J. Membrane Biol. 137, 261–270 (1994)

The Journal of
Membrane
Biology

Analysis of the Distribution and Phosphorylation State of ZO-1 in MDCK and Nonepithelial Cells

A.G. Howarth[1], K.L. Singer[2], B.R. Stevenson[1]

[1]Department of Anatomy and Cell Biology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7
[2]Department of Molecular and Cellular Biology, University of California at Berkeley, Berkeley, California 94720-0001

Received: 6 August 1993/Revised: 6 October 1993

Abstract. We have examined the distribution and extent of phosphorylation of the tight junction-associated protein ZO-1 in the epithelial MDCK cell line, and in three cell types that do not form tight junctions: S180 (sarcoma) cells, S180 cells transfected with E-cadherin (S180L), and primary cultures of astrocytes. In short-term calcium chelation experiments on MDCK cells, removal of extracellular calcium caused cells to pull apart. However, ZO-1 remained concentrated at the plasma membrane and no change in ZO-1 phosphorylation was observed. Maintenance of MDCK cells in low calcium medium, conditions where no tight junctions are found, resulted in altered ZO-1 distribution and lower total phosphorylation of the protein. In S180 cells, ZO-1 was diffusely distributed along the entire cell surface, with concentration of the antigen in motile regions of the cell. Cell-cell contact was not a prerequisite for ZO-1 localization at the plasma membrane in this cell type, and the phosphate content of ZO-1 was found to be lower in S180 cells relative to MDCK cells. Expression of E-cadherin in S180L cells did not alter either the distribution or phosphorylation of ZO-1. In contrast to S180 cells, ZO-1 in primary cultures of astrocytes was concentrated at sites of cell-cell contact, and the phosphorylation state was the same as that in control MDCK cells. Comparison of one-dimensional proteolytic digests of ^{32}P-labeled ZO-1 revealed the phosphorylation of two peptides in control MDCK cells that was absent in both MDCK cells grown in low calcium and in S180 cells.

Key words: Cell adhesion molecules — E-cadherin — Intercellular junction — Paracellular pathway — Protein phosphorylation

Correspondence to: B.R. Stevenson

Introduction

The tight junction forms a selectively permeable barrier to the movement of substances in the paracellular space between both epithelial and endothelial cells. This intercellular junction is also believed to play a role in restricting the movement of proteins and lipids between the compositionally distinct apical and basolateral membrane domains of these cell types. The identities of several tight junction-associated proteins has been determined, including ZO-1, cingulin, ZO-2, and 7H6. In addition, actin filaments have been localized to the tight junction [4, 23, 27]. While all of these proteins are found at the tight junction, the only defined interaction is that between ZO-1 and ZO-2, which have been shown to co-immunoprecipitate [13]. Knowledge of the molecular organization and regulation of the junctional components in vivo is still limited (for review, *see* [7, 12, 36]).

ZO-1 is a high molecular weight (~220 kD) protein that associates closely with the cytoplasmic surface of tight junctions in epithelial and endothelial cells. ZO-1 was recently found to occur in a wide variety of cell types in vitro that are not believed to form tight junctions [15]. Further work has shown that a previously identified 220 kD protein believed to be derived from adherens junctions is identical to ZO-1 [16]. Itoh and colleagues [16] found that this protein is localized to intercalated discs in heart muscle, suggesting a role for ZO-1 at both tight and some types of adherens junctions.

The permeability of the tight junction can be regulated through a number of treatments. For example, treatment of a mammary epithelial cell line with synthetic glucocorticoids causes a large transepithelial resistance (TER) increase [39]. Modulation of the actin cytoskeleton also affects junctional permeability [4, 24,

27], and the removal of extracellular calcium causes dissociation of epithelial monolayers and disruption of the tight junction [6, 25, 34]. There are considerable data indicating that pharmacologic manipulation of various protein kinase systems can affect the permeability properties of the tight junction [2, 10, 11, 18, 28, 31]. In addition, protein kinase inhibitors have been shown to inhibit the opening of tight junctions induced by the removal of extracellular calcium [8] and the resealing of tight junctions after the readdition of calcium [30]. However, no direct information on the phosphorylation state of tight junction-associated proteins under these conditions has been reported. Preliminary information on ZO-1 phosphorylation was provided by an analysis of the phosphate content of the protein in two strains of MDCK cells which differ significantly in TER. ZO-1 in low resistance strain II cells contained twice as much phosphate as that from high resistance strain I [37].

Here, we have examined the distribution and phosphorylation state of ZO-1 in MDCK cells that have had their junctional complexes disrupted by removal of extracellular calcium and in cell types that do not express tight junctions. We report significant differences in ZO-1 phosphorylation that correlate with changes in ZO-1 localization at sites of cell-cell contact. Evidence is presented that these differences correspond to detectable specific phosphorylation events on the ZO-1 molecule. In addition, we find that expression of E-cadherin alone is not sufficient to elicit changes in the phosphorylation or localization of ZO-1.

