

# Identification of ZO-1: A High Molecular Weight Polypeptide Associated with the Tight Junction (Zonula Occludens) in a Variety of Epithelia

Bruce R. Stevenson,\* Janet D. Siliciano,† Mark S. Mooseker,\* and Daniel A. Goodenough‡

\*Department of Biology, Yale University, New Haven, Connecticut 06511-8112; †Department of Anatomy and Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

**Abstract.** A tight junction-enriched membrane fraction has been used as immunogen to generate a monoclonal antiserum specific for this intercellular junction. Hybridomas were screened for their ability to both react on an immunoblot and localize to the junctional complex region on frozen sections of unfixed mouse liver. A stable hybridoma line has been isolated that secretes an antibody (R26.4C) that localizes in thin section images of isolated mouse liver plasma membranes to the points of membrane contact at the tight junction. This antibody recognizes a polypeptide of ~225,000 D, detectable in whole liver homogenates as well as in the tight junction-enriched membrane fraction. R26.4C localizes to the junctional

complex region of a number of other epithelia, including colon, kidney, and testis, and to arterial endothelium, as assayed by immunofluorescent staining of cryostat sections of whole tissue. This antibody also stains the junctional complex region in confluent monolayers of the Madin-Darby canine kidney epithelial cell line. Immunoblot analysis of Madin-Darby canine kidney cells demonstrates the presence of a polypeptide similar in molecular weight to that detected in liver, suggesting that this protein is potentially a ubiquitous component of all mammalian tight junctions. The 225-kD tight junction-associated polypeptide is termed "ZO-1."

**E**PITHELIA are sheets of cells that stand at the interface of two biologically distinct compartments. These cell layers form a semipermeable boundary at this interface and act to regulate the transit of ions and molecules between compartments. For epithelial cells to form a complete permeability barrier, they must generate and maintain a gasket-like seal to occlude the intercellular route between compartments. This sealing function is carried out by the zonula occludens, or tight junction. Much morphological and physiological evidence suggest that the tight junction is a highly dynamic intercellular junction that plays a critical role in normal epithelial cell structure and function. However, very little information is available regarding the biochemical composition and molecular dynamics of this structure.

Questions have been raised about whether the branching and anastomosing fibrils visible in freeze-fracture images of the tight junction are composed of protein or lipid (24, 34, 38, 43). It has been demonstrated that protein synthesis inhibitors block the development of normal transepithelial resistance, a parameter determined largely by the tight junction, in freshly plated trypsinized Madin-Darby canine kidney (MDCK)<sup>1</sup> cells (8, 16), an epithelial cell line that

forms tight junctions in vitro (9, 32). Also, tight junction structure is preserved after exposure to either Triton X-100 or the anionic detergent sodium deoxycholate (DOC), treatments that would be expected to disrupt the normal lipidic environment (40, 41).

Very little direct information is available regarding proteins potentially involved in or associated with the tight junction. Although a large body of literature concerns molecules involved in cell adhesion (see reference 11 for review), none of these have been localized to the tight junction. It has been shown that a monoclonal antibody directed against a uvomorulin-like, 130-kD polypeptide can disrupt normal MDCK cell polarity and the tight junction, and shows a localized distribution at the junctional complex region of some epithelial cells upon immunofluorescent staining (2, 22). Gumbiner and Simons (17) have demonstrated that a monoclonal antibody specific for a molecule similar to uvomorulin can inhibit the formation of normal transepithelial resistance in MDCK monolayers. These authors localize this molecule to the lateral surface of this cell type, whereas uvomorulin has been localized to the zonula adherens in intestinal epithelial cells using immunoelectron microscopy (6). Subcellular fractionation work from a number of laboratories has extensively catalogued protein markers and enzyme activities specific for either the sinusoidal, lateral, or canalicular membrane domains in the hepatocyte (1, 10, 19-21, 29, 35-37). None of the markers, however, have been shown to be associated with the tight junction.

1. *Abbreviations used in this paper:* DOC, sodium deoxycholate; DOC-JR, DOC-insoluble junctional ribbons; DTT, dithiothreitol; FBS, fetal bovine serum; MDCK, Madin-Darby canine kidney; TBS, Tris-buffered saline; TIU, trypsin inhibitor units.

The relationship of cytoskeletal proteins to the tight junction is as yet undetermined, although alpha-actinin and vinculin have been localized to the cytoplasmic area near the tight junction by immunocytochemistry (14). Implications of tight junction-cytoskeletal interactions also come from studies which show that drugs that disrupt cytoskeletal elements can also affect tight junction morphology and physiology (5, 30, 31). It has been demonstrated that contraction of isolated intestinal brush borders at the level of the zonula adherens can result in alteration of tight junction structure (7, 25), leading to the hypothesis that this contraction may act *in vivo* to alter tight junction permeability.

An alternative approach to studying the biochemical characteristics of the tight junction was established by the development of a protocol for the isolation of tight junction-enriched membrane fractions from mouse hepatocytes (40). Through selective subcellular fractionation and extraction with the detergent DOC, a preparation is obtained that contains ribbon-like remnants of the junctional complex. Embedded within these DOC-insoluble junctional ribbons (DOC-JR) are the structural elements characteristic of the tight junction. While the number of polypeptides present in the DOC-JR, as determined by SDS PAGE, is markedly reduced relative to whole hepatocyte plasma membrane fractions, the gel profile remains complex. Attempts to further enrich subcellular preparations for tight junction components have proven unsuccessful, largely because the only assay for the tight junction up to this point has been morphological.

In light of these problems, we have attempted to obtain monoclonal antisera specifically reactive with elements of the tight junction. We report here that by using the DOC-JR as immunogen, a monoclonal antibody (R26.4C) has been generated which recognizes a polypeptide, of ~225,000 D, that is localized by immunoelectron microscopy to precisely the points of membrane contact of the hepatocyte tight junction. In addition, R26.4C shows immunofluorescent staining of the tight junction region in frozen sections from a variety of mouse and rat epithelia. This protein has also been identified in the MDCK epithelial cell line. We have called the 225-kD protein "ZO-1" to indicate that it is the first polypeptide demonstrated to be exclusively associated with the tight junction.

## Materials and Methods

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

### Cell Fractionation

**Liver Tight Junction-enriched Fraction.** The DOC-JR was isolated from mouse livers according to the protocol described by Stevenson and Goodenough (40) with the following modifications: Livers were homogenized in 1 mM NaHCO<sub>3</sub>, pH 8.0, containing 2.5 µg/ml leupeptin, 0.5 µg/ml chymostatin, 0.5 µg/ml pepstatin, 30 µg/ml soybean trypsin inhibitor (1 µg inhibits 1.4 µg trypsin), 0.1 trypsin inhibitor units (TIU)/ml aprotinin, and 0.2 mM dithiothreitol (DTT). The same ingredients were also added to the detergent extraction solution (10 mM imidazole, pH 8.0, 1 mM EGTA, 0.5% DOC). All other solutions contained 0.02 TIU/ml aprotinin and 0.2 mM DTT. The purification of the canalculus-enriched fraction was done with a two-step (41%/sample-25%) sucrose gradient in 12 × 35-ml tubes and spun 90 min at 83,000 *g<sub>av</sub>* in two SW-28 rotors (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The particular DOC-JR used for the im-

munization resulting in the production of R26.4C was made with 1 µg/ml leupeptin, 2 µg/ml antipain, 0.01 TIU/ml aprotinin, 10 µg/ml benzamide, 1 µg/ml chymostatin, 1 µg/ml pepstatin, 5 mM phenylmethylsulfonyl fluoride (Eastman Kodak Co., Rochester, NY) and no DTT. All subsequent solutions, including the detergent extractions, contained 0.01 TIU/ml aprotinin.

**MDCK.** To prepare a plasma membrane-enriched fraction from MDCK cells, four 25-cm<sup>2</sup> flasks (Corning Glass Works, Corning, NY) of confluent cells were rinsed twice with PBS (10 mM phosphate, pH 7.4, 150 mM NaCl) and the cells removed by scraping with a rubber policeman in 2 ml/flask buffer II (10 mM imidazole, pH 7.4, 4 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 TIU/ml aprotinin, 0.2 mM DTT, 2.5 mg/ml leupeptin, 0.5 µg/ml pepstatin, 30 µg/ml soybean trypsin inhibitor). Cells were pooled and spun for 8 min at 400 *g<sub>av</sub>*. All further steps were carried out at 4°C. The cells were resuspended in 7 ml buffer II and homogenized in a dounce homogenizer using five strokes with a tight fitting pestle. Cells, but not nuclei, were broken by this procedure and apices from several cells held together by their junctional complexes were visible by phase-contrast microscopy. The homogenate was spun for 10 min at 8,000 *g<sub>av</sub>*, and this low-speed supernatant was set aside. The pellet was resuspended in 10 ml buffer II and spun 10 min at 2,500 *g<sub>av</sub>*. The supernatant was discarded and the pellet resuspended in 1 ml buffer II for analysis by SDS PAGE. The original low-speed supernatant was spun 1 h at 100,000 *g<sub>av</sub>* in a type 65 rotor (Beckman Instruments, Inc.) and both high-speed pellet and supernatant prepared for SDS PAGE analysis.

