

Molecular Characterization and Tissue Distribution of ZO-2, A Tight Junction Protein Homologous to ZO-1 and the *Drosophila* Discs-Large Tumor Suppressor Protein

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Abstract. ZO-1 is a 210–225-kD peripheral membrane protein associated with cytoplasmic surfaces of the *zonula occludens* or tight junction. A 160-kD polypeptide, designated ZO-2, was found to coimmunoprecipitate with ZO-1 from MDCK cell extracts prepared under conditions which preserve protein associations (Gumbiner, B., T. Lowenkopf, and D. Apatira. 1991. *Proc. Natl. Acad. Sci. USA*. 88: 3460–3464). We have isolated ZO-2 from MDCK cell monolayers by bulk coimmunoprecipitation with ZO-1 followed by electroelution from preparative SDS-PAGE gel slices. Amino acid sequence information obtained from a ZO-2 tryptic fragment was used to isolate a partial cDNA clone from an MDCK library. The deduced amino acid sequence revealed that canine ZO-2 contains a region that is very similar to sequences in human and mouse ZO-1. This region includes both a 90-amino acid repeat domain of unknown function and guanylate kinase-like domains which are shared among members of the family of proteins that includes ZO-1, erythrocyte p55, the product of the lethal(1)discs-large-1 (*dlg*) gene of *Drosophila*, and a synapse-associated protein from rat brain, PSD-95/SAP90. The *dlg* gene product has been shown

to act as a tumor suppressor in the imaginal disc of the *Drosophila* larva, although the functions of other family members have not yet been defined. A polyclonal antiserum was raised against a unique region of ZO-2 and found to exclusively label the cytoplasmic surfaces of tight junctions in MDCK plasma membrane preparations, indicating that ZO-2 is a tight junction-associated protein. Immunohistochemical staining of frozen sections of whole tissue demonstrated that ZO-2 localized to the region of the tight junction in a number of epithelia, including liver, intestine, kidney, testis, and arterial endothelium, suggesting that this protein is a ubiquitous component of the tight junction. Double-label immunofluorescence microscopy performed on cryosections of heart, a non-epithelial tissue, revealed the presence of ZO-1 but no ZO-2 staining at the *fascia adherens*, a specialized junction of cardiac myocytes which has previously been shown to contain ZO-1 (Itoh, M., S. Yonemura, A. Nagafuchi, S. Tsukita, and Sh. Tsukita. 1991. *J. Cell Biol.* 115:1449–1462). Thus it appears that ZO-2 is not a component of the *fascia adherens*, and that unlike ZO-1, this protein is restricted to the epithelial tight junction.

EPIETHELIA serve as selectively permeable barriers between body compartments. Free diffusion of substances between epithelial cells is prevented by a specialized region of cell–cell contact, the *zonula occludens* or tight junction, which encircles cells at their apices and closes the intercellular space (Farquhar and Palade, 1963; Diamond, 1977; Gumbiner, 1987). In addition to its sealing function, the tight junction plays an important role in polarization of the epithelial cell surface by preventing mixing of membrane lipids and proteins between distinct plasma membrane domains (Simons and Fuller, 1985). Tight junctions

are dynamic structures, showing differences in permeability and structure among different epithelia (Frömter and Diamond, 1972; Claude and Goodenough, 1973), modulation under physiologic conditions in response to extracellular signals, and rapid assembly and disassembly during morphogenesis, wound healing, and migration of leukocytes across endothelia and epithelia (Madara et al., 1992; Schneeberger and Lynch, 1992). Although the mechanisms of tight junction regulation are not known, protein kinases A and C, together with G-protein mediated signaling pathways have been implicated in tight junction assembly and disassembly in cultured cells (Balda et al., 1993; Citi, 1992; Balda et al., 1991; Nigam et al., 1991).

The tight junction is visualized by thin section electron microscopy as the most apical member of a tripartite junctional

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complex, which includes the *zonula adherens* and *macula adherens* in addition to the *zonula occludens*. The tight junction is characterized by a series of membrane contacts at the border between the apical and lateral plasma membranes (Farquhar and Palade, 1963) which appear in freeze-fracture images as branching and anastomosing fibrils encircling cell apices (Goodenough and Revel, 1970). The junctional fibrils are thought to be composed of rows of integral membrane proteins (Stevenson et al., 1988), although another model of tight junction molecular structure has been proposed (Pinto da Silva and Kachar, 1982).

ZO-1 (*zonula occludens-1*) is a 210–225 kD phosphoprotein which associates with the cytoplasmic surfaces of the tight junction close to the membrane contacts (Stevenson et al., 1986; Anderson et al., 1988). ZO-1 has been found associated with all tight junctions examined to date, and in two additional locations, adherens junctions in myocardial intercalated discs (Itoh et al., 1993) and cultured astrocytes (Howarth et al., 1992). Recent cloning and sequencing of a complete cDNA (Willott et al., 1993; Itoh et al., 1993; Tsukita et al., 1993) revealed that ZO-1 is a member of the discs-large family of proteins, including the product of the lethal(1) discs-large-1 (*dlg*) gene of *Drosophila* (Woods and Bryant, 1991), erythrocyte p55 (Ruff et al., 1991), and a presynaptic junction protein from rat brain (PSD-95/SAP90; Cho et al., 1992; Kistner et al., 1993). Members of this family contain a 90-amino acid internal repeat domain of unknown function, an SH3 (*src* homology 3) domain, a motif first identified in the *src* family of nonreceptor protein tyrosine kinases and thought to mediate protein interactions, and a guanylate kinase-like domain. Mutations of the *dlg* gene have been shown to result in neoplastic overgrowth of imaginal discs, indicating that the product of this gene acts as a tumor suppressor in *Drosophila* larvae (Woods and Bryant, 1989, 1991), although the functions of other family members are not known. The Dlg-A protein has been immunocytochemically localized to the cytoplasmic surfaces of the septate junction (Woods and Bryant, 1991), the arthropod counterpart of the vertebrate tight junction (Green and Bergquist, 1982; Wood, 1990). Two other proteins have been immunolocalized to the region of the vertebrate tight junction, Cingulin ($M_r \cong 140$ kD; Citi et al., 1988) and the 7H6 antigen ($M_r \cong 155$ –170 kD; Zhong et al., 1993).

ZO-2, a 160-kD protein, was identified as a peripheral membrane protein which coimmunoprecipitated with ZO-1 from MDCK cell extracts prepared under conditions which maintain protein associations (Gumbiner et al., 1991). We have obtained amino acid sequence data from purified ZO-2, allowing the isolation of a partial cDNA clone. An antiserum was raised against a ZO-2 fusion protein and used to demonstrate that ZO-2 colocalizes with ZO-1 at tight junction membrane contacts, indicating that ZO-2 is a component of the tight junction. Immunofluorescent staining of the tight junction region was observed in a variety of epithelial tissues as well as in arteriolar endothelium using the ZO-2 antibody, suggesting that ZO-2, like ZO-1, is a ubiquitous component of the tight junction. Amino acid sequence comparison of ZO-2 with proteins in the Genbank/EMBL database revealed that this protein is very similar to, though distinct from, ZO-1. ZO-2 also shares sequence homology with other members of the discs-large family. These results indicate that the tight junction has bound to its cytoplasmic surfaces two

structurally related and physically associated proteins, ZO-2 and ZO-1. Further, the membrane association of structural homologs at the mammalian tight junction and the arthropod septate junction reveals molecular similarity between these functionally related intercellular junctions.

Materials and Methods

Reagents

Tissue culture reagents were purchased from GIBCO BRL (Gaithersburg, MD), except for FCS which was obtained from Hyclone Labs. (Logan, UT). Alpha- 32 P]dCTP ($>3,000$ Ci/mmol) and alpha- 35 S]dATP (1,000–1,500 Ci/mmol) were from New England Nuclear (Boston, MA), and Tran 35 S-label was from ICN (Cleveland, OH). Trasylol was obtained from Miles FBA Division (West Haven, CT). Molecular biological reagents were from New England Biolabs (Beverly, MA). All other chemicals were obtained from Sigma Chem. Co. (St. Louis, MO) unless otherwise specified.