Materials and Methods

Cell Culture

MDCK cells (low resistance clone 8; [29]), a canine kidney epithelial cell line, S180 (sarcoma) cells, S180 cells transfected with E-cadherin (S180L; [26]) and primary cultures of astrocytes were grown as described previously [15]. Three to five days before harvesting, MDCK cells were plated onto 24 mm Transwell porous cell culture supports (Costar, Cambridge, MA) at 1.4×10^6 cells/filter, and grown with daily medium replacement. For low calcium experiments, cells were initially plated in normal medium (1.3 mM Ca^{2+}) for 90 min and then rinsed three times with phosphate-buffered saline without added Ca^{2+} or Mg^{2+}. Cells were then grown in medium with Ca^{2+} omitted from the DMEM formulation containing fetal bovine serum dialyzed extensively against 0.9% saline (low calcium medium, LCM). The calcium concentration in this medium, as determined by mass spectrophotometry, is ~5 μM (*data not shown*). LCM-grown cells returned to calcium-containing medium after three days regained a normal TER of approximately 200 Ωcm^2 within 24 hr (*data not shown*). In the calcium chelation experiments, a 0.5 M stock of BAPTA (Molecular Probes, Eugene, OR) was added directly to apical and basolateral compartments of MDCK filters with constant agitation to give a final concentration of 4 mM at $t = 0$ [8]. S180 and S180L cells were plated on 35 mm tissue culture dishes two to three days before cell harvesting. Type I rat astrocyte cultures were prepared as described [22], maintained in serum-free DMEM with 6 mg/ml glucose and utilized within one to three weeks of isolation. These cultures were judged to contain >95% astrocytes by GFAP staining ([15]; *data not shown*).

Transepithelial Resistance

TER was determined using a Millicell-ERS apparatus (Millipore, Bedford, MA). Electrodes were stored in (mM) 10 HEPES pH 7.4, 5.4 KCl, 137 NaCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 5.6 glucose (HBSG) for at least 24 hr prior to experiments. TER measurements were made at 37°C, and TER of blank collagen-coated filters was subtracted from all samples. Experimental TER values are expressed as a percentage of readings taken at $t = 0$.

^{32}P Labeling of ZO-1

Cells were rinsed three times with HBSG (Ca^{2+} and Mg^{2+} omitted for LCM and BAPTA-treated MDCK cells), incubated at 37°C in HBSG for 30 min to starve cells for phosphate, and then labeled for 1 to 2 hr (4 hr for proteolytic digests) in HBSG containing 250 μCi/ml ^{32}P-orthophosphate (ICN Biomedicals, Irvine, CA). For calcium chelation experiments, BAPTA was added for the final 5 min of the labeling period. Cultures were removed from the incubator and placed on ice, and all further operations took place at 4°C. The labeling medium was removed and cells rinsed two times with Tris-buffered saline (10 mM Tris pH 8.0, 150 mM NaCl), scraped from the dishes with a rubber policeman and pelleted at $300 \times g$ for 5 min.

Immunoprecipitation of ZO-1

All solutions contained protease and phosphatase inhibitors as follows: 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 μl/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 5 mM tetrasodium pyrophosphate. Cell pellets were solubilized in lysis buffer (10 mM Tris pH 7.8, 1% SDS; approximately 0.1 ml/cm^2 of monolayer) with vigorous vortexing at 100°C. Lysates were diluted with four volumes of buffer A (50 mM Tris pH 7.4, 190 mM NaCl, 10 mM EDTA, 2.9% Triton X-100), DNA was sheared by repeated passage through a 20-G needle, and the cell lysates centrifuged at $11,000 \times g$ for 10 min. R40.76 anti-ZO-1 monoclonal antibody [1] coupled to Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) was added to the supernatant and the samples rotated for 3 hr. Beads were washed as described previously [32] and resuspended in 1 × gel sample buffer. Gel samples were split into equal volumes and subjected to SDS-PAGE [20] on duplicate gels. One of the duplicate gels was dried, the corners marked with radioactive ink, and exposed to Kodak X-OMAT AR film (Kodak, Rochester, NY) overnight. The resulting autoradiogram was used as a template to excise ZO-1 containing bands from the dried gel which were solubilized using Solvable Tissue and Gel Solubilizer (NEN Research Products, Boston, MA) and ZO-1 phosphate determined by scintillation counting. The duplicate gel was processed for immunoblotting.