SDS PAGE samples were also made from whole MDCK cell sheets. A confluent 25-cm<sup>2</sup> dish was rinsed three times with ice-cold PBS containing 1 mM EGTA and 0.2 mM phenylmethylsulfonyl fluoride (PBS'). Cells were removed from the dish by gentle scraping with a rubber policeman in 5 ml PBS', and pelleted by centrifugation for 5 min at 400 *g<sub>av</sub>*. The cell pellet (~100 µl) was mixed with 100 µl of PBS' and resuspended by flicking the tube with a finger. Clumps of cells were clearly visible at this point. 80 µl of the cell suspension was slowly added to 20 µl of 10× gel sample buffer (see below) that was sitting in a boiling water bath. Samples were boiled for an additional 2 min.

### Cell Culture

The mouse myeloma cell line P3X63 Ag8U.1 (ATCC CRL 1597) was obtained from Drs. Victoria Lewis and Ira Mellman (Dept. of Cell Biology, Yale University School of Medicine). Cells were grown in either Dulbecco's modified Eagle's medium (DME) (cat. No. DM-326, KC Biological, Inc., Lenexa, KA) supplemented weekly with 2 mM glutamine, or RPMI-1640 (KC Biological, Inc.) supplemented with 1 mM sodium pyruvate, plus 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 100 U penicillin and 100 µg streptomycin/ml (Gibco Laboratories, Grand Island, NY). Cell density was maintained at 1-6 × 10<sup>5</sup> cells/ml. Hybridomas were grown in DME or RPMI-1640 supplemented with 20% FBS, 10% NCTC-109 (KC Biological, Inc.) and penicillin-streptomycin. Hypoxanthine/aminopterin/thymidine (HAT) and hypoxanthine/thymidine (HT) from 50× lyophilized stocks (final concentrations 0.1 mM hypoxanthine, 4 × 10<sup>-4</sup> mM aminopterin, 1.6 × 10<sup>-2</sup> mM thymidine) were added when appropriate. MDCK cells were grown in DME, 10% FBS, penicillin-streptomycin, and passaged weekly. All cells were maintained at 37°C in a humidified incubator with 95% air/5% CO<sub>2</sub>.

### Monoclonal Antibodies

**Immunization.** 10% SDS (BDH Chemicals Ltd., Poole, England) was added to the DOC-JR suspension to a final concentration of 1% and boiled for 2 min. It was then combined with an equal volume of complete Freund's adjuvant (Gibco Laboratories) and emulsified. A female, 21-d-old CD rat (Charles River Breeding Laboratories, Inc., Wilmington, MA) was injected *i.p.* with 0.5 mg total protein for the primary immunization. After a 2-wk rest the rat was injected *i.p.* with 0.5 mg of the identical SDS-denatured antigen emulsified with an equal volume of incomplete Freund's adjuvant. 1 wk later the animal was bled from the tail and a positive response to the immunogen verified by dot assay (see below). The rat was again boosted *i.p.* 4 d before fusion with 0.4 mg antigen without adjuvant.

**Fusion.** Rat splenocytes were fused with the P3 myeloma cells according to the techniques of Fazekas de St. Groth and Scheidegger (12). 50% polyethylene glycol (PEG 4000, Boehringer Mannheim Biochemicals, Indianapolis, IN) in RPMI-1640 containing 5% dimethylsulfoxide was used as the fusogen. The initial fusion products were plated in two 24-well plates (Linbro, Inc., Hamden, CT) in HAT medium and fed every 4 d. Cells were grown in media containing HAT during week 1 and media containing HT

during week 2. Fusion plates were screened for antibody production starting on day 7.

**Screening.** The original fusion master wells were first screened for positive activity against SDS-denatured DOC-JR on a modified ELISA assay (42). Approximately 0.2  $\mu\text{g}$  antigen was dotted on small ( $\sim 9 \text{ mm}^2$ ) nitrocellulose squares (0.2  $\mu\text{m}$ , Schleicher & Schuell, Inc., Keene, NH). These squares were then blocked with PBS-BLOTTO (23, 5% nonfat dry milk, Carnation Co., Los Angeles, CA) and sequentially incubated with culture well supernatants, peroxidase-conjugated rabbit anti-rat Ig (Boehringer Mannheim, diluted 1:500 in PBS), and 0.05% 3,3' diaminobenzidine, 0.01%  $\text{H}_2\text{O}_2$  in PBS. A rat monoclonal antibody (graciously provided by Dr. Ira Mellman) dotted directly on the nitrocellulose served as a positive control.

Culture wells that showed positive activity on the dot assay were then tested on frozen sections of unfixed mouse or rat liver. Small pieces of fresh liver from a young animal were rapidly frozen in isopentane with a minimum of handling and stored in liquid nitrogen. 5–10  $\mu\text{m}$  frozen sections were cut and applied to gelatin-coated multispot microscope slides (Shandon Southern Instruments Inc., Sewickley, PA), dipped in absolute acetone at  $-20^\circ\text{C}$  for 2 min, stained with the culture supernatant and fluorescein isothiocyanate-conjugated rabbit anti-rat Ig (Boehringer Mannheim, diluted 1:200 in PBS), and viewed with a Zeiss light microscope (Carl Zeiss, Inc., Oberkochen, West Germany) equipped with epifluorescent illumination and either 40 $\times$  or 63 $\times$  planapo objectives.

**Cloning and Propagation.** Those wells that showed positive activity on both screens were immediately expanded and then plated out at clonal density (0.8 cell/well) in a 96-well dish with round bottom wells into which a feeder layer of peritoneal macrophages (12) had previously been plated. Clones were tested for antibody activity with the double screen as before. Positive wells were expanded and cloned a total of three times.

Antibody-rich supernatants that were used in experiments were obtained from hybridoma cultures that were grown to a density of  $\sim 1 \times 10^6$  cells/ml in standard culture media containing 20% FBS. For some experiments the hybridomas were grown to a density of 6–10  $\times 10^5$  cells/ml in standard culture media, centrifuged 8 min at 400  $g_{av}$ , and resuspended at the same density in RPMI-1640 containing 1% Nutridoma-SP (Boehringer Mannheim) and no FBS. After 24–48 h the supernatant was harvested by spinning first at 400  $g_{av}$  for 8 min to remove whole cells and then 15 min at 21,000  $g_{av}$  to remove any other debris. All supernatants were made 0.02% in sodium azide to prevent bacterial growth.

Supernatants from cultures containing only the parent myeloma line, culture media alone, culture media containing nonspecific rat IgG (100  $\mu\text{g}/\text{ml}$ ), or an anti-gap junction rat monoclonal antiserum (R5.21C) were used as negative controls.

## Immunohistochemistry

**Light Microscopy.** Frozen sections of various unfixed mouse or rat tissues were prepared for immunofluorescence as described above for liver. In some cases the secondary fluorescein isothiocyanate-conjugated rabbit anti-rat antibody was adsorbed before use with mouse liver plasma membranes from the canaliculus-enriched fraction (non-detergent treated). This involved mixing undiluted secondary antibody solution with an equal volume of the canaliculus-enriched membrane pellet, incubated on ice for 1 h, and spun for 15 min at 15,000  $g_{av}$ . The supernatant was again mixed with an equal volume of fresh membrane pellet, and incubated and spun as before. MDCK cells to be used for immunofluorescent staining were grown on sterile coverslips and rinsed with PBS before dipping in acetone and staining. After staining, all samples were mounted with PBS/60% glycerol, 0.4% *n*-propyl gallate. Photographs were taken on Tri-X film (Kodak) and developed with D-76.

