

# Localization of the Tight Junction Protein, ZO-1, Is Modulated by Extracellular Calcium and Cell-Cell Contact in Madin-Darby Canine Kidney Epithelial Cells

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**Abstract.** Using the monoclonal antibody R26.4, we have previously identified a ~225-kD peripheral membrane protein, named ZO-1, that is uniquely associated with the tight junction (zonula occludens) in a variety of epithelia including the Madin-Darby canine kidney (MDCK) epithelial cell line (Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. *J. Cell Biol.* 103:755-766). In this study we have analyzed the effects of cell-cell contact and extracellular calcium on the localization and the solubility of ZO-1. In confluent monolayers under normal calcium conditions, ZO-1 immunoreactivity is found exclusively at the plasma membrane in the region of the junctional complex. If MDCK cells are maintained in spinner culture under low calcium conditions, ZO-1 is diffusely organized within the cytoplasm. After the plating of suspension cells at high cell density in medium with normal calcium concentrations, ZO-1 be-

comes localized to the plasma membrane at sites of cell-cell contact within 5 h in a process that is independent of de novo protein synthesis. However, if suspension cells are plated at high density in low calcium medium or if suspension cells are plated at low cell density in normal calcium growth medium, ZO-1 remains diffusely organized. ZO-1 localization also becomes diffuse in monolayers that have been established in normal calcium medium and then subsequently switched into low calcium medium. These results suggest that both extracellular calcium and cell-cell contact are necessary for normal localization of ZO-1 to the plasma membrane. An analysis of the solubility properties of ZO-1 from suspension cells and monolayers revealed that high salt, nonionic detergent, and a buffer containing chelators were somewhat more effective at solubilizing ZO-1 from suspension cells than from monolayers.

**T**HE tight junction is the apical-most member of the junctional complex, forming a continuous, belt-like structure that joins epithelial cells at the intersection of their apical and lateral surfaces, thereby creating a selective permeability barrier (5, 7, 8).

Until recently, little information was available regarding the biochemical composition of the tight junction. Using as an immunogen a mouse liver plasma membrane preparation enriched in tight junctions, we generated a monoclonal antibody (R26.4) against a zonula occludens-specific polypeptide (27). The molecule recognized by R26.4 was named ZO-1 and has an apparent molecular mass of ~225 kD (in mouse liver) on SDS gels (27). Immunofluorescence studies with R26.4 demonstrate the presence of ZO-1 in the region of the junctional complex in a variety of epithelia, including the Madin-Darby canine kidney (MDCK) epithelial cell line, where the molecule has an apparent molecular mass of ~210 kD (1, 27). Immunoelectron microscopy using isolated bile canaliculus-enriched plasma membrane fractions reveals a close association of ZO-1 with the cytoplasmic surface of the points of junctional membrane contact (27). Biochemical

characterization of ZO-1 demonstrates that the molecule is a peripheral membrane protein that is effectively solubilized from MDCK or liver plasma membranes with 6 M urea, high salt, or high pH buffers (1).

The tight junction is a dynamic intercellular junction that responds to various conditions such as transport stimuli, transepithelial migration of neutrophils, spermatocyte migration, and mechanical tension by changing the organization of the intramembrane fibrils (4, 6, 12, 14, 17, 22, 23). In addition, both the structure and function of the tight junction may be modulated by extracellular calcium. When calcium-free, EGTA-containing buffers are added to confluent epithelial cells, tight junctions open as observed in electron micrographs and as measured by a decrease in electrical resistance (3, 15, 25). In certain epithelia, the intramembrane fibrils of the tight junction disassemble or are displaced under these conditions (2, 9, 16, 22, 23). If low calcium medium is added to confluent monolayers, resistance also drops, albeit more slowly than when calcium is chelated (10). Both effects are reversible if calcium is added back to the monolayers (3, 15).

In this study we have explored the effects of extracellular calcium and cell-cell contact on ZO-1 localization and solubility in MDCK cells.

## Materials and Methods

### MDCK Monolayers

MDCK cells (passage 15–35) were grown to confluence on plastic petri dishes in MEM (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 U/ml streptomycin (Gibco Laboratories), 10 mM Hepes (Gibco Laboratories). This medium is referred to as high calcium medium (HCM)<sup>1</sup> throughout the text. The calcium concentration in HCM is  $\sim$ 1.8 mM. Cells were grown at 37°C in a 5% CO<sub>2</sub> humidified incubator.

For calcium studies, MDCK cells were grown to confluence on glass coverslips in HCM. Once confluency had been established, the medium was replaced with low calcium medium (see below) and the cells were incubated further for 15–24 h.

### MDCK Suspension Cultures

Confluent MDCK monolayers were rinsed thoroughly in calcium, magnesium-free Dulbecco's PBS (Gibco Laboratories), trypsinized with trypsin-EDTA (Gibco), and resuspended in suspension culture medium (Gibco) supplemented with 10 mM Hepes, 5% dialysed FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin. This medium will be referred to as low calcium medium (LCM) throughout the text. The calcium concentration in LCM is  $\sim$ 5  $\mu$ M. Cells were resuspended in this medium at a density of  $1-2 \times 10^6$  cells/ml and added to spinner flasks (Bellco Glass, Inc., Vineland, NJ). The medium was gassed with 5% CO<sub>2</sub>/95% air and gently stirred on a stirplate in a 37°C warm room for 18–20 h. For calcium studies, suspension cells were pelleted, resuspended, and plated onto glass coverslips in HCM medium. After 30 min adherence to coverslips and at various time points thereafter coverslips were processed for indirect immunofluorescence as described below. Alternatively, suspension cells were plated in HCM for 30 min which allowed cells to adhere to the substratum. Adherent cells were either rinsed and incubated in LCM for various lengths of time or rinsed, incubated in LCM for 3–5 h, then switched into HCM for 1 h, before being processed for indirect immunofluorescence.

The involvement of de novo protein synthesis in ZO-1 redistribution was examined in the following manner: suspension cells from a 24-h culture were plated at high cell density ( $2 \times 10^6$  cells/ml) in HCM with 30  $\mu$ g/ml cycloheximide. Cultures were incubated for 5 h and then processed for indirect immunofluorescence with R26.4 as the primary antibody.

### Antibody Characterization

Rat monoclonal antibody R26.4 was generated against a crude mouse liver plasma membrane preparation as described previously (27). The specificity of the antibody for ZO-1 in MDCK monolayers and suspension cultures was demonstrated as follows: 1 ml SDS extraction buffer (50 mM Tris, pH 8.8, 2% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM iodoacetamide [Sigma Chemical Co., St. Louis, MO], 100 U/ml Trasylol [Mobay Chemical Corp., Pittsburgh, PA], 20  $\mu$ g/ml each of chymostatin, leupeptin, and pepstatin; Sigma Chemical Co.) was added directly to washed and pelleted cells ( $2 \times 10^6$  cells). DNA was sheared by passing the extract through a 22-gauge needle attached to a syringe. 200  $\mu$ l of sample buffer (40% sucrose, 50 mM dithiothreitol [DTT], bromophenol blue) was added to 600  $\mu$ l of the extract. The extract was heated for 3 min at 95°C. Aliquots were subjected to electrophoresis on 6% SDS Laemmli gel (13). Proteins were electrophoretically transferred to nitrocellulose and then probed with R26.4 culture supernatant as described below.