Immunoblotting

Quantitative immunoblots were performed as described (Stevenson, B.R., Richards, C.L., Howarth, A.G., Maraj, V.A., Hibbard, J.G. 1994. Quantitative immunoblot detection of rare proteins in whole cell extracts using biotin-streptavidin reagents. *J. Exp. Zool., in press*). Regions of the gel containing the proteins of interest, as delineated

by prestained molecular mass standards (Bio-Rad Laboratories, Richmond, CA), were excised and transferred onto 0.2 μm pore size nitrocellulose (Schleicher and Schuell, Keene, NH) as described [15]. ZO-1 blots were incubated in primary anti-ZO-1 monoclonal antibody R40.76 ascites diluted 1:1,000 overnight at room temperature. Biotin-conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:1,000 was then applied for 2 hr. Finally, blots were incubated for 30 min in 0.1 μCi/ml ^{125}I-streptavidin (Amersham Canada, Oakville, Ontario). Blots were air dried, marked with radioactive ink, and exposed to film. Autoradiograms were aligned with the nitrocellulose and protein bands of interest were marked by punching a needle through the film and nitrocellulose. Bands were cut out and ZO-1 quantified by direct counting of ^{125}I. Whole MDCK cell lysate samples were run on each gel to provide a standard curve to ensure linearity of response. Nonradioactive prestained standard bands on the same nitrocellulose were excised, counted and subtracted as background from all samples. Beta emissions from sample ^{32}P were not detectable in the gamma counter.

Quantification of Results

Degree of ZO-1 phosphorylation was determined from the ratio of ^{32}P counts to ^{125}I gamma counts for each sample. Data were normalized to the mean value of the ^{32}P/^{125}I ratios of MDCK cells grown in normal medium which were used as internal standards in each experiment. This mean was arbitrarily assigned a value of 1.0. Data were plotted with GraphPad InPlot 4.03 graphics software and unpaired Student's *t*-tests were carried out using GraphPad InStat 2.0 statistical software (GraphPad Software, San Diego, CA).

Proteolytic Digestions

^{32}P-labeled ZO-1 from MDCK cells grown in normal and low calcium media and from S180 cells was immunoprecipitated and resolved by SDS-PAGE. ZO-1 bands within the dried gel were visualized by autoradiography, excised, and the gel fragments placed into wells of an 8–18% gradient gel. V8 endoproteinase (Calbiochem, San Diego, CA) was added to the wells and digestion and electrophoresis performed as described [9, 14]. Gels were then dried and autoradiographed.

Immunofluorescence

Cells were paraformaldehyde fixed, methanol permeabilized and processed for immunofluorescence as described [15]. ZO-1 was visualized using the R40.76 monoclonal antibody (mAb) and an anti-rat IgG rhodamine conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Cells were examined on a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany) and photographed on TMAX ASA 400 film (Kodak) pushed to ASA 1600.

Results

Measurement of ZO-1 Phosphate Content

We have developed a simple and reproducible technique for quantifying the total phosphate content of ZO-1. The procedure outlined in Fig. 1 allows parallel determination of total protein and ZO-1 phosphorylation and takes advantage of the ability to segregate gamma radiation emitted by ^{125}I from the beta emissions of ^{32}P. Briefly, ZO-1 is immunoprecipitated from cells after incubation of the cells in ^{32}P-orthophosphate. The sample is split into equivalent aliquots, and one aliquot is processed for determination of radioactive phosphate bound to the ZO-1, while the other is immunoblotted to determine total protein. Immunoblots are incubated with monoclonal antibodies directed against ZO-1, biotinylated secondary antibody and ^{125}I-labeled streptavidin. The ^{32}P/^{125}I ratio determined for each sample represents the amount of phosphate on the protein. To eliminate variation due to differences in phosphate labeling and transfer efficiency of immunoblots, MDCK cells grown in calcium-containing medium were used in each experiment as internal standards. The ^{32}P/^{125}I ratios in all other cell types and conditions were then normalized to the mean of the MDCK cell ratios.

Calcium Chelation

A recent report indicates that the opening of tight junctions induced by removal of extracellular calcium is attenuated by protein kinase inhibitors [8]. Hence, we were interested in examining the effect of calcium chelation on ZO-1 phosphorylation. Exposure of MDCK cells grown on permeable substrates to 4 mM BAPTA causes TER to drop to approximately 20% of initial values within 5 min of treatment (Fig. 2*A*). Comparison of the phosphate content of ZO-1 in MDCK cells before and 5 min after BAPTA treatment reveals no significant change in the degree of ZO-1 phosphorylation (Fig. 2*B*).

Examination of the immunofluorescent distribution of ZO-1 in control MDCK cells shows the typical discrete and continuous network of ZO-1 staining surrounding each cell at the level of the tight junction (Fig. 2*C*). In BAPTA-treated cells, ZO-1 remains concentrated at the plasma membrane, but cells are beginning to pull apart (Fig. 2*D*). These gaps between cells are consistent with the observed drop in TER.

