

ZO-1 mRNA and Protein Expression during Tight Junction Assembly in Caco-2 Cells

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Abstract. We previously identified and characterized ZO-1 as a peripheral membrane protein specifically associated with the cytoplasmic surface of tight junctions. Here we describe the identification of partial cDNA sequences encoding rat and human ZO-1 and their use to study the assembly of tight junctions in the Caco-2 human intestinal epithelial cell line. A rat cDNA was isolated from a lambda-gtl1 expression library by screening with mAbs. Polyclonal antibodies were raised to cDNA-encoded fusion protein; several properties of these antibodies support this cDNA as encoding ZO-1. Expression of ZO-1 mRNA occurs in the rat and Caco-2 cells with a major transcript of ~7.5 kb. To disrupt tight junctions and study the subsequent process of assembly, Caco-2 cells were grown in suspension for 48 h in Ca⁺⁺/Mg⁺⁺-free spinner medium during which time they lose cell-cell contacts, become round, and by immunofluorescence microscopy show diffuse and speckled localization of

ZO-1. Within hours of replating at confluent density in Ca⁺⁺/Mg⁺⁺-containing media, attached cells show discrete localization of ZO-1 at cell-cell contacts. Within 2 d, fully confluent monolayers form, and ZO-1 localizes in a continuous gasket-like fashion circumscribing all cells. ZO-1 mRNA levels are highest in cells in spinner culture, and upon replating rapidly fall and plateau at ~10% of initial levels after 2–3 wk in culture. ZO-1 protein levels are lowest in contact-free cells and rise five- to eightfold over the same period. In contrast, mRNA levels for sucrase-isomaltase, an apical membrane hydrolase, increase only after a confluent monolayer forms. Thus, in this model of contact-dependent assembly of the tight junction, there is both a rapid assembly beginning upon cell-cell contact, as well as a long-term modulation involving changes in expression of ZO-1 mRNA and protein levels.

LITTLE information exists about the structural components, assembly, and regulation of the tight junction or its developmental relationship to other cell structures. As a characteristic feature of polarized, transporting epithelial cells, the tight junction forms the intercellular seal which limits the movement of water and solutes between cells (14, 18, 34, 40) and may play a role in establishing and maintaining the separation of biochemically distinct apical and basolateral membrane components (2, 43). By thin-section electron microscopy the junction appears as a set of close membrane contacts positioned apically to the other intercellular junctions; i.e., desmosomes and the adherens and gap junctions (15). In freeze-fracture electron micrographic images the membrane appositions are revealed to correspond to interconnecting fibrils in the plane of the membrane. Fibrils appear composed of rows of intramembrane particles (16, 37) giving rise to the notion that the junction is composed of rows of transmembrane proteins which seal the extracellular space by their association with like proteins on adjacent cells.

We have recently identified (38) and characterized ZO-1 (1), the first protein shown to be uniquely a component of the tight junction in all epithelial and endothelial tissues. ZO-1 is a 225-kD peripherally associated membrane protein located under the points of membrane contact. This physically asymmetric protein is multiply phosphorylated and present in low abundance. More recently a second junction-associated protein, termed cingulin, has been identified (12); this protein appears to be present at higher levels and while also located on the cytoplasmic surface of intercellular contacts is positioned farther from the membrane than ZO-1 (39).

Much work has recently been directed toward enumerating components of each intercellular junction type, describing how they assemble, and how junctions contribute to generation of structural polarity in epithelial cells (19). Manipulation of extracellular calcium is frequently used to induce reversible disassembly of intercellular junctions. Specifically, it has recently been demonstrated in MDCK cell cultures that removal of calcium causes ZO-1 to become diffusely distributed throughout the cytoplasm and that calcium-dependen-

dent relocation to the plasma membrane occurs at cell-cell contacts and does not require new protein synthesis (35). Similar inducible assembly has been demonstrated for protein components of desmosomes (32), focal contacts, and the basolaterally positioned, cytoskeletal membrane protein, fodrin (30, 31). Suggestive evidence (19) has also been presented that the initiating event in assembly of intercellular junctions is the Ca^{++} -dependent association of cell adhesion molecules, variously termed CAMs (6), uvomorulin (19), and E-cadherin (40). Additional evidence suggests that formation of stable cell contacts can influence the subsequent expression of cytoskeletal proteins and pattern of cellular differentiation (4).

We have chosen to investigate the assembly of tight junctions and acquisition of cellular polarity in cultured Caco-2 cells, a human colonic adenocarcinoma cell line. As these cells reach confluence they form morphological and electrophysiologically measurable tight junctions and undergo a fetal enterocyte-like differentiation which includes the formation of an apical microvillar brush border and associated enzymes. This process takes up to three weeks for full expression (17, 20, 33).

In this article we describe temporal expression of mRNA and protein for ZO-1, during reassembly of the tight junction in Caco-2 cells.

Materials and Methods

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Isolation and Characterization of cDNAs Encoding Rat and Human ZO-1

All mAbs used for expression library screening were generated in rats against tight junction-enriched membranes from mouse liver, as previously described (38). These are designated R26.4C (38), R40.76, and R40.40D₃ (1). Two additional anti-ZO-1 mAbs, designated R40.40E8 and R40.5, were produced in the laboratory of Dr. Daniel A. Goodenough (Harvard Medical School). A pooled collection of all five mAbs, derived from protein-depleted Nutridoma (Boehringer-Mannheim Biochemicals, Indianapolis, IN) hybridoma culture supernatant, was used to screen a rat kidney-derived cDNA library (Clontech Laboratories, Inc., Palo Alto, CA) constructed in the lambda-gt11 expression vector (45). By screening $\sim 1.5 \times 10^6$ plaque forming units (pfu) a single immunopositive clone containing a 1-kb insert was identified. The insert was subcloned into pGEM-3Z (Promega Biotech, Madison, WI) for use as Northern blot probes and for DNA sequencing (24). A cross-hybridizing human cDNA was identified in a human brain stem lambda-gt11 library (gift of Dr. Robert Levenson, Department of Cell Biology, Yale School of Medicine) by hybridization screening with the rat rZ1 sequence and use of lower stringency washing conditions; i.e., twice at room temperature in $2\times$ SSC, 0.1% SDS, followed by twice at 42°C in $0.2\times$ SSC, 0.1% SDS ($1\times$ SSC is 0.15 M NaCl, 0.015 M NaCitrate, pH 7.0). The rZ1 cDNA insert was used to generate probes by the random priming labeling method (Boehringer Mannheim Biochemicals). Out of $\sim 5 \times 10^5$ pfu screened, two hybridizing plaques were identified; these contained Eco RI inserts of 0.7 and 1.8 kb. The larger fragment, designated hZ1, was subcloned into the Eco RI site of pGEM-3Z and used for the experiments presented here.

Production of Polyclonal Antibodies against Rat cDNA-encoded Protein Sequence

Lysogenized strains of *Escherichia coli* CAG-456 were created with phage containing rZ1, grown, and induced as described by Snyder et al. (36). Fusion protein (fp) was electroeluted from preparative SDS-PAGE gels (8) and antisera produced in rabbits by standard techniques. Anti-fp antibodies were affinity purified by passage over an affinity column of fp coupled to

CNBr-activated Sepharose. Coupling was done by the manufacturer's recommended technique (Pharmacia Fine Chemicals, Piscataway, NJ), and typically ~ 0.5 mg of fusion protein (fp)¹ per milliliter of Sepharose was coupled. After washing unbound protein through with 10 column volumes of PBS, bound Abs were eluted with 4 M guanidinium chloride in PBS. Eluted antibodies were dialyzed against PBS, and anti-beta-galactosidase activity was removed by passage over a beta-galactosidase-Sepharose column (8). Affinity-purified antibodies were pure IgGs, as determined by SDS-PAGE, and were stored in PBS with 0.05% NaN_3 .

