The Tight Junction Protein ZO-1 Is Concentrated Along Slit Diaphragms of the Glomerular Epithelium

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Abstract. The foot processes of glomerular epithelial cells of the mammalian kidney are firmly attached to one another by shallow intercellular junctions or slit diaphragms of unknown composition. We have investigated the molecular nature of these junctions using an antibody that recognizes ZO-1, a protein that is specific for the tight junction or zonula occludens. By immunoblotting the affinity purified anti–ZO-1 IgG recognizes a single 225-kD band in kidney cortex and in slit diaphragm–enriched fractions as in other tissues. When ZO-1 was localized by immunofluorescence in kidney tissue of adult rats, the protein was detected in epithelia of all segments of the nephron, but the glomerular epithelium was much more intensely stained than any other epithelium. Among tubule epithelia the signal for ZO-1 correlated with the known fibril content and physiologic tightness of the junctions, i.e., it was highest in distal and collecting tubules and lowest in the proximal tubule. By immunoelectron microscopy ZO-1 was found to be concentrated on the cytoplasmic surface of the tight junctional membrane. Within the glomerulus ZO-1 was localized predominantly in the epithelial foot processes where it was concentrated precisely at the points of insertion of the slit diaphragms into the lateral cell membrane. Its distribution appeared to be continuous along the continuous slit membrane junction. When ZO-1 was localized in differentiating glomeruli in the newborn rat kidney, it was present early in development when the apical junctional complexes between presumptive podocytes are composed of typical tight and adhering junctions. It remained associated with these junctions during the time they migrate down the lateral cell surface, disappear and are replaced by slit diaphragms.

The distribution of ZO-1 and the close developmental relationship between the two junctions suggest that the slit diaphragm is a variant of the tight junction that shares with it at least one structural protein and the functional property of defining distinctive plasmalemmal domains. The glomerular epithelium is unique among renal epithelia in that ZO-1 is present, but the intercellular spaces are wide open and no fibrils are seen by freeze fracture. The presence of ZO-1 along slit membranes indicates that expression of ZO-1 alone does not lead to tight junction assembly.

THE visceral epithelial cells or podocytes of the renal glomerulus constitute a highly specialized epithelium structurally adapted to facilitate bulk flow of the glomerular filtrate through the intercellular spaces or filtration slits. In contrast to typical polarized epithelial cells that posses junctional complexes with tight junctions or occluding zonules (zonulae occludentes) which seal off the intercellular spaces and prevent passage of molecules >8Å (6, 22), mature podocytes lack such junctions, and the intercellular spaces or filtration slits formed by their interdigitating foot processes allow passage of molecules up to ∼70Å effective molecular radius. The opening of the intercellular or urinary spaces takes place during glomerular development where the presumptive podocyte begins as a typical polarized epithelial layer with apical occluding and adhering junctions. As the glomerulus matures the junctions migrate down the lateral cell surface, are gradually eliminated, and their intercellular spaces open and become bridged at their narrowest point by tenuous (4 × 230 nm) slit diaphragms or filtration slit membranes (16). The slit diaphragm is a continuous structure in three dimensions that serves to firmly attach the foot processes to one another (7, 18). The molecular nature of these slit diaphragms bridging the filtration slits and connecting the podocyte foot processes is still unknown.

We have previously demonstrated (17, 19) that the slit diaphragm serves as a fence to maintain distinctive apical and basal plasmalemmal domains on the podocyte. Based on this finding and its developmental history, we suggested that the slit diaphragm most likely represents a shallow variant of either an adhering or an occluding junction (19). To investigate the derivation of the slit diaphragms, we have taken advantage of the availability of a specific antibody directed against ZO-1, a 225-kD polypeptide associated exclusively

with tight junctions or zonulae occludentes (24), and have used the antibody to localize ZO-1 in the kidney. We here report that in newborn and adult rat kidneys there is a significant pool of ZO-1 in glomeruli concentrated along the slit diaphragms, suggesting that the slit diaphragm is a derivative of the tight junction.

Materials and Methods

Materials

Polyvinyl alcohol (mol wt 10,000) and polyvinylpyrrolidone (mol wt 10,000) were from Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit IgG conjugated to HRP was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN), and sheep anti-rabbit IgG conjugated to alkaline phosphatase was from Promega Biotec (Madison, WI). F(ab)'$_2$ fragments of goat anti-rabbit IgG conjugated to rhodamine and FITC-conjugated sheep anti-mouse IgG were obtained from TAGO Inc. (Burlingame, CA). Fab fragments of sheep anti-rabbit IgG conjugated to HRP were kindly provided by Dr. J. Kim of TAGO. Goat anti-rabbit IgG coupled to 5 nm colloidal gold (GAR 5) was from Janssen Life Science Products (Piscataway, NJ). Protein A (5 nm)-gold conjugates were prepared as described (21).

Animals

Male adult Sprague-Dawley rats (150-170 g), and pregnant females or mothers with litters were from Charles River Breeding Laboratories, Inc. (Wilmington, MA).

Antibody Characterization

Rabbit polyclonal antiserum to ZO-1 was produced against a recombinant β-galactosidase fusion protein containing 38 kD of rat ZO-1. Details of the production, affinity purification and characterization of this antiserum (rabbit No. 6139) has been reported (1). After affinity purification, this IgG recognized a single antigen of $\sim$225 kD in rat liver (Fig. 1) and human Caco-2 cells (1). Mouse monoclonal anti-podocalyxin IgG (Clone 5A) was characterized in detail elsewhere (14, 19).

Preparation of Glomeruli and Basement Membrane-Slit Diaphragm-Enriched Fractions

Rat glomeruli were isolated by graded sieving at 4°C in the presence of protease inhibitors (1 mM each antipain, pepstatin A, leupeptin, benzamidine, and diisopropylfluorophosphate) as previously described (12). Fractions enriched in basement membranes with attached slit membranes (see Fig. 5 B) were prepared as detailed elsewhere (14). In brief, glomerular fractions were extracted at 20°C in 0.2% Triton X-100 in PBS for 10 min. The pellet was collected by centrifugation (600 g for 3 min), washed, and either used for immunoblotting analysis, or it was resuspended for 15 min in aldehyde fixative (3% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M sodium phosphate buffer), transferred to 400 μl tubes, pelleted in a microfuge, and prepared for cryosectioning as described below for kidney tissue.

Immunoblot Analysis

Rat liver kidney cortex and slit diaphragm-enriched fractions, prepared from isolated glomeruli as described above, were solubilized in SDS-PAGE sample buffer, transferred to nitrocellulose and subjected to immunoblot analysis as described elsewhere (1, 12, 14). Affinity-purified polyclonal anti-ZO-1 IgG was used at $\sim$1 μg/ml and detected with either HRP-conjugated goat anti-rabbit IgG or alkaline phosphatase-conjugated sheep anti-rabbit IgG.