Cell Culture, Metabolic Labeling, and Immunoprecipitation

Strain II MDCK cells (kindly provided by Dr. Karl Matlin) were grown in MEM supplemented with 5% FCS, 100 U penicillin and 100 mg/ml streptomycin (P/S), and 10 mM Hepes, pH 7.3 at 37°C in a humidified atmosphere of 5% CO₂. Cells were metabolically labeled for 18–24 h at 37°C with 150 mCi/ml Tran 35 S-label (35 S]met/ 35 S]cys), in labeling medium (methionine-free MEM, 5% dialyzed FBS, P/S, 10 mM Hepes pH 7.3, supplemented with 10% complete growth medium). At the end of the labeling period monolayers were rinsed twice in ice cold PBS⁺ (PBS, 1 mM CaCl₂, 0.5 mM MgCl₂) and extracted in situ by addition of a modified RIPA buffer, designated IP buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, 2 mM EDTA, 10 mM Tris, pH 7.4, 140 K.I.U./ml Trasylol, 0.01% diisopropyl fluorophosphate (DIFP),¹ 50 mg/ml benzamide, 1 mg/ml chymostatin, 1 mg/ml pepstatin, 1 mg/ml leupeptin, and 1 mM iodoacetic acid) to the tissue culture plate. After a 5-min incubation on ice, solubilized components were removed from the monolayer and fresh IP buffer added for an additional 15 min. At the end of the second incubation, the two extracts were combined and centrifuged at 13,000 g for 1 h at 4°C. Supernatants were immunoprecipitated overnight at 4°C with either the rat anti-ZO-1 monoclonal antibody R40.76 (Anderson et al., 1988) and rabbit anti-rat IgG-Sepharose (Cappel, West Chester, PA), or with anti-ZO-2 rabbit polyclonal antiserum R9989 and protein A-Sepharose (Sigma Chem. Co.). The following antibodies were used in control immunoprecipitations: the negative control for R40.76 was R5.21, a rat monoclonal of the same isotype as R40.76 that is directed against gap junction connexin32 (Stevenson et al., 1986). R9989 preimmune serum served as the negative control for R9989. R40.76 and R5.21 culture supernatants (C.S.) were used for immunoprecipitations at a concentration of 0.4 ml C.S./ml extract, whereas the R9989 immune and preimmune antisera were diluted 80-fold with hybridoma growth medium (Stevenson et al., 1986) before addition to the cell extract (400 ml/ml extract). 30 ml of a 1:1 slurry of conjugated Sepharose beads were used per ml of extract. Immunoprecipitates were washed with IP buffer three times, once with 0.5 M NaCl, 10 mM Tris, 2 mM EDTA, pH 7.4, and finally with 10 mM Tris, 2 mM EDTA pH 7.4. After denaturation in SDS sample buffer by boiling for 5 min in the presence of β -mercaptoethanol, immunoprecipitates were analyzed by 7.5% SDS-PAGE and fluorography.

ZO-2 Purification and Amino Acid Sequencing

A total of 100 roller bottles (1585 cm²/bottle; Bellco, Vineland, NJ) of confluent MDCK cells were used for the isolation of ZO-2; 10 bottles were processed at a time. Bottles were rinsed two times each with 50 ml of ice cold PBS⁺ and scraped up in 15 ml/bottle of 4°C IP buffer. The cell extracts were spun at 1,300 g for 60 min at 4°C and supernatants immunoprecipitated with R40.76 and rabbit anti-rat-Sepharose as described above to isolate ZO-1/ZO-2 complexes. Proteins were eluted from Sepharose beads by boiling in ~ 1.5 times the packed bead volume of SDS sample buffer

1. Abbreviations used in this paper: DHR, discs-large homology region; DIFP, diisopropyl fluorophosphate; GST, glutathione S-transferase.

(~3.5 ml total) and stored at -70°C . SDS samples prepared from a total of 100 roller bottles were thawed, passed through 0.2- μm filter (Nalgene, Rochester, NY), concentrated fivefold by ultrafiltration (Centricon 100; Amicon, Beverly, MA), and run on five 0.75 mm 7.5% preparative SDS-polyacrylamide gels to separate ZO-2 from contaminating proteins. Gels were stained with 0.25 M KCl at 4°C , after which the ZO-2 bands were excised, and protein electroeluted into dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) in the presence of 0.5% SDS, 2 mM EDTA, 40 mM Tris Acetate, pH 8.6. The eluate (~23 ml) was passed through a 0.2- μm filter, concentrated 20-fold by ultrafiltration, and brought to a final concentration of 20% methanol, 0.003% bromophenol blue, and 4% ultra pure sucrose. The eluate was then electroblotted onto nitrocellulose (Schleicher and Schuell, Keene, NH) using a custom-made apparatus composed of upper (cathode) and lower (anode) buffer chambers separated by nitrocellulose and filled with 0.1% SDS, 20% methanol, 2 mM EDTA, 40 mM Tris acetate buffer, pH 8.6. The sample was loaded in the bottom of the upper chamber and 100 V were applied until the dye passed through the nitrocellulose to the lower chamber, about 30 min. Approximately 70 pmol of ZO-2 protein on 37 mm² nitrocellulose were submitted to the Harvard Microchemistry Facility (Harvard University, Cambridge, MA) for trypsin digestion and amino acid sequencing as described by Aebersold et al. (1987) and Lane et al. (1991). The following amino acid sequences were obtained from two different tryptic polypeptides: LAGGNDVGIQEGTSAEQEGL was obtained from the first tryptic fragment with high confidence; LISDFEDT(D)GE(G)(G)AYTDNELD(E)P(A)(E) was obtained from the second fragment (parentheses indicate residues determined with lower confidence).

Screening of a cDNA Library

A nondegenerate ZO-2 hybridization probe was produced using PCR. Degenerate oligonucleotide primers were made corresponding to the NH₂ terminus (5'-GCX GGX GAX AAC/T GAC/T GT-3') and COOH terminus (3'-CGX CTC/T GTC/T CTC/T CCX A/GA-5') of the amino acid sequence, AGGNDVGIQEGTSAEQEGLR, obtained from direct sequencing of a ZO-2 tryptic polypeptide (see above). The template for the reaction was cDNA made by reverse transcription of MDCK total RNA according to the method of Sambrook et al. (1989); RNA was isolated from confluent MDCK cells by the guanidium isothiocyanate/CsCl method of Sambrook et al. (1989). A 72-bp PCR product resulting from this PCR amplification was subcloned into the EcoRV site of pBluescript II SK⁺ to produce the plasmid p6, and sequenced on both strands (Sequenase, Un. States Biochemical, Cleveland, OH) to confirm that it encoded the amino acid sequence of the ZO-2 tryptic fragment. To prepare probe, plasmid p6 was digested with XbaI and XhoI and an ~140 bp fragment containing ZO-2 coding sequence was isolated and random-prime labeled with [³²P]dCTP according to manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN). This labeled probe was then used to screen an oriented oligo(dT)-primed MDCK cDNA library in λ -ZAP (kindly provided by Dr. Marino Zerial) using the method of Sambrook et al. (1989). Twelve positively hybridizing phage were isolated after screening of ~500,000 plaques. pBluescript was excised from plaque-purified phage according to the manufacturer's instructions (Stratagene, La Jolla, CA) and the longest insert, 3.2 kb, contained in the plasmid pB6C, was subjected to double-stranded sequencing (Sequenase, see above or TaqTrack, Promega, Madison, WI). This partial sequence is available from the GenBank/EMBL database under accession #L27152.

Sequence Analysis

Amino acid sequences were compared using the GAP alignment program in the University of Wisconsin Genetics Computer Group Software Package. The internal repeat domains of ZO-2 (amino acids 96-176) and mouse ZO-1 (amino acids 423-503) were compared with each other and those of Dlg-A (amino acids 486-566, third repeat), PSD-95 (amino acids 313-393, third repeat), and p55 (amino acids 92-149). The putative SH3 regions of ZO-2 (amino acids 193-246) and ZO-1 (amino acids 520-576) were compared with each other and with those of Dlg-A (amino acids 604-622), PSD-95 (amino acids 432-495), and p55 (amino acids 162-225). The guanylate kinase domains of ZO-2 (amino acids 315-466) and ZO-1 (amino acids 644-794) were compared with each other and with those of Dlg-A (amino acids 770-948), PSD-95 (amino acids 534-712), and p55 (amino acids 282-454). Default alignment parameters of the Gap program were used to determine percent identity and percent similarity of sequences being compared. Sequences were obtained from the GenBank/EMBL and Protein Identification Resource data bases.

Northern Analysis

Total RNA was isolated from MDCK cells by the one-step method of Ausubel et al. (1992). Poly(A)⁺ RNA was isolated from total RNA using the PolyATtract mRNA Isolation System III kit (Promega). RNA transfer was performed as described previously (White et al., 1992). A 725-bp EcoRI/PstI restriction fragment generated from a human ZO-1 cDNA clone (kindly provided by Dr. J. M. Anderson) was random prime-labeled to generate a ZO-1 probe. A ZO-2 probe was made by PCR amplification of nucleotides 2-850 of the pB6C insert (see above) and random priming. Blots were hybridized and washed as described by White et al. (1992) except that the ZO-1 blot was washed for an additional 10 min at 65°C in fresh 1% SDS, 0.3 M sodium phosphate, pH 7.2 buffer whereas the blot probed with ZO-2 was washed for an additional 30 min in 15 mM sodium phosphate, pH 7.2, 0.05% SDS at 65°C .

Antibody Production

Antibodies were raised against a glutathione S-transferase (GST)/ZO-2 fusion protein. To construct the fusion protein, nucleotides 2-295 of the pB6C insert were PCR-amplified and subcloned into the EcoRI-BamHI sites of the expression vector pGEX-2T (Smith and Johnson, 1988). Expression of fusion protein and isolation by affinity chromatography on immobilized glutathione were performed as described in Smith and Johnson (1988). Rabbits and guinea pigs were immunized by popliteal lymph node injection using 50 mg protein/injection (Pocono Rabbit Farm and Laboratory, Canadensis, PA). Crude serum from rabbit R9989 and guinea pig GP753 were used for immunoblots and immunoprecipitations. For immunocytochemistry, the R9989 antiserum was affinity purified on a fusion protein-Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) column.