**Electron Microscopy.** Canaliculus-enriched mouse hepatocyte plasma membranes were obtained from a 41%/25% sucrose step gradient as described above. These membranes were washed free of sucrose by centrifugation in Tris-buffered saline (TBS; 10 mM Tris, pH 8.0, 150 mM NaCl). Membrane pellets were resuspended by trituration in undiluted tissue culture supernatant containing either R26.4C or R5.21, the anti-gap junction monoclonal antibody. After a 3-h incubation at room temperature, the membranes were washed three times by centrifugation at 10,000  $g_{av}$  for 10 min in TBS, the final pellet resuspended directly in undiluted, preadsorbed rabbit anti-rat secondary antiserum coupled to 5-nm gold (GARa G-5EM, Janssen Life Sciences Products, Piscataway, NJ). Preadsorption was carried out using mouse canaliculus membranes as described for fluorescent secondary antibody for 2 h at room temperature, then washed three times with TBS as above. The final pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, postfixed in aqueous 2%  $\text{OsO}_4$ , stained with

1% aqueous uranyl acetate, and embedded in Epon. Sections were stained with lead citrate before viewing in a Siemens Elmiskop 101 (Siemens-Allis Inc., Cherry Hill, NJ).

## Biochemistry

SDS PAGE (27) was performed using 5–16% gradient minigels (28). 7.5% regular sized slab gels were used for the anti-gap junction immunoblot. Gel sample buffer was made as a 10 $\times$  stock containing 12.5 mM Tris, pH 6.8, 10% SDS, 20%  $\beta$ -mercaptoethanol, 20% sucrose, 0.5% bromphenol blue. Immunoblots were run according to a modification of the techniques of Towbin et al. (42). Gels were electrophoretically transferred (Trans-Blot Cell, Bio-Rad Laboratories, Inc., Richmond, CA, or TE Series Transphor Electrophoresis Unit, Hoefer Scientific Instruments, San Francisco, CA) onto 0.2  $\mu\text{m}$  pore nitrocellulose paper (Schleicher & Schuell, Inc.) in 25 mM Tris, pH 7.0, 192 mM glycine, 0.1% SDS, 20% methanol at 40 V for 24 h. The nitrocellulose was rinsed with  $\text{dH}_2\text{O}$ , stained for total protein with 0.2% ponceau S in 3% trichloroacetic acid, destained with  $\text{dH}_2\text{O}$ , and trimmed into appropriate lane groupings. Nonspecific protein binding was blocked with TBS/BLOTTO. Hybridoma culture supernatant containing the primary antibody was used undiluted, and the secondary antibody and developing solutions were as described above for the dot assay. TBS was used throughout. Molecular weight standards run on the same gels included human erythroid spectrin (240 and 220 kD) and chicken brush borders containing myosin (200 kD), villin (95 kD), fimbrin (68 kD), and actin (42 kD). Photographs of both gels and immunoblots were taken with Kodak Ektapan film (4162). Protein determinations were made according to the method of Hartree (18).

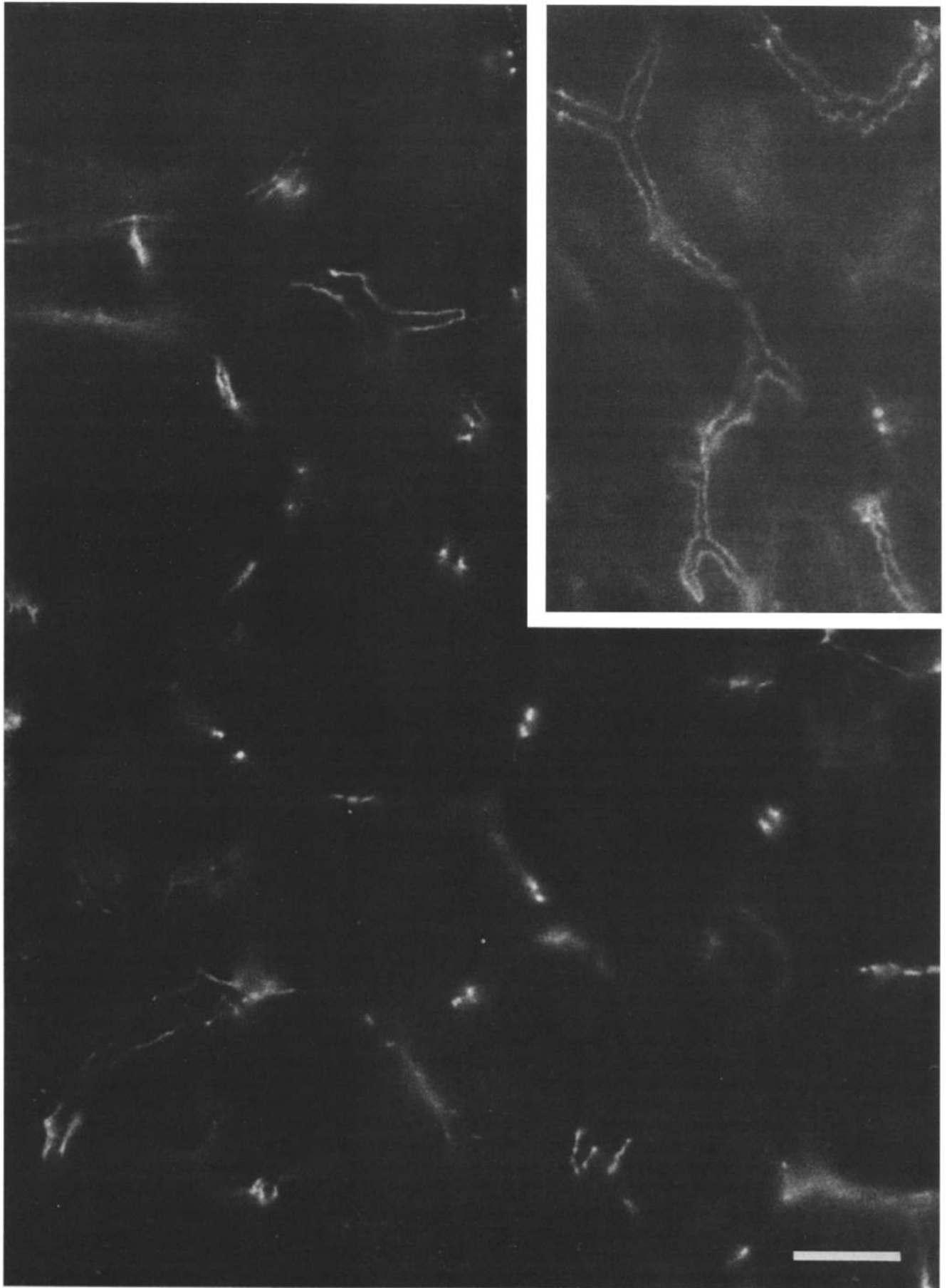
## Results

### Monoclonal Antibody Generation

A rat-mouse fusion was performed and screened for the ability to react with both the SDS-denatured ZO-enriched fraction DOC-JR and the junctional complex region on a frozen section of unfixed liver. The junctional complexes in this epithelial tissue are easily visualized in their obligatory position immediately adjacent to and on either side of the lumen of the bile canaliculus where they delineate the boundary between the apical, or canalicular, and basolateral, or serosal, compartments. Of the initial 48 fusion master wells from rat 26, 41 showed positive activity for both SDS-denatured antigen and the junctional complex region on frozen liver sections. Several wells were immediately expanded and cloned and from these a single stable hybridoma line, R26.4C, has been obtained. The localization of R26.4C using indirect immunofluorescence on a cryostat section of liver is shown in Fig. 1. Characteristic staining of the junctional complex region is revealed with this technique, visible in cross-section as a distinct pair of dots on either side of the bile canaliculus, and in longitudinal section as "railroad tracks," or two continuous lines, running along the lateral hepatocyte surface, again straddling the bile canaliculus. Branching of the bile canaliculi is also frequently observed, visible as a triad of paired lines (inset, Fig. 1). No other hepatocyte membrane surface or cytoplasmic domain was stained above background levels with R26.4C.

### Ultrastructural Localization

The immunofluorescent staining indicates that antibody activity is localized to the area of the junctional complex, but does not identify the specific reacting component. Standard approaches to this problem, such as ultrathin frozen sections, are made difficult by the fact that R26.4C does not react with tissue that has undergone even mild fixation.



Therefore, isolated liver plasma membranes from the canaliculus-enriched fraction were used to immunolocalize R26.4C at the electron microscopic level according to the techniques of Paul and Goodenough (33). As seen in Fig. 2, *a* and *b*, this antibody specifically stains the cytoplasmic surface of the tight junction. Labeling is observed either directly over or immediately adjacent to the points of membrane contact characteristically visible in thin-section images of this junction. No labeling is detected on nonjunctional membranes, or at zonulae adherentes, desmosomes, or gap junctions. Another rat-derived monoclonal antibody, specific for the gap junction 27-kD polypeptide, was used as a functional negative control. This antibody exclusively labels the cytoplasmic surface of the gap junction and not the tight junction or any other membrane or intercellular junction (Fig. 2 *c*).