Preparation of Kidney Tissue for Immunocytochemistry

Adult rat kidneys were flushed with DME and fixed in situ by retrograde perfusion. The kidneys of newborn rats, 2-3 d old, were similarly fixed by perfusion through the left ventricle as described elsewhere (19). For cryosectioning 3% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M sodium phosphate buffer was used as fixative. The kidneys were removed, cut

into pieces, and further fixed by immersion for a total of 1 h, after which they were cryoprotected by infiltration with 2.3 M sucrose in phosphate buffer containing 50% polyvinylpyrrolidone, mounted on aluminum nails, and frozen in liquid nitrogen (26). For immunoperoxidase experiments, 2% paraformaldehyde, 0.075 M lysine, and 0.01 M sodium periodate in phosphate buffer (13) was used, and the tissue was fixed for a total of 4-5 h, cryoprotected in 10% DMSO, frozen in liquid nitrogen-cooled isopentane, and similarly frozen (2).

Immunofluorescence

Semithin (0.5 μm) frozen sections were prepared (25) from aldehyde-fixed adult and newborn rat kidneys and transferred to glass slides coated with poly-L-lysine (mol wt 40,500; 1 mg/ml). They were then incubated with affinity-purified polyclonal anti-ZO-1 IgG (2-5 μg/ml) overnight at 4°C, followed by TRITC-conjugated goat anti-rabbit F(ab')$_2$ (diluted 1:50) for 1 h. For double labeling they were subsequently incubated with mouse monoclonal anti-podocalyxin IgG (5-10 μg/ml) for 2 h at room temperature, followed by FITC-conjugated sheep anti-mouse IgG (diluted 1:50) for 1 h. Sections were mounted in 90% glycerol in PBS containing 0.1% p-phenylenediamine (to retard fading), examined by epifluorescence, and photographed using a Zeiss Axiophot or Photomicroscope III and Kodak Tri-X Pan (ASA 400) film.

Immunogold Labeling on Ultrathin Cryosections

Ultrathin cryosections were with an Ultracut E equipped with the FC-4E cryoattachment (Reichert Scientific Instruments, Buffalo, NY) at −110°C following the techniques of Tokuyasu (25, 26). Sections were transferred to hexagonal nickel grids (200 mesh), which had been coated with formvar and carbon. Subsequent incubations and washing steps were carried out by floating the grids on droplets of the filtered solutions. After quenching free aldehyde groups with 10% FCS containing 0.01 M glycine, sections were incubated for 2 h with affinity-purified anti-ZO-1 IgG (2-5 μg/ml), followed by goat anti-rabbit IgG coupled to 5 nm gold (diluted 1:50) for 1 h. Sections were then postfixed with 2% glutaraldehyde (10 min) and 2% OsO$_4$ (15 min), contrasted with 2% uranyl acetate (15 min), and absorption stained with 2.2% polyvinyl alcohol containing 0.002% lead citrate (5 min).

Immunoperoxidase Labeling of Cryostat Sections

Cryostat sections (20-30 μm) were incubated overnight at room temperature with affinity-purified polyclonal anti-ZO-1 IgG (2-5 μg/ml) followed by incubation for 2 h in Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase. The sections were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, reacted with DAB, and processed for electron microscopy as described elsewhere (2).
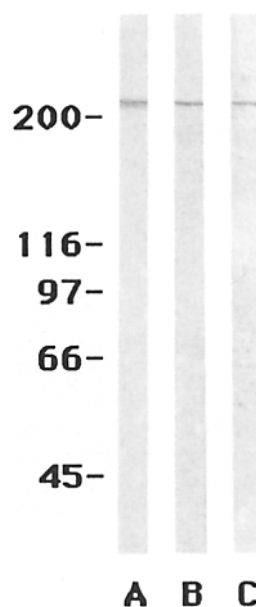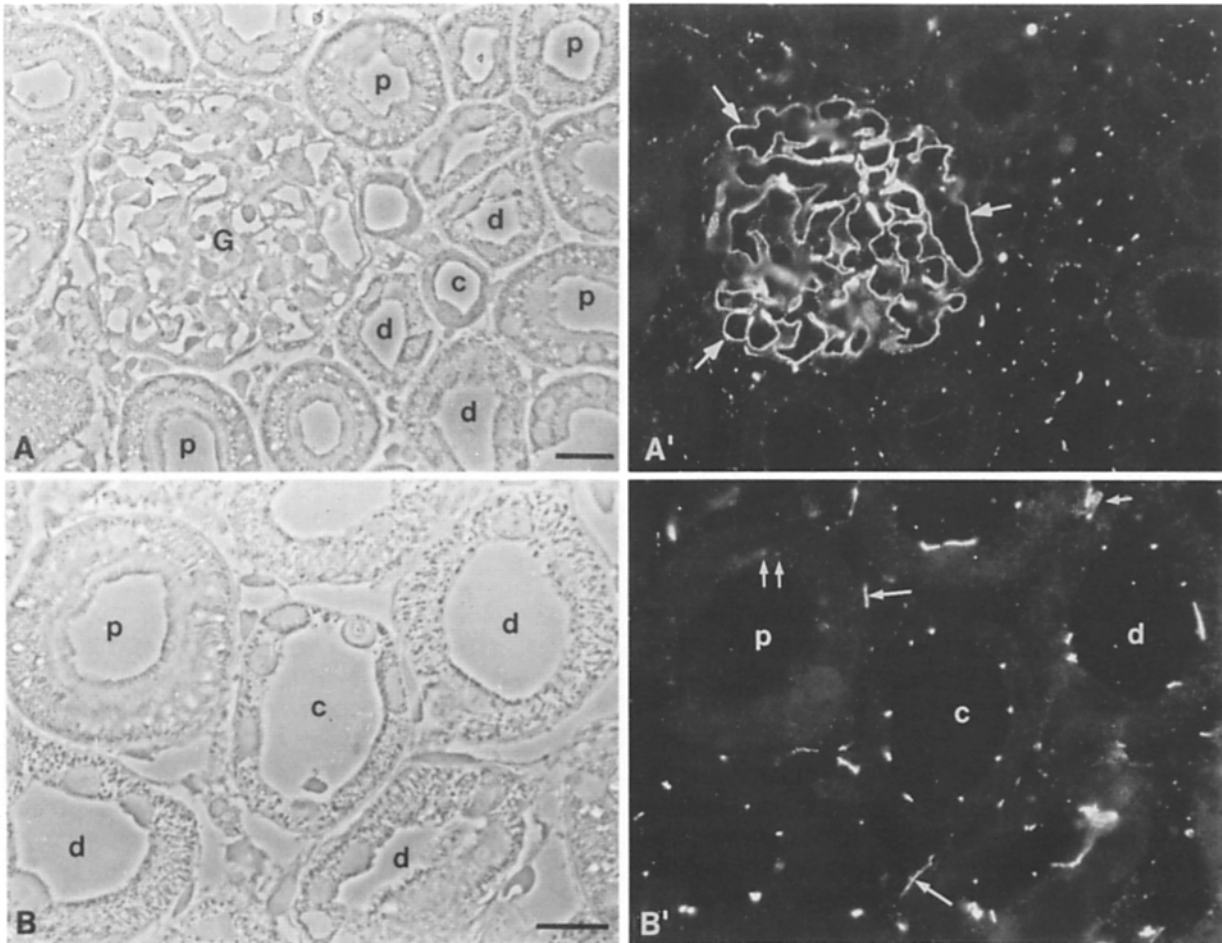
200—