Immunoblotting

MDCK whole cell extracts were prepared by rinsing monolayers with ice cold PBS⁺, scraping in IP buffer, and boiling in 1/10 vol 10 \times SDS sample buffer with β -mercaptoethanol. Proteins were separated by SDS-PAGE on either 6% minigels or 7.5% standard gels and electrophoretically transferred (Towbin et al., 1979) onto Immobilon (Millipore Corporation, Bedford, MA). Incubation of blots with antibodies was carried out as described previously (Stevenson et al., 1986). Culture supernatants of R40.76 (Anderson et al., 1988) were used undiluted; the polyclonal antisera R9989, GP753, and rabbit anti-cingulin (generously provided by Dr. S. Citi) were used at 1:2,500, 1:500, and 1:5,000, respectively. Alkaline phosphatase-conjugated secondary antibodies (anti-rabbit IgG, Promega; anti-rat IgG, Pierce, Rockford, IL) and HRP-conjugated goat anti-guinea pig IgG (Chemicon, Co., Temecula, CA) were used at 1:5,000, 1:2,000, and 1:500, respectively.

Immunofluorescence and Immunoelectron Microscopy

Double-label immunofluorescence microscopy was performed with confluent MDCK cells grown on glass coverslips. Cells were rinsed with PBS⁺, fixed for 10 min at room temperature in 2% paraformaldehyde in PBS⁺, and permeabilized in 0.2% Triton X-100-containing PBS. Cells were then incubated sequentially with R40.76 culture supernatant, affinity-purified R9989 diluted 1:20 in PBS containing 5% normal goat serum, and a mixture of FITC-conjugated goat anti-rat IgG (Cappel) and rhodamine-conjugated goat anti-rabbit IgG (Pierce). After staining, samples were mounted with 60% glycerol, 0.4% n-propyl galate in PBS. Frozen sections of various unfixed canine tissues (kindly provided by Dr. Taichi Takeda) were prepared for immunofluorescence as described previously (Stevenson et al., 1986). Heart sections were double-labeled as described for MDCK cells, except that the secondary antibodies were adsorbed with heart plasma membranes (Paul and Goodenough, 1983) before use to eliminate non-specific staining. All other tissue sections were stained with R9989 using rhodamine-conjugated goat anti-rabbit IgG as the secondary antibody. Epifluorescence microscopy was performed with a Zeiss Axioskop and photographed with either Tmax 400 or Ektar 1000 film.

For immunogold electron microscopy, membranes were prepared by homogenizing four 10-cm plates of confluent MDCK cells with 15 strokes in a 7-ml Dounce tissue homogenizer (Belco Glass, Inc.) in ice-cold 1 mM NaHCO₃, pH 8.0, containing the protease inhibitors 2.5 mg/ml leupeptin, 0.5 mg/ml pepstatin, 0.5 mg/ml chymostatin, 0.01% DIFP, 140 K.I.U./ml Trasylol, and 1 mM DTT. Protease inhibitors were used in all subsequent buffers and antisera. The homogenate was spun at 1,500 g for 10 min at 4°C to pellet membrane sheets. The pellet was resuspended in 0.1% BSA/PBS

and incubated for 10 min on ice to block non-specific binding of antisera. The membrane sheets were then incubated overnight at 4°C in either undiluted R40.76 culture supernatant or in undiluted affinity-purified R9989 in 0.1% BSA/PBS. Membranes were washed, incubated in the appropriate undiluted preadsorbed (Grujters et al., 1987) 5-nm gold-conjugated secondary antibodies (Goat anti-rat IgG or goat anti-rabbit IgG; Janssen Life Sciences Products, Piscataway, NJ) and processed for electron microscopy as described previously (Stevenson et al., 1986).

Results

Isolation of ZO-2

Lane 1 of Fig. 1 demonstrates that the anti-ZO-1 monoclonal antibody R40.76 coimmunoprecipitated ZO-2 from metabolically labeled MDCK cell extracts prepared with a mixture of Triton X-100, deoxycholate, and SDS (modified RIPA buffer). ZO-2 was not observed in immunoprecipitations performed using a control monoclonal antibody (lane 2). A specific 130-kD polypeptide (arrowhead, Fig. 1) was also seen in ZO-1 immunoprecipitates, but the level of this protein varied greatly between different experiments. To determine whether the R40.76 monoclonal recognized ZO-2, an ³⁵S-labeled ZO-1/ZO-2 coimmunoprecipitate identical to that in lane 1 was boiled in SDS and β-mercaptoethanol to dissociate proteins, diluted in a Triton X-100 containing buffer to create conditions compatible with antibody-binding, and re-immunoprecipitated with R40.76. As shown in

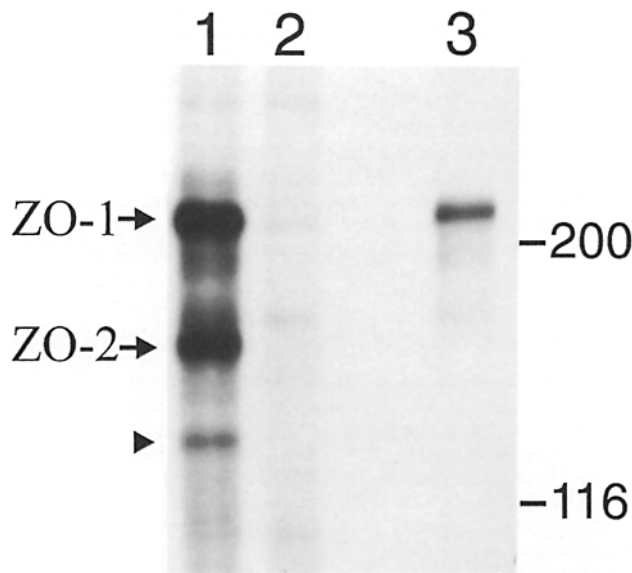


Figure 1. ZO-2 coimmunoprecipitates with ZO-1. Confluent MDCK cell monolayers were metabolically labeled with [³⁵S]methionine/cysteine overnight, extracted with 0.5% Triton X-100, 0.5% deoxycholate, 0.2% SDS, and immunoprecipitated with anti-ZO-1 monoclonal antibody R40.76 (lane 1) or a control monoclonal antibody of the same isotype (lane 2), followed by rabbit anti-rat conjugated Sepharose. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Both ZO-1 and ZO-2 are seen in the coimmunoprecipitate in lane 1 together with an $M_r \cong 130$ kD band (arrowhead). A ³⁵S-labeled coimmunoprecipitate identical to that in lane 1 was boiled in 2% SDS to dissociate the ZO-1/ZO-2 complex and reimmunoprecipitated with the R40.76 antibody (lane 3), yielding only ZO-1. Molecular weights of protein standards are given in kiloDaltons (kD).

lane 3, although ZO-1 was immunoprecipitated after denaturation with SDS, the 160-kD polypeptide was not detected, demonstrating that ZO-2 did not contain the R40.76 epitope. These results are in keeping with those of Gumbiner et al. (1991) who have previously shown that anti-ZO-1 antibodies do not react with coimmunoprecipitated ZO-2 in a Western blot after SDS-PAGE.

R40.76 was used to purify ZO-2 by bulk coimmunoprecipitation of MDCK cell extracts followed by preparative SDS-PAGE and excision and electroelution of the ZO-2 band. Fig. 2 shows a Coomassie blue-stained gel of the bulk coimmunoprecipitate (lane 1) and ~0.1 and 0.5 pmoles (lanes 2 and 3, respectively) of the gel-isolated ZO-2 protein. Approximately 70 pmol of purified ZO-2 was directly blotted onto nitrocellulose and submitted to the Harvard Microchemistry Facility for trypsin digestion and amino acid sequencing. Sequence data were obtained from two ZO-2 tryptic fragments (see boxed regions, Fig. 4). The sequence from the first fragment was determined with a high degree of certainty over 25 amino acid residues and thus was used to clone a cDNA for ZO-2.

ZO-2 Is Related to ZO-1 and the Discs-Large Tumor Suppressor Protein

Degenerate oligonucleotide primers based on the NH₂- and COOH-termini of the amino acid sequence from the first

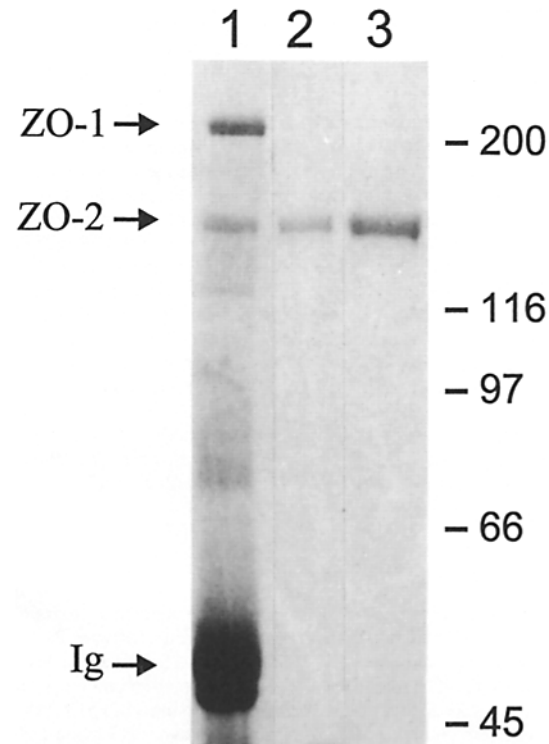


Figure 2. Isolation of ZO-2 from MDCK cells. ZO-2 was bulk-coimmunoprecipitated from MDCK cells and isolated on preparative SDS polyacrylamide gels as described in Materials and Methods. Lane 1 shows a Coomassie brilliant blue-stained polyacrylamide gel of the bulk-coimmunoprecipitate. Lanes 2 and 3 show gel-isolated ZO-2 at 0.1 and 0.5 pmol estimated loading amounts, respectively. Molecular weight standards are given in kD.

tryptic fragment (see boxed residues, Fig. 3) were used in PCR reactions containing MDCK cDNA. A 72-bp product was amplified and subcloned into pBluescript for DNA sequencing. The deduced amino acid sequence matched that of the ZO-2 tryptic fragment. The 72-bp product was used to screen an MDCK cDNA library in λ -Zap under stringent conditions. A 3.2-kb clone was isolated and sequence analysis showed that it included both previously generated internal amino acid sequences of ZO-2 (see boxed residues, Fig. 3).