Cell Culture and Replating Protocol

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and used between passage numbers 34 and 62. Cells on plates were maintained in DME (Hazelton Systems, Inc., Aberdeen, MD) supplemented with glutamine (2 mM; Hazelton Systems, Inc.), transferrin (10 $\mu\text{g}/\text{ml}$, Boehringer-Mannheim Biochemicals), penicillin/streptomycin, and 10% FCS (HyClone Laboratories, Logan, UT) and grown at 37°C in 5% CO_2 . Cells were passaged every 4–5 d. Short term spinner cultures were established from trypsinized confluent monolayers by suspension at 5×10^5 cells/ml in MEM spinner media supplemented as above except that FCS was thoroughly dialyzed against 0.9% saline to remove Ca^{++} and Mg^{++} . Spinner bottles (Bellco Glass, Inc., Vineland, NJ) were tightly closed and cultures maintained at 37°C with gentle spinning. Cells were replated from spinner culture at a density of 4.8×10^5 cells/cm² after first centrifugation and resuspension in the DME with 10% FCS. Cells were plated onto 9.6-cm² plastic culture wells for use in protein and DNA analysis, 21.2-cm² wells for RNA analysis, or sterilized glass coverslips for immunofluorescence microscopy. In preliminary experiments cells were plated directly onto glass or plastic tissue culture surfaces. On these unprepared surfaces, cells adhered poorly and formed large aggregates for up to a week before attaching as a monolayer. To accelerate monolayer formation, all culture surfaces for experiments reported here were coated with secreted matrix components of the mouse EHS sarcoma line. This was either purchased as Matrigel (lot No. 86-1356; Millipore Continental Water Systems, Bedford, MA) and used as described by the manufacturer or obtained as a gift from Dr. Joseph Madri (Pathology Department, Yale School of Medicine). The density of Caco-2 cells at "confluence" is time dependent, however, at the high plating density used in these experiments cells could adhere and regain intercellular contacts and apparent confluence in <2 d, without significant change in cell number per plate. The media was never changed before 4 d. This was done to prevent cell loss or selection of a subpopulation of more tightly adherent cells. Cells were processed directly from spinner media or at specified times after replating to determine (a) the DNA content per culture well as a measure of cell number, (b) the content of ZO-1 mRNA relative to total RNA, (c) ZO-1 protein content, and (d) the immunofluorescent localization of ZO-1.

Strain II MDCK (Madin-Darby canine kidney) cells were maintained as previously described (38).

Immunoblot Analysis

Methods for SDS-PAGE, electrophoretic transfer of proteins to nitrocellulose paper, and immunoblot analysis using mAbs and peroxidase-conjugated secondary antibodies have been described in detail elsewhere (38) and are modifications of the methods of Laemmli (26) and Towbin et al. (42). Affinity-purified polyclonal rabbit antibodies were used at ~ 1 $\mu\text{g}/\text{ml}$ and detected with peroxidase-conjugated goat anti-rabbit IgG (1:500; Cappel Laboratories, Malvern, PA) as described above for mAbs.

Quantitative immunoblots were performed on Caco-2 samples in the following manner. A known number of cells from spinner culture or cells scraped from culture wells plated at known density were pelleted, resuspended in 0.7 ml of TBS (25 mM Tris, pH 7.4, 150 mM NaCl) and rapidly solubilized in 0.3 ml of $10\times$ SDS-PAGE sample buffer by placing tubes in boiling water for 3 min ($1\times$ is 1.25 mM Tris, pH 6.8, 1% SDS, 2% mercaptoethanol, 2% sucrose). Samples, in duplicate, were subjected to electrophoresis on 7% SDS-PAGE gels; the proteins were transferred to nitrocellulose and incubated with affinity-purified polyclonal antibodies. After washing, blots were incubated in ¹²⁵I-protein A (2 $\mu\text{Ci}/\text{ml}$, 92.3 $\mu\text{Ci}/\mu\text{g}$; ICN Radiochemicals, Irvine, CA) for 1 h, washed extensively in TBS, dried, and autoradiographed (X-OMAT AR film; Eastman Kodak Co., Rochester, NY). ¹²⁵I-protein A bound to each ZO-1 gel band was measured by scanning densitometry and shown to be a linear function of sample loaded over the range analyzed. ZO-1 protein content per cell as a function

1. *Abbreviation used in this paper:* fp, fusion protein.

of culture time is presented in arbitrary units as densitometric area per culture well divided by the DNA content per culture well. Total DNA per culture well was quantitated, after purification (28), by spectrofluorometry (25).

Northern Analysis

RNA was isolated from freshly dissected tissues of adult male Sprague Dawley rats by guanidine isothiocyanate extraction as described by Chirgwin et al. (9). Caco-2 cell RNA was prepared from cultured cells by the acid-guanidinium method of Chomczynski and Sacchi (10). RNA was quantified by determining OD₂₆₀ and equal amounts of RNA were loaded in each lane. Samples were electrophoresed in formaldehyde-containing agarose gels (28), stained with acridine orange, and transferred to nitrocellulose (41). Prehybridization and hybridization was performed as described by Wahl et al. (44) without dextran sulfate. After hybridization, the nitrocellulose was washed four times at 22°C in 2× SSC, 0.1% SDS, and two times at 65°C in 0.1× SSC, 0.1% SDS. Hybridization probes were prepared from gel-purified plasmid inserts and labeled by the random priming technique (Boehringer Mannheim Biochemicals). A cDNA corresponding to the full length coding region of rabbit sucrase-isomaltase was generously provided by Dr. Walter Hunziker (Department of Cell Biology, Yale School of Medicine), and shown to hybridize to a 6-kb mRNA band in Caco-2 cell RNA (22). The relative content of specific mRNAs in each sample was quantified from the autoradiographs using a scanning densitometer.

In Situ Hybridization

Freshly dissected mouse jejunum was fixed in acetone, paraffin embedded, and sectioned for histologic study. Techniques for sense and antisense cRNA probe preparation, hybridization, and autoradiography followed the methods of Jones et al. (23). The cDNA used to direct ³⁵S-cRNA probe synthesis was an Exonuclease III-generated sequence (21) corresponding to the 3'-most 290 bp of the original rZ1 cDNA insert. Photomicroscopy was performed on a Zeiss light microscope, illuminated through a dark-field condenser, using a 25× objective and Tri-X film (Eastman Kodak Co.).

Other Methods

Immunoprecipitation of ZO-1, solubilized from confluent monolayer cultures of MDCK cells, was done as previously described (1) using the R40.76 mAb covalently coupled to Sepharose beads. Methods for immunofluorescent localization of ZO-1 in frozen sections of rat liver, using mAbs, have been described (38). For immunostaining with the affinity-purified antifusion protein antibodies the same procedure was followed, using 0.1 μg/ml polyclonal antibody in 5% normal goat serum and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG as the secondary antibody (1:100; Cappel Laboratories). Live cells were observed and photographed using contrast modulation (Hoffman) microscopy to better define cell borders.