### Tissue Cross-reactivity

The potential presence of the R26.4C epitope in the junctional complexes from other epithelia was assessed by immunofluorescent staining of cryostat sections of a variety of mouse and rat tissues. These results, demonstrating R26.4C staining in the epithelia of colon, kidney, and testes are shown in Fig. 3 and 4. In the colon, staining is observed in cross-section as bright spots at the apical-most aspect of the lateral surface of the absorptive epithelial cell (Fig. 3 *a*). In oblique or longitudinal section, belt-like or circumferential staining is seen around the apical pole of each cell (Fig. 3 *b*). R26.4C antibody activity is also detected in the junctional complex region in cross-sections of kidney tubule epithelia and in the junctional regions between contiguous arterial endothelial cells (Fig. 4 *a*). Similar endothelial staining is observed in an arteriole in the tunica propria between adjacent seminiferous tubules of the mouse testis. Sertoli-Sertoli cell junctions are also brightly stained where they separate the basal and adluminal testicular compartments (Fig. 4 *b*).

To determine the presence of R26.4C activity in epithelia from a different species, confluent monolayers of the canine-derived MDCK epithelial cell line were stained for immunofluorescence. Fig. 5 shows distinct antibody binding in these cells, visible as a network of staining surrounding each cell where it meets its neighbors. Optical sectioning reveals that this staining pattern is produced by a discreet band of fluorescence at the apical-lateral border of these cells, rather than continuous staining of the entire lateral surface.

### Biochemistry

Immunoblot analysis was performed to determine which of the polypeptides present in the DOC-JR fraction is recognized by the R26.4C antibody. When samples from sequential steps in the DOC-JR isolation protocol are run on SDS PAGE, transferred to nitrocellulose, and reacted for R26.4C activity, clear staining of a polypeptide at ~225 kD is observed (Fig. 6 *b*). This protein migrates between the  $\alpha$  and  $\beta$  subunits of human red blood cell spectrin. Successive fractions from the isolation protocol show a definite enrichment

for this protein, ZO-1. No obvious enrichment of a polypeptide in this molecular weight range is detected on the Coomassie Blue-stained gel of the same fractions. Minor bands of lower molecular mass (150–200 kD) and a general smeared staining in the region above ZO-1 are also visible in the immunoblots of DOC-JR. The higher molecular mass smearing can be quantitatively removed by passing the gel sample through a 0.2  $\mu$ m filter before SDS PAGE (Fig. 6, *c* and *d*). To verify both the presence and molecular weight of the R26.4C antigen in the starting material, an undiluted sample was taken from the initial liver homogenate and processed for R26.4C activity. Fig. 6, *c* and *d* shows definite staining of a similar size polypeptide, indicating that ZO-1 can be detected in whole liver and that gross proteolysis is not occurring during the isolation protocol. The anti-gap junction rat monoclonal antiserum (R5.21C) reacts with a 27-kD polypeptide and its 47-kD aggregate present in the DOC-JR fraction (Fig. 6 *e*).

ZO-1 was also detected in a biochemical analysis of MDCK cells. Samples of either whole, intact epithelial cell sheets added directly to boiling SDS or a low-speed pellet fraction enriched for MDCK plasma membranes isolated in the presence of protease inhibitors was prepared and run on an immunoblot for R26.4C activity. In both cases a band comigrating with ZO-1 from liver is stained (Fig. 7). No activity was detected in either the high speed pellet or supernatant fractions from the MDCK plasma membrane isolation protocol.

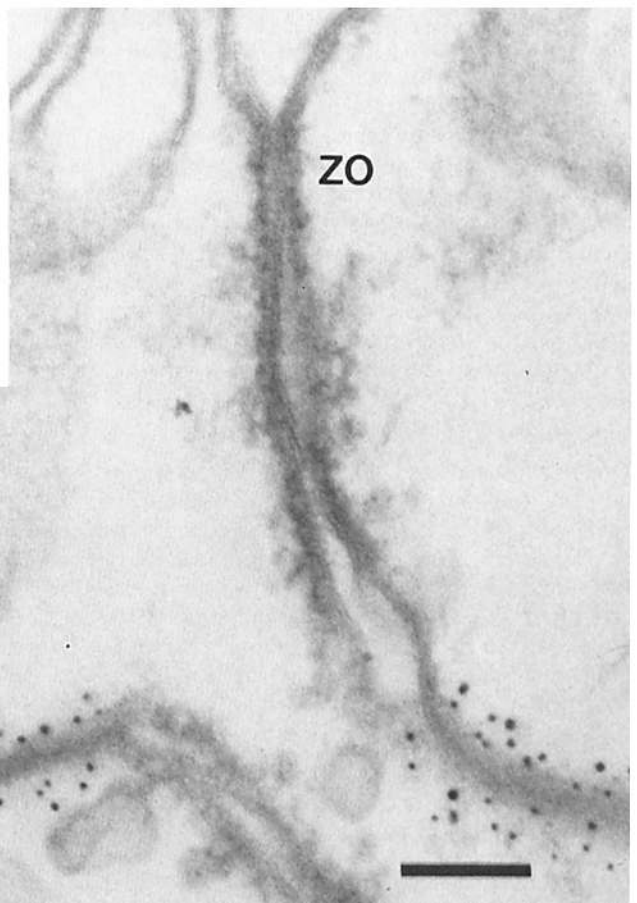
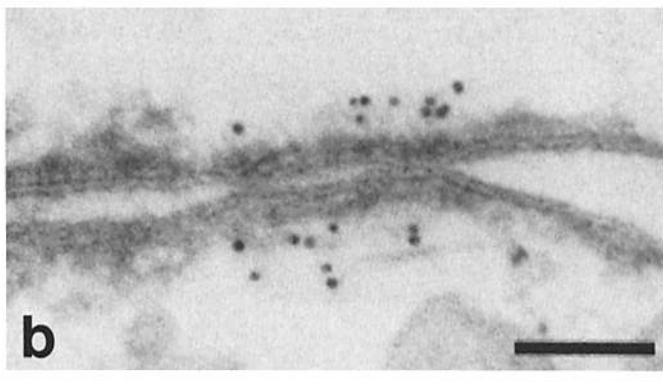
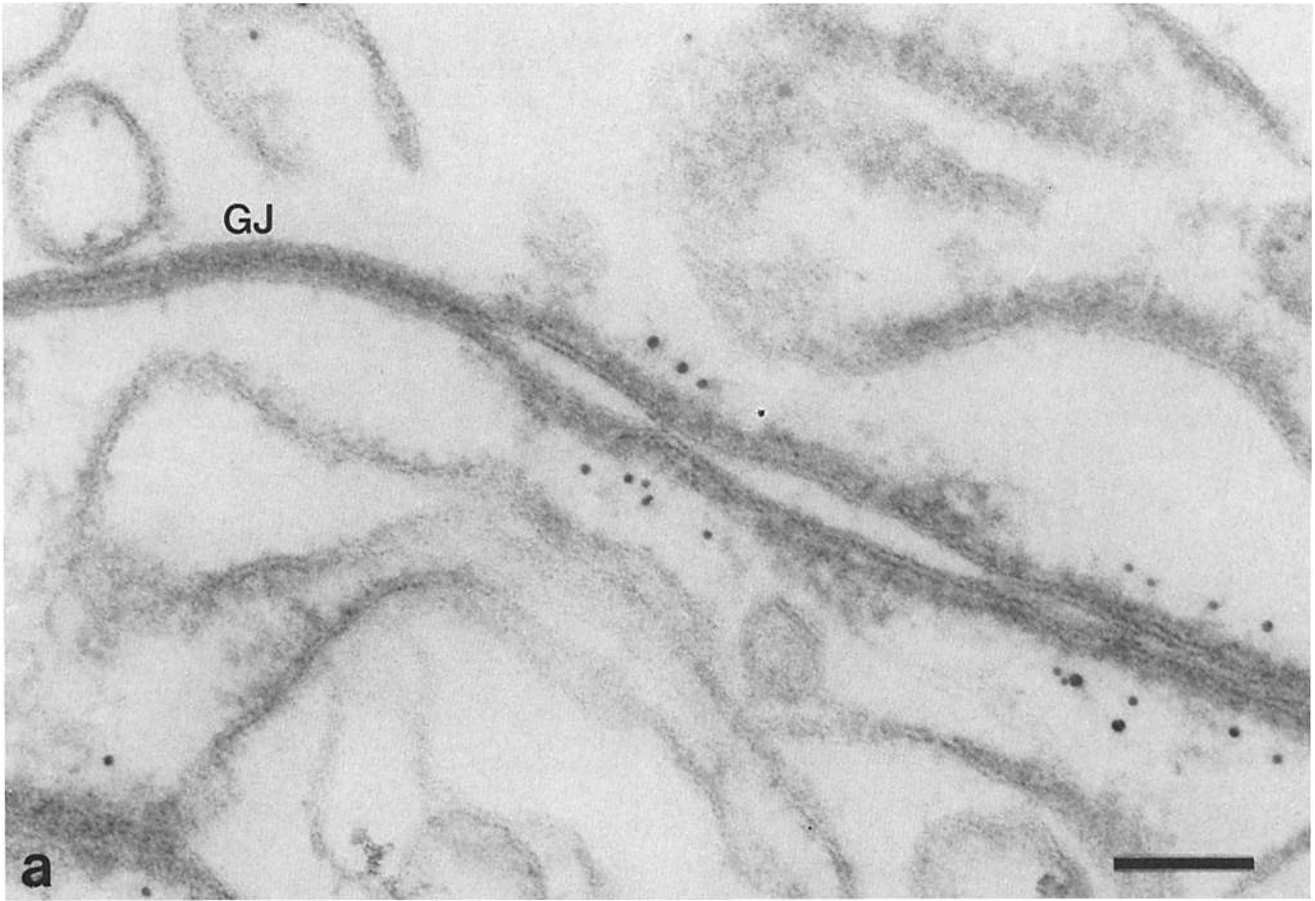
### Discussion