We conclude that the 3.2-kb ZO-2 clone is not a full length cDNA. The nucleotide sequence did not contain a consensus initiation codon as defined by Kozak (1989). In addition, a single open reading frame beginning at the second nucleotide was found to encode 775 amino acid residues with a predicted molecular mass of 86.9 kD, significantly less than the $M_r \cong 160$ kD observed on SDS gels. Northern analysis (presented below) further supported our conclusion that the clone is a partial cDNA. The presence of a poly(A)⁺ tail, preceded by a polyadenylation signal in the 3' noncoding region of the clone, indicated that this cDNA encodes carboxy-terminal amino acid residues.

A search of the GenBank/EMBL protein data base revealed strong similarity between ZO-2 and ZO-1 from both mouse (54% identical; Itoh et al., 1993) and human (49% identical; Willot et al., 1993). Mouse and human ZO-1 are 88% identical over their entire length. Alignment of the predicted carboxy-terminal amino acid sequences of ZO-2 and mouse ZO-1 (Fig. 3) revealed that ZO-2 exhibited greatest similarity with ZO-1 (~70% identical from residue 96 to 541 of ZO-2) in a region previously shown to share sequence homology with members of the discs-large family of proteins (Dlg-A, PSD-95/SAP90, p55; Willot et al., 1993). The sequence homologies of these family members are clustered in several domains, including a novel 90-amino acid internal repeat region (disc-large homology region, or DHR) of unknown function (Cho et al., 1992), an SH3 domain (Musacchio et al., 1992), and a region similar to yeast guanylate kinase (Berger et al., 1989). While there are up to three internal repeat (DHR) domains in members of the discs-large family, the available sequence for ZO-2 contains a single DHR domain which is most closely related to the third internal repeat. The close relationship exhibited between ZO-2 and ZO-1 indicated that the former was also a member of the discs-large family of proteins.

The region of high similarity between ZO-2 and ZO-1 also encompassed sequences not shared by other members of the discs-large family, including an acidic domain (Willot et al., 1993) and a heptad repeat of leucine residues (Itoh et al., 1993), characteristic of the leucine zipper-type dimerization motif (Landschulz et al., 1988). Another feature shared uniquely by ZO-2 and ZO-1 was a proline-rich carboxyl terminus (13% from residue 560 of ZO-2, 14.4% from residue 883 of ZO-1; Willot et al., 1993). These carboxy-terminal sequences, however, displayed significantly less similarity to each other (~33% identical from residue 528 of ZO-2) than the preceding domains. ZO-1 has been shown previously to contain an alternatively spliced 80-amino acid domain, designated α -motif (see Fig. 3, Willot et al., 1992). It is not possible to determine from the amino acid sequence alignment alone whether ZO-2 also has an α -motif.

Direct comparison of amino acid sequences indicated that ZO-2 is also related to other members of the discs-large fam-

ily (Table I). ZO-2 is most closely related to ZO-1, but also displayed homology to Dlg-A and PSD-95. The greatest identity between these proteins was found in the region of the third internal repeat and guanylate kinase domains. Less identity is shown between the SH3 domains of either Dlg-A or PSD-95 and ZO-2, though the relatively high level of conserved amino acid residues suggests a possible evolutionary relationship.

Northern analysis suggested that the 3.2-kb clone is a partial cDNA and revealed that ZO-2 and ZO-1 are encoded by distinct messenger RNAs in MDCK cells. Under stringent conditions, a ZO-2 probe hybridized to a single band of 5.2 kb in both total and poly(A)⁺ RNA prepared from MDCK cells (Fig. 4, lanes 1 and 2). In contrast, the ZO-1 probe hybridized to a 7.9 kb species (lane 3), similar in size to that previously reported for human (Anderson et al., 1989) and rodent ZO-1 (Howarth et al., 1992; Itoh et al., 1993).

ZO-2 Antibodies Coimmunoprecipitate ZO-1

To generate an antiserum specific for ZO-2, a fragment of the 3.2-kb clone encoding sequences displaying the least similarity to ZO-1 (amino acids 1-88) was fused to the gene encoding GST. Fusion protein isolated on GST-conjugated Sepharose was used to immunize rabbits, resulting in the polyclonal antiserum R9989. In Fig. 5, an anti-ZO-1 coimmunoprecipitate containing ZO-1 and ZO-2 proteins (lanes 1, 3, and 5, labeled "Ip") and a whole MDCK-cell extract (lanes 2, 4, and 6, labeled "E"), were run on an SDS-PAGE gel, transferred to Immobilon, and reacted with either anti-ZO-2, anti-Cingulin, or anti-ZO-1 antibodies. The polyclonal antiserum R9989 recognized ZO-2 as a $M_r \cong 160$ kD band in the coimmunoprecipitate but not the $M_r \cong 210$ kD ZO-1 (lane 1). In the whole cell extract, a doublet at $M_r \cong 160$ kD was observed in addition to a faint band at $M_r \cong 190$ kD (lane 2). Anti-cingulin antiserum (the generous gift of S. Citi) recognized a $M_r \cong 140$ kD band in whole cell extracts (lane 4) which was not detectable in the immunoprecipitates (lane 3), while anti-ZO-1 labeled the $M_r \cong 210$ kD protein in both specimens (lanes 5 and 6). These data demonstrated that ZO-2 was immunologically distinct from both cingulin and ZO-1, and suggested that cingulin was not associated with the ZO-2/ZO-1 complex in MDCK cell extracts.

In a reciprocal experiment, the R9989 antiserum was demonstrated to coimmunoprecipitate ZO-1 with ZO-2 from MDCK extracts. Metabolically labeled MDCK cell extracts were prepared in the Triton X-100, DOC, SDS detergent mixture to preserve protein interactions and immunoprecipitated with either the anti-ZO-1 monoclonal or the anti-ZO-2 R9989 polyclonal. As shown previously, both ZO-1 and ZO-2 were immunoprecipitated by the ZO-1 antibody (Fig. 6, lane 1). Bands comigrating with ZO-1 and ZO-2 were observed when the anti-ZO-2 antibody was used for the immunoprecipitation (lane 3). Positive identification of these polypeptides was made by immunoblot analysis of the R9989 coimmunoprecipitate using either rat anti-ZO-1 or guinea pig anti-ZO-2 antibodies (Fig. 6, lanes 5 and 6); guinea-pig anti-ZO-2 antiserum (GP753) was used for the immunoblot in lane 6 to avoid staining of the rabbit immunoglobulin (R9989) used for the coimmunoprecipitation by the alkaline phosphatase-conjugated secondary antibodies. In lane 3, two additional bands at $M_r \cong 180$ and 240 kD were observed in the R9989 coimmunoprecipitate. The absence of these poly-

ZO-2 1ARERRQYSDYDYHSSNEKLERPNSREDMQRNRSR 36
 ZO-1 281 ANASERDDISEIQSLASDHSGRSHDRPPRRRSQSRSPDQRSEPSDHTQSPQPSNGSLRSREE..ERM SK 348

37 MGATPTPFKSMGDIASVVGTE NSKEPRYQEPPAPQPKA.....APRTFLRPSPEDEAIY 91
 349 PGAISTPVKHHVDDHPPKAVEEVTVEKNEKQTPTLPEPKPVYAQVGQPDVLDVSPSDGALPNSAHEDGIL 418

92 GPNTK MVRFKKGDVGLR LAGGNDVGT FVAGTQEGTSAEQEGLDEGDQILKVNTQDFRGLVREDAVLYLL 161
 419 RPSMKLVKFRKGDSVGLRLAGGNDVGT FVAGVLEDSPAAKEGLEEGDQILRVNNVDF TNI IREEAVLFL 488

162 EIPKGMVITILAQSRADVYRDILACGRGDSFFIRSHFECEKETPQSLAFSRGEVFRVVD TLYDGKLGHWL 231
 489 DLPKGEVITILAQKKKDVYRRIVESDVGDSFYIRTHFEYKESPYGLSFNKGEVFRVVD TLYNGKLGSWL 558