Results

Identification and Characterization of ZO-1-encoding cDNAs

A rat kidney-derived cDNA library in lambda-gt11 was screened with a pooled collection of five anti-ZO-1 mAbs. Out of 1.5 × 10⁶ pfu screened, a single immunoreactive clone, containing an insert of ~1 kb, was identified and designated rZ1. After plaque purification it was found that four out of five of the individual mAbs reacted with the fusion protein expressed from rZ1 (data not shown). Reactivity was assessed by both plaque screening and on immunoblots of purified fusion protein. This highly antigenic sequence represents 38 out of 225 kD in the intact ZO-1 protein.

Fusion protein was produced from lysogenized strains of *E. coli* and used to produce antisera in three rabbits. Although we have been unable to verify this cDNA as ZO-1 by direct comparison of protein and DNA sequences, characteristics of the affinity-purified anti-fp antibodies demonstrate that the cDNA codes for a portion of the ZO-1 protein. These

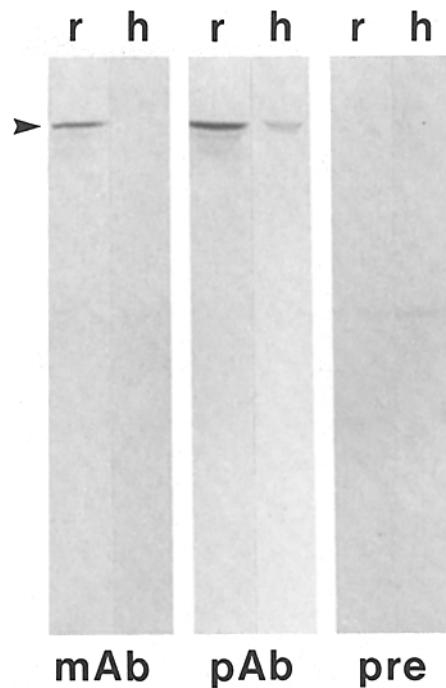


Figure 1. Immunoblot analysis of rat and human samples probed with anti-ZO-1 mAb and anti-fp antibodies. Samples from rat liver (r) or human Caco-2 cells (h), were probed with either an anti-ZO-1 mAb (R.40.76, *mAb*), affinity-purified anti-fp antibodies (rabbit No. 6139, *pAb*), or with preimmune serum (rabbit No. 6139, *pre*). The mAb recognizes rat ZO-1, indicated by the arrow, but not human ZO-1. pAb recognizes a band in rat liver and Caco-2 cells at the same relative molecular mass. Preimmune serum shows only lower molecular weight, nonspecific reactivity.

characteristics are listed below. (a) By immunoblot analysis, all five mAbs recognize ZO-1 as a single 225-kD protein in samples of whole rat liver, but fail to recognize ZO-1 in human Caco-2 cell samples (e.g., R40.76 in Fig. 1). In contrast, affinity-purified anti-fp antibodies recognize a 225-kD band in both rat liver and Caco-2 cells. Preimmune sera does not recognize ZO-1 in either species. Thus, antibodies produced against the 38-kD fragment encoded by this rat cDNA recognize a protein of the appropriate molecular weight. Further, the rZ1 cDNA encodes epitopes present in human ZO-1 but not recognized by any of the mAbs used to initially screen the expression library. Identical results were obtained with antisera from all three rabbits. (b) By immunoprecipitating ZO-1, which had been solubilized from cultured MDCK cells, it can be demonstrated that mAb and anti-fp polyclonal Abs recognize exclusively the same protein. As shown in the immunoblots of Fig. 2, mAb coupled to CNBr-activated Sepharose can quantitatively immunoprecipitate ZO-1 from total solubilized proteins of MDCK cultured cells. Under these conditions all of the ZO-1 in the cultured cells is present in the solubilized protein sample (1). After centrifugation of the immune complexes, the pellet contains all the ZO-1. When portions of the same mAb-precipitated ZO-1 samples are immunoblotted for protein reacting with the polyclonal antisera, the anti-fp antibodies recognize the same protein precipitated by mAb and react with nothing remaining in the supernatant. (c) The subcellular localization of antigen rec-

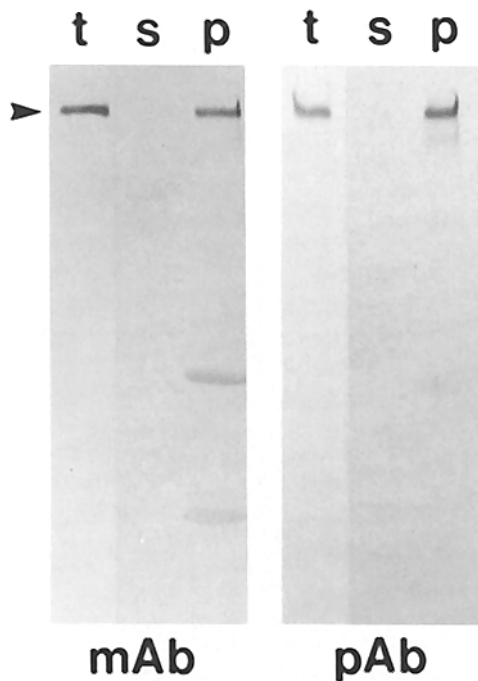


Figure 2. Immunoblot analysis of immunoprecipitated MDCK protein samples, probed with either mAb or pAb. Total (*t*) soluble protein from confluent monolayer cultures of MDCK cells were subjected to immunoprecipitation with mAb coupled to Sepharose and protein in the pellet (*p*) and postcentrifugation supernatant (*s*) analyzed for immunoreactivity with either the mAb (R40.76) or the anti-fp polyclonal antibodies (No. 6139, *pAb*). The position of ZO-1 is marked. *pAb* recognizes only the protein which is quantitatively immunoprecipitated by the mAb. Pellet samples developed with anti-mAb secondary antibody visualize heavy and light chain IgG bands released from mAb-Sepharose in SDS-PAGE sample buffer.

ognized by affinity-purified anti-fp antibodies is the same as that defined by anti-ZO-1 mAbs. Identical immunofluorescent localization is seen at the junction complex region of epithelial tissues and endothelia in several animal species and