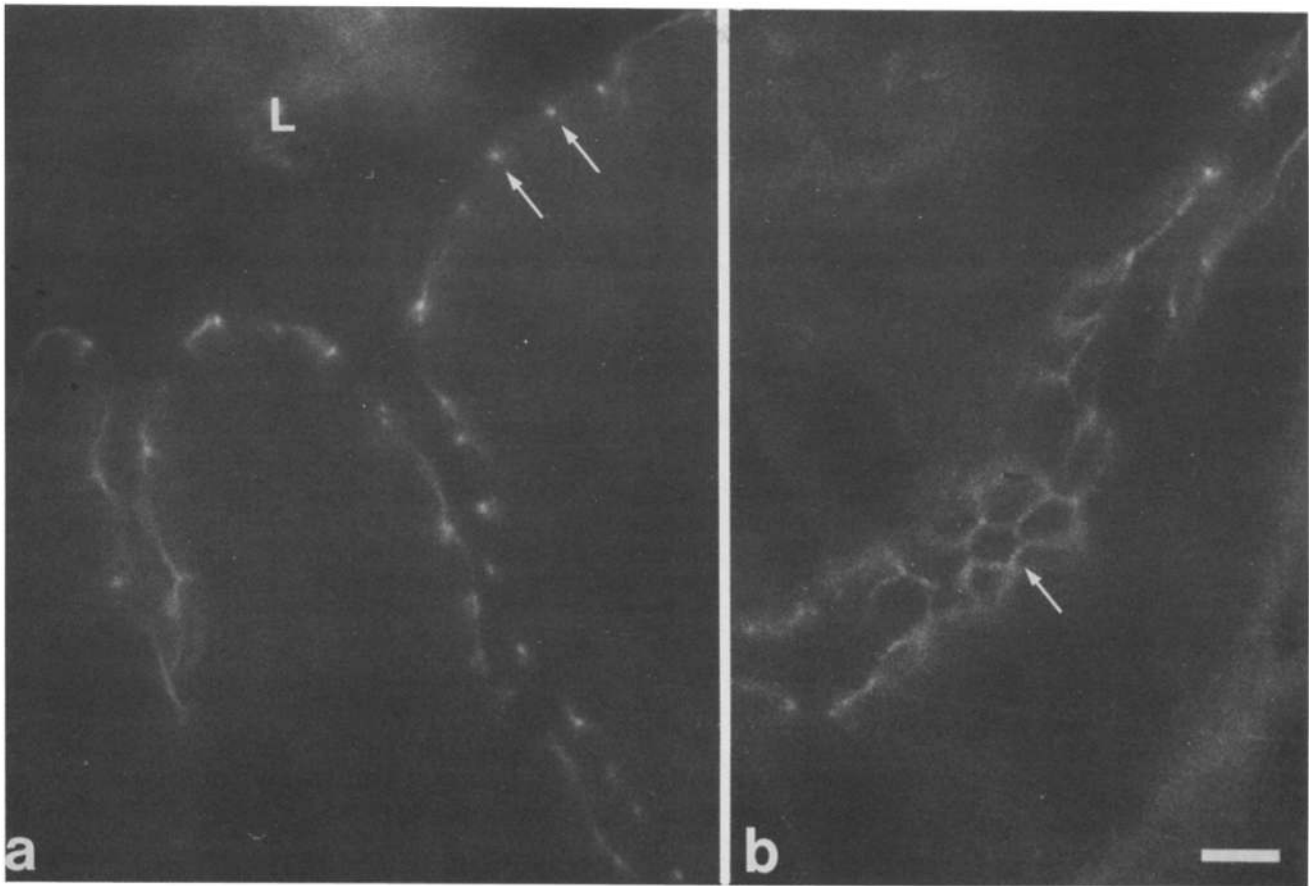
We have identified a 225-kD polypeptide, ZO-1, present in membrane fractions of the hepatocyte, which is specifically associated with the tight junction. Immunoreactive forms of ZO-1 are present in the junctional regions of other epithelial and endothelial cell types as well, suggesting that ZO-1 is a ubiquitous component of the mammalian tight junction.

The principle evidence for the identification of ZO-1 as a tight junction component is the ultrastructural localization of this protein in preparations of canaliculus-enriched plasma membrane fractions from liver (Fig. 2). The epitope recognized by R26.4C is localized to the cytoplasmic surface of the tight junction membrane, providing evidence for the exclusive localization of ZO-1 to the tight junction in these isolated membrane preparations. However, it is possible that some rearrangement or selective loss of ZO-1 from other membrane surfaces during isolation could have occurred. While this possibility must be examined by immunolocalization of ZO-1 intact hepatocytes, the discrete nature of the staining patterns seen both by light microscopy in intact tissue and electron microscopy argues against the artifactual association of ZO-1 with the tight junction during membrane isolation.

The relationship of ZO-1 to the tight junction fibrils visualized in freeze-fracture (13, 26, 39) and in negatively stained preparations (15, 40) cannot be discerned from these ultra-

*Figure 1.* Immunofluorescent localization of ZO-1 in frozen sections of rat liver. The monoclonal antibody R26.4C specifically stains the junctional complex region of the hepatocytes. In cross-section, a pair of dots on either side of the bile canaliculus is observed, while in longitudinal section a pair of parallel lines running along the lateral cell surface is visible. Inset shows numerous bile canaliculi in longitudinal section. At points where they branch, a triad of paired lines is displayed. Bar, 10  $\mu$ m.





**Figure 3.** Immunofluorescent localization of ZO-1 in frozen sections of mouse colon. As in the liver (Fig. 1), R26.4C staining is restricted to the junctional complex of the enterocyte. (a) A cross-section view of adjacent intestinal villi and a portion of the intestinal lumen (L). Staining appears as bright dots at the apical-most aspect of the lateral cell surface (arrows). (b) An oblique section through an intestinal crypt showing continuous belt-like fluorescence around the apical cell borders (arrow). Bar, 5  $\mu\text{m}$ .