116—
97—

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Figure 1. Immunoblots demonstrating the specificity of affinity-purified rabbit anti-ZO-1 IgG. Lane *A*, liver homogenate. Lane *B*, whole kidney cortex. Lane *C*, glomerular slit diaphragm-enriched fraction prepared as described in Materials and Methods. In all cases a single $\sim$225-kD band is recognized.

A   B   C

Figure 2. Localization of ZO-1 by immunofluorescence in semithin (0.5 μm) cryosections of adult rat kidney. (*A*) The glomerulus (*G*) is intensely labeled for ZO-1. Staining for ZO-1 outlines each of the glomerular capillary loops (*arrows*). Comparison of the phase-contrast (*A*) and immunofluorescence (*A'*) images suggests that staining is concentrated in the foot process layer. ZO-1 is also seen as bright fluorescent dots and short strings between cells of the distal (*d*) and collecting (*c*) tubule that have deep tight or occluding junctions (see *A* and *B*). However, it is barely detectable between cells of the proximal tubule (*p*) which have very shallow tight junctions (see Fig. 3 *C*). (*B*) Comparative staining of proximal (*p*), distal (*d*), and collecting (*c*) tubules for ZO-1. In the proximal tubule ZO-1 appears as tiny, faint dots at the base of the brush border (*short arrows*), whereas in the distal tubule (*d*), collecting tubule (*c*), and peritubular capillaries (*long arrows*) it appears as coarser dots or strands. Bars, 50 μm.

Results

### ZO-1 Is Detectable by Immunoblotting in Kidney Cortex and Slit Diaphragm–Enriched Fractions

By immunoblotting analysis the affinity-purified polyclonal anti–ZO-1 IgG recognized a single 225-kD protein in samples of whole rat liver and kidney cortex (Fig. 1, lanes *A* and *B*). It also recognized a similar 225-kD band in slit diaphragm–enriched fractions prepared by detergent extraction of isolated rat glomeruli (Fig. 1, lane *C*). Thus, ZO-1 appears to be a normal constituent of the kidney cortex and, more surprisingly, of the glomerulus.

ZO-1 Is Detectable by Immunofluorescence in All Variants of Junctional Complexes in the Adult Rat Kidney Tubule

Epithelia from different regions of the kidney tubule vary widely in their structural and functional properties. The or-

ganization and physiologic properties of their intercellular junctional complexes likewise varies considerably. Epithelia of the distal tubule and the collecting duct have the highest transepithelial potential difference, the tightest tight junctions, and are sealed by a junctional complex which consists of a deep occluding junction (6) (see Fig. 3, *A–B*) with multiple fibrils by freeze fracture (3) followed by a shallow adherens or intermediate junction. By contrast, the proximal tubule epithelium is more leaky and its junctional complex consists of a very shallow occluding junction (6) with only one fibril by freeze fracture (3) and a more extensive adhering junction (see Fig. 3 *C*). The endothelial cells of peritubular and glomerular capillaries are also connected by rather extensive tight junctions (see Fig. 3 *D*).

It was previously shown that by immunofluorescence ZO-1 typically appears as fluorescent dots between neighboring cells or sometimes short strings if a junction is cut at an oblique angle (24). In kidney sections stained for ZO-1 (Fig. 2, *A–B*) there was a striking correlation between the im-
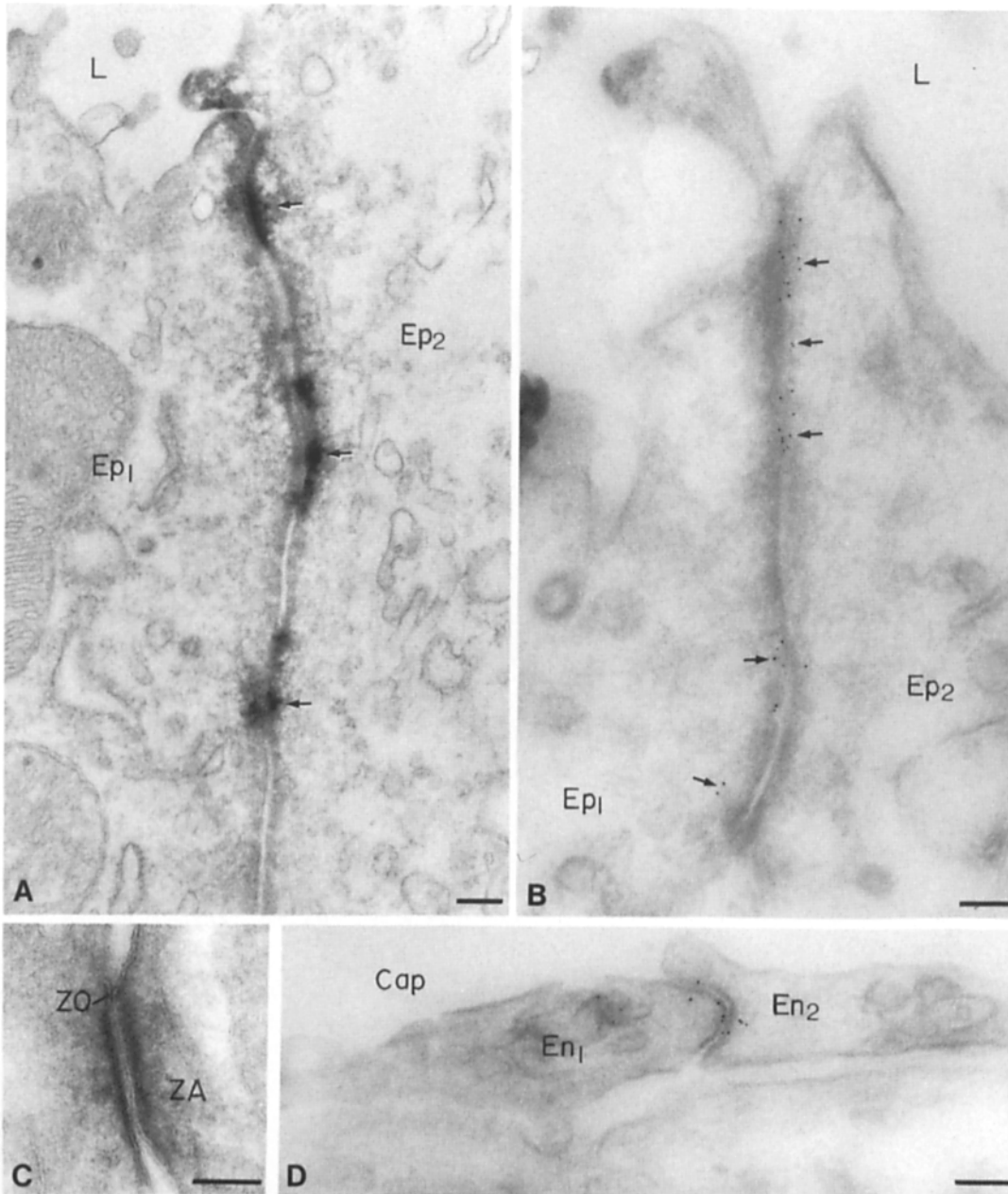
Figure 3. Junctional complexes between cells of the distal tubule (*A* and *B*), proximal tubule (*C*), and peritubular capillary endothelium (*D*) of the adult rat kidney. (*A*) Immunoperoxidase localization of ZO-1 in an extensive tight junction between two epithelial cells (*Ep$_1$* and *Ep$_2$*) of a distal tubule which is cut in grazing section. The section passes in and out of the tight junction several times, and reaction product is seen in the cytoplasm immediately alongside the junction wherever the plane of section passes through it (*arrows*). (*B*) Similar section through a distal tubule tight junction after immunogold labeling of an ultrathin cryosection for ZO-1. Gold particles are concentrated in the cytoplasm on both sides of the tight junction (*arrows*). The section cuts broadly through the junction near the luminal (*L*) surface of the cell and grazes through the junction in two other places below. (*C*) Junctional complex between two cells of the proximal tubule epithelium as seen in an ultrathin cryosection. A shallow tight junction or zonula occludens (*ZO*) and an extensive adherens or intermediate junction (*ZA*) are found in this segment of the renal tubule. (*D*) Immunogold localization of ZO-1 to a tight junction between two endothelial cells (*En$_1$* and *En$_2$*) of a peritubular capillary (*Cap*). Colloidal gold particles are concentrated along the tight junction. Bars, 0.1 μm.