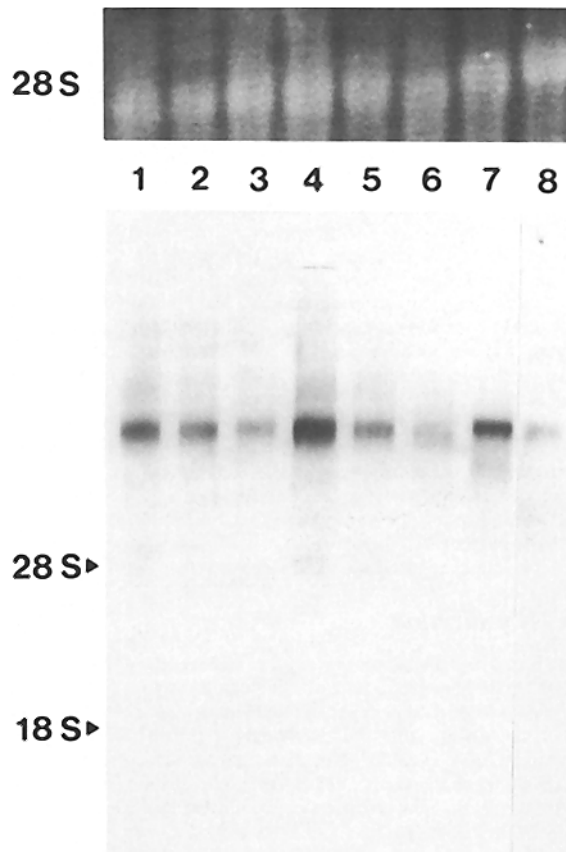


Figure 4. Northern blot analysis of total RNA from rat tissues and the Caco-2 cell line hybridized with a ^{32}P -labeled ZO-1 cDNA probe. 20 μg per lane of total RNA was electrophoresed on a 1.2% agarose/formaldehyde gel, stained with acridine orange, and transferred to nitrocellulose. Acridine orange staining of 28 S RNA bands, before transfer (*top section*), shows approximately equal RNA loading per lane. Samples are from lane 1, whole brain; lane 2, lung; lane 3, liver; lane 4, stomach; lane 5, colon; lane 6, kidney; lane 7, testis; and lane 8, Caco-2 cells. The autoradiograph of the Caco-2 sample was exposed four times longer than the rat samples. Positions of 28 S and 18 S RNAs are indicated.

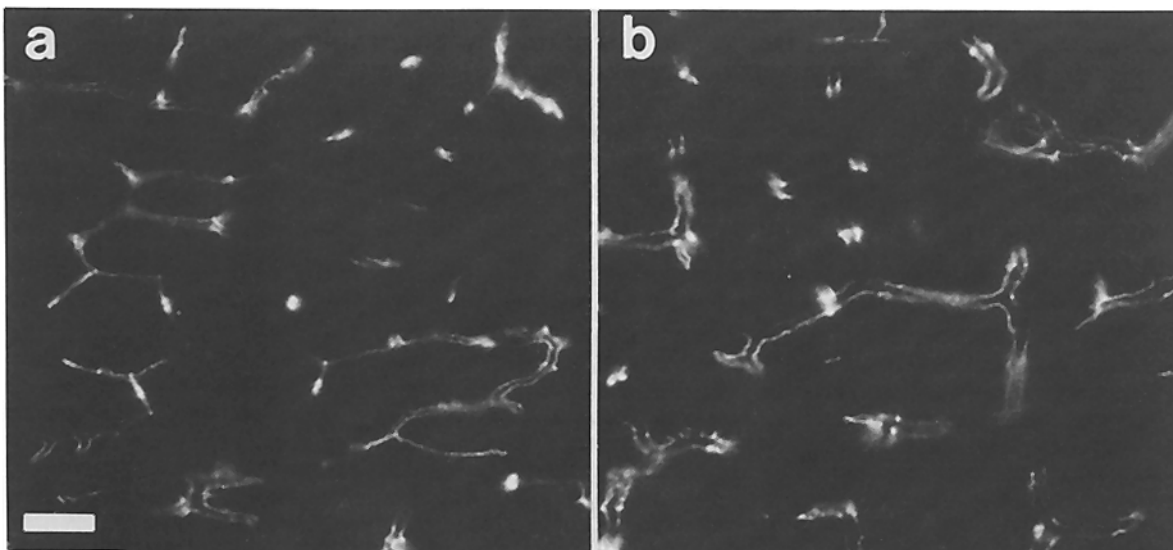


Figure 3. Indirect immunofluorescent staining of rat liver sections using either mAb (*a*) or affinity-purified polyclonal anti-fp antibodies (*b*). Both antibodies show identical localization to the parallel junctions bordering the canalicular domains. Bar, 10 μm .

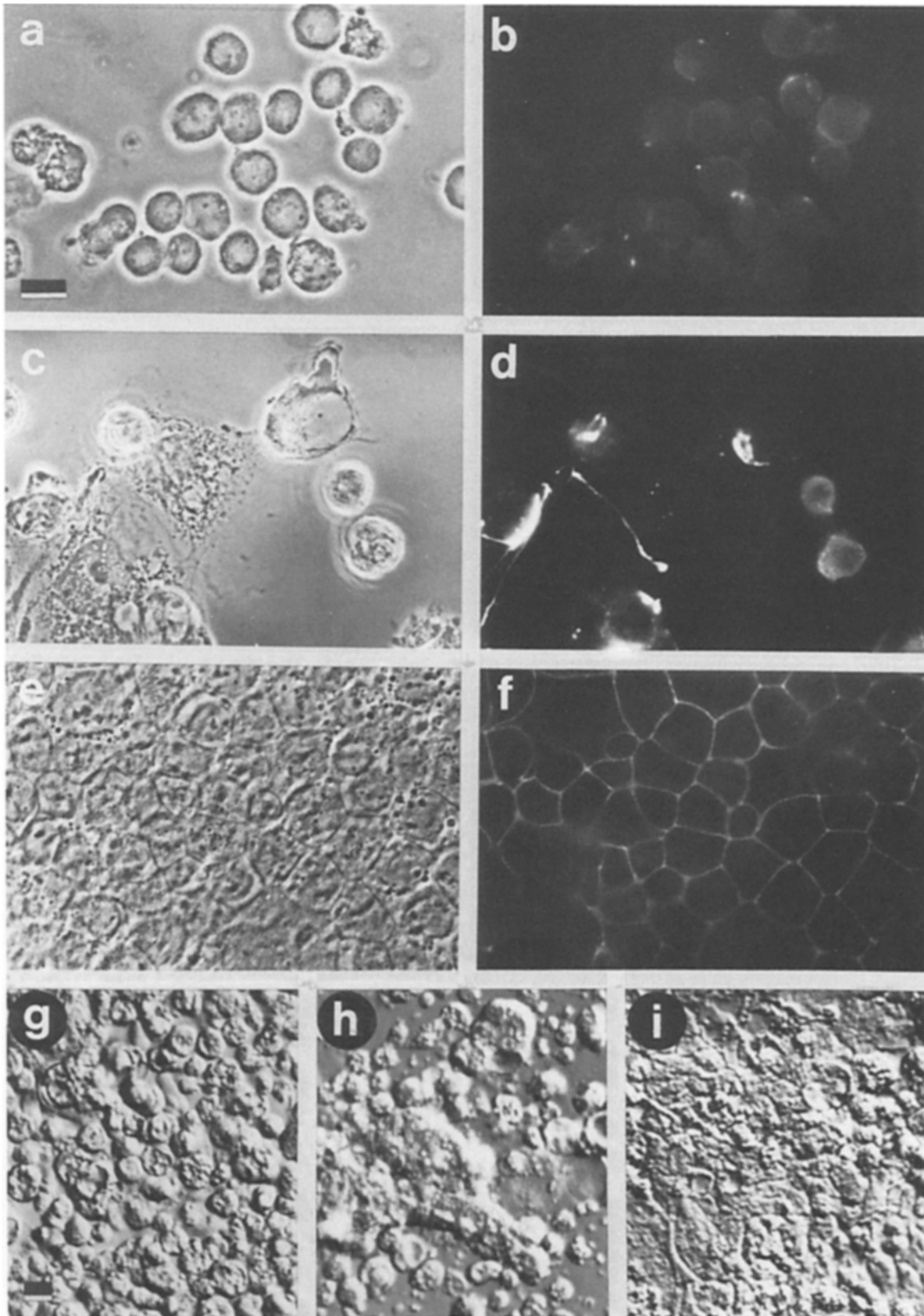


Figure 5. Indirect immunofluorescent localization of ZO-1 in Caco-2 cells at various times after replating from spinner culture. Phase contrast micrographs of cells plated onto coverslips and permeabilized with methanol (*a*) immediately after, (*c*) 3 h after, and (*e*) 21 d after 48 h in spinner culture. Corresponding micrographs (*b*, *d*, and *f*) demonstrate the indirect immunofluorescent localization of ZO-1 in these cells. Contrast modulation micrographs (*g*, *h*, and *i*) demonstrate the plating density and morphology of living cells (*g*) immediately, (*h*) 3 h, and (*i*) 2 d after replating. Bars, 10 μm .