### Immunoblotting

Proteins were transferred electrophoretically from 6% SDS polyacrylamide gels to nitrocellulose for 2 h at 75 V at 4°C (28). Nonspecific binding was blocked by incubating the nitrocellulose with 5% nonfat dry milk in PBS. The nitrocellulose was washed in PBS and incubated overnight with R26.4 culture supernatant. After extensive washing in PBS, the nitrocellulose was

1. *Abbreviations used in this paper:* HCM, high calcium medium; LCM, low calcium medium.

incubated with a 1:200 dilution of peroxidase-conjugated rabbit anti-rat IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN). After washing in PBS, the reaction was developed by a solution containing 0.05% 3,3'-diaminobenzidine, 0.01% H<sub>2</sub>O<sub>2</sub> in PBS.

### Immunofluorescence

MDCK cells were plated onto sterile glass coverslips and processed for indirect immunofluorescence as follows: coverslips were rinsed with PBS, fixed, and permeabilized with  $-20^\circ\text{C}$  methanol for 3 min. Coverslips were rinsed in PBS and incubated with undiluted R26.4 culture supernatant for 2 h in a humidified chamber at room temperature. After rinsing in PBS, coverslips were incubated with a 1:200 dilution of fluorescein-conjugated rabbit anti-rat IgG (Boehringer Mannheim Biochemicals) for 1 h. Coverslips were washed in PBS, mounted in a solution containing PBS, 50% glycerol, 0.4% *n*-propyl gallate (Sigma Chemical Co.), and viewed with a 63 $\times$  objective on a Zeiss microscope equipped with epifluorescence illumination. Photographs were taken on T Max film (Eastman Kodak Co., Rochester, NY).

### Solubility Properties of ZO-1 from Monolayer and Suspension Cultures

Monolayers were rinsed twice with PBS, scraped into the same buffer with a rubber policeman, and then pelleted at 1,200 rpm for 10 min. Aliquots of suspension cells were centrifuged in the same manner. All subsequent manipulations were done at 4°C. 200  $\mu$ l of an extraction buffer containing protease inhibitors (20  $\mu$ g/ml each of pepstatin, leupeptin, and chymostatin; 3  $\mu$ l/ml diisopropylfluorophosphate [Sigma Chemical Co.]; 200 U/ml Trasylol) was added directly to the cell pellet. The following extraction buffers were used in these studies: (a) 10 mM Tris, 0.1% NP-40, 2 mM MgCl<sub>2</sub>, pH 7.4 (low salt); (b) 0.5 M Tris, 0.1% Triton X-100, 2 mM MgCl<sub>2</sub>, pH 7.4 (high salt); (c) 0.5 M KCl, 10 mM Tris, 0.1% Triton X-100, 2 mM MgCl<sub>2</sub>, pH 7.4 (high salt); (d) 1% Triton X-100, 10 mM Tris, 2 mM MgCl<sub>2</sub>, pH 7.4 (non-ionic detergent); (e) 6 M urea, 10 mM imidazole, 2 mM MgCl<sub>2</sub>, 0.1% Triton X-100, pH 7.4 (denaturing agent); (f) 0.1 M acetate buffer, 0.1% Triton X-100, 2 mM MgCl<sub>2</sub>, pH 5.6 (low pH); (g) 1% sarcosine, 0.1% Triton X-100, 2 mM MgCl<sub>2</sub>, 10 mM imidazole, pH 8 (anionic detergent); (h) 50 mM sodium bicarbonate buffer, 0.1% Triton X-100, 2 mM MgCl<sub>2</sub>, pH 10.7 (high pH); (i) 0.6 M KI, 0.1% Triton, 2 mM MgCl<sub>2</sub>, 10 mM Tris, pH 7.4 (chaotropic agent); (j) 0.25 M KCl, 0.1% Triton X-100, 2 mM MgCl<sub>2</sub>, pH 7.4 (intermediate ionic strength buffer); (k) 5 mM EDTA, 5 mM EGTA, 10 mM Tris, 2 mM MgCl<sub>2</sub>, pH 7.4 (chelators). Extracts were centrifuged at 100,000 g for 60 min at 4°C. Aliquots of supernatants and pellets were subjected to electrophoresis on 6% SDS-PAGE, electrophoretically transferred to nitrocellulose, and Western blotted as described above. The nitrocellulose was photographed and the negatives scanned on a laser densitometer (model Ultrascan XL; LKB Instruments Inc., Bromma, Sweden) to quantitate the amount of ZO-1 in supernatants and pellets.