232 AVRIG...NELEKGLIPNKSRAEQMASVQNA.QRDNAGDRADFWRMGRGQRSGMKNLRKSREDLTA AVSV 297
 559 AIRIGKNHKEVERGIIPKNRAEQLASVQYTLPKTAGGDRADFWFRGLRSS.KRNLRSREDLSAQ.PV 626

298 STKFPAYERVLLREAGFKRPVVLFGPIADIALEKLANELPDLFQTAKTEPKDAGSE.KSSGVVRLN TVRQ 366
 627 QTKFPAYERVLLREAGFLRPVTIFGPIADVAREKLAREEPDIYQIAKSELRDAGTDHRSSGIIRLHTIKQ 696

367 IIEQDKHALLDVTPKAVDLLNYTQWFPIVIFFPNPSRQGVKTMQRQLNPTSNKSSRKL YDQANKLKKTC A 436
 697 IIDQDKHALLDVTPNAVDRNLNYAQWYPIVVF LNPDSKQGVKTMRMRLCPESRKSARKLYERSHKLRKNH 766

437 HLFTATINLNSANDSWFGSLKDTIQHQGGEAVVWVSEGMKMEGDDD...PEDRMSYLTAMGADY..LSCD 500
 767 HLFTTTINLNSMNDGWYGALKEAIQQQQNLVWVSEGKADGATSDDLDLHDDRLSYLSAPGSEYSMYSTD 836

501 SRLTSDFEFDTGEGGAYTDNELDEPAEPLV...SSITRSSEPVOHEESIR.....KPSPEPRA 556
 837 SRHTSDYEDTDTGEGGAYTDQELDETLDNEVGTPEESAITRSSEPVREDSSGMHHEHQTYPPYSPQAQPQA 906

557 QMR.....RAASRDQLRDSSPPPAFKPEPPKAKTQN.....REESFDISRSH...DYKSN 603
 907 IHRIDSPGLKPASQKAEASSPVYLSPETTPASSASAVNHVSVTNVSL EEPAPAPPTSHASQPGCLGA 976

604 PSAVAGN..... 610
 977 PSAEAAHVGLRGEGPPLPHADPAKVYRKEPYSEEMMRQNHILKQPALGHPGQRPDKEPNLAYEPQLPYI 1046

611EVSG..... 614
 1047 EKQASRDLEQPSYRYEVSSYTDQFSRNYDHRLRFEDRIPTYEDQWSYDDKQPYQPRPFENQHPRDLDSR 1116

615ASTRSC..... 620
 1117 QHP EEA SERGYFQRFE EPAPLSYDSRTRYEQLPRTSTLRHEEQPAPAYEVHNRYRPEAQPSSTGPKSSE 1186

621PPPIAAK.....PSFGRSILKP...STPVSPSEEEVGE GSEEQ 656
 1187 PKQYFDQYPRSYEQVPPPGFTSKTGHYEPLHGAAVVPLIPSSQKPEVLR SATKPPPPPTL TEEEDP 1256

657 EGAPKSVLKGKVKIFEKMDHKARLQRMQELQE.AQNARIEIAQKHPDIYAVPIKTHKPD PGLSQHTSS..R 723
 1257 AMKQSVLTRVKMFEN.KRSASLENKKDVNDTASFKPEVASKPPGA.SLAGPKVPQSQFSEHDKTL YR 1324

724 PPEPQKGPSRLYQDPRGS..YGSDAE EEEYRQQLSEHSKRGYYSQPSRYRDT EL..... 775
 1325 LPEPQKPVKPPEDIVRSNHYPDEE EYRQQLSYFDRRSFESKPSAHL PAGHHSEPAKPVHSQSQP NF 1394

Figure 3. Comparison of ZO-2 and ZO-1 predicted amino acid sequences. A vertical line indicates identity, two dots a conservative amino acid change, and one dot a less conservative change. ZO-2 tryptic fragment sequences are boxed. The third internal repeat (DHR) is identified with a thin solid line; SH3 domain, with a thin dashed line; guanylate kinase domain, with a thick solid line; acidic domain, with a dotted line; alternatively spliced α -motif of ZO-1, with two thin solid lines. The four leucine residues of the leucine zipper motif are marked by arrowheads. The ZO-2 cDNA sequence data are available from EMBL/GenBank DDBJ under accession number L27152.

Table 1. ZO-2 Is a Member of the Discs-Large Family of Proteins

	ZO-2	ZO-1	
ZO-1	69 (86)	100	
<i>dlg</i>	37 (63)	41 (69)	Internal repeat
PSD-95	39 (64)	43 (67)	
p55	16 (43)	27 (54)	
ZO-1	70 (87)	100	
<i>dlg</i>	17 (59)	28 (60)	SH3 domain
PSD-95	26 (56)	39 (61)	
p55	19 (50)	29 (58)	
ZO-1	67 (83)	100	
<i>dlg</i>	27 (50)	32 (54)	GK domain
PSD-95	32 (51)	27 (55)	
p55	22 (51)	26 (49)	

Amino acid residues contained in the internal repeat, SH3, and guanylate kinase domains of ZO-2 and ZO-1 were compared with those of other discs-large family members. Percent identity and percent similarity (percent identical residues plus conservative substitutions, in parentheses) were calculated using the GAP alignment program, based on the algorithm of Needleman and Wunsch (1970). The residues compared are given in Materials and Methods.

peptides in both the anti ZO-1 immunoprecipitate (lane 1) and in a coimmunoprecipitate performed using the guinea pig anti-ZO-2 fusion protein antiserum (GP753, data not shown) makes it unlikely that the $M_r \cong 180$ and 240 kD bands are polypeptides associated with ZO-1/ZO-2 complex.

ZO-2 Is a Component of the Tight Junction

The cellular distributions of ZO-2 and ZO-1 were compared by double-label immunofluorescence microscopy in confluent MDCK cell monolayers (Fig. 7). ZO-1 (left panel) and ZO-2 (right panel) colocalized at the apical cell borders, resulting in indistinguishable, honeycomb-like staining patterns. The affinity-purified anti-ZO-2 antibodies also showed some diffuse cytoplasmic staining not observed for ZO-1. Both the cytoplasmic and cell border staining seen with the anti-ZO-2 antibodies could be competed with ZO-2/GST fusion protein but not with GST alone (data not shown).

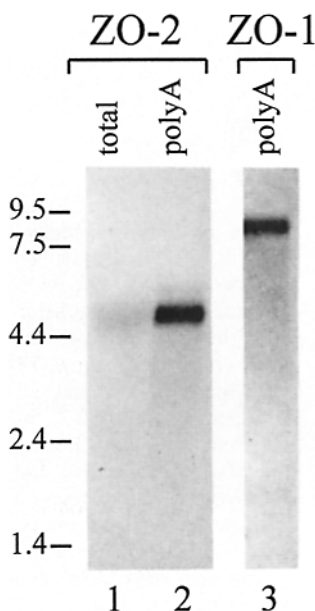


Figure 4. Northern blot analysis. 10 μ g of total RNA isolated from confluent MDCK cells were loaded in lane 1. Lanes 2 and 3 each contain 2.5 μ g of MDCK poly(A)⁺ RNA. A ZO-2 probe hybridized to a \sim 5.2-kb transcript in both total and poly(A)⁺ RNA (lanes 1 and 2). A 7.9-kb transcript was detected in poly(A)⁺ RNA using a human ZO-1 probe (lane 3). The positions of RNA standards are indicated in kilobases.

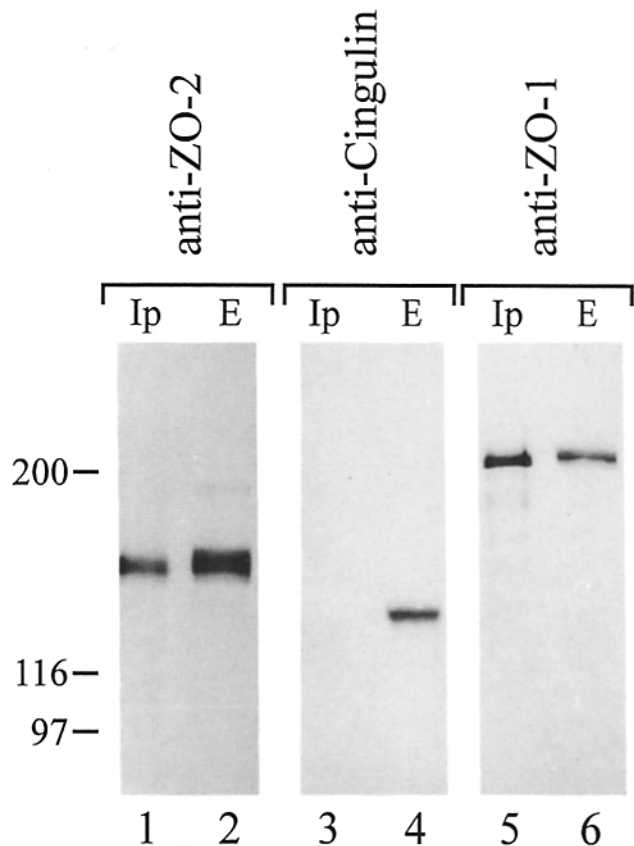


Figure 5. Immunoblot analysis of whole cell extracts and the ZO-1/ZO-2 immunoprecipitated complex with various antisera. Anti-ZO-1 coimmunoprecipitate (lanes 1, 3, and 5, labeled "Ip," prepared as in Fig. 2, lane 1) and whole MDCK cell extract (lanes 2, 4, and 6, labeled "E," prepared as described in Materials and Methods) were subjected to SDS-PAGE, electroblotted onto Immobilon, and reacted with the following antisera: (lanes 1 and 2) anti-ZO-2 fusion protein polyclonal R9989; (lanes 3 and 4) anti-cingulin polyclonal antiserum; (lanes 5 and 6) anti-ZO-1 monoclonal R40.76. Molecular weight standards are given in kD.

