Catenins and Zonula Occludens-1 Form a Complex during Early Stages in the Assembly of Tight Junctions

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Abstract. We characterized the role of the E-cadherin adhesion system in the formation of epithelial tight junctions using the calcium switch model. In MDCK cells cultured in low (micromolar) calcium levels, the tight junctional protein Zonula Occludens-1 (ZO-1) is distributed intracellularly in granular clusters, the larger of which codistribute with E-cadherin. Two hours after activation of E-cadherin adhesion by transfer to normal (1.8 mM) calcium levels, ZO-1 dramatically redistributed to the cell surface, where it localized in regions rich in E-cadherin. Immunoprecipitation with ZO-1 antibodies of extracts from cells kept in low calcium and 2 h after shifting to 1.8 mM Ca^{2+} demonstrated the association of ZO-1 with α-, β-, and γ-catenins. E-cadherin was not detected in the ZO-1 immunoprecipitates but it was found in β-catenin immunoprecipitates that excluded ZO-1, suggesting that the binding of ZO-1 to catenins may weaken the interaction of these proteins with E-cadherin. Immunofluorescence and immunoelectron microscopy confirmed a close association of β-catenin and ZO-1 at 0 and 2 h after Ca^{2+} switch. 48 h after Ca^{2+} switch, upon complete polarization of the epithelium, most of the ZO-1 had segregated from lateral E-cadherin and formed a distinct, separate apical ring. The ZO-1–catenin complex was not detected in fully polarized monolayers. MDCK cells permanently transformed with Moloney sarcoma virus, which expresses low levels of E-cadherin, displayed clusters of cytoplasmic ZO-1 granules and very little of this protein at the cell surface. Upon transfection with E-cadherin into Moloney sarcoma virus-MDCK cells, ZO-1 redistributed to E-cadherin-rich lateral plasma membrane but later failed to segregate into mature tight junctions. Our experiments suggest that catenins participate in the mobilization of ZO-1 from the cytosol to the cell surface early in the development of tight junctions and that neoplastic transformation may block the formation of tight junctions, either by decreasing the levels of E-cadherin or by preventing a late event: the segregation of tight junction from the zonula adherens.

THE tight junction (*zonula occludens*), a member of the tripartite junctional complex that includes the *zonula adherens* and the *macula adherens*, forms a continuous belt at the boundary between the apical and lateral plasma membrane domains of neighboring epithelial cells (Farquhar and Palade, 1963). Structurally characterized by the fusion of the exoplasmic leaflets of contiguous plasma membranes (Farquhar and Palade, 1963; Goodenough and Revel, 1970), tight junctions (TJ)[1] selectively regulate the passage of molecules across the paracellular pathway (*gate function*), and passively separate molecules in the apical and basolateral plasma membrane domains (*fence function*) (Diamond, 1977; Gumbiner, 1987; Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989; Cereijido, 1992; Citi, 1993). Experimental evidence indicates that tight junctions are dynamic in nature, changing their structural and functional properties under different physiological conditions (Madara et al., 1992; Schneeberger and Lynch, 1992) and are subject to modulation by a variety of cellular and metabolic regulators (Balda et al., 1991, 1993; Nigam et al., 1991; Citi, 1992; Singer et al., 1994).

Several tight junctional proteins have been characterized. *Zonula occludens-1* (ZO-1), a 210–225-kD phosphoprotein, bears close structural similarity to the product of the lethal (l) discs large-1 *dlg*, a tumor suppressor gene in *Drosophila* (Woods and Bryant, 1991; Itoh et al., 1993; Willot et al., 1993), and is thought to act as a tumor suppressor in mammalian cells (Tsukita et al., 1993). ZO-1 has been observed in the TJ of epithelial and endothelial cells (Stevenson et al., 1986, 1989; Anderson et al., 1988). In cells lacking TJ such as fibroblasts and cardiac muscle cells, a colocalization of ZO-1 with cadherins has been re-

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1. *Abbreviations used in this paper*: l, lethal; MSV, Moloney sarcoma virus; SMEM, MEM modified for suspension culture; TJ, tight junctions; wt, wild type.

ported (Itoh et al., 1991, 1993; Howarth et al., 1992; Tsukita et al., 1993). Electron microscopy immunolabeling experiments have shown that ZO-1 is the peripheral TJ protein closest to the membrane (Stevenson et al., 1989; Itoh et al., 1991), followed by 7H6 (Zhong et al., 1993) and cingulin (Citi et al., 1988; Stevenson et al., 1989). Immunoprecipitation experiments revealed that ZO-1 associates with another TJ protein, ZO-2 (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994) and with a recently identified transmembrane component, occludin (Furuse et al., 1993; Furuse et al., 1994). Because ZO-1 interacts with spectrin tetramers, it has been postulated that the latter play a role in the architecture of tight junctions (Itoh et al., 1991). Recently, association of the GTP binding protein rab13 to the TJ has been demonstrated (Zahraoui et al., 1994). Furthermore, an actin-based cytoskeleton lies in the vicinity of TJ and experiments with F-actin depolymerizing drugs such as cytochalasins suggest a role for actin in the maintenance of junctional tightness (Meza et al., 1980; Madara, 1987).

The organization and step-wise assembly of the various components of the tight junction have not yet been elucidated. Early experiments by several groups have shown that disruption of adhesive cell–cell contacts between epithelial cells prevents the assembly of tight junctions (Gonzalez-Mariscal et al., 1985; Gumbiner and Simons, 1986; Behrens et al., 1985). Cell–cell contacts in epithelial cells are initially mediated by E-cadherin, a 120-kD transmembrane protein concentrated in the adherent junctions and lateral cell membranes, but normally excluded from the TJ region (Boller et al., 1985; Vestweber and Kemler, 1985; Edelman, 1986; Takeichi, 1991). E-Cadherin plays a critical role in epithelial cell recognition and adhesion and in the establishment of the epithelial cell phenotype (Vestweber and Kemler, 1984; Rodriguez-Boulan and Nelson, 1989; McNeill et al., 1990; Takeichi, 1990; Birchmeier and Birchmeier, 1993; Marrs et al., 1995). Antibodies against the ectodomain of E-cadherin block its adhesive function and inhibit the assembly of functional TJs (Behrens et al., 1985; Gumbiner and Simons, 1986; Gumbiner et al., 1988). Removal of extracellular calcium leads to the opening of TJs (Cereijido et al., 1978; Madara et al., 1987; Sedar and Forte, 1964) and the release of ZO-1 and cingulin into the cytoplasm (Anderson et al., 1989; Citi, 1992; Siliciano and Goodenough, 1988). In spite of this wealth of circumstantial evidence on the role of E-cadherin in TJ assembly, specific interactions between tight junction components and E-cadherin or E-cadherin–associated proteins have not yet been identified.

In the present study, we have examined in detail the redistribution of ZO-1 from cytosol to cell–cell contact sites and to its final site in the TJ at various stages of TJ assembly using the Ca^{2+} switch assay in normal MDCK cells (Gonzalez-Mariscal et al., 1985) and after transfection of E-cadherin into E-cadherin–deficient MDCK cells transformed with Moloney sarcoma virus (MSV). We report that activation of E-cadherin function in MDCK cells by calcium causes redistribution of ZO-1 to cell–cell adhesion sites which is followed later by its segregation from E-cadherin rich regions in the lateral membrane to form mature TJ, and present evidence for a role of catenins in this process. In contrast, we show that transformed MDCK cells fail to target ZO-1 to the cell surface because of their low levels of E-cadherin expression; however, upon transfection of E-cadherin, they redistribute ZO-1 to the cell surface but nonetheless fail to form mature TJ. Our results are compatible with a model in which transient association of TJ components with catenins mediates their anchoring to the cell surface and the early assembly of TJs.

Materials and Methods

Cell Lines and DNA Transfection

The MSV-transformed MDCK cell line, DoCl1 (American Type Culture Collection, Rockville, MD), designated in this paper as MSV-MDCK, and MDCK cells, were grown in DME containing 10% fetal calf serum, glutamine, nonessential amino acids, penicillin, and streptomycin (GIBCO BRL, Gaithersburg, MD) in an air-5% CO_2 atmosphere at constant humidity. MSV-MDCK cell lines expressing levels of E-cadherin comparable to those of MDCK cells (MSV-MDCK-E-cad1 and MSV-MDCK-E-cad2) were obtained by transfecting MSV-MDCK cells with a full length dog E-cadherin cDNA. Cells (1×10^6/100 mm Petri dish), grown overnight at 37°C were trypsinized, and incubated for 9–12 h in the presence of a calcium phosphate precipitate containing 20 μg of E-cadherin cDNA cloned into the eukaryotic expression vector pR2 (Overly et al., 1991) and 3 μg $pMV6^{neo}$ (Graham and Van der Eb, 1973). Cells were then osmotically shocked with 15% glycerol for 2 min, washed four times in PBS, incubated in complete medium for 48 h, and selected in 400 μg/ml G418 (Geneticin, GIBCO BRL). Stable clones of control (pR2 only) transfected MSV-MDCK cells obtained after G418 treatment were pooled and used for experiments. Clones expressing E-cadherin were identified by immunofluorescence as described below.