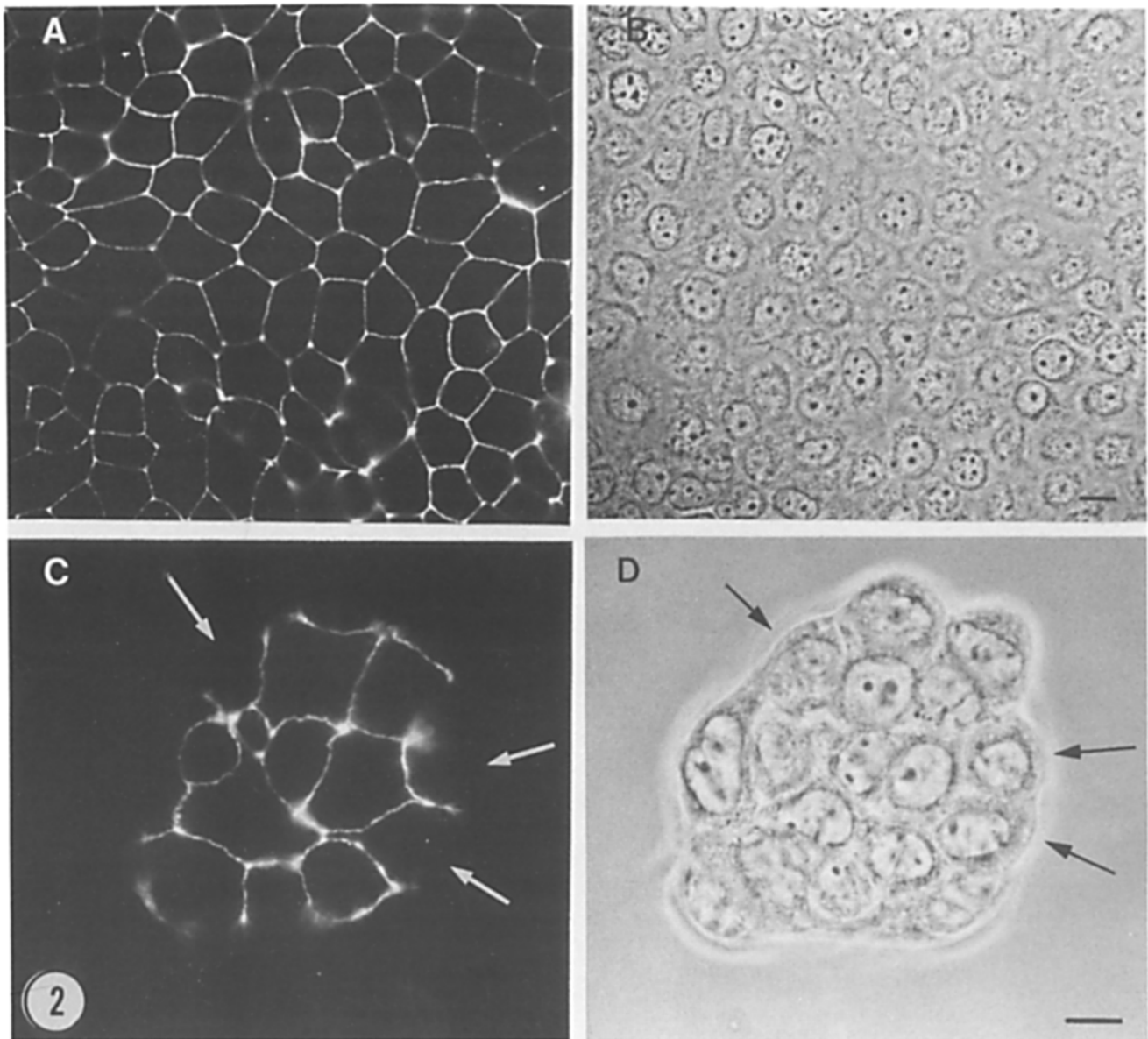
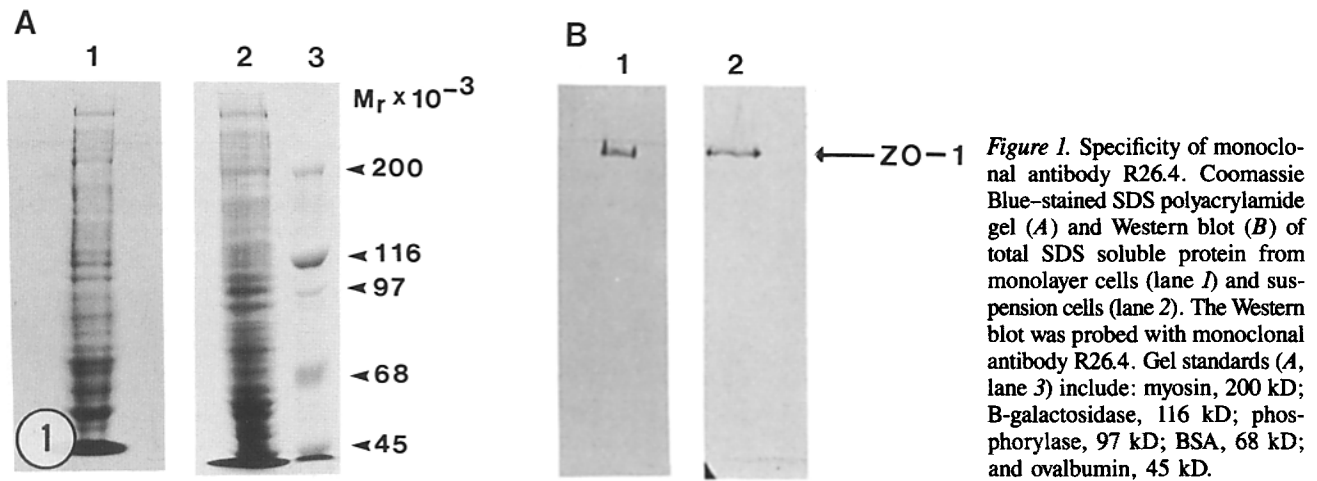
## Results

### Specificity of Monoclonal Antibody R26.4

In the experiments described below, we have used monoclonal antibody R26.4 as a probe for ZO-1 localization. Western blot analysis (Fig. 1) of total cell protein demonstrates that R26.4 recognized a single,  $\sim$ 210-kD band in both MDCK monolayer (Fig. 1 B, lane 1) and suspension culture (lane 2) extracts. A comparable amount of ZO-1 was extracted from equal numbers of suspension and monolayer cells.

### Immunofluorescent Localization of ZO-1 in Confluent MDCK Monolayers

ZO-1 immunoreactivity in confluent MDCK monolayers was found exclusively at the plasma membrane of adjacent cells (Fig. 2, A and B). Diffuse, cytoplasmic staining was never observed. Before reaching confluency, small islands of cells were observed (Fig. 2 D). ZO-1 was also found at sites of



cell-cell contact in these clusters (Fig. 2 C). However, in cells at the periphery of these clusters, there was often no ZO-1 membrane staining in areas that did not abut another cell (Fig. 2, C and D, arrows). Diffuse, cytoplasmic ZO-1 immunofluorescence was not observed in these small clusters of cells.

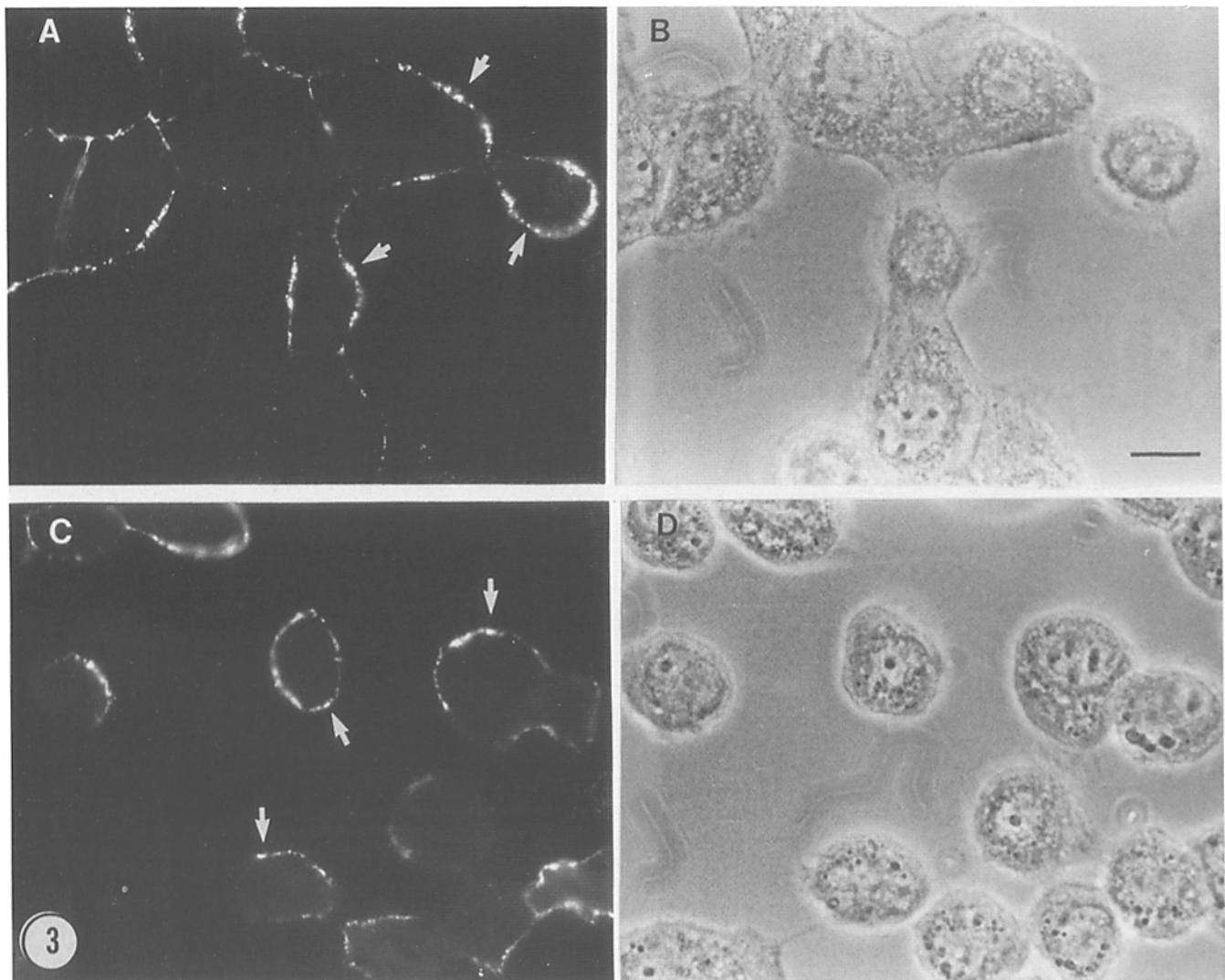
#### **Effect of Extracellular Calcium Chelation on ZO-1 Localization**