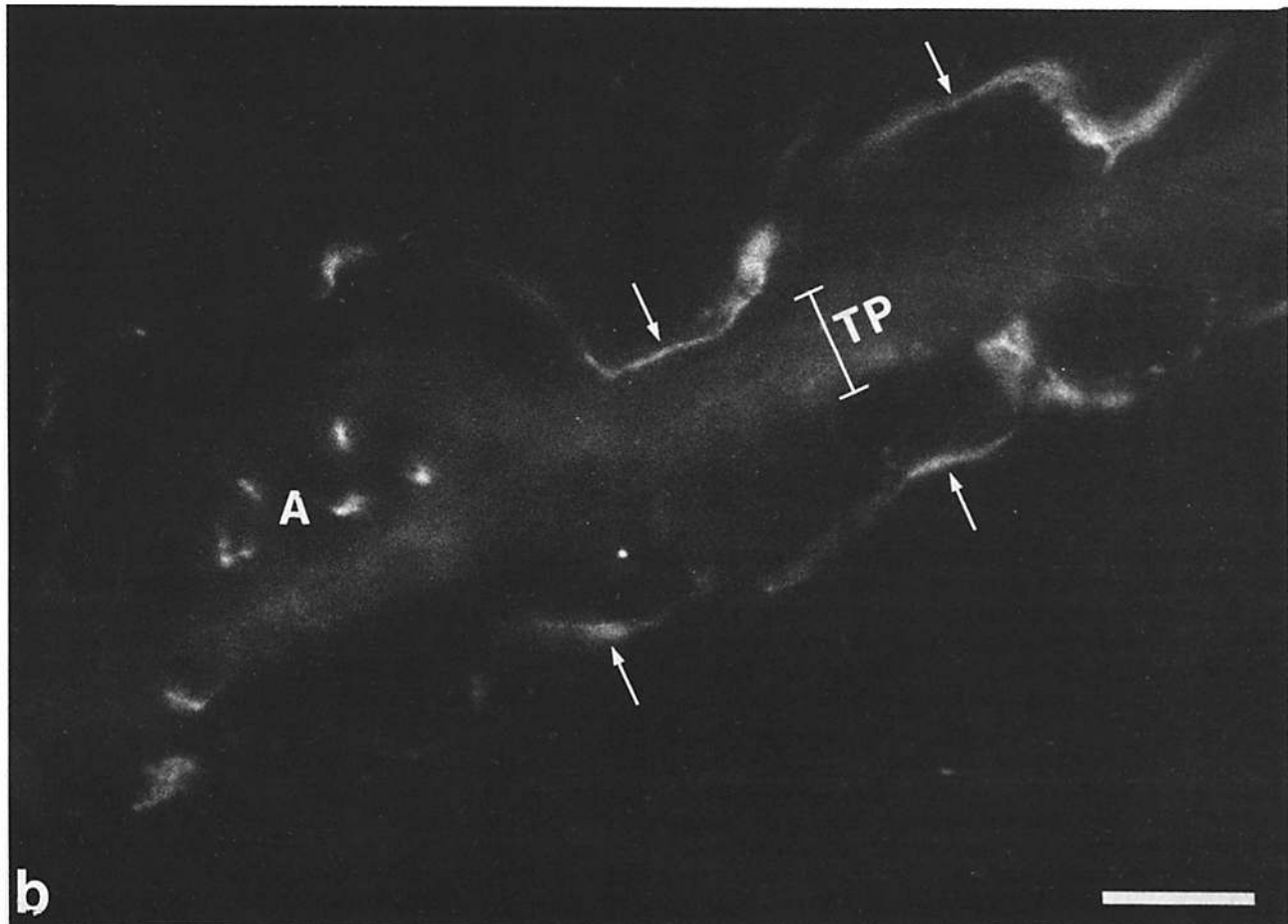
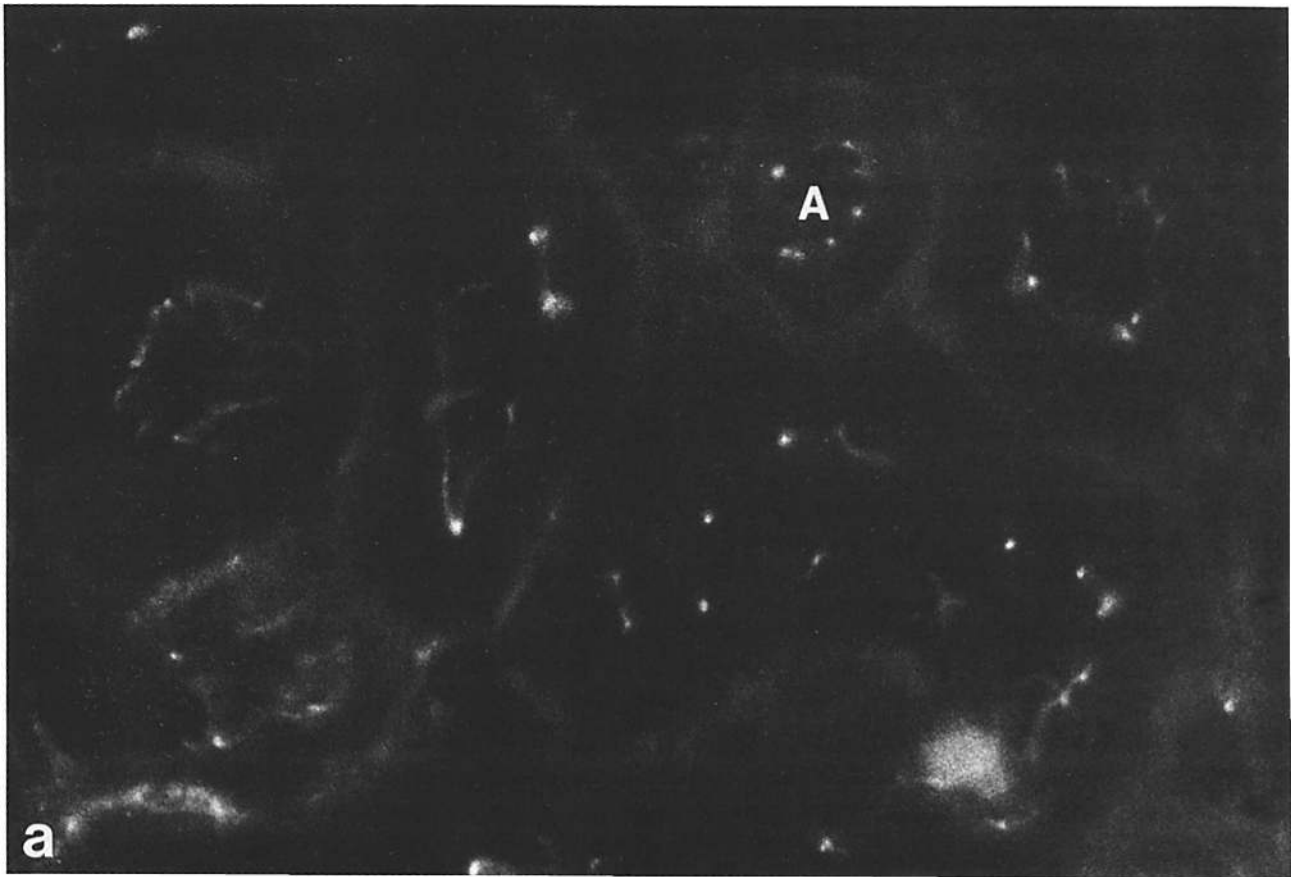
structural studies. The secondary antiserum-labeling method used to visualize the monoclonal antibody R26.4C is limited in resolution by the size of the sandwiched immunoreagents. The gold particles are observed to cluster around the points of membrane contact characteristically seen in thin-section images of the tight junction. With these data, however, it can only be concluded that ZO-1 is positioned close to the tight junction fibrils; no conclusions about the nature of the relationship of ZO-1 to the fibrils or the properties of the fibrils themselves can be drawn.

The results obtained from the immunofluorescent staining of various rat and mouse epithelial tissues and the canine cell line MDCK with R26.4C strongly suggest that ZO-1 is a ubiquitous element of the tight junction in mammalian epithelia. Ultrastructural localization of ZO-1 will be required to positively identify it as a tight junction component in these other cell types. Nevertheless, the presence of an R26.4C immunoreactive polypeptide in MDCK cells which co-migrates with mouse liver ZO-1, indicates that ZO-1 is a conserved protein which may play key roles in tight junction structure, function, and/or assembly.

Although the biochemical and functional characterization of ZO-1 must await future study, the results presented here do provide some clues regarding its properties as well as some directions for future experiments. The immunoblot analysis of successive steps in the purification of the DOC-JR membrane fraction (Fig. 6, a and b) indicates that the 225-kD polypeptide reactive with R26.4C is significantly enriched by these methods. It is also clear from comparison of the immunoblot with the Coomassie Blue-stained gel that ZO-1 is a relatively minor component of the final DOC-JR fraction. In fact, unambiguous assignment of the immunoreactive 225-kD band to one of the several high molecular weight bands detectable by protein staining has thus far not been possible.