Antibodies

Rabbit polyclonal (1:200) and mouse monoclonal (1:1,000) antibodies directed against the ectodomain of E-cadherin, rabbit polyclonal anti α- and β-catenin antibodies (1:1,000) were kindly provided by Dr. Barry Gumbiner (Memorial Sloan Kettering Institute, New York, NY) and used at the dilutions indicated. A rat monoclonal antibody to ZO-1 kindly donated by Dr. Daniel Goodenough (Harvard Medical School, Boston, MA) and a rabbit polyclonal antibody to ZO-1 (Zymed, S. San Francisco, CA) were used at dilutions of 1:100. A rabbit polyclonal antibody to cingulin was a gift from Dr. Sandra Citi (Universita' di Padova, Padova, Italy). Anti-plakoglobin (γ-catenin) antibody PG 5.1 was purchased from American Research Products, Inc. (Belmont, MA). FITC and Texas red labeled, affinity purified secondary antibodies, were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Ca^{2+} Switch Assay

The Ca^{2+} switch assay was performed as previously described (Gonzalez-Mariscal et al., 1985; Vega-Salas et al., 1988). Briefly, confluent MDCK monolayers were trypsinized in the presence of EDTA until a single cell suspension was obtained. Cells (6×10^5) were plated on glass coverslips (12 mm) in a 12-well cell culture dish (Costar, Cambridge, MA) and allowed to attach for about 2 h in DME, 10% FCS containing a normal concentration of Ca^{2+} (1.8 mM). Thereafter, the cells were washed gently in MEM modified for suspension culture (SMEM), which lacks $CaCl_2$ and contains <5 μM Ca^{2+} [(low-Ca^{2+} (LC) medium]. This was followed by an 18-h incubation in SMEM containing 5% FCS that had been dialyzed extensively against PBS and 0.2 mM EDTA (Gumbiner and Simons, 1986). The switch was begun by replacing the SMEM by DMEM (1.8 mM Ca^{2+}) containing 10% FCS. At different time points the cells grown on coverslips were fixed with cold methanol and processed for immunofluorescence and confocal microscopy as described below. Cells on culture dishes were homogenized and the lysate processed for immunoprecipitation.

Immunofluorescence

Cells plated at confluency (6×10^5/well) on 12-mm-round glass coverslips in 12-well culture dishes, were processed for immunofluorescence after 2 d in culture. The cells were fixed in cold methanol at −20°C for 20 min, rehydrated in PBS containing $MgCl_2$ (1 mM) and $CaCl_2$ (0.1 mM) (PBS-

CM), incubated with rat antibody against ZO-1 or mouse antibody against E-cadherin for 1 h at room temperature, washed three times for 10 min in PBS-CM, and then incubated for 30 min at room temperature with Texas red–conjugated goat anti–rat and FITC-conjugated goat anti–mouse secondary antibodies (1:100 dilution in PBS-CM) before mounting in Vecta shield (Vector Labs, Burlingame, CA). Fluorescent samples were examined with a Nikon Optiphot epifluorescence microscope and photographed using Tmax 400 ASA film (Eastman Kodak Co., Rochester, NY).

Laser Scanning Confocal Microscopy

The relative distributions of ZO-1 and E-cadherin in MDCK and MSV-MDCK-E-cad-1 cells were examined using a Phoibos 1000 laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA), as described in Rajasekaran et al. (1994). Briefly, to detect simultaneously FITC- and Texas red–labeled antigens, samples were excited at 514 nm with an Argon laser and the light emitted between 525 and 540 nm was recorded for FITC and above 630 nm for Texas red. 30–40 horizontal (X-Y) confocal sections were obtained for each cell type and used to generate three-dimensional images using the Image Space software (version 3.01) from Molecular Dynamics on an Iris Indigo workstation (Silicon Graphics, Mountain View, CA). Photographs were taken on Fujicolor Super HG II (100 ASA) print film.

Cell Homogenization and Immunoprecipitation

Cells (3.7×10^6) were plated in a 100 mm plastic culture dish (Corning Glassware, Corning, NY) and were subjected to the Ca^{2+} switch as mentioned above. At indicated time points, cells from each dish were homogenized in 500 μl of the homogenization buffer (PBS containing 1% Triton X-100, 40 mM β-octyl glucopyranoside, 1 mm PMSF, benzamidine, iodoacetamide, aprotinin, trypsin inhibitor, and a cocktail containing pepstatin, antipain, and leupeptin) using a recently invented low pressure shear type cell homogenizer (A. K. Rajasekaran, U.S. Patent No. 5,390,859) developed by General Valve Corp. (Fairfield, NJ). A detailed description of this homogenizer will be published elsewhere. Briefly, cells in a 100 mm dish are quickly disrupted (<1 min) by applying mild homogenization pressures applied by a flat pestle that fits exactly the culture surface of the 100 mm culture dishes, under the control of an electromechanical processor. Cells from each culture dish were homogenized in 500 μl of lysis buffer. The lysates were rotated gently at 4°C for 30 min and centrifuged at 10,000 rpm for 10 min in a microfuge. The supernatant was precleared using albumin-coated protein A agarose beads (10 mg/ml) and subjected to immunoprecipitation using either ZO-1 or β-catenin antibody-coated protein A beads (10 mg/ml). The immunoprecipitates were washed once with the lysis buffer and 3× with wash buffer I (500 mm NaCl, 50 mM Tris/HCl, pH 7.5, 0.05% NP-40, and 0.2% BSA) followed by two washes in wash buffer II (50 mM Tris/HCl, pH 7.5). The precipitates were boiled in sample buffer for 10 min and subjected to electrophoresis and immunoblotting as described below.

Gel Electrophoresis and Immunoblotting

Samples were boiled in 1× Laemmli buffer at 100°C for 10 min before loading onto a 7.5% SDS–polyacrylamide gel (Laemmli, 1970). Proteins were electrophoretically transferred to a nitrocellulose membrane (Millipore Corp., Bedford, MA) at 200 mA for 12–15 h and the blots were blocked for 1 h in buffer A (10% Carnation® milk in PBS-CM), incubated for 2 h with primary antibodies in buffer B (buffer A containing 0.3% Tween 20), washed (eight times for 5 min) in buffer C (PBS-CM containing 0.3% Tween 20), and incubated 60–90 min in buffer B containing 1×10^6 cpm/ml of ^{125}I-labeled protein A. After washing (eight times, 5-min each) in buffer C, the blots were exposed to Kodak X-Omat AR film at −80°C. Exposed film was analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoelectron Microscopy

MDCK cells, 2 and 48 h after Ca^{2+} switch on glass coverslips, were fixed in cold methanol and incubated with ZO-1 and β-catenin antibodies as described above for immunofluorescence. Cells were further incubated with 5 and 15 nm gold conjugated goat anti–rat and anti–rabbit antibodies (Amersham Life Science Inc., Arlington Heights, IL), respectively, for 1 h. Cells were washed extensively and fixed in 2.5% glutaraldehyde for 1 h at room temperature, gently scraped, pelleted, and processed for electron microscopy as described in Rajasekaran et al. (1995). Electron micrographs were taken using a Jeol 100 CX electron microscope.

Transepithelial Electrical Resistance

To measure transepithelial electrical resistance, cells were plated onto polycarbonate filters (Transwell; Costar Corp., Cambridge, MA), and the resistance of the monolayers was determined with a Millicell ERS Volt-ohmeter (Millipore Corp., Bedford, MA). Values were normalized for the area of the filter (4.7 cm^2), after subtracting the background resistance of a filter without monolayer.

Results

Localization of E-Cadherin and ZO-1 during the Early Stages of Development of the Tight Junction in MDCK Cells

Earlier observations have implicated E-cadherin in the formation of tight junctions in MDCK cells (Behrens et al., 1985; Gumbiner et al., 1988). To understand the role of E-cadherin in this process, we studied the localization of ZO-1 and E-cadherin at early stages in the development of tight junctions using the Ca^{2+} switch assay (Gonzalez-Mariscal et al., 1985; Gumbiner and Simons, 1986). In this assay, MDCK monolayers formed in the presence of low calcium medium, which prevents E-cadherin–mediated cell–cell interactions and the development of tight junctions, are transferred to medium containing normal calcium levels, which allows E-cadherin–mediated cell–cell adhesion and tight junction assembly (Gumbiner et al., 1988; Nigam et al., 1991). Three-dimensional images of monolayers of MDCK cells, doubly labeled with antibodies against ZO-1 and E-cadherin, were generated from serial optical sections obtained by laser scanning confocal microscopy. En face views of monolayers fixed 48 h after the Ca^{2+} switch detected E-cadherin and ZO-1 as distinctly separate rings (Fig. 1 *A*). Latitudinal rotation by 56° (Fig. 1 *B*) and 88° (Fig. 1 *C*) resolved more clearly the apical ZO-1 ring (*red*) from the lateral E-cadherin staining (*green*). The segregated staining of ZO-1 and E-cadherin 48 h after the Ca^{2+} switch is similar to the staining observed in MDCK monolayers plated under regular conditions (data not shown). This staining pattern differs strikingly from the more diffuse cytoplasmic staining pattern observed at time 0, when the cells are dome shaped and fail to make contact with each other (Fig. 2). At this time, E-cadherin was predominantly localized in the cytoplasm as large and small clusters of granules with only a small amount irregularly distributed at the cell surface (Fig. 2 *B*); a similar pattern has been described by Gumbiner et al. (1988). Very low levels of ZO-1 were detected at the cell surface; instead, it was found as small intracellular granules distinct from E-cadherin and in large clusters of granules codistributing with E-cadherin (Fig. 2 *A*). Interestingly, 2 h after the Ca^{2+} switch, all of ZO-1 had redistributed to the cell surface whereas a large proportion of E-cadherin was still intracellular (Fig. 1 *D*, en face view). Latitudinal rotation by 56° (Fig. 1 *E*) and 88° (Fig. 1 *F*) indicated that a large fraction of ZO-1 colocalized with surface E-cadherin at this time point, as indicated by the inability to separate ZO-1 and E-cadherin staining upon rotation of the monolayer.