To examine factors that might modulate the cellular localization of ZO-1, we first studied the effect of transient chelation of extracellular calcium on ZO-1 localization. Confluent MDCK monolayers were incubated in PBS containing 2 mM EGTA for various lengths of time up to 15 min and then processed for indirect immunofluorescence using R26.4 as the primary antibody. As shown in Fig. 3, cells pulled apart from each other and many had completely rounded up as in-

tercellular junctions were disrupted (Fig. 3, B and D). Single unattached cells were also observed. In all cells, ZO-1 remained associated with the plasma membrane in a bright, punctate staining pattern (Fig. 3, A and C, arrows). Replacing the calcium-free EGTA medium with HCM caused a complete reversal of the above observations within 30 min. During this interval, cells spread and made contact with their neighbors. ZO-1 immunofluorescence was now indistinguishable from that of control monolayers (data not shown; see Fig. 2 A).

#### **Effect of Long-Term Calcium Depletion on ZO-1 Localization**

Having established that transient calcium depletion did not appear to affect ZO-1 localization despite disruption of cell contacts, we next examined the effect of long term  $\text{Ca}^{+2}$  depletion on ZO-1 localization. For these studies we took ad-



**Figure 3.** Effect of brief, extracellular calcium chelation on ZO-1 localization in confluent MDCK monolayers. Established, confluent monolayers were incubated for 15 min in PBS containing 2 mM EGTA and then processed for indirect immunofluorescence using R26.4 as the primary antibody. After 15 min, the majority of cells have pulled apart from their neighbors, with many cells completely detached from the monolayer (B and D). In all cells, ZO-1 remains associated with the plasma membrane in a bright, punctate staining pattern (A and C, arrows). Bar, 10  $\mu\text{m}$ .

vantage of the observation that MDCK cells may be maintained as single cells in low calcium, spinner culture (24). Under these conditions, most cells remain single and have a rounded morphology (Fig. 4). In contrast to the membrane localization of ZO-1 in confluent MDCK monolayers (Fig. 2 A), ZO-1 immunoreactivity in single suspension cells appeared diffuse within the cytoplasm (Fig. 4, A, C, E, and F). In many instances, bright, punctate cytoplasmic staining was also observed (Fig. 4, A, C, E, and F, arrows). Variability in this punctate staining pattern was seen (Fig. 4, E and F). Occasionally, small aggregates or pairs of cells were also seen in suspension cultures. These adjoining cells may never have separated after trypsin dissociation of the initial monolayer. Two staining patterns were discernable in these cells. ZO-1 was found both in the region of contact between neighboring cells as well as diffuse within the cytoplasm (Fig. 4, A and C; open arrows). Suspension cells that had been stained with preimmune serum as the primary antibody or just with secondary antibody alone showed no immunofluorescence staining (data not shown).

The observations on ZO-1 localization in suspension cells were also seen with confluent monolayers grown in LCM. Confluent monolayers were established in HCM and then incubated further for 15–24 h in LCM. All of the cells had diffuse ZO-1 localization with occasional punctate staining (Fig. 4 G). In addition, some cell pairs also had remaining concentrated ZO-1 membrane staining (Fig. 4 G, open arrows). In contrast to what was observed when suspension cells were placed in low calcium medium, ZO-1 immunoreactivity disappeared more slowly when confluent monolayers were switched to low calcium medium. However, after 24 h in LCM, diffuse ZO-1 immunoreactivity had disappeared in the majority of these cells (data not shown).

#### ***Kinetics of ZO-1 Redistribution in Suspension Cells That Have Been Allowed to Adhere to the Substratum***

As shown in Fig. 4, long-term maintenance of MDCK cells in low calcium medium did influence ZO-1 localization. To examine whether cell contact might influence the redistribution of ZO-1 localization in these cells, suspension cells were plated at either high or low cell density in HCM, allowed to adhere to coverslips, and then processed for indirect immunofluorescence at various time points to monitor the changes in ZO-1 localization (Fig. 5).

At high plating density ( $2 \times 10^6$  cells/ml) most cells made contact with one or more cells (Fig. 5, A–F). 60 min after attachment to the substratum, most cells did not have localized ZO-1 membrane staining in the region of cell–cell contact (Fig. 5, A and D). ZO-1 still had a diffuse, cytoplasmic localization. Some pairs of cells did have localized ZO-1 at sites of contact (Fig. 5 D, arrows). After 180 min of adherence, many more cells within a small cluster had highly localized membrane staining in contact regions (Fig. 5, B and E, arrows), however cytoplasmic staining was still apparent in some cells. By 5 h cells were well-spread and ZO-1 was almost completely localized to the junctional complex area (Fig. 5, C and F). With overnight incubation these cells were indistinguishable from control monolayers (data not shown). Redistribution of ZO-1 to the plasma membrane did not require de novo protein synthesis. When suspension cells were plated at high cell density in HCM containing cy-

cloheximide, ZO-1 became localized to sites of cell–cell contact in a time course identical to that observed above (data not shown).

In contrast, when suspension cells were plated at low cell density ( $1-2 \times 10^5$  cells/ml) in HCM, ZO-1 did not redistribute to the plasma membrane in single cells (Fig. 5, G–L). By 5 h in culture ZO-1 immunoreactivity in single cells was diminished (Fig. 5, H and I, open arrows). With overnight incubation, ZO-1 immunofluorescence had completely disappeared in single cells (data not shown). In those cells that made contact with a neighboring cell, ZO-1 did become concentrated at membrane contact areas with a time course identical to that observed in cells plated at high density (Fig. 5, H and I, arrows).