cultured cell types (data not shown). Comparative staining is demonstrated in the rat liver, where tight junctions define the parallel borders of the canalicular/apical surface domains (Fig. 3). In addition, at the ultrastructural level, anti-fp antibodies and anti-ZO-1 mAbs both localize discretely on the cytoplasmic surface of the tight junction's intercellular membrane contacts (39).

We conclude from the evidence presented above that the cDNA identified and used in these studies does code for a portion of ZO-1.

When Northern blots of total mRNA prepared from various rat tissues are probed with rZ1 cDNA, hybridization to a major mRNA size of ~ 7.5 kb is seen in all tissues studied, including the human Caco-2 cell line (Fig. 4). This mRNA length is determined by extrapolating migration distance vs. log (kb), based on the positions of human alpha-fodrin mRNA (8 kb; reference 11), sucrase-isomaltase mRNA (6 kb; reference 22), 28 S RNA (4.9 kb), and 18S RNA (1.8 kb). This is clearly sufficiently large to encode a 225-kD protein. Expression of ZO-1 mRNA, relative to total RNA varies significantly between organs. This, however, is difficult to interpret since none of these organs contains a homogeneous epithelial cell population and each contains a varying content of capillary endothelial cells, which also express ZO-1. Expression of this single mRNA size was detected in a wide range of cultured epithelial and endothelial cell types. However it was absent in cultured T-lymphocyte lines (gift of Dr. Nancy Ruddle, Yale School of Public Health), which do not form intercellular junctions (data not shown).

The nucleic acid sequences were determined for both the rat ZO-1 cDNA and a cross-hybridizing human cDNA (see Materials and Methods) to verify that the human cDNA also encoded ZO-1. They are 80% identical and absent from sequence databanks (Genbank). Of the 335 amino acids encoded by the single open reading frame of the rat cDNA, 15% are proline residues. These are partial sequences and thus can not be presented here.

Contact-dependent Localization of ZO-1 in Caco-2 Cells

Our goal was to establish a uniform contact-free cell population in which we could observe de novo reassembly of the tight junction. Short-term growth in spinner culture was used because in preliminary experiments, we observed that even after removal of extracellular calcium from plated confluent monolayers, linear ZO-1-containing remnants of the tight junction persisted on the plasma membrane for several days (data not shown). In contrast we observed that passage through spinner culture would eliminate ZO-1 from localizing at residual cell-cell contacts and generate a more uniform and structurally "depolarized" cell population for synchronous immunolocalization and biochemical observations.

When confluent Caco-2 cells were trypsinized and placed in $\text{Ca}^{++}/\text{Mg}^{++}$ -free spinner culture they gradually round and lose stable intercellular contacts. After 24 h a few pairs and small groups of cells were still present, which as determined by immunofluorescent staining, retained ZO-1 along cell contacts. After 48 h the percent of single cells approached 100% while viability also remained near 100%, based on trypan blue exclusion. At longer times viability fell; consequently 48 h was used for all experiments. Cells at this time were round and, by light microscopy, showed no obvious

brush border microvillar remnant (Fig. 5). By immunofluorescence the cytoplasm showed diffuse staining for ZO-1; however, unambiguous subcellular localization is difficult without confocal or ultrastructural imaging. In some cells ZO-1 localization appeared to be at a single membrane point reminiscent of a cap. Cells were returned to $\text{Ca}^{++}/\text{Mg}^{++}$ -containing media and plated at a density characteristic of confluent monolayers (4.8×10^5 cells/cm²) so that cell-cell contacts could form immediately (Fig. 5g). Cell number was determined over the subsequent 3 wk in culture by determining the total DNA content per culture well. We resorted to this measure of cell number because cells proved difficult to dissociate into single cells for reliable counting. Between 1 and 3 d in culture the DNA content routinely began to increase and doubled by ~ 14 d in culture.

Within 1–2 h after replating ZO-1 localized to points of cell-cell contact (Fig. 5). Localization was observed at fine points of contact, irregularly shaped surfaces or the linear localization characteristic of mature monolayers. Reassembly was never seen at points of cell-substrate contact or on plasma membrane borders lacking contact with another cell. In the first day minor discontinuities in linear cell-cell localization were commonly seen. Within 2 d after replating all cells had reattached and the characteristic gasket-like staining pattern of ZO-1 in monolayers did not change over the subsequent 3 wk in culture.

Expression of ZO-1 mRNA and Protein in Replated Caco-2 Cells

A previous study has shown that new protein synthesis is not required for the reappearance of ZO-1 at the membrane when MDCK cells are switched from low to normal calcium-containing media (35). This suggests that an intracellular pool of ZO-1 is capable of relocating to the membrane when triggered by cell-cell contact. We observed no change in the continuous intercellular appearance of ZO-1 at the tight junction of Caco-2 cells over a period of 2 d to 3 wk in culture. Therefore, it was not obvious what effect, if any, cell contacts and culture time would have on the expression of ZO-1 mRNA and protein levels. The relative content of ZO-1 protein was determined as a function of time after replating from spinner culture and then normalized to DNA content per culture well. This represents the relative content of ZO-1 per cell by taking into account the small increase in cell number per culture well which occurs over 3 wk despite the high initial plating density (Fig. 6D). The relative content of ZO-1 protein/DNA typically increased five- to eightfold, beginning between 1 and 3 d and plateauing between 2 and 3 wk (Fig. 6B). The increase began after cells had reestablished a monolayer, and paralleled, but was not accounted for by the increase in cell number. ZO-1 levels in cells which had not been subjected to spinner culture also increased with time since last passage, but were never as low as those after 48 h in spinner media (data not shown).