Quantitative analysis of the confocal immunofluorescence

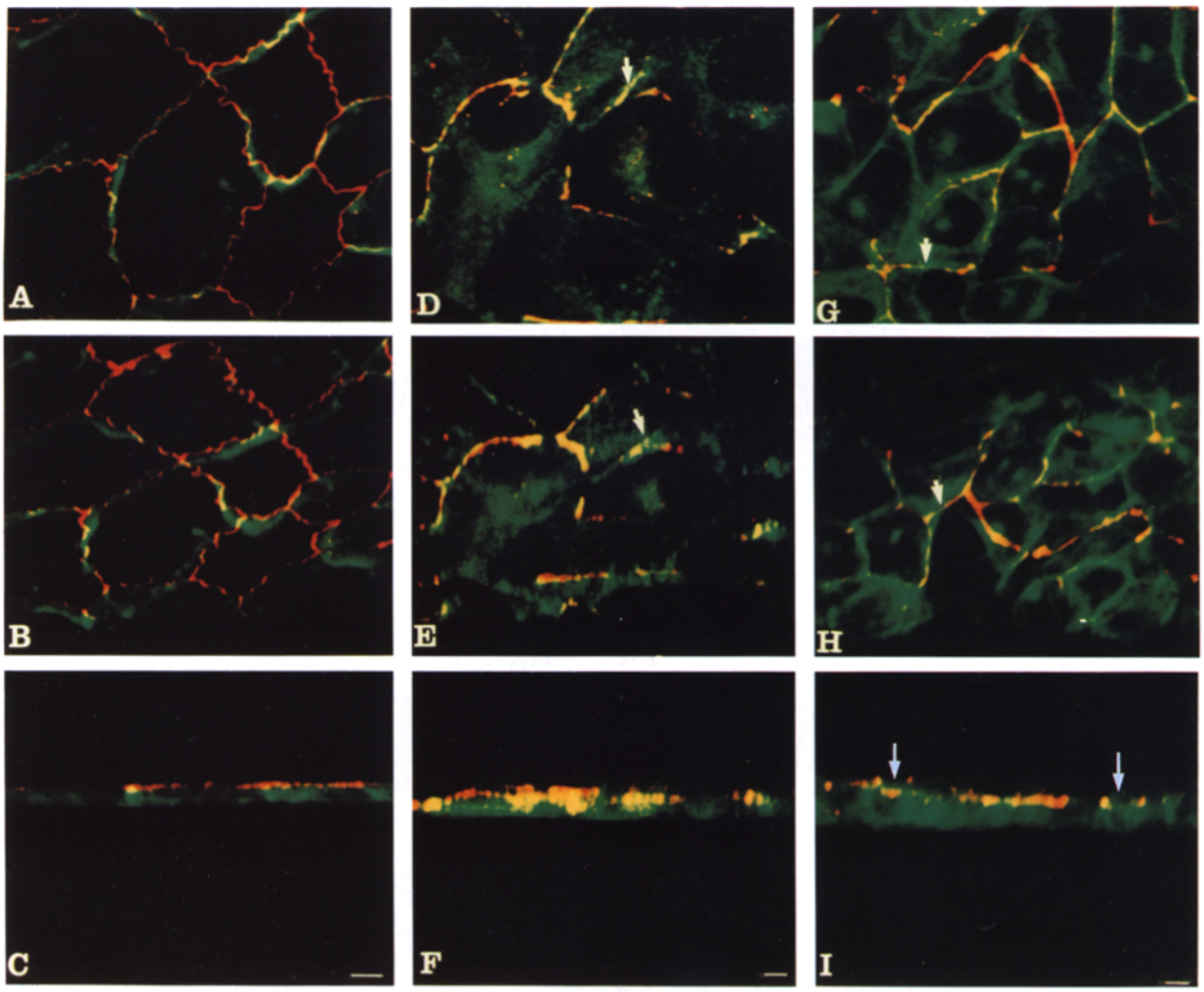

Figure 1. Confocal microscope images of MDCK and MSV-MDCK-E-cad1 showing the distribution of E-cadherin and ZO-1. Confluent monolayers of MDCK cells 48 h (*A–C*) or 2 h of Ca^{2+} switch (*D–F*), and MSV-MDCK-E-cad1 (*G–I*) were grown on glass coverslips and fixed in cold methanol. Cells were labeled with a rat monoclonal antibody against ZO-1 and a mouse monoclonal antibody against E-cadherin followed by Texas red–conjugated anti–rat and fluorescein-conjugated anti–mouse antibodies. Serial optical sections of the monolayers were recorded at 0.4-μm intervals. Three-dimensional images were generated using Image space software (3.01) (Molecular Dynamics, Sunnyvale, CA) and processed using an Iris Indigo work station (Silicon Graphics, Mountain View, CA). Noise levels from the serial optical sections were reduced by Gaussian filtration using the kernel size 3 × 3 × 3. Look through projections were created from filtered serial optical sections of the cell layers. To visualize the distribution of ZO-1 and E-cadherin, images were rotated at different angles. Images in *A*, *D*, and *G* are unrotated *en face* views; images *B*, *E*, *H*, and *C*, *F*, *I* are rotated latitudinally by 56° and 88°, respectively. Note the distinct staining pattern of ZO-1 (*red*) and E-cadherin (*green*) in a tight MDCK monolayer (*A–C*) and the colocalization (*yellow*) of these proteins in MDCK cells after 2 h of Ca^{2+} switch (*D–F*) and MSV-MDCK-E-cad1 (*G–I*) after 48 h of Ca^{2+} switch. The arrows show E-cadherin staining above the ZO-1 staining. Bar, 5 μm.

data was performed by measuring the levels of E-cadherin fluorescence (*green*) and ZO-1 fluorescence (*red*) in each optical section. The ratios of these two values for monolayers fixed and stained 2 and 48 h after Ca^{2+} switch are represented in Fig. 3. In monolayers fixed at 2 h after the Ca^{2+} switch, the ratio of E-cadherin and ZO-1 fluorescence intensities was constant (close to 1) in all optical sections indicating that the two proteins had overlapping distributions from bottom to top. In contrast, after 48 h of Ca^{2+} switch, the E-cadherin/ZO-1 fluorescence ratio decreased gradually from 7 to 1 from the basal to the apical optical sections of the monolayer indicating that E-cadherin had acquired a predominantly basal localization away from ZO-1, now concentrated in apical TJ.

Analysis of the Interaction of ZO-1 and Catenins in MDCK Cells

The colocalization of E-cadherin and ZO-1 at early times in the assembly of tight junctions suggested that these two proteins might interact and play a role in the targeting of ZO-1 to the cell surface. Other workers have indeed suggested functional interactions between E-cadherin and ZO-1 on the basis of colocalization data (Itoh et al., 1993; Watabe et al., 1994), although they failed to co-immunoprecipitate E-cadherin and ZO-1, which led them to suggest that the interaction between these two proteins might be very weak or unstable (Watabe et al., 1994). In agreement with these reports, we found that E-cadherin did not

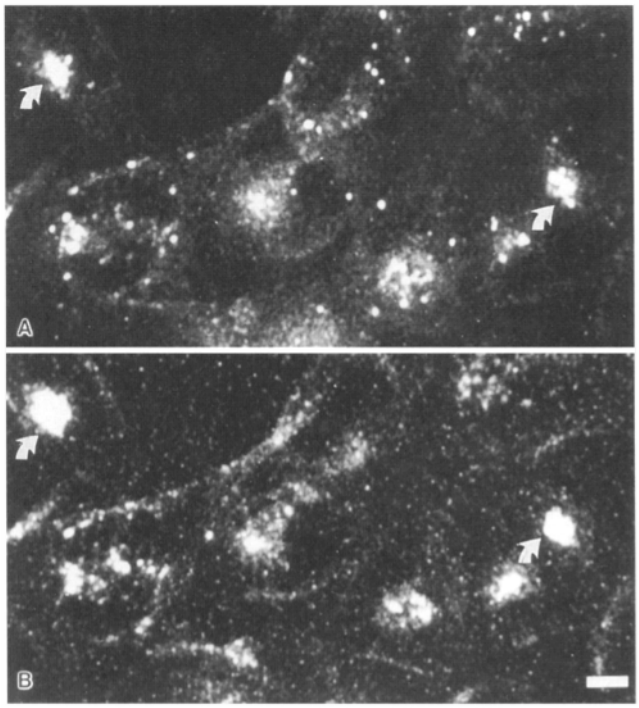

Figure 2. Distribution of E-cadherin and ZO-1 at zero time point after the Ca^{2+} switch. MDCK cells after growth in low Ca^{2+} medium over night were fixed and stained for ZO-1 (*A*) and E-cadherin (*B*) were used to create a projection (en face view) as described in figure legend 1. Note large spots (*arrows*) of E-cadherin codistributing with ZO-1. Bar, 5 μm.