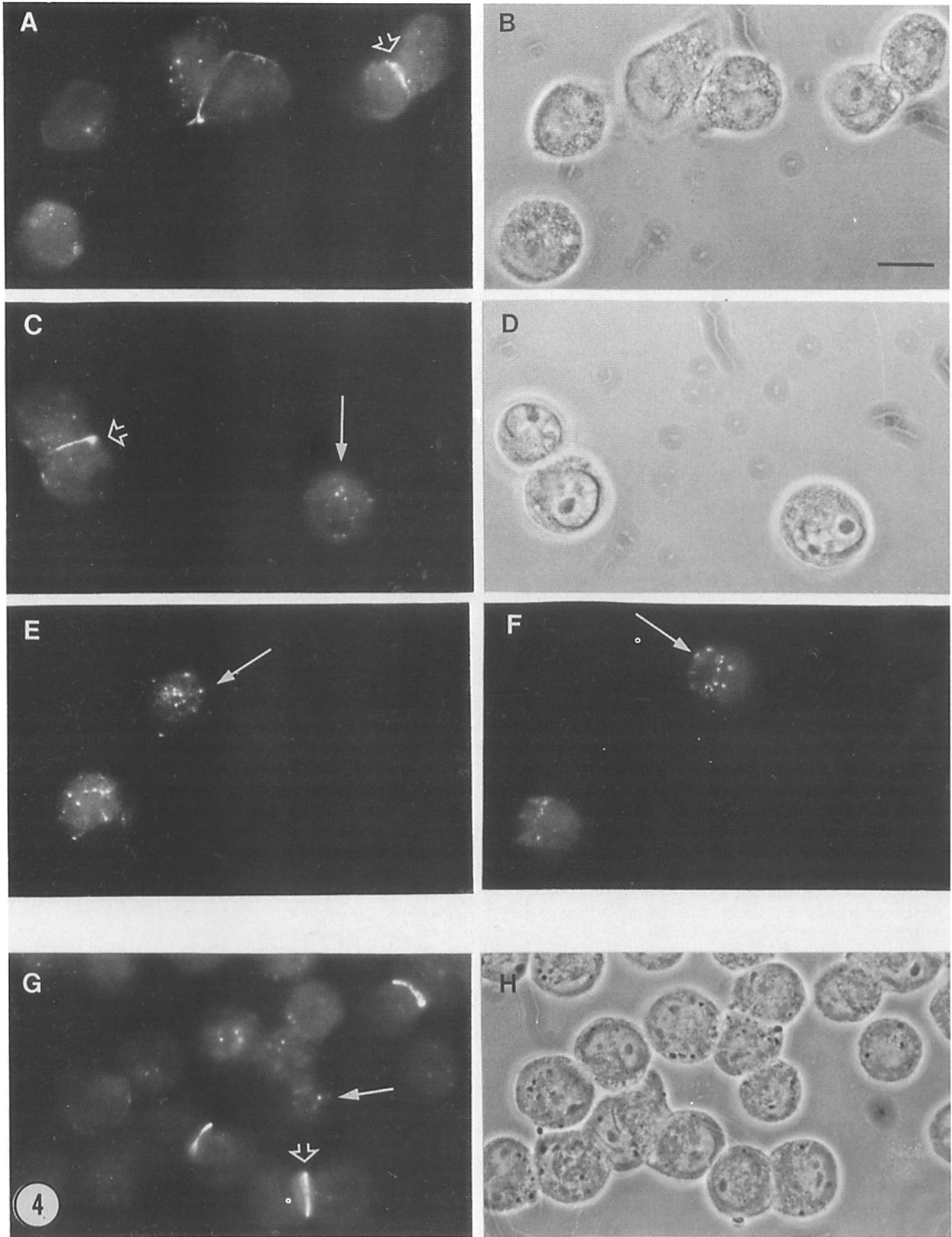
These findings suggested that cell–cell contact in the presence of calcium was critical for ZO-1 localization at the region of the tight junction. To examine the effect of cell–cell interaction in low calcium, overnight cultures of suspension cells were plated at high cell density in HCM for 30 min to allow cells to adhere to the substratum. The cultures were rinsed, incubated for various lengths of time in LCM, and then processed for indirect immunofluorescence. ZO-1 immunofluorescence remained diffuse and punctate even after 5 h in LCM, although most of this immunoreactivity disappeared after 12-h incubation in LCM (Fig. 6, A–D). ZO-1 was never seen to redistribute to sites of cell–cell contact. The reversibility of this phenomenon was tested in the following manner. Suspension cells were attached as before and then incubated in LCM for 3 h. Cultures were rinsed in HCM and incubated in the same medium. After only 1 h in HCM, ZO-1 had redistributed to the region of the tight junction in many of the cells that were contacting one another (Fig. 6, E and F). After 2 h in HCM the cells were well-spread and all had concentrated ZO-1 plasma membrane immunoreactivity at sites of cell–cell contact (Fig. 6, G and H).