In contrast to the increasing levels of ZO-1 protein, the relative content of mRNA for ZO-1 was highest in contact-free cells, and declined rapidly after reestablishment of cell contacts (Fig. 6, A and C). Levels relative to total RNA declined to 11–25% (in separate experiments) of initial levels by 3 d and $\sim 10\%$ by 3 wk in culture. The relative elevation in the ZO-1 mRNA level resulted from exposure to spinner culture since simple trypsinization and passage of cells did not result

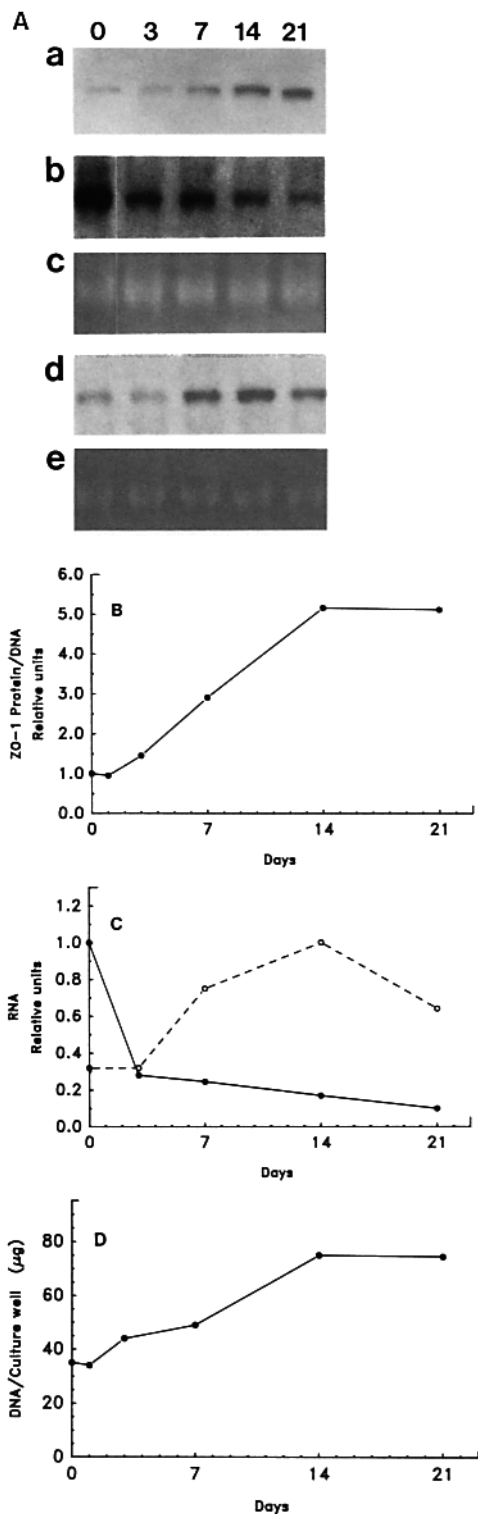


Figure 6. Immunoblot analysis of ZO-1 protein and Northern analysis of ZO-1 and sucrase-isomaltase mRNA expression at various times after replating Caco-2 cells from spinner culture. Samples were prepared from cells at the time of plating (0), 3, 7, 14, and 21 d after plating. (A) Each lane in immunoblot (a) represents protein collected from 0.3 cm² of culture plate area, developed with ¹²⁵I-protein A and visualized by autoradiography. Each lane of both Northern blots contains 20 μg of total RNA probed for ZO-1 (b) or sucrase-isomaltase (d). c and e presents acridine orange staining of 28 S ribosomal RNA bands on gels b and d, respectively, to demonstrate approximately equal loading of RNA. (B–D) ZO-1 protein

in this elevation. Measurements of the level of cyclophilin mRNA, an unrelated cytosolic protein (13), did not show this large and rapid decrease although it did decline to 70–80% of initial levels by 14 d in culture (data not shown). By contrast, the relative mRNA levels for sucrase-isomaltase increased after appearance of a confluent monolayer and then declined after 2 wk (Fig. 6, A and C).

In Situ Expression of ZO-1 mRNA in the Intestine

Enterocytes normally undergo structural and functional differentiation as they move from the mitotically active crypts up to the villus tip where they are sloughed into the intestinal lumen. An increase in tight junction fibril content has been noted in cells along the crypt-villus axis (29). To determine whether regulation of ZO-1 mRNA occurs during differentiation along the crypt-villus axis we performed *in situ* hybridization with radiolabeled ZO-1 cRNA probes in mouse jejunum. Control, sense cRNA demonstrated no specific hybridization above background, while antisense cRNA revealed intense hybridization above some crypt, but not villus, cells (Fig. 7).

Discussion

In this report we have examined patterns of expression of mRNA and protein for ZO-1 in Caco-2 cells as they develop from single cells to a confluent monolayer. The major findings are that ZO-1 is capable of rapid relocation onto the plasma membrane at points of cell-cell contact and that, coincident with reestablishment of cell contacts, there is a rapid fall in the relative ZO-1 mRNA levels followed by a slow rise in total cell levels of the protein.

Identification of Partial cDNAs for ZO-1

To perform these experiments we first identified cDNAs for ZO-1 and produced antibodies which, unlike our original mAbs, would recognize human ZO-1. Properties of antisera produced to protein encoded by the rZ1 cDNA suggest it is a partial sequence for ZO-1. First, polyclonal antibodies to the fusion protein recognize, on immunoblots, only a polypeptide of the appropriate molecular weight and recognize, as might be expected of polyclonal antibodies, epitopes on ZO-1 in animal species not recognized by mAbs. Second, when ZO-1 is quantitatively immunoprecipitated out of solubilized MDCK cell proteins with the mAbs, the immunoprecipitated protein is recognized by antifusion protein antibodies. More importantly, no protein immunoreactive with polyclonal antibodies is left unprecipitated. Finally, the cellular localization of antigen recognized by anti-ZO-1 mAbs and

levels per cell (B) are presented in arbitrary units normalized to 1.0 at *t*₀, by dividing the densitometric area of scanned immunoblot autoradiograms by the content of DNA per culture well on that day, as a measure of ZO-1 protein per cell. ZO-1 mRNA (C, ●) and sucrase-isomaltase (C, ○) levels are derived from scanned Northern blots normalized to 1.0 at their highest levels. DNA content per culture well (D) represents the increase in DNA over time in culture wells plated at the same initial cell density, 4.6 × 10⁶ cells/9.6-cm² culture well.

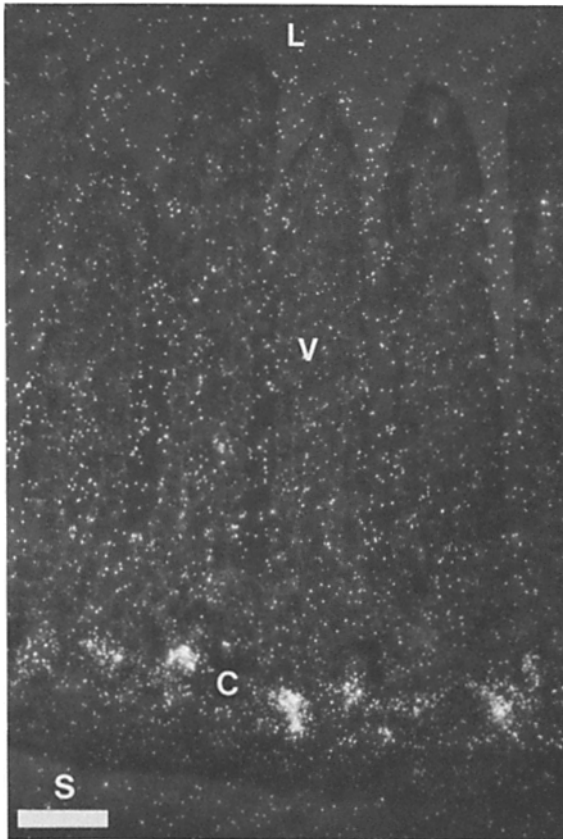


Figure 7. Autoradiomicrograph (dark-field illumination) of mouse intestine (jejunum) showing hybridization of ^{35}S -labeled ZO-1 cRNA probe. ZO-1 antisense RNA probe hybridizes predominantly over the crypt cells. Tissue is oriented with the serosal surface (S) at the bottom; above this is the mitotically active crypt (C) zone and the villi (V). The intestinal lumen (L) is uppermost. Bar, 300 μm .

by polyclonal anti-fusion protein antibodies is identical in all tissues and cell types tested. We have also confirmed this at the ultrastructural level (unpublished observations). These results strongly suggest we have identified an authentic ZO-1 cDNA and not a sequence encoding a protein which shares cross-reacting epitopes with ZO-1.