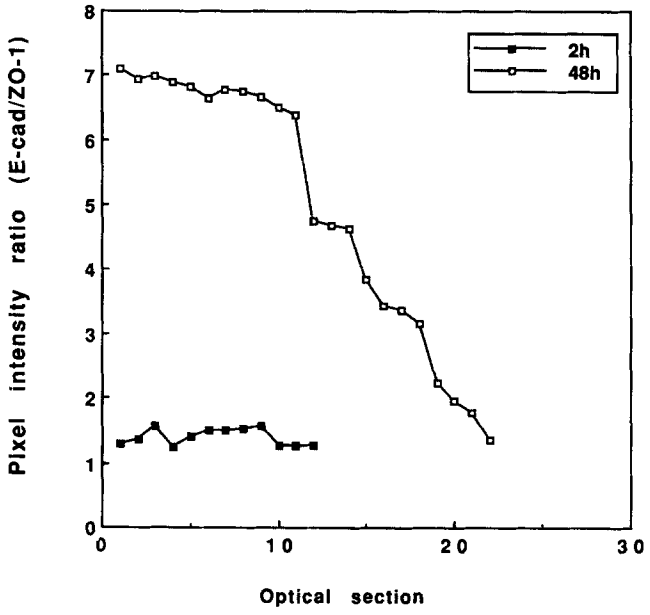

Figure 3. Quantitation of the pixel intensities of E-cadherin and ZO-1 in MDCK and MSV-MDCK-E-cad1 cells. The fluorescence intensity in confocal sections obtained from MDCK monolayers double stained for E-cadherin (*green*) and ZO-1 (*red*) were quantified by measuring the average pixel intensity in each section using Image space software version 3.01. The ratio of the pixel intensity of E-cadherin/ZO-1 was determined and plotted.

co-precipitate with ZO-1 at any time during the Ca^{2+} switch (Fig. 4 *E*). We hypothesized that the interaction of ZO-1 with E-cadherin could be indirect, mediated by cytoplasmic proteins. Because E-cadherin specifically associates with cytoplasmic catenins (Ozawa and Kemler, 1989) and because β-catenin has been recently shown to interact with two surface molecules, the APC gene product (Rubinfield et al., 1993; Su et al., 1993) and the EGF receptor (Hoschuetzky et al., 1994), a scenario could be imagined in which catenins might bridge E-cadherin and ZO-1.

To test this idea, we sought for an interaction between ZO-1 and β-catenin at different times during the assembly

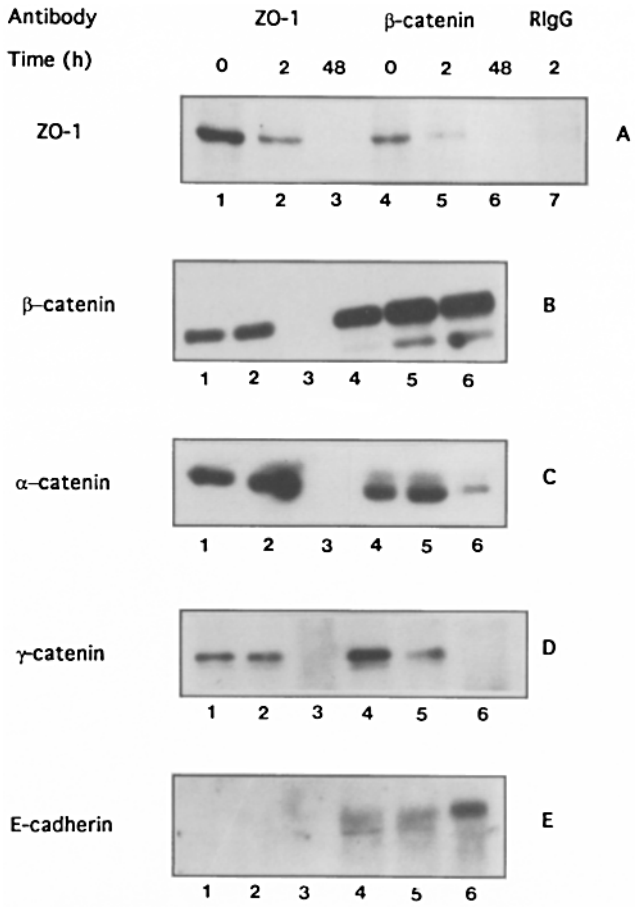

Figure 4. Immunoprecipitation analysis of ZO-1 and β-catenin in MDCK cells at different stages during the formation of tight junctions. MDCK cells grown on 100 mm culture dishes were subjected to Ca^{2+} switch. At 0, 2, and 48 h after Ca^{2+} switch cells were homogenized and cell lysates prepared as described in Materials and Methods. For ZO-1 detection (*A*) cells labeled for 16 h with ^{35}S express protein labeling mix (0.5 mCi/plate) were used. ZO-1 (lanes *1–3*), β-catenin (lanes *4–6*), and the control rabbit IgG (lane 7) were immunoprecipitated using polyclonal antibodies from the cell lysates and subjected to SDS-PAGE and autoradiography. To detect α-,β-,γ- catenins, and E-cadherin, the immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting with β-catenin (*B*), α-catenin (*C*), γ-catenin (*D*), and E-cadherin (*E*) antibodies. Blots were visualized by ^{125}I-labeled protein A and autoradiography. ZO-1 and β-catenin blots were exposed for 4 h at −80°C and the rest of the blots were exposed for 2–3 d and quantified with a PhosphorImager.

of tight junctions. For this purpose, MDCK monolayers plated in low calcium medium were homogenized before (0 h) and at two time points of the Ca^{2+} switch (2 and 48 h), and the presence of β-catenin in the ZO-1 immunoprecipitates was analyzed by autoradiography and Western blot with a monospecific antibody to β-catenin. As shown in Fig. 4 *A*, the ZO-1 was detected by immunoprecipitation of the detergent extracts of cells kept in low Ca^{2+}, but the amount of ZO-1 in the immunoprecipitates decreased 2 h after shift to normal Ca^{2+} and was almost undetectable at 48 h of shift (Fig. 4 *A*, lanes *1*, *2*, and *3*). Strikingly, a sizable fraction of total extractable β-catenin was precipitated by ZO-1 antibodies (23%) at 0 h but this amount decreased to 14% (at 2 h) and 3.7% (at 48 h), even when the total β-catenin stayed constant, determined by Western blot of the β-catenin immunoprecipitates at all three times. Similarly, the amount of ZO-1 in the β-catenin immunoprecipitates decreased at 2 h and was undetectable at 48 h. Although ZO-1 band was detected at 0 h of Ca^{2+} switch by Western blot analysis, at 2 h after Ca^{2+} switch it was barely detectable by this analysis. Therefore we resorted to metabolic labeling and immunoprecipitation to reveal ZO-1 at these time points. Control experiments with non-specific antibodies against chloramphenicol acetyltransferase (data not shown) demonstrated the specificity of the coprecipitation. These experiments indicate that ZO-1 forms a complex with β-catenin in cells kept in low Ca^{2+} and this complex progressively disappears as ZO-1 becomes unextractable upon incorporation to the membrane during the Ca^{2+} switch.

Immunoblot experiments similar to those described above also demonstrated the presence of α- and γ-catenins in the ZO-1 immunoprecipitates at 0 and 2 h, but not after 48 h, of Ca^{2+} switch (Fig. 4, *C* and *D*, lanes *1–3*). This was exactly the same behavior observed with β-catenin (Fig. 4 *B*). Control immunoprecipitation experiments with β-catenin antibody detected reduced amounts of α- and γ-catenins in the β-catenin immunoprecipitates at 48 h (Fig. 4, *C* and *D*, lanes *6*); this reduction may reflect recruitment of these proteins into insoluble membrane complexes. Surprisingly, immunoblot analysis of the ZO-1 immunoprecipitates with E-cadherin antibody did not reveal this protein at 0, 2, and 48 h time points after Ca^{2+} switch (Fig. 4 *E*, lanes *1–3*). However E-cadherin was present in the β-catenin immunoprecipitates at all these time points. Since a clear codistribution of ZO-1 and E-cadherin was visualized in the immunofluorescence, the absence of E-cadherin in the ZO-1 immunoprecipitates suggests that the association of E-cadherin with the ZO-1–catenin complex is either weak or unstable.