munofluorescence signal intensity and the documented tight junction fibril content and physiologic tightness of renal tubule epithelia, i.e., the signal was strongest between distal and collecting tubules where bright fluorescent dots and strings were seen and weakest between proximal tubule cells where the signal was limited to tiny dots (Fig. 2, *A* and *B*).

A strong signal was also found between endothelial cells of peritubular capillaries (Fig. 2, *A* and *B*).

## ZO-1 Staining Is Most Intense in the Renal Glomerulus

Within the normal glomerulus the only epithelial cells that
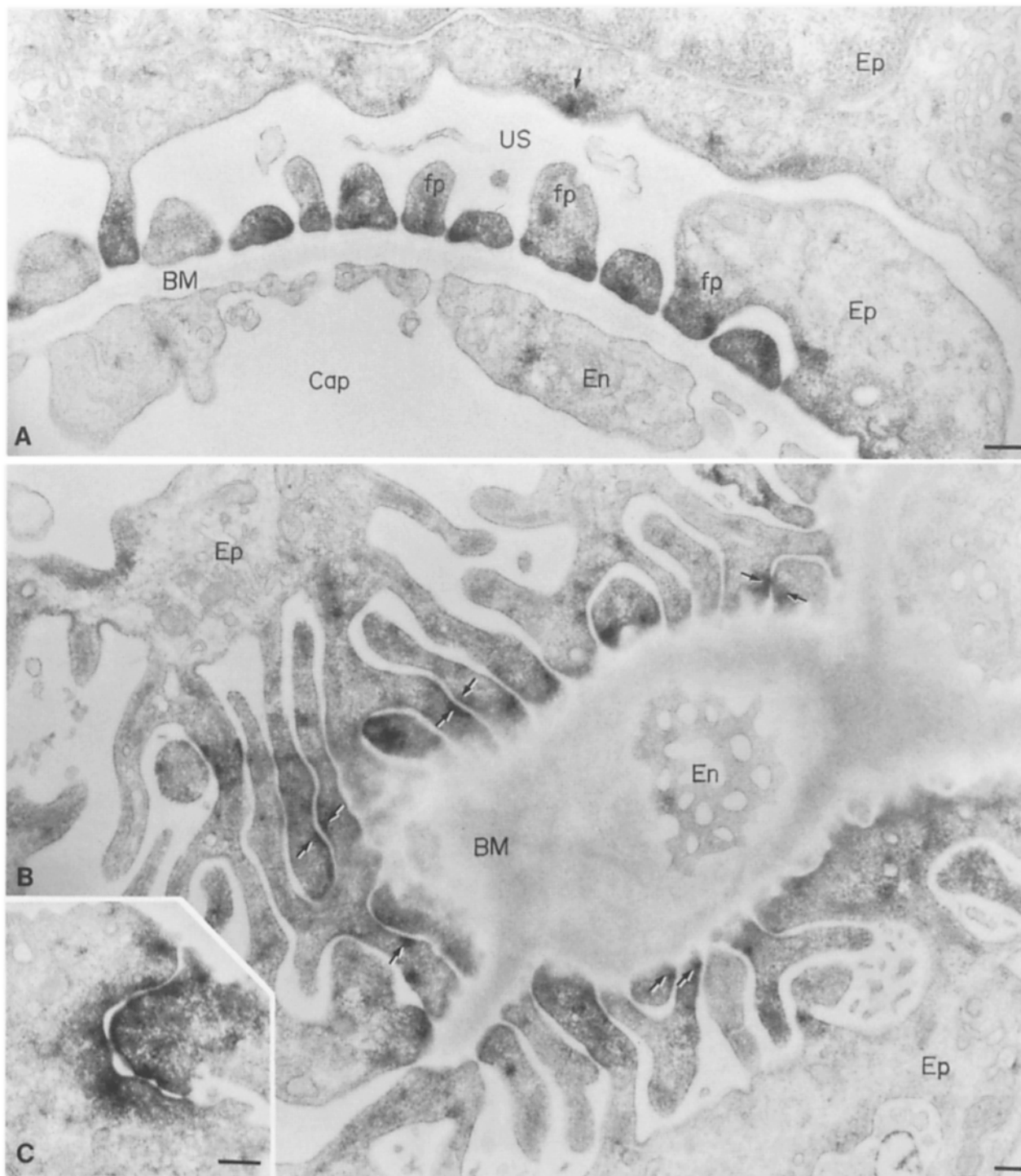
Figure 4. Immunoperoxidase localization of ZO-1 in the adult rat glomerulus. (*A*) DAB reaction product is concentrated in the foot processes (*fp*) of the glomerular epithelium (*Ep*). Sometimes small immunoreactive areas are also seen in the cell body of the podocyte close to the cell membrane (*arrow*). (*B*) Oblique section through the foot process layer showing the endothelium (*En*), basement membrane (*BM*), and the interdigitating epithelial foot processes cut in grazing section. In many places (*arrows*) it can be seen that DAB reaction product is most concentrated in those regions of the foot processes where the slit diaphragms insert. (*C*) Section through a more extensive tight junction between two epithelial cells of Bowman's capsule which is heavily labeled for ZO-1. Bars, 0.2 μm.