Immunogold electron microscopy was used to determine the ultrastructural localization of ZO-2. Plasma membranes isolated from confluent MDCK cells were used to permit accessibility of the antibodies to the cytoplasmic surfaces of the tight junction. Fig. 8 shows electron micrographs of plasma membrane pairs labeled with either anti-ZO-1 monoclonal antibody (upper panel) or affinity-purified R9989 (lower panel). 5-nm gold-conjugated secondary antibodies were used to detect the primary antibodies. The labeling patterns observed for ZO-1 and ZO-2 were indistinguishable. The gold particles clustered along the cytoplasmic surfaces of the tight junction, which is seen as closely apposed regions of plasma membrane proximal to other members of the junctional complex, and were absent from other areas of the membranes.

ZO-2 Is Present in a Variety of Epithelia and in Endothelia

Affinity purified R9989 antibodies were used to examine the distribution of ZO-2 in a number of different epithelia. Intense staining of junctional complexes was observed in liver,

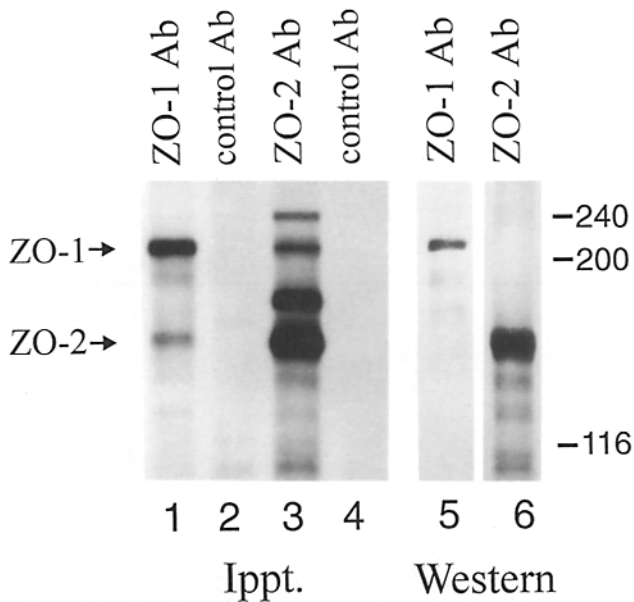


Figure 6. Coimmunoprecipitation of ZO-1 using anti-ZO-2 fusion protein serum. Lanes 1-4 show fluorographs of immunoprecipitates of MDCK extracts performed with different antibodies. (Lane 1), anti-ZO-1 monoclonal antibody R40.76; (lane 2) control monoclonal antibody of the same isotype as R40.76; (lane 3) anti-ZO-2 fusion protein serum R9989; (lane 4) R9989 preimmune serum. Lanes 5 and 6 show immunoblots of an R9989 immunoprecipitate, identical to that shown in lane 3, reacted with either anti-ZO-1 monoclonal R40.76 (lane 5) or guinea pig anti-ZO-2 fusion protein GP753 (lane 6).

intestine, kidney, and testis (Fig. 9). Junctional complex staining between adjacent hepatocytes (*a*) appeared as pairs of dots (*arrows*) and continuous lines (*arrowhead*) on either side of the bile canaliculi as has been described for anti-ZO-1 staining (Stevenson et al., 1986). In intestinal epithelium (*b*), ZO-2 localized to the junctional complexes (*arrows*) adjacent to the intestinal lumen (L) along both the villi and crypts (C). Diffuse cytoplasmic staining seen in intestine resulted from non-specific binding of secondary antibodies on the frozen section (data not shown). Cross-sections of kidney tubules (*c*) revealed staining at the apical-most lateral borders of both proximal and distal tubule epithelial cells. In cryosections of testis (*d*), the distinctive staining pattern of Sertoli-Sertoli cell junctions (*arrowheads*) was observed in seminiferous tubules (T) and bright dots of fluorescence appeared between contiguous arterial endothelial cells (A). Thus ZO-2 is present in the junctional region of all epithelia examined in this study.

Previous studies revealed that ZO-1 immunoreactivity localizes to the *fascia adherens*, a specialized intercellular junction, unrelated to the tight junction, that plays a cohesive role between opposing ends (intercalated discs) of cardiac muscle cells (Itoh et al., 1993). Double-label immunofluorescence microscopy was performed on cryosections of heart to examine the relative distributions of ZO-1 and ZO-2 in cardiac tissues. Prominent ZO-1 staining (Fig. 10, *upper panel*) was seen in both blood vessels (*arrows*) and intercalated discs (*arrowheads*). In contrast, ZO-2 was localized exclusively in blood vessels (*arrows*, Fig. 10, *lower panel*), demonstrating that although ZO-1 and ZO-2 colocalize in en-

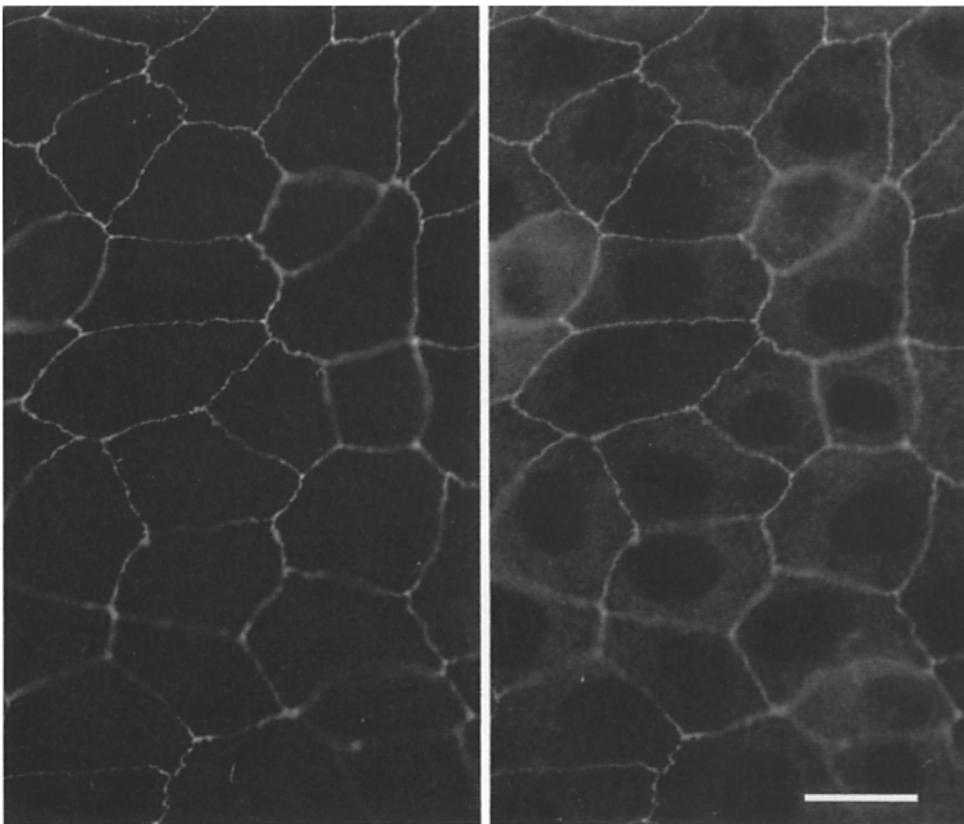


Figure 7. ZO-1 and ZO-2 colocalize to apical cell borders in the MDCK epithelial cell line. Cells grown on glass were fixed with paraformaldehyde, permeabilized with Triton X-100, and stained with a mixture of anti-ZO-1 monoclonal R40.76 and affinity-purified anti-ZO-2 fusion protein polyclonal R9989. Both ZO-1 (*left panel*) and ZO-2 (*right panel*) delineate a thin band around cell apices. Diffuse ZO-2 immunoreactivity was also seen in the cell cytoplasm. Bar, 20 μ m.