The rZ1 cDNA hybridizes to a major 7.5-kb mRNA band in RNA from all organs tested. Taken together with the known presence of a single protein weight in all tissues (1), this does not suggest the existence of ZO-1 isoforms, the presence of which might account for the wide tissue variability in physiologic and ultrastructural properties of the junction. The protein sequences deduced from both rat and human ZO-1 cDNA sequences are novel and contain a very high proline content. But, unlike other proline-rich proteins such as collagen, these residues are not positioned in an obviously repeating sequence. Although the functional properties of this, or any part of the ZO-1 protein, have not been defined, this 38-kD region would be incapable of maintaining significant amounts of stable alpha helix or beta sheet secondary structure. Consistent with its potential flexibility, this region of ZO-1 appears to be highly antigenic since it is recognized by four of five different mAbs. We have previously demonstrated that some of these mAbs recognize sin-

gle, and not repeating, epitopes in the full length protein further supporting the high antigenicity of this proline-rich region (1).

Contact-dependent Localization of ZO-1

Our results on the relocalization of ZO-1 to cell-cell contacts in Caco-2 cells confirm recent work performed in MDCK cells (35). This previous study also demonstrated that new protein synthesis was not needed for this relocalization, and that relocalization has an absolute requirement for intercellular contacts and restoration of millimolar external calcium concentrations. The tight junction is often defined physiologically by its ability to form a continuous resistive seal between adjacent epithelial cells. In this context it is interesting to note that the assembly of the tight junction, as monitored by the localization of ZO-1, is a very early event in the formation of intercellular contacts. This cellular structure is organizing long before cells become confluent and the junction capable of demonstrating its paracellular sealing function. The initial recognition or adhesive event in intercellular contact formation is thought to be mediated by the transmembrane cell adhesion proteins (40). Several of the proteins found at these initial focal contacts, such as uvomorulin and vinculin, subsequently localize at the zonula adherens (7), a junction invariably positioned contiguous to the tight junction in differentiated epithelia. It seems reasonable to speculate that initial uvomorulin-mediated contacts confer the temporal and spatial information for subsequent assembly of other cell-cell junctions, including the tight junction (19). The signals controlling assembly are unknown; ZO-1 is a multiply phosphorylated protein (1) and it would be of interest to determine whether this covalent modification was involved in controlling subcellular localization.

Expression of ZO-1 mRNA and Protein in Replated Caco-2 cells

Our biochemical studies suggest that cell contact not only affects subcellular localization of ZO-1, but also has profound effects on the relative levels of ZO-1 mRNA and protein expressed. When contacts are eliminated in spinner culture the levels of ZO-1 mRNA are elevated above those ever observed in plated cultures. When cells are replated ZO-1 mRNA levels rapidly decline and continue to decline over 3 wk in culture. The signal to decrease mRNA levels could result from restoring intercellular contacts. This down-regulation is opposite to the positive influence that cell contacts have on vinculin mRNA and protein levels observed in fibroblasts (3). It is not clear why the effects of contacts on ZO-1 and vinculin levels are in opposite directions since as a cytoplasmic membrane component of focal contacts and the adherens junction, vinculin has at least a superficial resemblance to ZO-1. On the other hand we have not eliminated the possibility that the primary stimulus for reducing mRNA levels is simply the return to normal extracellular calcium levels. It also remains to be determined whether the elevation of ZO-1 mRNA as a fraction of total cellular RNA results from increased transcription or mRNA stabilization. Ribosomal RNA accounts for most of the total RNA; so if in calcium-free spinner culture rRNA levels are preferentially decreased relative to ZO-1 mRNA, then the latter would appear to rise even if absolute ZO-1 mRNA levels were un-

changed. Previous studies have shown that suspension culture alone does not change the ratio of ribosomal RNA to polyA+ RNA in 3T3 fibroblasts (5); however it is possible that the removal of calcium and magnesium from the medium may result in selective stabilization of specific mRNA species or selective degradation of rRNA. Over 3 wk in culture the absolute amount of total RNA per cell does increase ~2.5-fold. An increase in rRNA content relative to ZO-1 mRNA content could contribute to, but can not account for, the 10-fold decline in relative ZO-1 mRNA levels over this time. The much smaller change observed in the level of cyclophilin mRNA is consistent with the large drop in ZO-1 mRNA levels being specific for that mRNA. As an additional internal control we assessed changes in mRNA for the apical hydrolase sucrase-isomaltase. Our observations confirm previous studies performed in Caco-2 cells showing that relative mRNA levels for this protein increase only after cells reestablish a confluent monolayer (6). The kinetics of mRNA levels which we present here clearly indicate ZO-1 and sucrase-isomaltase are under very different controls.

ZO-1 protein levels are dramatically reduced when cell contacts are lost and only very slowly return after contacts are reestablished. The decline is explained either by a reduction in translation, an increase in degradation, or both. Other investigators have made measurements of the effect of cell contacts on the half-life of the peripherally associated membrane cytoskeletal protein fodrin (31). When cell contacts between MDCK cells are established by restoration of extracellular calcium, fodrin returns to a membrane-bound state and demonstrates a significantly increased stability. ZO-1 may also be stabilized by membrane association and thus continue to accumulate despite the declining mRNA levels observed over several weeks in culture. It will be of interest, in future studies, to determine the mechanism by which cell contacts regulate the noncoordinate expression of ZO-1 protein and mRNA levels.

We have not measured any functional consequence of the continued accumulation of ZO-1. However, this increase is reminiscent of the culture time-dependent increase in the content of tight junction fibrils documented in monolayers of T84 cells, another human adenocarcinoma cell line (27). In this case the increasing fibril content is paralleled by an increase in transmonolayer electrical resistance. It also remains unanswered whether the protracted changes in mRNA levels documented here would occur in other cultured epithelial cell types or is unique to cells with an intestinal lineage which normally undergo a crypt to villus differentiation in situ.

The observation that in situ ZO-1 mRNA expression is highest in the crypt cells is consistent with the notion that expression is highest either around the time of mitosis or when cells are least differentiated. Perhaps alteration in cytoskeletal organization or intercellular contacts coincident with cell division plays a role in controlling ZO-1 mRNA expression as it does in cultured Caco-2 cells deprived of intercellular contacts. However, without better defining the controls operative in differentiating Caco-2 cultures and normal crypt to villus enterocyte differentiation these similarities should be considered only superficial.

In summary, we have presented observations regarding the cell-cell contact dependent assembly of tight junctions and the regulation of ZO-1 protein and mRNA levels in cultured

Caco-2 cells. These observations suggest that cell contacts initiate rapid relocalization of ZO-1 to the membrane and that long term modulation of this structure, at the mRNA and protein level, also occurs. Our long term goal is to characterize the mechanisms governing intercellular junction assembly and what role these processes play in generation of structural polarity of epithelial cells.