are connected by typical tight junctions are those lining Bowman's capsule (see Fig. 4 *C*). The visceral epithelial cells or podocytes lack a typical apical junctional complex, and the intercellular spaces between their interdigitating cell pro-

cesses are wide open (see Fig. 5 *A*). Their narrowest gap located on the lateral cell surface near the base of the cell measures ∼250 nm across and is spanned by a so-called slit diaphragm (see Fig. 5 *B*), which delineates the apical from
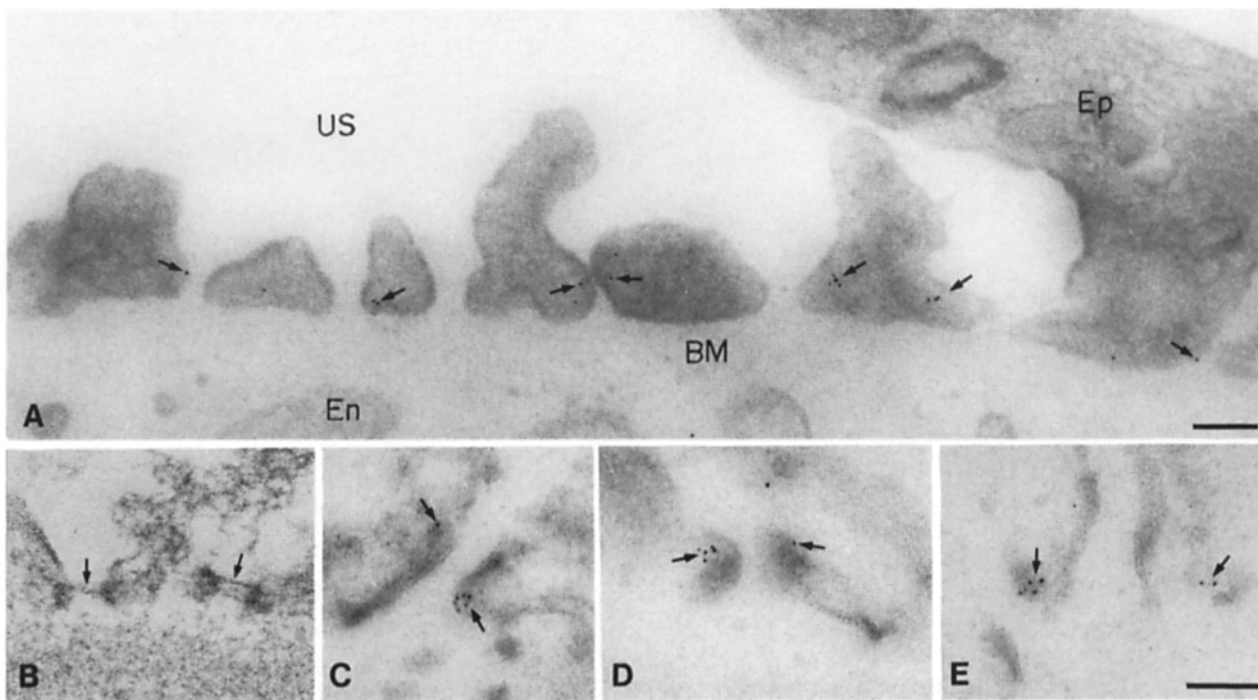
Figure 5. (*A*) Immunogold labeling for ZO-1 in an ultrathin cryosection of an adult rat glomerulus. Gold particles (5 nm) are found in the cytoplasm at the level of the slit diaphragms (arrows). (*B*) Small field from a detergent-extracted glomerular fraction enriched in slit membranes. Most of the contents of the foot processes have been extracted, and only the slit diaphragms (*arrows*) and the basement membrane remain. (*C–E*) Immunogold labeling of ultrathin cryosections prepared from the preparation shown in *B*. Clusters of one to six gold particles are found on the cytoplasmic side of the podocyte membrane over the accentuated insertion points (*arrows*) of the slit diaphragms. ZO-1 is retained under these conditions despite the fact that the membranes of the podocyte foot processes are disrupted and the soluble cytoplasmic proteins have been extracted. Bars, 0.1 μm.

the basolateral plasmalemmal domain of the podocyte. Surprisingly, by immunofluorescence the strongest staining for ZO-1 is seen in the glomerulus where each of the capillary loops is outlined by a bright, linear fluorescent signal (Fig. 2 *A*).

ZO-1 Is Located Along Tight Junctions between Tubular Epithelia and the Peritubular Capillary Endothelium

When ZO-1 was localized in renal tubule epithelia at the electron microscope level by immunoperoxidase labeling, reaction product was found in the cytoplasm alongside the tight junctions wherever they were present (Fig. 3 *A*). The amount of DAB reaction product was proportional to the extensiveness of the tight junction, being greatest in distal and collecting tubules (Fig. 3 *A*). The fact that the peroxidase reaction product was located in the cytoplasm rather than on the outside of the cell membrane is in keeping with the previous demonstration (22) that ZO-1 is a peripheral rather than an integral membrane protein distributed on the cytoplasmic side of the tight junction. Similarly, in immunogold experiments a linear array of gold particles was found outlining the junctions of distal (Fig. 3 *B*) and collecting tubules.

By contrast, the tight junctions between neighboring proximal tubule cells showed few gold particles, i.e., only one to two per section, if any, which is in keeping with their shallow nature (Fig. 3 *C*) and weak fluorescence signal (Fig. 2 *B*). With the immunoperoxidase technique, little or no reaction product was localized on this shallow occluding junction,

probably due to the fact that access of ZO-1 IgG to its antigen is limited by the densely packed cytoskeletal proteins of the brush border.