Additional evidence for the association of ZO-1 and β-catenin was provided by laser scanning confocal microscopy and immunoelectron microscopy experiments. As previously shown for E-cadherin and ZO-1 (Fig. 1), confocal microscopy demonstrated overlapping distributions of ZO-1 and β-catenin at 2 h after Ca^{2+} switch (Fig. 5, *D–F*),

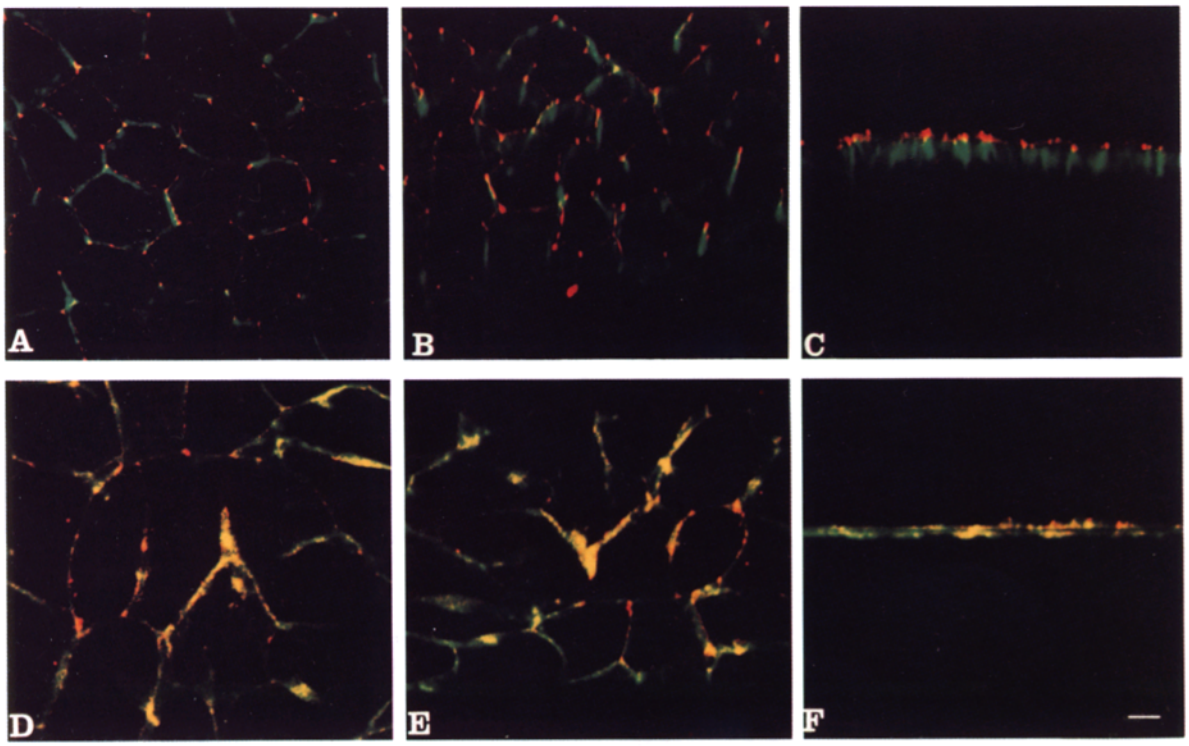

Figure 5. Confocal microscope images of MDCK cells showing the distribution of β-catenin and ZO-1 at an intermediate and final stages of tight junction formation. MDCK cells after 48 (*A–C*) and 2 h (*D–F*) of Ca^{2+} switch were fixed by cold methanol. Cells were labeled with a rat monoclonal antibody against ZO-1 and a rabbit polyclonal antibody against β-catenin followed by Texas red–conjugated anti–rat and fluorescein-conjugated anti–rabbit antibodies. Three-dimensional confocal images were created as described in the legend of Fig. 1. Images in *A* and *D* are unrotated en face views, in *B* and *E* and *C* and *F* are images rotated latitudinally at 56° and 88°, respectively. ZO-1 is shown in *red* and β-catenin in *green*. *Yellow* color indicates colocalization of ZO-1 and β-catenin. Note the clear codistribution of ZO-1 and β-catenin (*yellow*) after 2 h of Ca^{2+} switch. Bar, 5 μm.

but very little overlap at 48 h: at this time ZO-1 formed a distinct ring more apically localized than β-catenin (Fig. 5, *A–C*). This segregation was confirmed by quantitative analysis of the ratios of the fluorescence intensities of both proteins at 2 and 48 h of Ca^{2+} switch, as described before (Fig. 3) for E-cadherin and ZO-1 (Fig. 6). Finally, double label immunoelectron microscopy using 15 nm gold particles for β-catenin and 5 nm gold particles for ZO-1 revealed, at the 2 h time point, clusters of small and large particles (Fig. 7, *A* and *C*) with a relatively constant distance (~68 + 5 A°) between the two particle sizes (Fig. 7, *A* and *C*). On the other hand, 48 h after the start of the Ca^{2+} switch, a distinctly separate localization of these proteins was observed: ZO-1 (small gold particles) was restricted to the apical tight junction while β-catenin (large gold particles) was localized to the lower lateral plasma membrane (Fig. 7 *B*). Quantitative analysis of the electron micrographs showed that 18.3% of the small gold particles (ZO-1) was associated with one or more large particles (β-catenin) at 2 h of Ca^{2+} switch, whereas only 2.7% of the small particles formed clusters with large particles at 48 h of Ca^{2+} switch (Fig. 8). These results strongly suggest that ZO-1 and β-catenin associate with each other at the earlier stages of development of the tight junction, but then segregate once the mature TJ is formed.

Localization and Interaction of ZO-1 with β-Catenin in a Transformed MDCK Cell Line Expressing E-Cadherin

In carcinoma cells, E-cadherin levels are frequently highly reduced, which leads to a disruption of the epithelial phenotype (Stetler-Stevenson et al., 1993). On the other hand, transfection of E-cadherin causes carcinoma cell lines to revert to a differentiated epithelial phenotype (Behrens et al., 1989; Navarro et al., 1991; Birchmeier and Birchmeier, 1993). To additionally characterize the role of E-cadherin

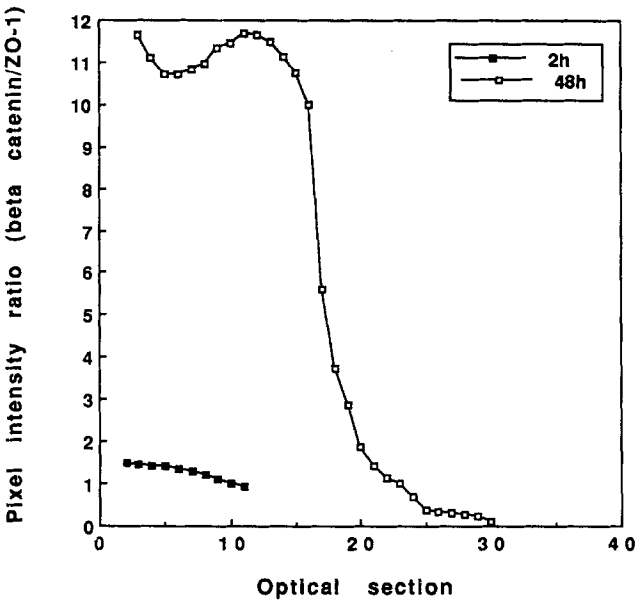

Figure 6. Quantitation of the pixel intensities of β-catenin and ZO-1. Pixel intensities were quantified as described in the Fig. 3 legend.

in the targeting of ZO-1 to the cell surface, MSV-transformed MDCK cells (MSV-MDCK), which show a fibroblastoid morphology and express trace amounts of E-cadherin (Behrens et al., 1985; data not shown), were transfected with a cDNA encoding dog E-cadherin. Two clones were selected that displayed 88 and 53% of the E-cadherin levels in wild-type (wt) MDCK cells (MSV-MDCK-E-cad1 and MSV-MDCK-E-cad2, respectively) and similar or somewhat higher levels of ZO-1 than wt MDCK cells (data not shown). Both of these cell lines exhibited a more epithelioid phenotype (Fig. 9, *C* and *D*) than the parental MSV-MDCK cell line (Fig. 9 *B*). Transfection of E-cadherin caused a striking redistribution of ZO-1 in these transformed cell lines (Fig. 9, *C* and *D*). Whereas in MSV-MDCK cells, most of the ZO-1 was localized intracellularly (Fig. 9 *B*), in MSV-MDCK-E-cad1 and MSV-MDCK-E-cad2, the ZO-1 staining had acquired the typical ring staining pattern observed in wt MDCK cells (Fig. 9, *A*, *C*, and *D*). However, the reversal to a differentiated phenotype was not complete since confluent monolayers of E-cadherin transfected cells showed discontinuous staining of ZO-1, failed to develop significant transepithelial resistance, and were permeable to macromolecules (data not shown). The redistribution of ZO-1 from a cytoplasmic to a membrane distribution upon expression of E-cadherin in MSV-MDCK cells provides additional support for the participation of the E-cadherin adhesion system in the early assembly of tight junctions.