#### ***Comparison of Solubility Properties of ZO-1 from Suspension Cultures and Monolayers***

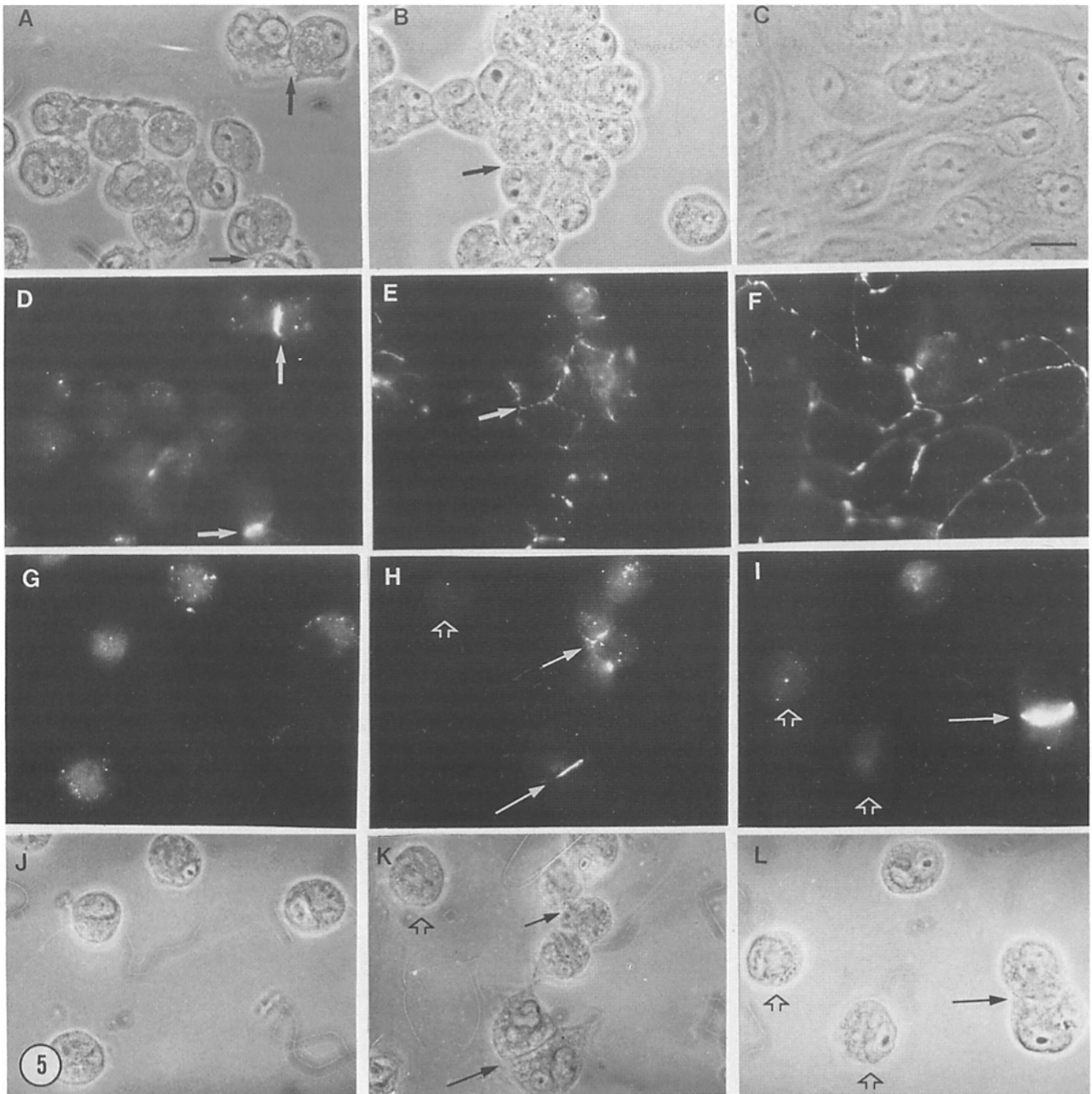
To determine whether changes in ZO-1 localization were accompanied by changes in the solubility properties of the molecule, the solubility of ZO-1 in monolayer and suspension cells was examined. A panel of extraction buffers designed to disrupt either electrostatic or hydrophobic interactions was used to probe for differences in ZO-1 solubility (Table I). Neither low salt (10 mM Tris, pH 7.4) nor low pH buffers (0.1 M acetate buffer, pH 5.6) solubilized ZO-1 from suspension cells or monolayer cells. In both cases ZO-1 was soluble in 6 M urea, 0.6 M KI, and sarcosine buffers. However, differences were found. ZO-1 from suspension cells was completely soluble in 0.5 M Tris or 0.5 M KCl buffers whereas only ~50–80% was extracted from monolayer cells in 0.5 M KCl or 0.5 M Tris buffers, respectively. Non-ionic buffers (1% Triton X-100, pH 7.4) or buffers with chelating agents (5 mM EDTA, 5 mM EGTA) partially solubilize ZO-1 from suspension cells but not from monolayers. In contrast, high pH buffer (50 mM sodium bicarbonate, pH 10.7) solubilized 20% of ZO-1 from monolayer cells but suspension cell ZO-1 was completely insoluble.

#### ***Discussion***

In this study we have shown that in MDCK cells, the local-

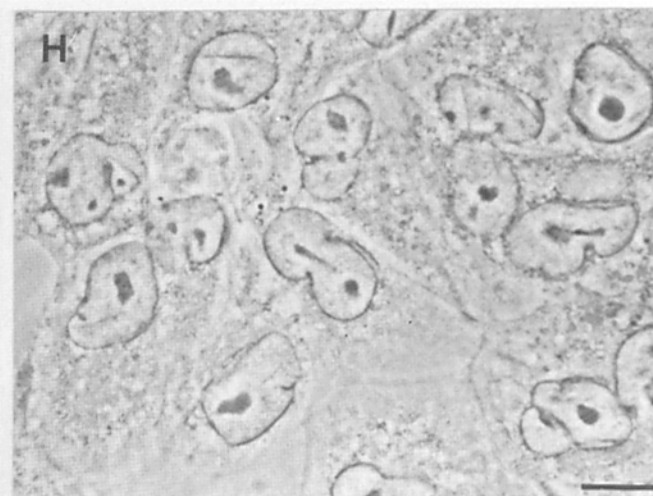
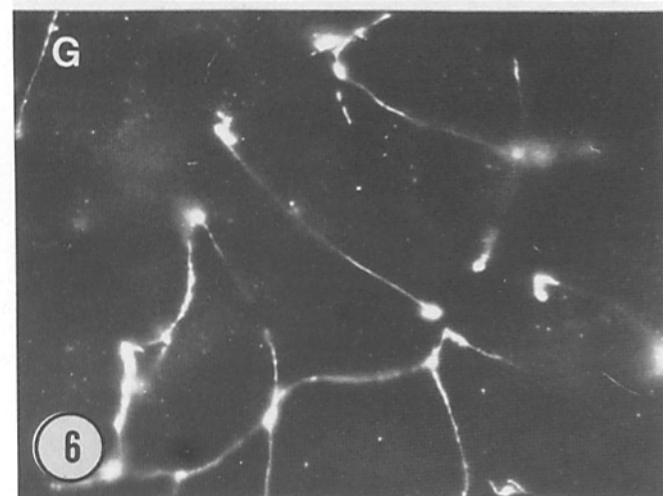
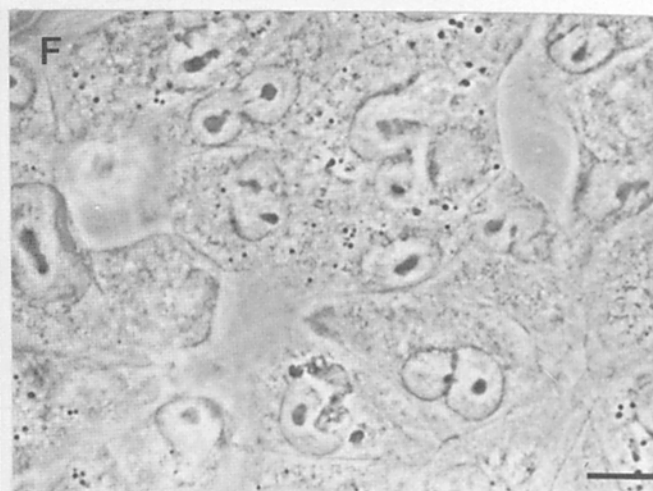
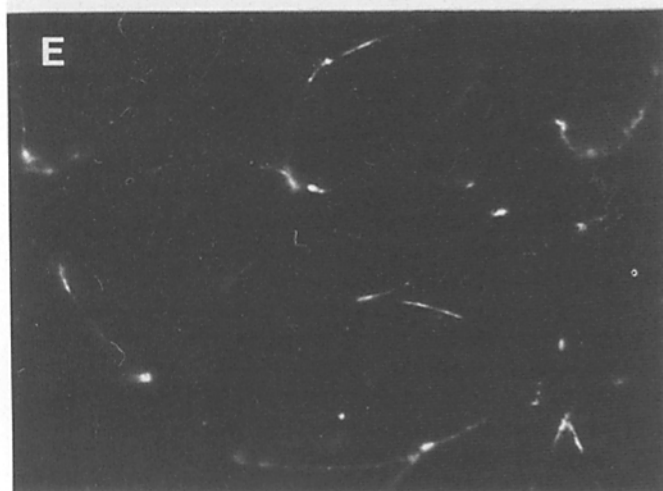
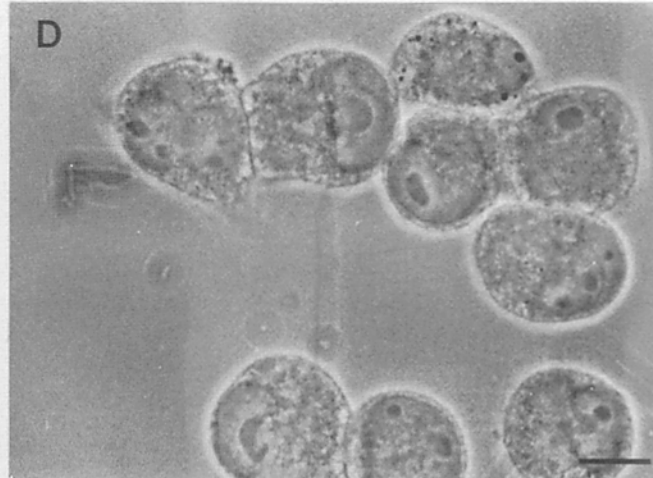
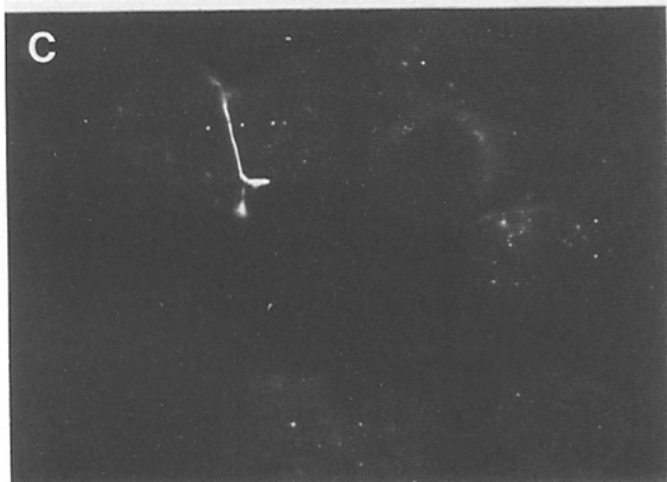
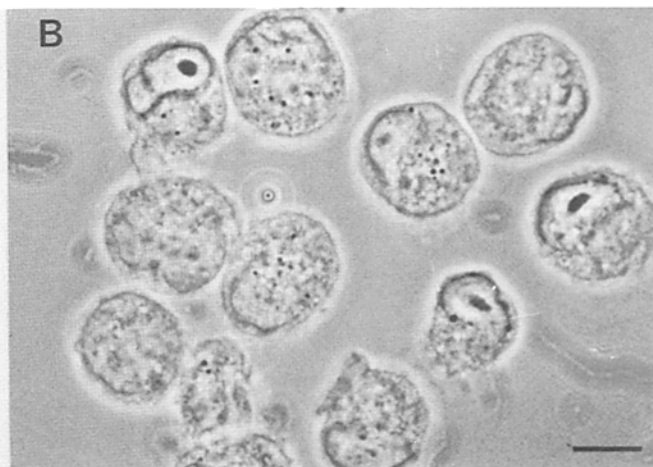
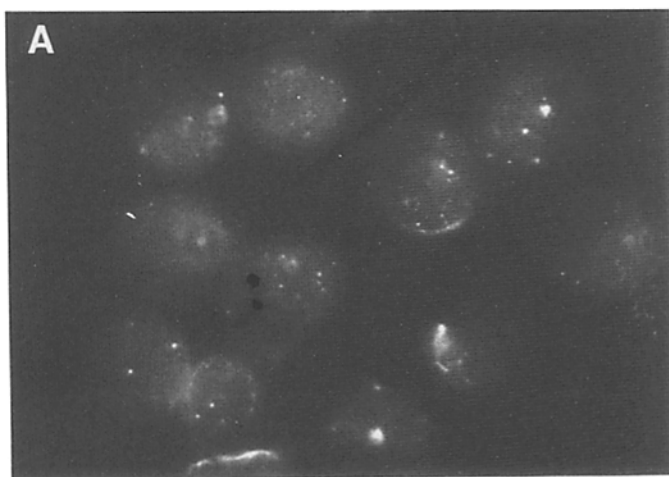