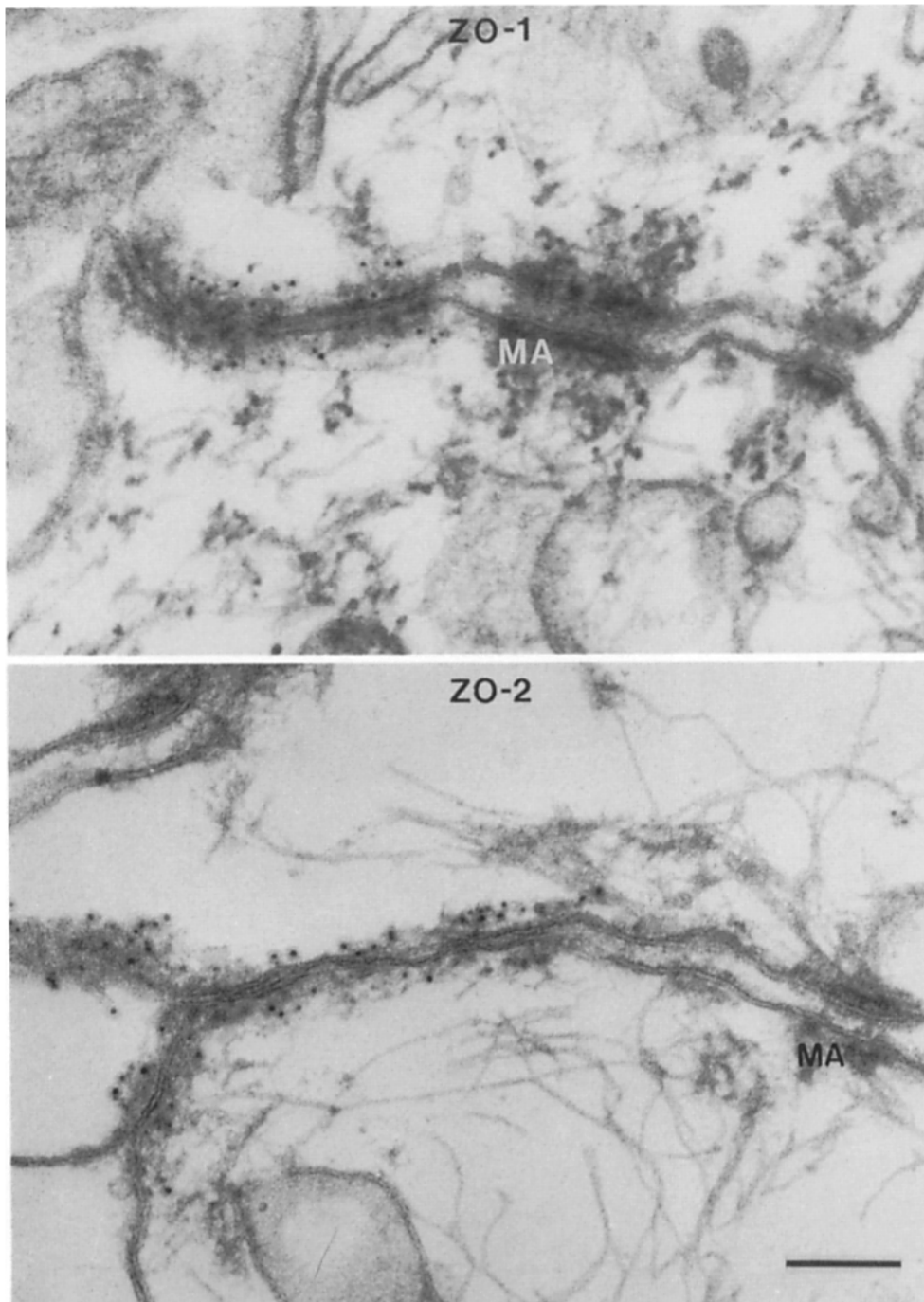


Figure 8. ZO-1 and ZO-2 localize to the tight junction in isolated MDCK plasma membranes. 5-nm gold particles coupled to the secondary immune reagents were seen along the cytoplasmic surfaces of tight junctions using either R40.76 monoclonal (anti-ZO-1, *upper panel*) or R9989 polyclonal serum (anti-ZO-2 fusion protein, *lower panel*). Maculae adherentes (MA) are not decorated with the gold label. Bar, 100 nm.

dothelial tight junctions, they are not obligate partners in the *fascia adherens*.

Discussion

We have shown that ZO-2, a 160-kD polypeptide associated with ZO-1 in MDCK cell detergent extracts, is a component of the tight junction. Ultrastructural localization performed on plasma membranes isolated from confluent MDCK cells demonstrated that ZO-2 was associated with the cytoplasmic surfaces of the tight junction membrane. Immunofluorescence microscopy revealed that ZO-2 was present in the junctional complexes of a number of different epithelia and

in endothelia, suggesting that this protein, like ZO-1, is a ubiquitous component of the tight junction.

The partial cDNA clone we obtained codes for ZO-2 protein based on two criteria. First, the 3.2-kb clone encoded the amino acid sequences obtained from two ZO-2 tryptic fragments. Second, antibodies prepared against a GST/ZO-2 fusion protein recognized ZO-2 by immunoblot analysis and immunoprecipitation. Comparison of the deduced amino acid sequence with those contained in the GenBank/EMBL data base revealed that ZO-2 was closely related to, but distinct from, ZO-1. ZO-2 was also found to exhibit homology to other members of the discs-large family of proteins, particularly with the Dlg-A protein of *Drosophila* and PSD-

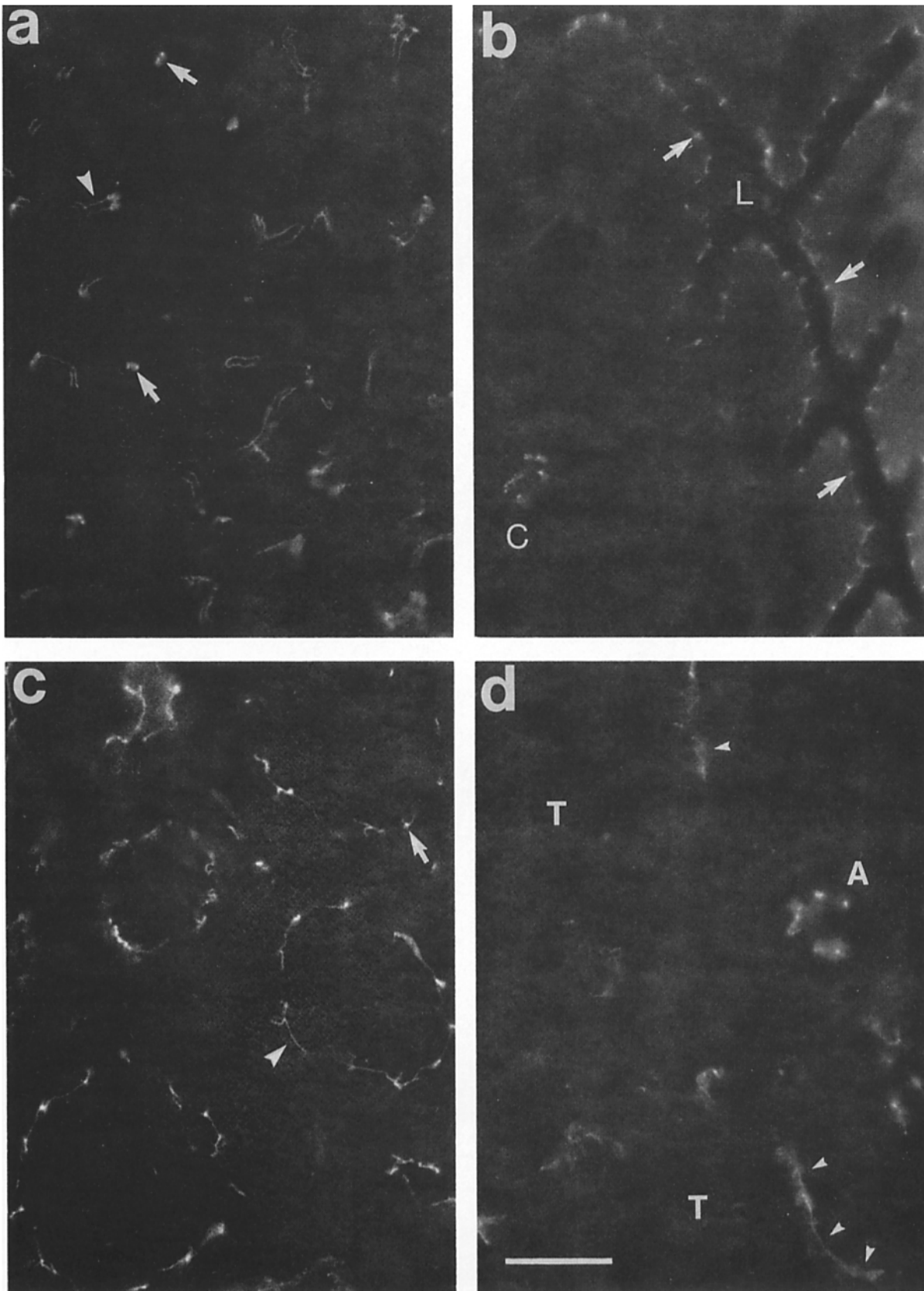


Figure 9. Immunofluorescent localization of ZO-2 frozen sections of liver, intestine, kidney, and testis. (a) Liver. ZO-2 localized to the junctional complexes of hepatocytes. Pairs of dots (*arrows*) were observed flanking bile canaliculi in cross section, while parallel lines were seen in longitudinal views (*arrowhead*). (b) Intestine. Dots of fluorescence were observed between enterocytes (*arrows*) adjacent to the intestinal lumen (*L*) along both the villi and crypts (*C*). (c) Kidney. Junctional complexes were stained in cross sections of kidney tubules which appeared either as dots (*arrow*) or continuous lines (*arrowhead*). (d) Testis. ZO-2 localized within Sertoli-Sertoli cell junctions (*arrowheads*) in the seminiferous epithelium of two seminiferous tubules (*T*). The endothelial junctions of an arteriole (*A*) also show ZO-2 immunoreactivity. Bar, 20 μ m.