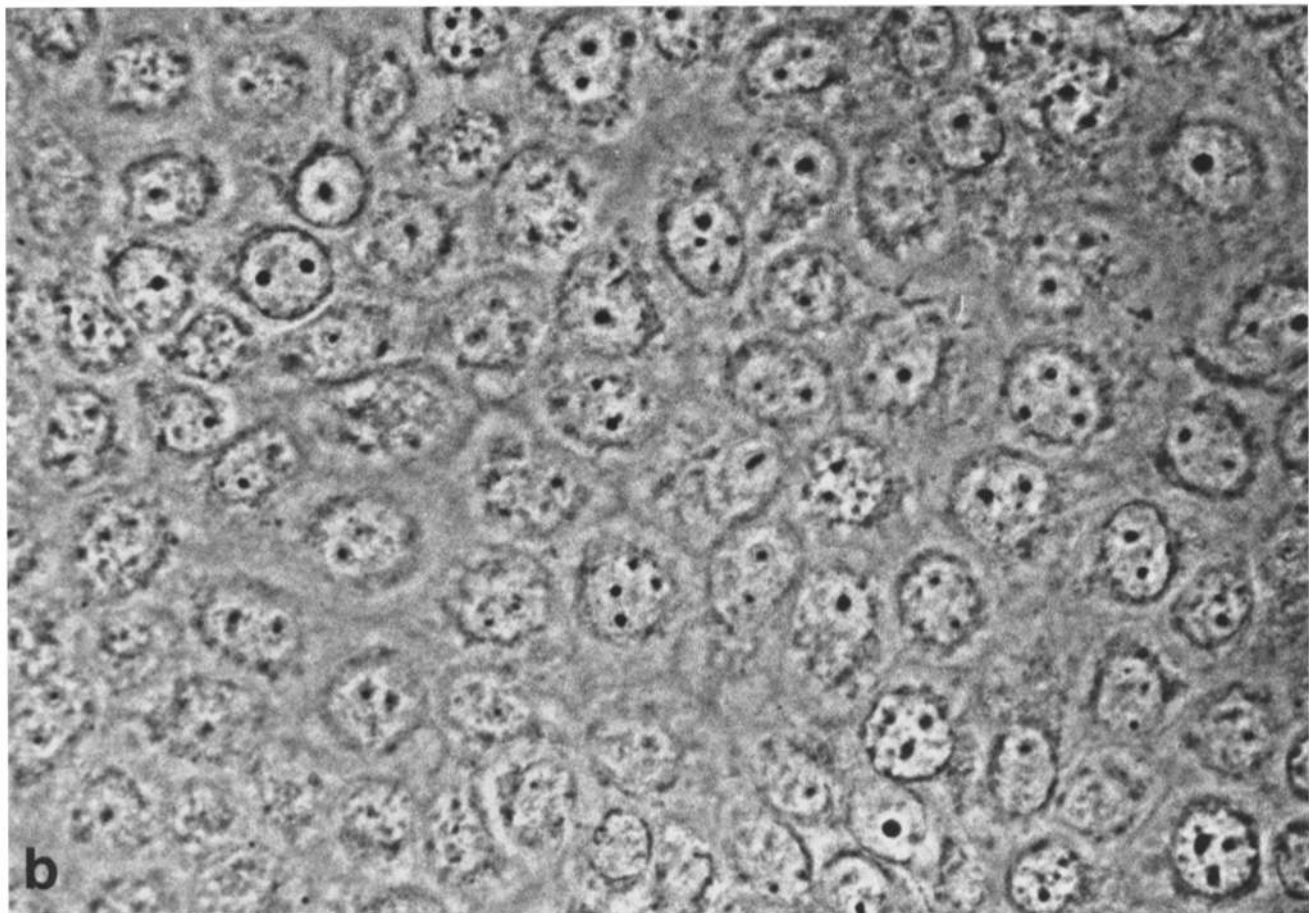
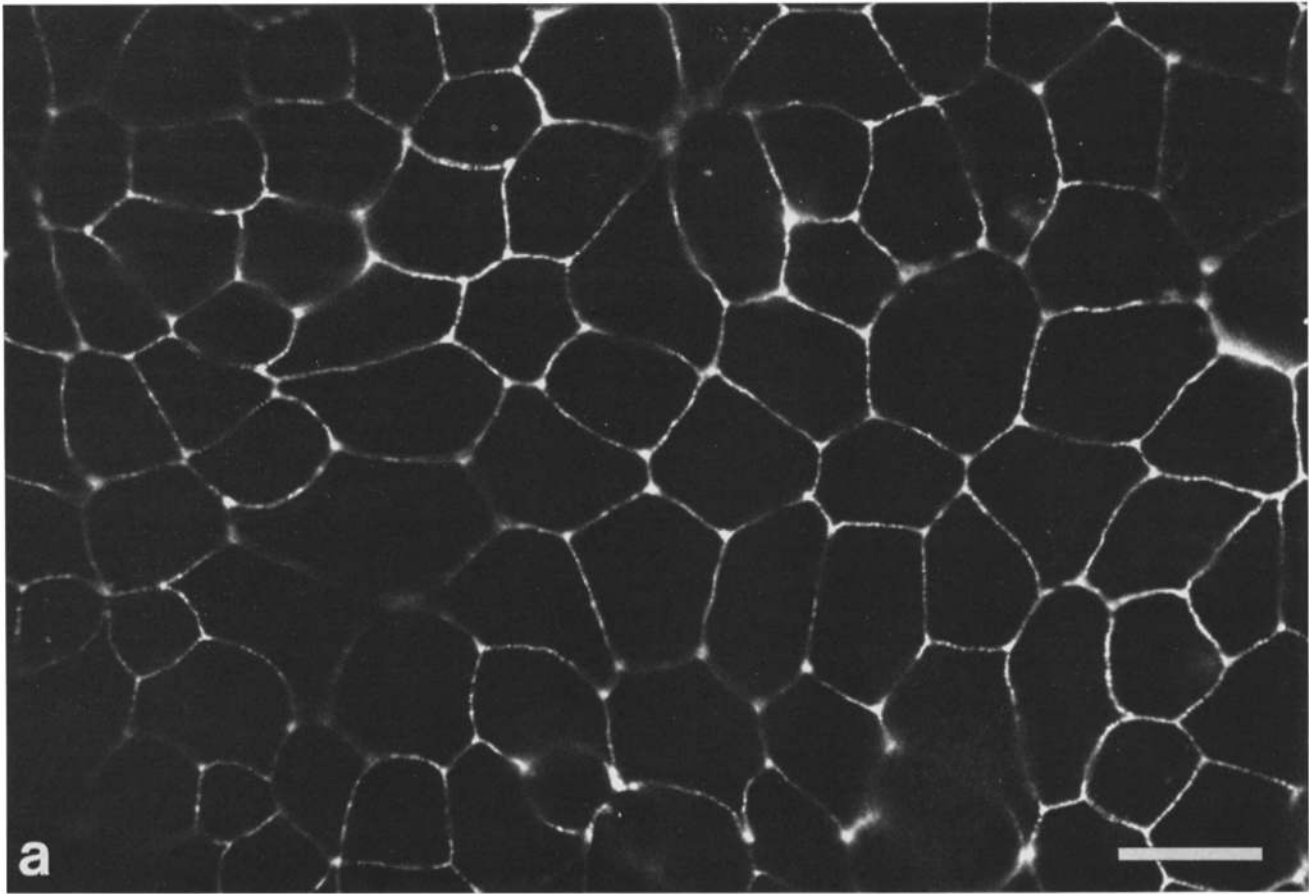
The observation that ZO-1 can be detected in the initial liver homogenate (Fig. 6, c and d) as well as in whole cell homogenates of MDCK cells (Fig. 7) suggests that this polypeptide does not arise *de novo* during membrane fractionation from a higher molecular weight precursor. Proteolysis is clearly a problem since numerous lower molecular weight polypeptides immunoreactive with R26.4C are observed in

**Figure 2.** (a and b) Ultrastructural localization of ZO-1. Thin-section electron micrographs of the canalculus-enriched plasma membrane fraction incubated with R26.4C and a colloidal gold-conjugated rabbit anti-rat secondary antibody before fixation and embedment. Gold particles decorate the cytoplasmic surface of the tight junction at the precise points of membrane contact. No labeling is detectable on any other membrane surface, including adjacent nonjunctional membrane and a gap junction (GJ) which has been obliquely sectioned. (c) Same as in a and b, but using the monoclonal antibody (R5.21C) that reacts with the 27-kD gap junction protein. Label is confined to the cytoplasmic surface of the gap junctions. No label is visible at the tight junction (ZO). Bars, 100 nm.