**Figure 4.** Effect of long-term calcium depletion on ZO-1 localization. MDCK cells were maintained in low calcium, long-term (18–20 h) suspension culture and then processed for indirect immunofluorescence. In single cells, ZO-1 was diffusely organized and was no longer found at the plasma membrane with variable amounts of punctate staining in the cytoplasm also seen (A, C, E, and F, arrows). ZO-1 was



**Figure 5.** Kinetics of ZO-1 redistribution in suspension cells plated in HCM. Suspension cells from an 18-h spinner culture were plated at either high cell density ( $2 \times 10^6$  cells/ml; A-F) or low cell density ( $2 \times 10^5$  cells/ml; G-L) in HCM. Coverslips were processed for indirect immunofluorescence at 1 (A and D; G and J), 3 (B and E; H and K), or 5 (C and F; I and L) h after plating. ZO-1 redistributes to sites of cell-cell contact within 3 h after plating MDCK suspension cells (E and H). By 5 h all cells that have formed contacts with adjacent cells have localized ZO-1 plasma membrane staining (F). In contrast, ZO-1 never was observed to redistribute to the plasma membrane in single suspension cells (H and I, open arrows). 5 h after plating cells at low cell density, ZO-1 immunoreactivity in most single cells has disappeared (I and L, open arrows). Bar, 10  $\mu$ m.

also found concentrated at sites of cell-cell contact in the occasional cell pair or small cluster found in suspension cultures (A and C, open arrows). The diffuse organization of ZO-1 was also observed when established, confluent monolayers were subsequently switched into LCM for 18 h and then processed for indirect immunofluorescence (G and H). Note that some cell pairs still have concentrated plasma membrane staining in regions of cell contact (G, open arrow). Bar, 10  $\mu$ m.





**Table I. Comparison of Solubility Properties of ZO-1 from Suspension Cells and Confluent Monolayers**

	Suspension culture (in percent)		Monolayer (in percent)	
	S	P	S	P
Low salt	0	100	0	100
0.5 M Tris	100	0	80	20
0.5 M KCl	100	0	50	50
1% TX-100	8	92	0	100
6 M Urea	100	0	100	0
High pH	0	100	20	80
Low pH	0	100	0	100
Anion Det.	100	0	100	0
0.6 M KI	100	0	100	0
0.25 M KCl	60	40	17	83
Chelators	33	67	0	100

Equal numbers of suspension and monolayer cells were extracted. Extracts were centrifuged at 100,000 g for 1 h. Aliquots of the supernatants (S) and pellets (P) were subjected to electrophoresis on 6% SDS gels, electrophoretically transferred to nitrocellulose, and probed with R26.4.

ization of the tight junction protein, ZO-1, was modulated both by extracellular calcium and cell-cell contact. Under normal growth conditions, this epithelial cell line becomes a confluent monolayer in which adjacent cells form junctional complexes upon contact with other cells. In such monolayers, ZO-1 had a membrane distribution in the region of the tight junction at sites of cell-cell contact.