The tight junctions between the endothelial cells of peritubular (Fig. 3 *D*) and glomerular capillaries were also regularly labeled.

ZO-1 Is Concentrated in the Epithelial Foot Processes of the Adult Glomerulus

When ZO-1 was localized in the glomerulus by immunoperoxidase labeling, reaction product was for the most part restricted to the cytoplasm of the epithelial foot processes concentrated in the regions where the slit diaphragms insert (Fig. 4, *A* and *B*). Oblique sections through the foot process layer emphasize that the reaction product was densest along the cytoplasmic surface of the lateral membrane of the foot processes (Fig. 4 *B*). Immunogold experiments on ultrathin cryosections from adult kidney confirmed that the protein is concentrated along the slit diaphragms. Gold particles were located in the cytoplasm of the podocyte foot processes at the same level as the insertion point of the slit diaphragms (Fig. 5 *A*). Relatively few gold particles (one to two) were seen per slit diaphragm insertion point. The distribution of ZO-1 in grazing sections suggests that the protein is distributed as a continuous band alongside the slit diaphragm which itself is a continuous structure (7, 18). It is difficult to be certain whether the protein is confined to the sites of slit diaphragm insertion or if it is also present at lower concentrations in other regions of the foot process.

With the immunoperoxidase method there is some general staining of the foot process cytoplasm which could be due to diffusion of reaction product away from the antibody binding site (4). With the immunogold method the gold particles are clearly concentrated along the slit membranes, but since labeling is never very heavy this method may not be sensitive enough to detect sites of lower concentration of the protein.

When immunogold labeling was carried out on ultrathin cryosections of slit diaphragm–enriched fractions prepared by detergent extraction of isolated glomeruli, labeling for ZO-1 was detected over the accentuated insertion points of the residual slit diaphragms (Fig. 5, C–E). The level of labeling was somewhat heavier (three to six vs. one to three gold particles per insertion point) than in situ. This provides further evidence that ZO-1 is concentrated along the slit diaphragms. The immunocytochemical findings together with our immunoblotting analysis (Fig. 1, lane C) demonstrate that at least some of the ZO-1 remains behind after the podocyte foot processes have been broken open and the soluble proteins extracted by detergent treatment.

ZO-1 Is Retained on the Membrane during Podocyte Development as Tight Junctions Migrate and Are Replaced by Slit Diaphragms

In 2-d-old rat kidneys immunofluorescence staining for ZO-1 was first seen during the S-shaped body stage (Fig. 6, A–C). This is the earliest stage at which the presumptive podocyte can be identified based on the expression of podocalyxin which is an apical domain marker for this cell type (19). At this time the presumptive podocytes occur as a typical polarized epithelial cell layer connected by junctional complexes at their apical–lateral cell membrane interface (16), and the junctions consist of a shallow occluding junction and a deeper adhering junction (16, 19) comparable in organization to those found between proximal tubule cells (Fig. 3 C). These junctions between the putative podocytes migrate as a wave down the cell membrane toward the basal cell surface as glomerular development progresses. At any single time the junctions in a given glomerulus are found progressively farther down the lateral cell borders between successive cells. The localization of ZO-1 to the intercellular contact point is clearly maintained throughout migration of the junctional complex (Fig. 6, A and C). In contrast to the adult glomerulus, the fluorescence signal between the differentiating glomerular epithelial cells at this stage of development is not as bright as between distal tubule cells. After double staining for ZO-1 and the apical domain marker, podocalyxin, the linear fluorescence signal typical for podocalyxin (19) outlined the apical and lateral cell surfaces (Fig. 6 B) down to the level where a fluorescent dot, i.e. the signal of ZO-1 (Fig. 6 C), marked the presence of a junctional complex.

The junctions reach their most basal location near the basement membrane and interdigitation between the basolateral plasmalemmal of adjoining cells begins during the capillary loop stage. At this time the punctate ZO-1 pattern is concentrated on the epithelial side of the glomerular basement membrane outlining the forming capillary loops (Fig. 6, D–E). With further interdigitation of the foot processes and the concomitant amplification of the basolateral membrane surface that takes place, staining for ZO-1 becomes increasingly more intense until it reaches its mature pattern

outlining all the forming capillary loops at the level of the foot process layer (Fig. 1 A).

Discussion

### The Slit Diaphragm Is a Variant of the Tight Junction

The molecular nature of the glomerular epithelial cell slit diaphragm bridging the filtration slits and its relationship to other intercellular junctions has remained a mystery. It was previously suggested, based on its developmental history and functional properties, that the slit diaphragm is a shallow variant of either an adhering or an occluding junction (19). In this paper we have shown that there is a structural relationship between the slit diaphragm and the tight junction based on the immunolocalization of the tight junction-associated protein, ZO-1, in glomeruli of developing and adult rat kidney. ZO-1 was detected in the cytoplasm along all tight junctions in the junctional complexes of all segments of the renal tubule, but it was most abundant in glomeruli where it was concentrated along the precise regions of the plasmalemma of the foot processes where the slit diaphragms insert. Therefore our observations support the concept that the slit membrane is a variant tight junction which is derived developmentally from the tight junction and shares with it at least one structural protein component and the functional property of defining the boundary between plasmalemmal domains. We assume the antigen recognized at slit membranes is authentic ZO-1 since immunoblots of kidney cortex and slit membrane enriched fractions revealed only a single 225-kD antigen (see Fig. 1) as in other tissues (1).

We have further shown that there is a close developmental relationship between the tight junction and the slit diaphragm. ZO-1 is present in glomerular epithelial cells early in their development when they possess typical tight and adhering junctions positioned at the apical–lateral membrane inflection; it remains present as the junctions migrate along the lateral surface and disappear coincident with the opening of the intercellular spaces and the appearance of slit diaphragms. The fact that ZO-1 remains present throughout this process provides further evidence of the close relationship between tight junctions and slit diaphragms.

Correlation between ZO-1 and the Fibril Content of Tight Junctions in Tubule Epithelia

ZO-1 is a peripheral membrane protein associated with the cytoplasmic surface of the tight junction or zonula occludens in all epithelial and endothelial cell types studied (24). It has been localized to the point of membrane contact within the junction (23) that corresponds closely to the distribution of fibrils seen by freeze fracture. In the present study we have noted that among tubule epithelia there is good correlation between the content of ZO-1 and the number of freeze-fracture fibrils. In the distal and collecting tubule where numerous (three to five) fibrils are present ZO-1 is abundant, whereas in the proximal tubule where there is only a single interrupted fibril ZO-1 is sparse.