In spite of the membrane localization of ZO-1, laser scanning confocal microscopy analysis in MSV-MDCK-E-cad1 monolayers (Fig. 1, *G–I*) revealed a different ZO-1/E-cadherin staining pattern from that observed in wt MDCK cells (Fig. 1, *A–C*). In en face views ZO-1 staining did not appear as a clear ring more apically localized than E-cadherin as in mature MDCK monolayers but, rather, as a continuous band codistributing with E-cadherin (Fig. 1 *G*). A 56° latitudinal rotation of the cell layer confirmed that ZO-1 staining colocalized with E-cadherin (Fig. 1 *H, yellow*) on the lateral membrane. When the cell layer was viewed from the side (88° latitudinal rotation), ZO-1 staining was localized to the upper half of the lateral domain stained by E-cadherin (Fig. 1 *I*). In some areas of the monolayer, E-cadherin staining was seen clearly more apical than the ZO-1 staining (Fig. 1, *H* and *I*, *arrows*), indicating that these two proteins had not fully segregated from each other as in wt MDCK cells. Furthermore, β-catenin was detected in ZO-1 immunoprecipitates at 0 and 2 h after Ca^{2+} switch (Fig. 10, lanes *4* and *5*), but not after 48 h of Ca^{2+} switch (Fig. 10, lane *6*). These results suggest that the molecular events involved in E-cadherin mediated translocation of ZO-1 to the plasma membrane are preserved in highly transformed MDCK cells. However, a main difference between wt and transformed MDCK cells is that the latter fail to segregate TJ from E-cadherin at later stages.

Discussion

Transient Overlapping Distributions of ZO-1 and E-Cadherin

Early reports by Behrens et al. (1985) and Gumbiner and

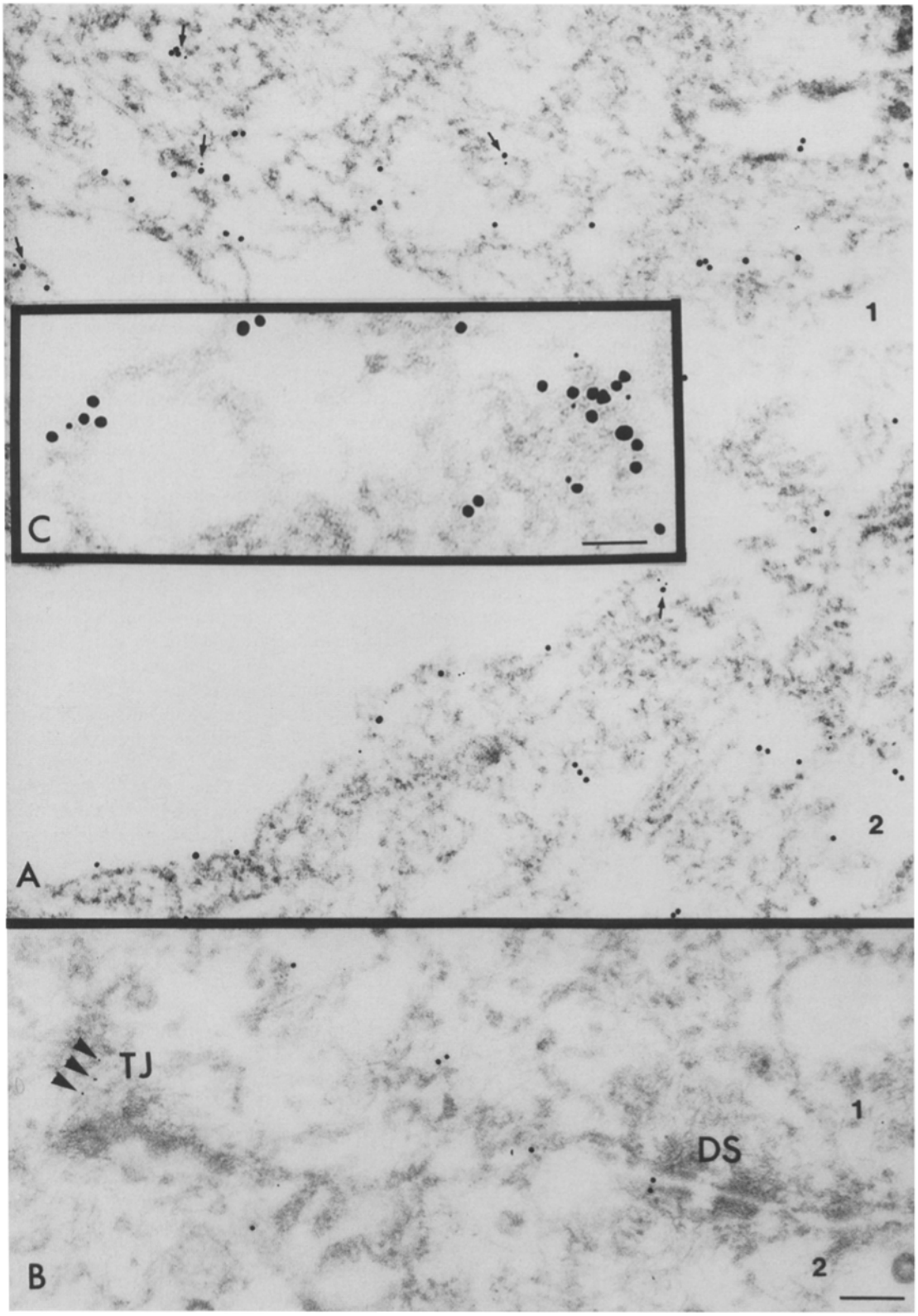

Figure 7. Immunoelectron microscopic detection of ZO-1 and β-catenin. MDCK cells 2 h (*A* and *C*) and 48 h (*B*) after Ca^{2+} switch were fixed in cold methanol and processed for immunoelectron microscopy as described in Materials and Methods. 5 and 15 nm gold particles correspond to ZO-1 and β-catenin, respectively. Note the constant distance between ZO-1 and β-catenin at 2 h time point. These micrographs are printed lightly in order to show the 5 nm beads more clearly. Adjacent cells are indicated by numbers *1* and *2*. TJ and DS indicate tight junction and desmosome, respectively. Bars: (*A* and *B*) 0.15 μm; (*C*) 0.08 μm.

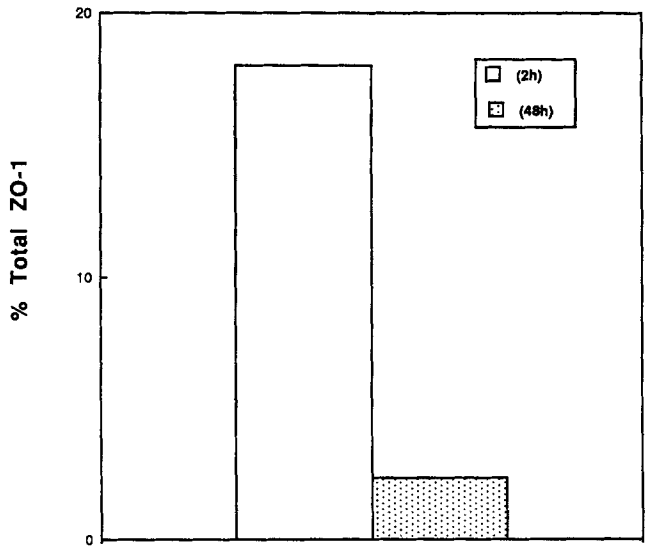

Figure 8. Quantification of the immunoelectron microscopic analysis. Small gold particles (ZO-1, 5 nm) from 15 electron micrographs for the 2 h time point and 10 electron micrographs from the 48 h time points were counted. The small particles observed in close proximity of the large particles were determined for both time points and plotted. The number of small particles counted for 2 and 48 h time points were 233 and 73, respectively.

Simons (1986), suggested a role for E-cadherin in the formation of functional TJ. Behrens et al. (1985) showed that an antibody against the MDCK surface antigen Arc-1, later shown to be E-cadherin, interfered with the formation of TJ. Gumbiner and Simons (1986), using a Ca^{2+} switch assay developed by Gonzalez-Mariscal et al. (1985), screened a monoclonal antibody library and identified an antibody, rrl, that specifically blocked the development of transepithelial resistance (Gumbiner and Simons, 1986). This antibody turned out to be directed against E-cadherin. In this paper, we have used biochemical and morphological techniques to characterize the role of E-cadherin in TJ assembly.

The inhibition of TJ formation by E-cadherin antibodies suggests a direct or an indirect interaction of the adhesive molecule with tight junctional components. To attempt to obtain evidence for such an interaction, we studied the relative subcellular distributions of E-cadherin and the cytoplasmic tight junctional protein ZO-1 in mature MDCK monolayers and during the gradual assembly of tight junctions in monolayers subjected to a sudden increase in extracellular calcium levels (Ca^{2+} switch). Previous work has shown that MDCK monolayers kept in micromolar calcium display very little surface E-cadherin and ZO-1 (Gumbiner et al., 1988). We confirmed these observations by double label immunofluorescence, which additionally showed a substantial co-distribution of both proteins in large intracellular clusters of "granules" (Fig. 2). Earlier workers have reported colocalization of E-cadherin and ZO-1, but the significance of this co-distribution remains unclear. For example, Itoh et al., (1993) have shown that, in E-cadherin transfected mouse L cells, ZO-1 co-localizes with N-, P-, and E-cadherin in immunofluorescence experiments. Furthermore, these authors have also shown that liver epithelial cells, with less well developed TJ, express ZO-1 not only in the *zonula occludens* region but also in the adherens junction, where it overlaps with E-cadherin, suggesting a close developmental relationship between adherent junctions and TJ.