The studies described in this paper took advantage of observations that extracellular calcium has a profound effect on the physiology and morphology of the tight junction. Occluding junctions of MDCK monolayers open when calcium-free, EGTA medium is added to the monolayers (2, 15). Opening of junctions occurs very rapidly (within ~15 min) as measured by a reduction in transepithelial electrical resistance. When EGTA-containing medium is removed and calcium restored, junctions reseal (15). Our data indicate that transient calcium chelation does not rapidly alter the plasma membrane localization of ZO-1, although clearly tight junctions are disrupted by EGTA. ZO-1 remains associated with the plasma membrane even in cells that have completely pulled away from all neighbors, although the morphology of this distribution is changed from a continuous to a beaded appearance. However, Stevenson et al. (26) have shown that placing confluent cells in low calcium medium for short periods (under 10 min) produces somewhat different results than the chelation data reported here. They observed the appearance of diffuse cytoplasmic ZO-1 immunoreactivity, in conjunction with membrane staining, within 1-10 min after addition of spinner medium.

However, long-term calcium depletion has a different

effect on ZO-1 localization. Single MDCK cells maintained in long-term (18-20 h), low calcium spinner culture had diffuse, cytoplasmic ZO-1 localization, with occasional bright, punctate cytoplasmic staining also observed. This diffuse staining appeared to be cytoplasmic and not plasma membrane associated since no cell, even highly rounded cells, exhibited an annular, ring-like staining pattern, which would be characteristic of a molecule having a plasma membrane localization. The punctate staining does not appear to be associated with lysosomes for several reasons. First, analysis of total SDS-soluble protein from suspension cells reveals a single ~210-kD band present in comparable amounts to that extracted from monolayer cells. No immunoreactive, proteolytic fragments were observed. Second, comparison of phase and fluorescent micrographs of suspension cells showed many areas where there were clearly no lysosomes but yet areas of punctate staining were seen.

This same fluorescent pattern that was seen in suspension cells was also seen under several other experimental conditions. Both established, confluent monolayers that were subsequently switched into LCM medium, as well as spinner cells plated at low cell density in HCM, exhibited diffuse, nonplasma membrane-associated ZO-1 immunoreactivity. Thus, it appeared that extracellular calcium alone did not regulate ZO-1 membrane localization. Rather, both the appropriate calcium concentration and cell-cell contact were essential for ZO-1 localization to the plasma membrane.

This hypothesis was examined directly by following the redistribution of ZO-1 upon plating of suspension culture cells. ZO-1 redistributed in suspension cells plated at high cell density in HCM. The diffuse immunofluorescence disappeared with a concomitant appearance at the plasma membrane in the region of cell-cell contact within a period of 5 h. This process was independent of de novo protein synthesis, suggesting that the diffuse ZO-1 observed in suspension cells is functional and able to assemble with other existing components at the appropriate membrane site when MDCK cells contact one another in the presence of the appropriate calcium concentration. Redistribution of ZO-1 to sites of cell contact was inhibited, however, when suspension cells were plated at high density in LCM, consistent with the morphological and physiological absence of tight junctions under these growth conditions.

These results provide further support for the hypothesis that both extracellular calcium and cell-cell contact are essential for the assembly of a functional tight junction as well as ZO-1 redistribution to the plasma membrane. These findings are also consistent with observations that the addition of cycloheximide to either established monolayers or to suspension cells at the time of plating has no effect on either the existing transepithelial electrical resistance (in the case of confluent monolayers), or the development of resistance

**Figure 6.** Localization of ZO-1 in suspension cells plated at high cell density in LCM. Suspension cells from an 18-h spinner culture were plated at high cell density ( $2 \times 10^6$  cells/ml), incubated for 1 (A and B) or 5 (C and D) h in LCM, and then processed for indirect immunofluorescence using R26.4. ZO-1 immunoreactivity remains diffuse (A) and eventually disappears (C). In contrast, when suspension cells are plated, incubated for 3 h in LCM, switched into HCM, and then processed for indirect immunofluorescence, ZO-1 redistributes to the plasma membrane in areas of cell-cell contact (E-H). Within 1 h, cells have spread and contacted adjoining cells with concomitant appearance of ZO-1 at the plasma membrane (E and H). After 2 h in HCM, most cells exhibit intense ZO-1 fluorescence in the region of the tight junctions (G and H). Diffuse, cytoplasmic staining is not apparent. Bar, 10  $\mu$ m.

(plated spinner culture cells) (11). This suggests that a stable pool of proteins involved in tight junction formation exists in the presence or absence of cell-cell contact and then is assembled under appropriate growth conditions.

Other studies have demonstrated that the localization of several cytoskeletal or junctional proteins is dependent on cell-cell contact and extracellular calcium. Desmoplakins I and II, components of the desmosome, and fodrin have a diffuse cytoplasmic distribution when MDCK monolayers are maintained in low calcium medium (19, 21). In low calcium growth medium, the cells remain adherent to the substratum but are unable to make contact with adjacent cells. When the calcium concentration is raised, cells form intercellular junctions and these proteins eventually become reorganized at the appropriate sites along the plasma membrane (19, 21). The diffuse organization of fodrin and desmoplakins I and II was also observed with single suspension MDCK cells (data not shown). In contrast, the distribution of intermediate filament proteins, vimentin and cytokeratin, and actin is similar in both single MDCK cells, small colonies, and confluent monolayers (18).

The studies in this paper demonstrated that in MDCK cells, distribution of ZO-1 was altered under different growth conditions, suggesting that the interaction of ZO-1 with other molecular components might also be affected. This was reflected in altered solubility properties of ZO-1. In both monolayer and suspension cells, ZO-1 was insoluble in low salt or low pH buffers but yet was completely solubilized in 6 M urea, 0.6 M KI, or sarcosine buffers. ZO-1 was not freely soluble in suspension cells, despite its apparent cytoplasmic localization. Extraction with high salt buffers, however, revealed that ZO-1 in suspension cells was completely solubilized while being only partially soluble in monolayer cells. A small amount (~8%) of suspension cell ZO-1 was extracted with non-ionic detergents whereas ZO-1 from confluent monolayers was completely insoluble in non-ionic detergent. Taken together, these results indicated that ZO-1 remained relatively insoluble despite its change to a cytoplasmic localization in suspension cells. Yet the molecular interactions of some of the population of ZO-1 molecules did change as partial solubility differences were observed. In particular, the entire ZO-1 pool in suspension cells was extracted with high salt buffers and partially extracted with a non-ionic buffer. We also found that under several extraction conditions, the ZO-1 pool partitioned into soluble and insoluble fractions that differed between monolayer and suspension cells. This suggested either that the association of ZO-1 with other molecular components of the tight junction had been altered when MDCK cells were maintained in low calcium growth medium, or that the total ZO-1 pool in low calcium conditions was partitioned into more than one subcellular compartment, each with a different set of molecular interactions resulting in different solubilities.

It will be interesting to see whether the differences in solubility and intracellular location of ZO-1 that we have observed in MDCK cells under different growth conditions are also reflected in differences in stability and turnover of the molecule as has been shown to be the case for fodrin, desmoplakins I, and desmoplakins II (18, 19, 20, 21). These experiments might provide insight on how ZO-1 associates with other proteins of the tight junction and how this process is

regulated in response to cell-cell contact and extracellular calcium.

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