The tight junction fibrils are believed to be composed of rows of integral membrane proteins responsible for creating the intercellular occlusion, but the identity of the putative occluding element remains unknown (9, 22). The function of ZO-1 is also unknown; however, based on its distribution
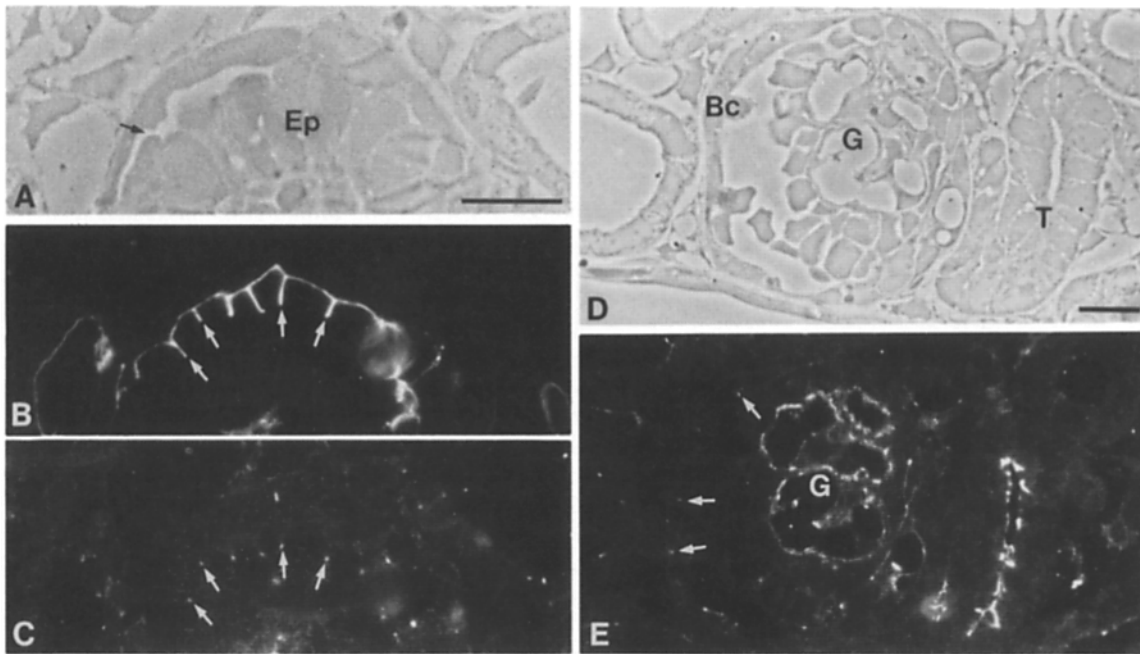
Figure 6. (A–C) Portion of a developing glomerulus (S-shaped body stage) seen by phase-contrast microscopy (*A*) or by immunofluorescence after double staining for podocalyxin (*B*) and ZO-1 (*C*). At this stage Bowman's space (*arrow* in *A*) is open and the presumptive podocytes are organized into a columnar epithelial cell layer (*Ep*). After labeling for podocalyxin (*B*), which is an apical differentiation marker for podocytes (19), bright linear staining outlines the apical cell surface of the latter and extends partially down their lateral surfaces (*arrows*). After labeling for ZO-1 (*C*), faint fluorescent dots are seen at exactly the points where the linear signal of podocalyxin ends (*arrows*). (*D–E*) Developing glomerulus (Capillary loop stage). The fluorescent signal for ZO-1 is concentrated on the epithelial side of the glomerular basement membrane of the forming capillary loops. ZO-1 labeling forms a series of dots or an interrupted line surrounding the capillary lumens. The level of the dots corresponds to the level of the forming foot processes. Arrows point to junctions between the epithelial cells of Bowman's capsule (*Bc*). Note also the prominent staining for ZO-1 in the developing tubule epithelium (*T*). Bars, 50 μm.

it is assumed to be closely associated with the occluding element and interact either directly or indirectly with its cytoplasmic domain.

Lack of Correlation between ZO-1 and the Fibril Content of the Glomerular Epithelium

The glomerular epithelium represents an exception among renal epithelia in that staining for ZO-1 is prominent, whereas no fibril-like intramembrane specialization has been noted by freeze fracture of normal podocytes (15). This demonstrates that expression of ZO-1 alone does not lead to tight junction assembly. However, it has been shown (20) that the slit diaphragms can be rapidly (within 1–2 min) converted into tight junctions experimentally by infusion of polycations into glomeruli which neutralizes the surface charge on the podocytes. Under these conditions the foot processes collapse, and the slit diaphragms are replaced by tight junctions with typical fibrils recognizable by freeze fracture (11, 20). Remarkably the conversion from slit diaphragm to tight junction is rapidly reversible by perfusion of polyanions (20). This suggests that (*a*) preformed pools of junctional proteins exist in the glomerulus which can be assembled and disassembled under appropriate conditions, and (*b*) cell surface charge normally serves to prevent their assembly.

A key question is, "Why is the immunofluorescence staining for ZO-1 much more prominent in glomerular than in tubule epithelia?" It seems likely that it is due at least in part to the redundancy of the highly infolded plasmalemma of the

foot processes and associated slit diaphragms which cause a greatly amplified fluorescence signal. By contrast the level of immunogold labeling was relatively low. However, it has been demonstrated (10) that immunogold labeling varies as a function of accessibility and has an efficiency of only ∼10–15%. The fact that heavier labeling for ZO-1 was seen in the slit diaphragm–enriched fractions suggests that accessibility is improved by extraction of soluble proteins from the foot processes.

Structural and Functional Relationships between Slit Diaphragms and Occluding and Adhering Junctions

Tight junctions of typical transporting epithelia are defined by several well recognized functions: (*a*) they are continuous in three dimensions and form a gasket that seals off the intercellular spaces and restricts the movement of proteins, water and solutes along the paracellular pathway (6, 9), and (*b*) they serve as a fence maintaining the polarized distribution of membrane proteins by restricting the intermixing of distinct apical and basolateral surface proteins (9, 22) and lipids (27) by diffusion in the plane of the membrane. The slit diaphragm, like the tight junction, is a continuous structure in three dimensions, but it clearly does not serve as a seal since there is a wide gap between foot processes which allows ultrafiltration of large fluid volumes and escape of other plasma components with an effective molecular radius <70 Å. However, the slit diaphragm does retain a fencelike function, because the podocyte is clearly polarized with its surface do-

mains demarcated by the slit membrane. During glomerular development, podocalyxin, a 140-kD membrane glycoprotein characterized previously (12), is restricted to the apical-lateral cell surface above the level of the tight junctions. After replacement of the latter with slit diaphragms podocalyxin remains confined to the apical–lateral domain above the slit diaphragms (19).