The intracellular co-localization of ZO-1 and E-cadherin in cells kept in low calcium suggested that these two molecules might be forming an intracellular complex at early times of TJ development. We used quantitative confocal microscopy imaging techniques in combination with the "Ca^{2+} switch" to determine the extent and fate of this association during junction formation. To precisely visualize the relative distributions of ZO-1 and E-cadherin, we created three-dimensional images of MDCK monolayers immunostained for these antigens at various stages in the formation of TJ. Analysis of these images at different angles showed that after 2 h of Ca^{2+} switch, when tight junctions are not yet fully assembled (Gonzalez-Mariscal et al., 1985), ZO-1 was still co-localized with E-cadherin at the lateral plasma membrane (Fig. 1, *D–F*). However, after 48 h of Ca^{2+} switch, when tight junctions are fully functional (Gonzalez-Mariscal et al., 1985; Nigam et al., 1991), ZO-1 experienced a dramatic redistribution to its final localization at the boundary between the apical and lateral membranes, largely separated from E-cadherin, which was concentrated immediately below, in the adjacent lateral membrane (Fig. 1, *A–C*). The dramatic segregation of ZO-1 and E-cadherin at the lateral plasma membrane during the 2 h Ca^{2+} switch could also be detected by the striking change in the profile of E-cadherin/ZO-1 fluorescence intensity ratios from flat to sigmoid between early and late stages of monolayer development (Fig. 3). These experiments suggest that E-cadherin may participate in the targeting and assembly of tight junctional components at the cell surface, triggered by cell–cell contacts during initial stages of tight junction development.

Association of ZO-1 with Catenins

The co-distribution between E-cadherin and ZO-1 before and during the Ca^{2+} switch suggested an interaction between the two molecules, either direct or indirect, mediated by E-cadherin associated molecules, the catenins. To provide biochemical evidence for these intermolecular associations, we carried out co-immunoprecipitation experiments with antibodies against ZO-1, E-cadherin, and catenins (Fig. 4). Immunoprecipitates of ZO-1 contained β-catenin at early (0 and 2 h) but not at late stages of monolayer development (Fig. 4, *A* and *B*). Conversely, β-catenin immunoprecipitates displayed ZO-1 also at early but not at late times of monolayer development (Fig. 4 *A*). It is not clear, however, whether the interaction of ZO-1 with β-catenin is direct or indirect, since α- and γ- catenins (which form a complex with β-catenin) were also detected in the immunoprecipitates and could mediate this interaction (Fig. 4, *C* and *D*). The presence of α- and γ- catenins in the immunoprecipitates suggests that either the α-, β-, γ-catenin complex or α/β or β/γ complexes of catenins interact with ZO-1. Further experiments will be necessary to discriminate between these possibilities.

If β-catenin is involved in the interaction between E-cadherin and ZO-1 it should follow, during Ca^{2+} switch an

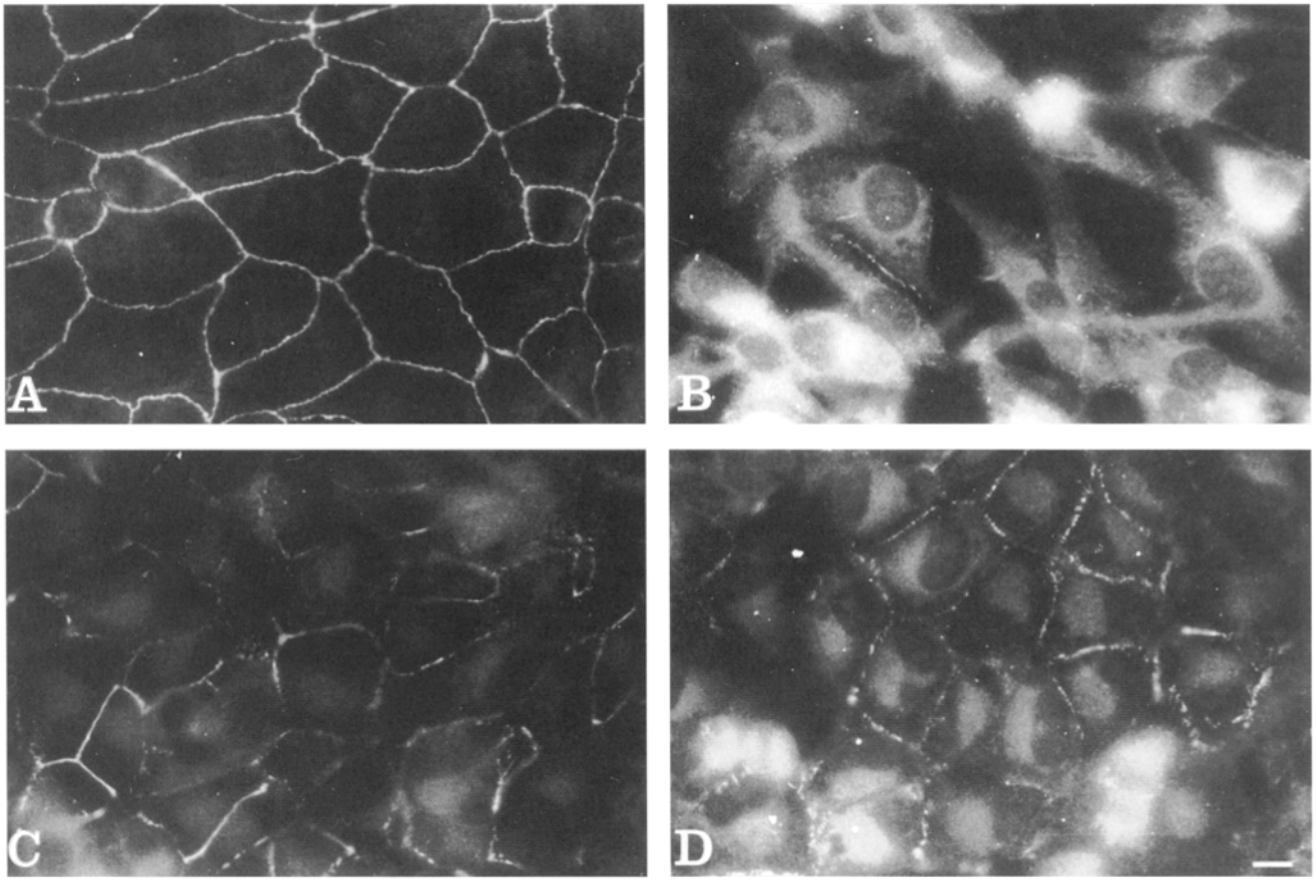

Figure 9. Immunofluorescence localization of ZO-1. Cell lines MDCK (*A*), MSV-MDCK (*B*), MSV-MDCK-E-cad1 (*C*), and MSV-MDCK-E-cad2 (*D*) were fixed, permeabilized, and stained using a rat monoclonal antibody raised against ZO-1. Note the striking redistribution of ZO-1 in E-cadherin–transfected MSV-MDCK-E-cad1 and MSV-MDCK-E-cad2 cells. Bar, 16 μm.

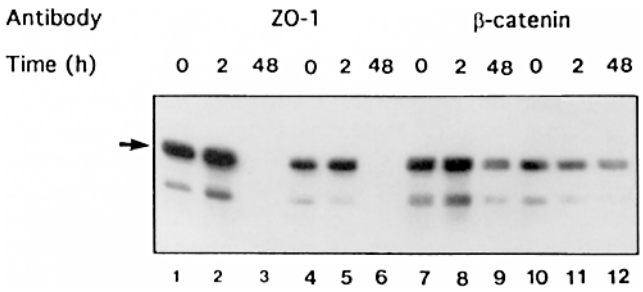

Figure 10. Immunoprecipitation analysis of ZO-1 and β-catenin in MSV-MDCK-E-cad1 cells at different stages during the formation of tight junctions. Ca^{2+} switch assay and immunoprecipitations were done as described in legend of Fig. 4. Immunoprecipitates obtained by ZO-1 or β-catenin antibodies were resolved on a 7.5% SDS-PAGE, probed with β-catenin antibody and visualized by ^{125}I-labeled protein A and autoradiography. β-Catenin is indicated by an arrow. The low molecular weight band corresponds to a degraded product. Note that ZO-1 immunoprecipitates from either wt MDCK (lanes *1–3*) or MSV-MDCK-E-cad1 (lanes *4–6*) cells contained β-catenin at 2 h after Ca^{2+} switch (lanes *1* and *2* and *4* and *5*) whereas at 48 h no β-catenin was detected (lanes *3* and *6*). However, β-catenin was coimmunoprecipitated at all these time points in MDCK (lanes *7–9*) and in MSV-MDCK-E-cad1 cells. Further analysis of the immunoprecipitates with α-, γ-, and E-cadherin antibodies gave similar results as shown for wt MDCK cells in Fig. 4 (data not shown).

identical redistribution pattern as E-cadherin. This is indeed what was observed. In cells plated in low calcium medium, β-catenin was present in large intracellular aggregates together with E-cadherin and ZO-1 (data not shown). Latitudinal rotation of three-dimensional confocal microscopy images revealed overlapping membrane distributions of ZO-1 and β-catenin after 2 h but not after 48 h, of Ca^{2+} switch (Fig. 5). Quantitation of fluorescence in series of confocal images at both time points detected a change from flat to sigmoidal in the β-catenin/ZO-1 fluorescence intensity ratio, similar to that previously described for the E-cadherin/ZO-1 ratio (Fig. 6). The co-localization of β-catenin and ZO-1 at early time points was also clearly seen by immunoelectron microscopy using double label immunoelectron microscopy (Fig. 7), which revealed the presence of clusters of small (ZO-1) and large (β-catenin) gold particles. The constant distance between the two particle sizes (68 A°) together with the transient detection of these clusters at 2 h but not at 48 h, is consistent with a specific association of these two molecules.