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References

- Anderson, J. M., B. R. Stevenson, L. A. Jesaitis, D. A. Goodenough, and M. S. Mooseker. 1988. Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J. Cell Biol.* 106:1141-1149.
- Balcarova-Stander, J., S. E. Pfeiffer, S. D. Fuller, and K. Simons. 1984. Development of cell surface polarity in the epithelial Madin-Darby canine kidney (MDCK) cell line. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2687-2694.
- Bendori, R., D. Salomon, and B. Geiger. 1987. Contact-dependent regulation of vinculin expression in cultured fibroblasts: a study with vinculin-specific cDNA probes. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2897-2905.
- Ben-Ze'ev, A. 1986. The relationship between cytoplasmic organization, gene expression and morphogenesis. *Trends Biochem. Sci.* 11:478-481.
- Benecke, B.-J., A. Ben-Ze'ev, and S. Penman. 1978. The control of mRNA production. Translation and turnover in suspended and reattached anchorage-dependent fibroblasts. *Cell.* 14:931-939.
- Bertolotti, R., U. Rutishouser, and G. M. Edelman. 1980. A cell surface molecule involved in aggregation of embryonic liver cells. *Proc. Natl. Acad. Sci. USA.* 77:4831-4834.
- Boller, K., D. Vestweber, and R. Kemler. 1985. Cell-adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. *J. Cell Biol.* 100:327-332.
- Carroll, S. B., and A. Laughon. 1987. Production and purification of polyclonal antibodies to the foreign segment of beta-galactosidase fusion proteins. *In* DNA Cloning. Vol. 3. A Practical Approach. D. M. Glover, editor. IRL Press Ltd. Oxford.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribosomes. *Biochemistry.* 18:5294-5301.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. *Anal. Biochem.* 162:156-159.
- Cioe, L., and P. Curtis. 1985. Detection and characterization of a mouse alpha-spectrin cDNA clone by its expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* 82:1367-1371.
- Citi, S., H. Sabanay, R. Jakes, B. Geiger, and J. Kendrick-Jones. 1988. Cingulin, a new peripheral component of tight junctions. *Nature (Lond.)* 333:272-275.
- Danielson, P. E., S. Forss-Petter, M. A. Brow, K. Calavetta, J. Douglass, R. J. Milner, and J. G. Sutcliffe. 1988. pIB15: a cDNA clone of the rat mRNA encoding cyclophilin. *DNA.* 7:261-267.
- Diamond, J. M. 1977. The epithelial junction: Bridge, gate and fence. *Physiologist.* 20:10-18.

15. Farquhar, M. G., and G. E. Palade. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* 17:375-412.
16. Friend, D. S., and N. B. Gilula. 1972. Variations in tight and gap junctions in mammalian tissues. *J. Cell Biol.* 53:758-776.
17. Grasset, E., M. Pinto, E. Dussaulz, A. Zweibaum, and J.-F. Desjeux. 1984. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. *Am. J. Physiol.* 247:C260-C267.
18. Gumbiner, B. 1987. The structure, biochemistry and assembly of epithelial tight junctions. *Am. J. Physiol.* 253:C749-C758.
19. Gumbiner, B., B. Stevenson, and A. Grimaldi. 1988. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* 107:1575-1587.
20. Hauri, H.-P., E. E. Sterchi, D. Bienz, J. A. M. Fransen, and A. Marxer. 1985. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. *J. Cell Biol.* 101:838-851.
21. Henikoff, S. 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. *Methods Enzymol.* 155:156-165.
22. Hunziker, W., M. Speiss, G. Semenza, and A. F. Lodish. 1986. The sucrase-isomaltase complex: primary structure, membrane orientation, and evolution of a stalked, intrinsic brush border protein. *Cell.* 46:227-234.
23. Jones, B., S. Carding, S. Kyes, S. Mjolsness, C. Janeway, Jr., and A. Hayday. 1988. Molecular analysis of T cell receptor gamma gene expression in allo-activated splenic T cells of adult mice. *Eur. J. Immunol.* 18:1907-1916.
24. Krawet, S. A. 1987. DNA sequencing with AMV (Avian Myoblastosis Virus) reverse transcriptase and a modified T7 DNA polymerase (Sequenase). *BioTechniques.* 5:620-627.
25. Labarca, C., and K. Paigen. 1979. A rapid, simple and sensitive DNA assay procedure. *Anal. Biochem.* 102:344-352.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.
27. Madara, J. L., and K. Dharmasathaphorn. 1985. Occluding junction structure-function relationships in a cultured epithelial monolayer. *J. Cell Biol.* 101:2124-2133.
28. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
29. Maricial, M. A., S. L. Carlson, and J. L. Madara. 1984. Partitioning of paracellular conductance along the crypt-villus axis: a hypothesis based on structural analysis with detailed consideration of tight junction structure-function relationship. *J. Membr. Biol.* 80:59-70.
30. Nelson, W. J., and P. J. Veshnock. 1986. Dynamics of membrane-skeleton (fodrin) organization during development of polarity in Madin-Darby canine kidney cells. *J. Cell Biol.* 103:1751-1765.
31. Nelson, W. J., and P. J. Veshnock. 1987. Modulation of fodrin (membrane skeleton) stability by cell-cell contact in Madin-Darby canine kidney cells. *J. Cell Biol.* 104:1527-1537.
32. Pasdar, M., and W. J. Nelson. 1988. Kinetics of desmosome assembly in Madin-Darby Canine kidney cells: temporal and spatial regulation of desmoplakin organization and stabilization upon cell-cell contact. II. Morphological analysis. *J. Cell Biol.* 106:687-695.
33. Pinto, M., S. Robine-Leon, M.-D. Appay, M. Kedinger, N. Triadou, E. Dussanly, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell.* 47:323-330.
34. Powell, D. W. 1981. Barrier function of epithelia. *Am. J. Physiol.* 241:G275-G288.
35. Siliciano, J. D., and D. A. Goodenough. 1988. Localization of the tight junction protein, ZO-1 is modulated by extracellular calcium and cell-cell contact in Madin-Darby canine kidney cells. *J. Cell Biol.* 107:2389-2399.
36. Snyder, M., S. Elledge, D. Sweeter, R. A. Young, and R. W. Davis. 1987. Lambda gt 11: gene isolation with antibody probes and other applications. *Methods. Enzymol.* 154:107-128.
37. Stevenson, B. R., J. M. Anderson, and S. Bullivant. 1988. The epithelial tight junction: structure, function, and preliminary biochemical characterization. *Mol. Cell. Biochem.* 83:129-145.
38. Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103:755-766.
39. Stevenson, B. R., M. B. Heintzelman, J. M. Anderson, S. Citi, and M. S. Mooseker. 1989. ZO-1 and Cingulin: tight junction proteins with distinct identities and localizations. *Am. J. Physiol.* In press.
40. Takeichi, M. 1987. Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. *Trends Genet.* 3:213-217.
41. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201-5205.
42. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
43. van Meer, G., and K. Simons. 1986. The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1455-1464.
44. Wahl, G. M., M. Stern, and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyl-oxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. USA.* 76:3683-3687.
45. Young, R. A., and R. W. Davis. 1983. *Proc. Natl. Acad. Sci. USA.* 80:1194-1198.