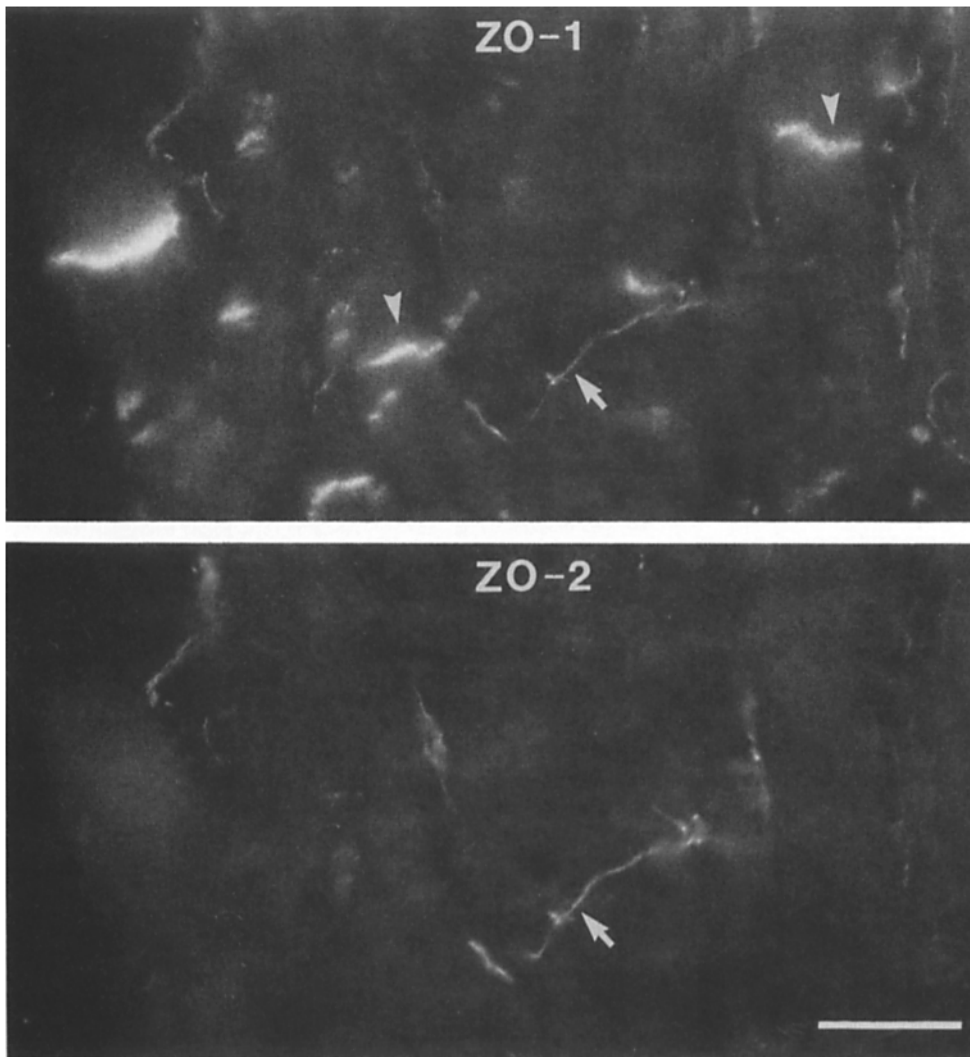


Figure 10. Double-label immunofluorescent localization of ZO-1 and ZO-2 in frozen sections of heart. Unfixed cryosections were stained simultaneously with R40.76 (anti-ZO-1, upper panel) and R9989 (anti-ZO-2, lower panel) followed by FITC-goat anti-rat (to visualize ZO-1) and rhodamine-goat anti-rabbit (to visualize ZO-2). ZO-1 and ZO-2 colocalized in endothelia of blood vessels (arrows). ZO-1 staining was uniquely observed in intercalated discs of myocytes (arrowheads). Bar, 20 μ m.

95/SAP90, a synapse-associated protein from rat brain. The homology between ZO-2 and these proteins was displayed within discrete domains shared among all family members, including an internal repeat (DHR) of unknown function and a region resembling yeast guanylate kinase, an enzyme which converts 5'-GMP to GTP. Taken together, the close relationship displayed with ZO-1 and the sequence homology shared with other discs-large family members indicates that ZO-2 is a member of the discs-large family of proteins. The roles of ZO-2, ZO-1, PSD-95/SAP90, and p55 have not yet been elucidated. Mutations in the *dlg* gene have been shown to result in neoplastic over-growth of imaginal discs of *Drosophila* larvae, suggesting that the *dlg* gene product acts as a tumor suppressor. Though it is not yet known how the *dlg* product carries out this function, both the guanylate kinase and amino-terminal regions are important for its tumor suppressing activity (Woods and Bryant, 1989, 1991).

Northern analysis reveals an mRNA size of \sim 5.2 kb, distinct from the 7.9-kb message for ZO-1. Our 3.2 kb of cloned ZO-2 cDNA contains 2325 bp of coding and 875 bp of 3'-untranslated cDNA. Thus, there are roughly 2.0 kb of missing 5' cDNA from our isolate. Since SDS gels reveal an $M_r \cong$ 160 kD for ZO-2, most of this missing 5' cDNA must be coding, with very little 5'-untranslated sequence.

SH3 domains have been shown to bind to proline-rich sequences (Ren et al., 1993). The proline-rich carboxyl terminus of ZO-2 together with the presence of an SH3-like domain in ZO-1, may offer an explanation for the association between these two proteins. Alternatively, it is possible that formation of ZO-2/ZO-1 complexes is mediated by the interaction of putative leucine zipper regions contained in these two proteins, since this motif has been shown to be both necessary and sufficient for heterodimerization of DNA-binding proteins (Baxevanis and Vinson, 1993). Another function which has been demonstrated for SH3 domains is the targeting of proteins to specific subcellular localizations (Bar-Sagi et al., 1993). Thus SH3 domains in ZO-2/ZO-1 complexes may be responsible for localization of these proteins to tight junctions.

From the sequence comparison of ZO-2 with ZO-1, it was not possible to determine whether ZO-2 also contained an alternatively spliced α -motif. Since we have only one cDNA isolate, we also have no evidence for alternative splicing from our DNA sequence data. Immunoblotting of whole MDCK cell extracts with the R9989 anti-ZO-2 polyclonal antibody revealed a doublet at 160 kD, as well as a slower migrating band at 190 kD (Fig. 5), suggesting that these cells may synthesize alternative forms of ZO-2. Recognition of the

160-kD doublet and 190-kD band by a second anti-ZO-2 antiserum, GP 753, raised in guinea pig (data not shown), lends support to the notion that these bands are related to ZO-2. In blots of ZO-1/ZO-2 coimmunoprecipitates (Fig. 5) only a single band comigrating with the lower band of the 160-kD doublet was observed, raising the interesting possibility that ZO-1 associates exclusively with one of several alternative ZO-2 species.

Cytoplasmic staining was observed with R9989 in MDCK cell monolayers which was not seen in cryosections of *in situ* epithelia. The cytoplasmic staining, which was inhibitable with immunizing polypeptide, could indicate the presence of cytoplasmic pools of ZO-2 not seen in other tissues. Alternatively, immunoprecipitation of MDCK cell extracts with R9989 yielded additional polypeptides at $M_r \cong 180$ and 240 kD not seen in immunoprecipitates of identical extracts with either R40.76 (anti-ZO-1 monoclonal) or with the anti-ZO-2 guinea-pig serum GP 753 (data not shown). Taken together, these results suggest that our R9989 reagent immunoprecipitates additional polypeptides in the MDCK cell, whose location is currently unknown. Thus it is not clear if the cytoplasmic staining observed in MDCK cells corresponds to cytoplasmic ZO-2, or if it corresponds to the cross-reacting species.

Itoh et al. (1993) have previously shown that ZO-1 localizes to the *fascia adherens* component of intercalated discs joining myocardial cells. The results presented here confirm the presence of ZO-1 immunoreactivity in intercalated discs by immunofluorescence microscopy. In contrast, ZO-2 staining was detected only in blood vessel endothelia in heart, suggesting that this protein is not associated with the *fasciae adherentes* of the intercalated discs. At the ultrastructural level, ZO-2 immunogold labeling was associated exclusively with tight junctions in MDCK epithelial cells. We did not observe ZO-2 or ZO-1 staining of the other members of the junctional complex, the *zonulae adherentes* and *maculae adherentes*, in these preparations.

ZO-2 and ZO-1 thus appear to be obligate partners at the cytoplasmic surfaces of vertebrate tight junctions while ZO-1 localizes, in the absence of ZO-2, at the myocardial intercalated disc. It will be of interest to investigate whether a related family member, the product of the *dlg* gene in *Drosophila*, also has an obligate partner in the structure of the septate junction. The tumor suppressor activity exhibited by the product of the *dlg* gene in insect imaginal disc epithelial cells raises the additional question as to whether ZO-2, ZO-1, or both proteins share this activity in vertebrate epithelia.

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