Besides serving as a fence, the slit membrane also functions in adhesion as it serves to firmly attach the foot processes to one another and to stabilize the foot process layer against the high filtration pressure ($\sim$55 mm Hg) to which these capillaries are subjected. Since the slit membrane has some morphological features (wide intercellular gap, presence of a central dense line in grazing sections [7, 18]) in common with adhering junctions, we cannot rule out the possibility that these junctions are also sites of concentration of adhering junction proteins. However, to date no desmosomal or other adhering junction protein has been detected in the visceral glomerular epithelium. In fact uvomorulin or L-Cam, which is the only well-characterized integral membrane protein associated with the zonula adherens, is detected in renal tubules but does not appear to be expressed in developing or mature glomeruli (28). Vinculin and talin, two cytoskeletal proteins found only at adhesive-type contacts (8), have been localized in the podocyte foot processes (5), but they were not detected along slit diaphragms. These proteins appear to be confined to the cytoplasm immediately adjacent to sites of attachment of the basal membrane of the foot processes to the basement membrane.

In summary, we have made the novel observation that ZO-1, a protein previously thought to be expressed exclusively as a component of the tight junction, is present along the slit diaphragm junctions of the glomerular epithelium. The implication of our studies is that the tight junction is transformed during glomerular development into the slit diaphragm and that in some pathologic conditions this process is reversed. It will be of interest to further define the molecular components that are unique to, or shared by each junctional type and to determine how the structural organization of these two intercellular junction types is controlled in development and disease.

References

1. Anderson, J. M., C. M. Van Itallie, M. D. Peterson, B. R. Stevenson, E. A. Carew, and M. S. Mooseker. 1989. ZO-1 mRNA and protein expression during tight junction assembly in Caco-2 cells. *J. Cell Biol.* 109:1047–1056.

2. Brown, W. J., and M. G. Farquhar. 1984. The mannose-6-phosphate receptor for lysosomal enzymes is concentrated in cis Golgi cisternae. *Cell.* 36:295–307.

3. Claude, P., and D. A. Goodenough. 1973. Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia. *J. Cell Biol.* 58:390–400.

4. Courtoy, P. J., D. H. Picton, and M. G. Farquhar. 1983. Resolution and limitations of the immunoperoxidase procedure in the localization of extracellular matrix antigens. *J. Histochem. Cytochem.* 31:945–951.

5. Drenckhahn, D., and R. P. Franke. 1988. Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man. *Lab. Invest.* 59:673–682.

6. Farquhar, M. G., and G. E. Palade. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* 17:375–412.

7. Farquhar, M. G., S. L. Wissig, and G. E. Palade. 1961. Glomerular Permeability: I. Ferritin transfer across the normal glomerular capillary wall. *J. Exp. Med.* 113:47–66.

8. Geiger, B., T. Volk, and T. Volberg. 1985. Molecular heterogeneity of adherens junctions. *J. Cell Biol.* 101:1523–1531.

9. Gumbiner, B. 1987. Structure, biochemistry, and assembly of epithelial tight junctions. *Am. J. Physiol.* 253:C749–C758.

10. Howell, K. E., U. Reuter-Carlson, E. Devaney, J. P. Luzio, and S. D. Fuller. 1987. One antigen, one gold? A quantitative analysis of immunogold labeling of plasma membrane 5'-nucleotidase in frozen thin sections. *Eur. J. Cell Biol.* 44:318–327.

11. Kerjaschki, D. 1978. Polycation-induced dislocation of slit diaphragms and formation of cell junctions in rat kidney glomeruli. *Lab. Invest.* 39:430–440.

12. Kerjaschki, D., D. J. Sharkey, and M. G. Farquhar. 1984. Identification and characterization of podocalyxin—the major sialoprotein of the renal glomerular epithelial cell. *J. Cell Biol.* 98:1591–1596.

13. McLean, W., and P. F. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077–1083.

14. Miettinen, A., G. Dekan, and M. G. Farquhar. 1990. Monoclonal antibodies against membrane proteins of the rat glomerulus: immunochemical specificity and immunofluorescence distribution of the antigens. *Am. J. Pathol.* In press.

15. Pricam, C., F. Humbert, A. Perrelet, M. Amherdt, and L. Orci. 1975. Intercellular junctions in podocytes of the nephrotic glomerulus as seen with freeze-fracture. *Lab. Invest.* 33:209–218.

16. Reeves, W., J. P. Caulfield, and M. G. Farquhar. 1978. Differentiation of epithelial foot processes and filtration slits. Sequential appearance of occluding junctions, epithelial polyanion, and slit membranes in developing glomeruli. *Lab. Invest.* 39:90–100.

17. Reeves, W., Y. S. Kanwar, and M. G. Farquhar. 1980. Assembly of the glomerular filtration surface. Differentiation of anionic sites in glomerular capillaries of newborn rat kidneys. *J. Cell Biol.* 85:735–753.

18. Rodewald, R., and M. J. Karnovsky. 1974. Porous substructure of the glomerular slit diaphragm in the rat and mouse. *J. Cell Biol.* 60:423–433.

19. Schnabel, E., G. Dekan, A. Miettinen, and M. G. Farquhar. 1989. Biogenesis of podocalyxin; the major glomerular sialoglycoprotein, in the newborn rat kidney. *Eur. J. Cell Biol.* 48:313–326.

20. Seiler, M. W., H. G. Rennke, M. A. Venkatachalam, and R. S. Cotran. 1977. Pathogenesis of polycation-induced alterations ("fusion") of glomerular epithelium. *Lab. Invest.* 36:48–61.

21. Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multilabeling cytochemistry. *Eur. J. Cell Biol.* 38:87–93.

22. Stevenson, B. R., J. M. Anderson, and S. Bullivant. 1988. The epithelial tight junction: structure, function, and preliminary biochemical characterization. *Mol. Cell Biochem.* 83:129–145.

23. Stevenson, B. R., M. B. Heintzelman, J. M. Anderson, S. Citi, and M. S. Mooseker. 1989. ZO-1 and cingulin: tight junction proteins with distinct identities and localizations. *Am. J. Physiol.* 257:C621–C628.

24. Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103:755–766.

25. Tokuyasu, K. T. 1986. Application of cryoultramicrotomy to immunocytochemistry. *J. Micros. (Oxf.).* 143:139–149.

26. Tokuyasu, K. T. 1989. Use of polyvinylpyrrolidone and polyvinyl alcohol for cryoultramicrotomy. *Histochem. J.* 21:163–171.

27. Van Meer, G., B. Gumbiner, and K. Simons. 1986. The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next. *Nature (Lond.).* 322:639–641.

28. Vestweber, D., R. Kemler, and P. Ekblom. 1985. Cell-adhesion molecule uvomorulin during kidney development. *Dev. Biol.* 112:213–221.