Unlike the typical continuous staining of ZO-1 seen along the plasma membrane in vehicle control cells (identical to that seen in Fig. 5A), ZO-1 activity in regions of the cytochalasin D-treated monolayers appears clustered along cell boundaries. The transepithelial resistance of this monolayer was 20% of a monolayer treated with vehicle alone. Bar = 5 μm.

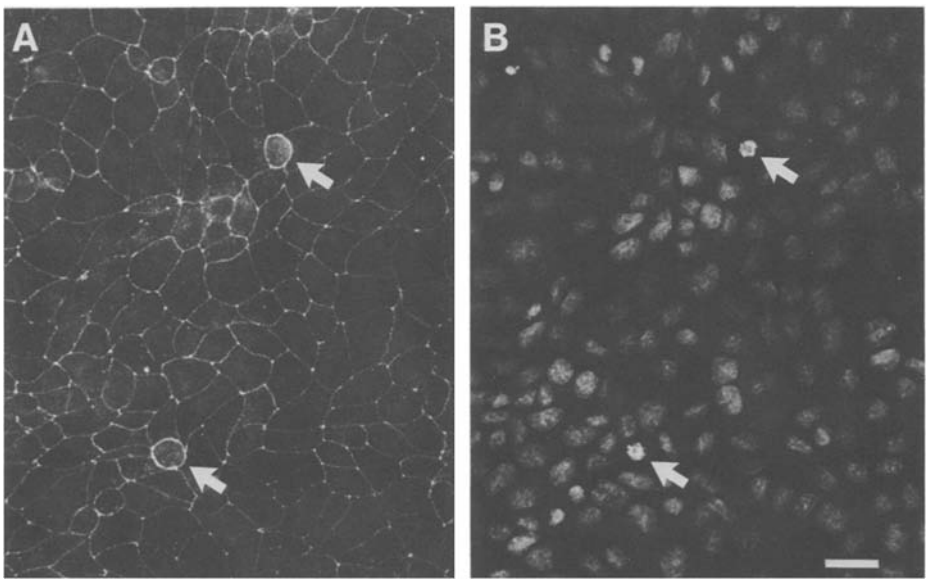

Fig. 7. Colocalization of ZO-1 and DNA in subconfluent monolayers of MDCK II cells grown on glass coverslips. (A) Immunofluorescent localization of ZO-1. Isolated cells (arrows) show a brighter ZO-1 staining both along the cell periphery and dispersed within the cytoplasm. (B) The identical field stained with a dye (Hoescht) specific for DNA. The cells which showed the altered ZO-1 staining pattern in A (arrows) here display condensed chromosomes characteristic of dividing cells. Bar = 25 μm.

to treatment with EGTA and the anionic detergent DOC. The ZO remains structurally intact in the absence of calcium ions, suggesting that the well-characterized breakdown of ZO structure and barrier function observed in whole cells in response to the removal of Ca^{2+} is the result of secondary cellular processes, perhaps involving calcium dependent cell adhesion molecules [125, 126], and not a direct action on the junction itself. ZO fibrils are also structurally stable following treatment with detergent conditions which would be expected to solubilize, or at least disrupt, lipid micellar structures. While the identity of the putative ZO occluding element remains unknown, these results suggest that it is of proteinaceous character. Alternative models which postulate a combination of lipid and protein elements can not be conclusively ruled out.

The identification and characterization of ZO-1 have shown it to be a high molecular weight, asymmetric, monomeric phosphoprotein peripherally associated with the cytoplasmic surface of the ZO. While its role in the biology of the ZO is undetermined, preliminary observations in two epithelia which differ in transepithelial resistance, a parameter directly dependent on ZO function, suggest that the gross amount of ZO-1 is not directly related to junctional permeability but is related to the amount of fibrils present. These investigations have also provided evidence that the controversy surrounding the relationship of ZO morphology to permeability will probably be resolved only upon molecular analysis of all the junctional elements. Finally, the localization of ZO-1 changes in response to calcium deprivation, disruption of the cytoskeleton, and cell division, indicating that the cell can alter the distribution of junctional components in different functional states.

The structure of the ZO as we currently envision it is shown in Fig. 8. This very preliminary model clearly depicts the peripheral location of the phosphoprotein ZO-1. More importantly, it also communicates that much remains to be learned about this important intercellular junction. The identity of the putative occluding element remains unknown and there is no direct information on the precise relationship of ZO-1 to either this occluding element or to cytoskeletal or enzymatic components within the cytoplasm which may act to control paracellular

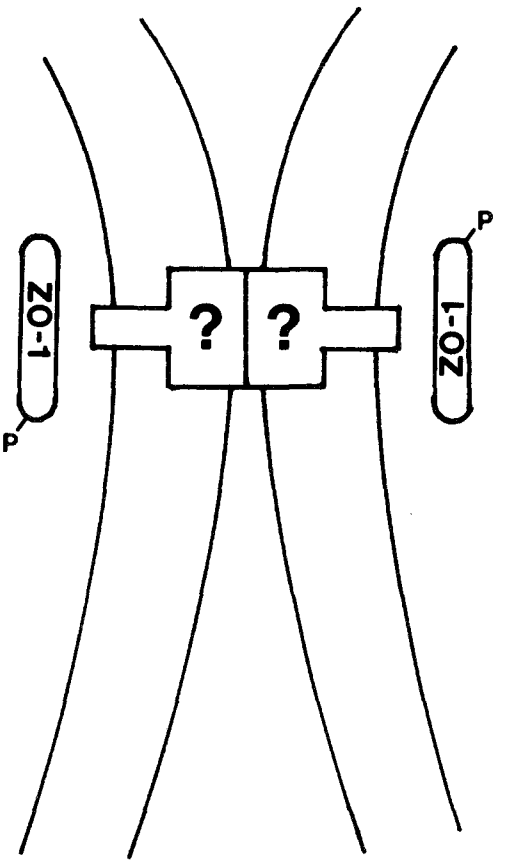

Fig. 8. Preliminary molecular model of the ZO depicting the peripheral relationships of the newly identified phosphoprotein ZO-1 to the site of cell-cell contact. The identity of the putative occluding element and its functional relationship to ZO-1 are the subject of continuing investigations.

permeability. The existence of a marker for the ZO should now allow identification of some of these other components and determination of where they fit in ZO structure and function. In the end this approach will hopefully yield a coherent picture of the role the ZO plays in epithelial cell biology.

An additional tight junction-associated component, termed 'cingulin', has been identified and purified from chicken intestinal brush borders using monoclonal antibody techniques [127]. Anti-cingulin mono- and polyclonal antibodies react on immunoblots with bands at 140 kD and 108 kD. The relationship between these two polypeptides is currently unknown. Cingulin, like ZO-1, also appears to be peripherally associated with the tight junction membrane.

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