Given the presence of all catenins in the ZO-1 immunoprecipitates, it is surprising that ZO-1 immunoprecipitates did not reveal any E-cadherin, which is known to form immunoprecipitable complexes with β-catenin. However, a similar result was reported in the case of interaction of β-catenin with the APC gene product: no E-cadherin was

detected in the APC–catenin complex (Rubinfield et al., 1993; Su et al., 1993). A possible explanation of both of these results is that the interaction of E-cadherin with ZO-1 may be indirect, mediated by catenins. It may be speculated that different complexes of individual catenins (αβ, βγ, αγ, αβγ) might have a differential affinity for different proteins (e.g., ZO-1, APC protein, EGF receptor) other than E-cadherin and that ZO-1 (like APC) may prevent β-catenin interaction with E-cadherin. Alternatively, signals generated by E-cadherin–mediated cell–cell contacts may result in posttranslational modification (e.g., phosphorylation/dephosphorylation) of any of the components of the ZO-1–catenin complex, thus regulating its assembly. Indeed, phosphorylation has been described for β-catenin (Behrens et al., 1993), α-catenin (Hamaguchi et al., 1993), γ-catenin (Hoschuetzky et al., 1994), and ZO-1 (Stevenson et al., 1989; Howarth et al., 1994), and recent studies indicate that the development of TJs may be regulated by phosphorylation (Singer et al., 1994; Kurihara et al., 1995; Stuart and Nigam, 1995). We propose that catenin–ZO-1 complexes that interact weakly with E-cadherin are involved in the shuttling of tight junctional components to the lateral membranes for the assembly of epithelial TJ. Interaction of catenins with the tight junctional protein ZO-1 suggests that catenins play not only an important role in the signaling pathway mediated by E-cadherin (Kemler, 1993; Gumbiner and McCrea, 1993; Peifer, 1995) but also in the development of TJ in polarized epithelial cells.

E-Cadherin Induces the Translocation of ZO-1 to the Plasma Membrane of MSV-transformed MDCK Cells

Transformation of epithelial cells frequently results in decreased adhesion due to decreased levels of E-cadherin expression (Stetler-Stevenson et al., 1993). In MDCK cells transformed with MSV (MSV-MDCK), ZO-1 was found to be largely intracellular, as previously shown for MDCK cells kept in low calcium medium (Gumbiner et al., 1988). However, upon transfection of MSV-MDCK cells with E-cadherin, the resulting cell lines, MSV-MDCK-E-cad1 and MSV-MDCK-E-cad2, efficiently translocated ZO-1 to the cell surface (Fig. 9). This process parallels the translocation of ZO-1 to the surface of wt MDCK cells upon activation of cadherin adhesion during the Ca^{2+} switch (Fig. 1, *C–E*). As in MDCK cells, a complex of ZO-1 and catenins was detected in the E-cadherin transfected MSV-transformed MDCK lines (Fig. 10). A main difference between the two systems is that, whereas wt MDCK cells segregate at a later time ZO-1 from E-cadherin, E-cadherin transfected transformed MDCK cells fail to segregate these two

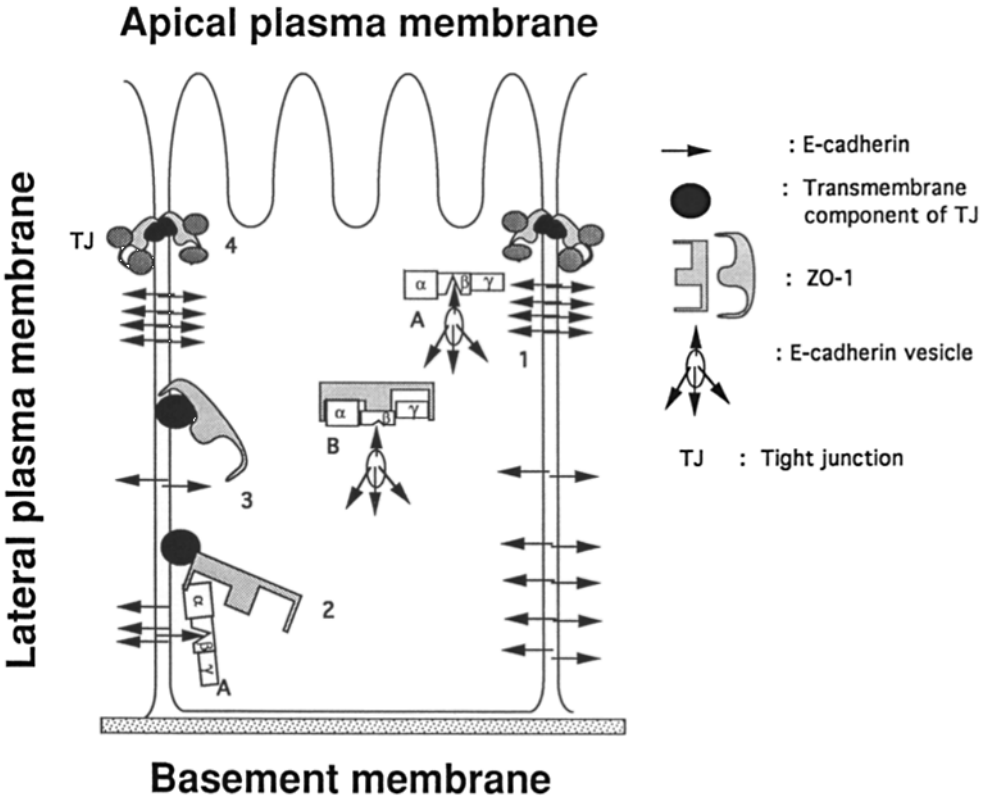

Figure 11. A model for the development of tight junction in epithelial cells. (*1*) E-cadherin mediated close cell–cell contact initiates the mobilization of ZO-1–catenin complexes to the plasma membranes. Two pools of catenins are represented: one that binds E-cadherin with high affinity but not ZO-1 (*A*) and another that binds ZO-1 with high affinity and E-cadherin with low affinity (*B*). The low affinity interaction between E-cadherin and the ZO-1–catenin complex (*B*) directs the complex to the plasma membrane. (*2*) Association of ZO-1 with the transmembrane component of the TJ changes conformation of ZO-1 which, together with a stabilization of E-cadherin–catenin complex at the cell surface result in the release of ZO-1 from the catenin complex. (*3*) ZO-1 interacts with additional components of the TJ and the complex segregates from E-cadherin rich regions at the lateral plasma membrane. (*4*) Maturation of TJ occurs as it sequentially interacts with other components of the tight junction to form a fully functional tight junction.

molecules into different areas of the lateral membrane (Fig. 1). Taken together, these results support the hypothesis that E-cadherin is involved in the initial targeting of TJ components to the cell surface. They also suggest that neoplastic transformation disrupts an E-cadherin–independent mechanism that controls the subsequent segregation of TJ from the adherens junction. This role of E-cadherin in ZO-1 translocation to the cell surface appears to involve cell-type specific factors since it has been shown that E-cadherin expression in S180 sarcoma cells does not promote redistribution of ZO-1 to the cell surface (Howarth et al., 1994). Additional work is required to elucidate the molecular details of this important function of E-cadherin.

A Model for the Development of Tight Junction

Our data are compatible with the model for the development of epithelial TJ summarized in Fig. 11. The establishment of tight E-cadherin mediated cell–cell contacts activates an intracellular signaling mechanism that mobilizes ZO-1–catenin complexes to the plasma membrane. E-cadherin may also play a direct role in the surface redistribution of catenin–ZO-1 complexes via a low affinity interaction that cannot be detected by immunoprecipitation but may be inferred from the intracellular codistribution of the adhesive molecule with the complex. It is possible that the other tight junction proteins follow the same pathway to the basolateral membrane. The development of tight intercellular contacts by E-cadherin and/or the establishment of specific interactions between ZO-1 and other tight junctional components at the cell surface (occludin?) results in the dissociation of ZO-1 from catenins. TJ assembly then proceeds by the incorporation of more peripheral components. As the TJ matures, it progressively segregates from E-cadherin rich areas of the plasma membrane to reach its characteristic locale at the apico-lateral border of fully polarized epithelial cells.

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