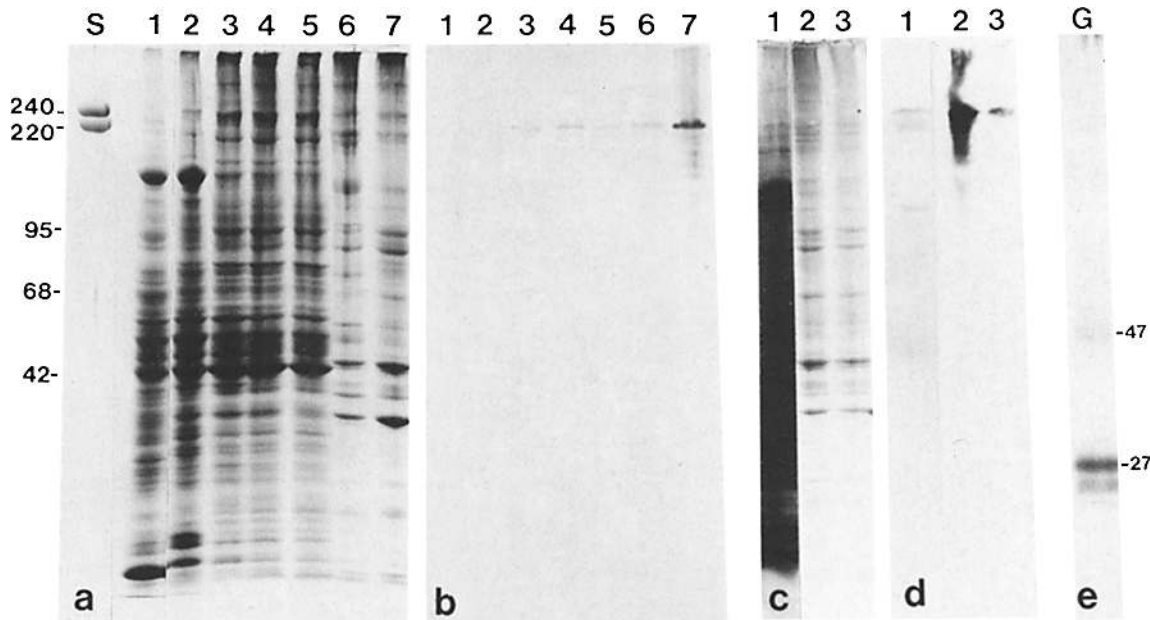


**Figure 4.** Immunofluorescent localization of ZO-1 in frozen sections of mouse kidney and testis. (a) Kidney. R26.4C staining is observed in the junctional complexes in profiles of tubules in the kidney cortex. The endothelial junctions of an arteriole (A) are also stained with this antibody. (b) Testis. Portions of two seminiferous tubules are shown separated by the tunica propria (TP) connective tissue layer. R26.4C antibody activity is localized in this tissue to the Sertoli-Sertoli cell junctions (arrows) of the seminiferous epithelium. The endothelial junctions of an arteriole (A) in the tunica propria are also stained. Bar, 10  $\mu$ m.





**Figure 5.** Immunofluorescent localization of ZO-1 in monolayers of the MDCK epithelial cell line. (a) Immunofluorescence. R26.4C activity is seen as a network of staining discretely localized to a thin band around the apical borders of the cells. (b) Phase-contrast image of the same field as in a. Bar, 20  $\mu\text{m}$ .



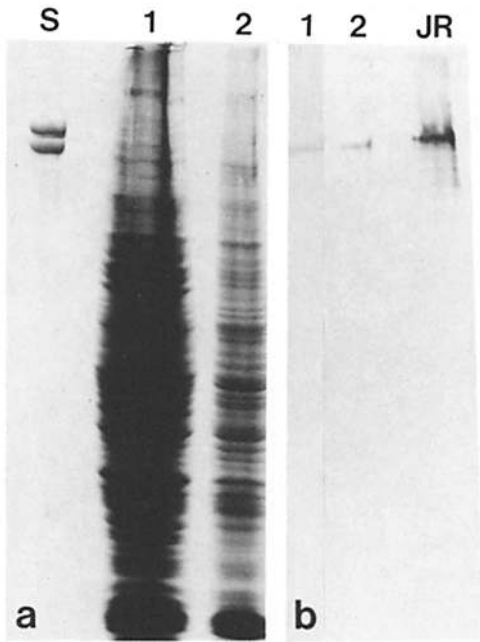
**Figure 6.** Immunoblot analysis of ZO-1 in fractions from the isolation of the tight junction-enriched preparation (DOC-JR). Molecular mass standards on the left are in kilodaltons. Lane S, human erythrocyte spectrin. (a) Coomassie Blue-stained SDS PAGE of (1) initial whole liver homogenate, (2) washed membrane pellet before loading on the sucrose step gradient, (3) plasma membrane pellet collected from the sucrose gradient, (4) purified plasma membrane "canaliculus-enriched" fraction, (5) supernatant from the first DOC wash, (6) DOC-extracted "low speed pellet" enriched for collagen and insoluble detritus, (7) DOC-JR. Equivalent amounts of protein were loaded in each lane. Lane 6 appears to contain less protein, probably because collagen does not run into the gel. (b) Immunoblot of an identical gel as that shown in a without the spectrin standard, stained with R26.4C. Enrichment of the 225-kD ZO-1 band is observed. (c) Coomassie Blue-stained SDS PAGE and (d) identical R26.4-stained immunoblot of (1) undiluted initial whole liver homogenate, (2) DOC-JR, and (3) DOC-JR gel sample that has been passed through a 0.2- $\mu$ m filter. (e) Immunoblot of a DOC-JR sample run on a 7.5% SDS PAGE and incubated with the monoclonal antibody R5.21C. Staining is detected at the gap junction 27-kD protein, its aggregate at 47-kD, and proteolysis fragments.

some of the membrane fractions, including the final DOC-JR fraction (Fig. 6 b). These lower molecular weight bands are most likely proteolytic fragments of the 225-kD protein since their relative intensity can be increased under conditions such as detergent extractions in the absence of protease inhibitors where proteolysis can be expected to be occurring. Final resolution of this issue will require either peptide map comparisons of the 225-kD proteins with these putative fragments or the generation of similar sized fragments by proteolysis of purified ZO-1. The R26.4C immunoblots also demonstrate the anomalous behavior of ZO-1 on SDS gels in membrane fractions that have been pretreated with DOC before solubilization in SDS. In such samples, an apparent aggregation of ZO-1 is observed, resulting in the presence of an amorphous smear of immunoreactive material extending from the 225-kD band up to the top of the gel (Fig. 6, b and d, and 7 b). Much of this smear can be removed by filtration of the gel sample before electrophoresis, although this also results in substantial loss of the 225-kD immunoreactive protein. The stability and aggregation behavior of ZO-1 in the presence of certain detergents may pose problems if these reagents are required for its solubilization and purification.

One striking feature of ZO-1 is its relatively large molecular weight. Its apparent molecular weight is comparable to the subunit molecular weights of the various isoforms of the membrane skeletal protein, spectrin, present in both erythroid and nonerythroid tissues including the liver (for review see reference 3). Given this similarity in molecular weight

and its membrane association, it is possible that ZO-1 might actually be a spectrin isoform. Two lines of evidence argue against this. First, the distribution of spectrin in the hepatocyte has been determined by immunohistochemical staining with anti-fodrin, the major isoform of spectrin present in mammalian, nonerythroid tissues (3). These studies indicate that unlike ZO-1, fodrin is present on all membranes of the hepatocyte, and shows no preferential localization to the junctional complex region (4). Second, R26.4C shows no reactivity with purified mouse or human erythroid spectrin subunits, purified from rat or mouse small intestine (data not shown). These results do not rule out the possibility that ZO-1 may be a novel tight junction-associated spectrin subunit.

The first issue to be addressed in future characterizations of ZO-1 will be the determination of whether this protein is a peripheral or integral component of the tight junction membrane. The ultrastructural studies reported here indicate that ZO-1 is found, at least in part, on the cytoplasmic surface of the junction. Results of preliminary studies to determine conditions required for extraction of ZO-1 from hepatocyte membranes suggest that this protein may be peripherally associated, since it is partially extracted by both high salt and alkaline pH (Anderson, J. M., and B. R. Stevenson, unpublished observations). However, given the susceptibility of ZO-1 to proteolysis, it is possible that the release of ZO-1 from the membrane could result from cleavage of a relatively small membrane insertion sequence from one end of the polypeptide.



**Figure 7.** Immunoblot analysis of ZO-1 in MDCK cells using R26.4C. (a) Coomassie Blue-stained SDS PAGE of (S) human erythrocyte spectrin (subunits at 240 and 220 kD), (1) whole MDCK cell sheets rapidly solubilized in boiling SDS, (2) MDCK plasma membrane-enriched fraction. (b) R26.4C immunoblot of the whole cell (1) and membrane fraction (2) from MDCK, as in a. A liver-derived DOC-JR sample (JR) was included as a molecular weight marker. The whole cell homogenate was run on a separate gel to ensure that no spill-over of the liver antigen occurred.

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