MDCK Cells Grown in Low Calcium

MDCK cells maintained in the absence of extracellular calcium have been shown to lack all intercellular contacts, including tight junctions [6, 25]. Upon comparison of MDCK cells grown in physiological levels of calcium with those grown three days in the absence of exogenously added calcium, a significant difference in the degree of ZO-1 phosphorylation is detected. The total phosphate content of ZO-1 in MDCK cells maintained in LCM is 85% of that in control cells (Fig. 3*A*).

Immunofluorescent localization of ZO-1 in MDCK cells grown in LCM shows a very different staining

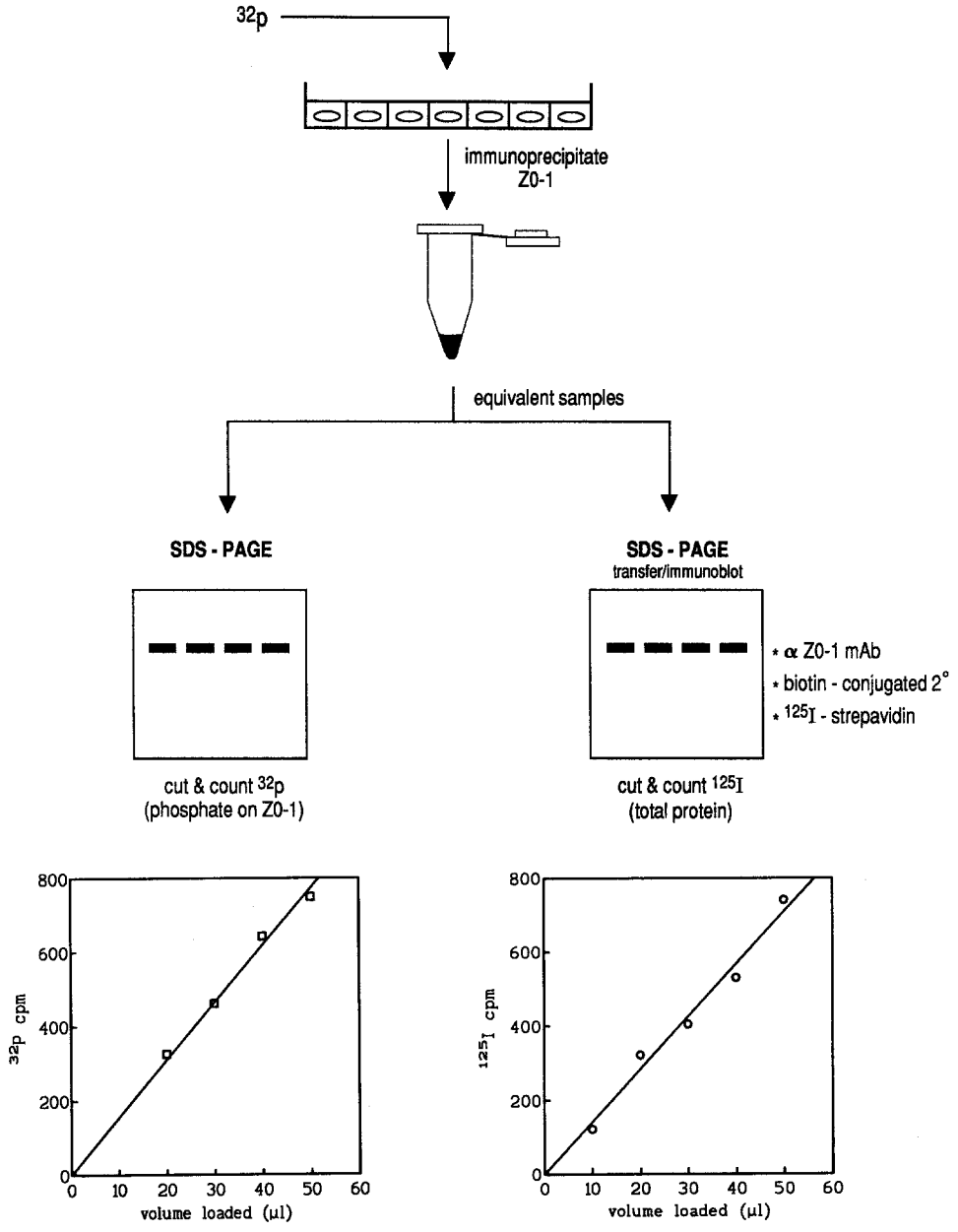

Fig. 1. Schematic diagram outlining the methodology for determining total phosphate content of ZO-1. Cells are labeled with ^{32}P-orthophosphate, and ZO-1 is immunoprecipitated from a total cell lysate. Samples are divided into equivalent aliquots and subjected separately to SDS-PAGE. ZO-1 bands from one gel are excised and counted to quantify ^{32}P. Protein from the second gel is transferred to nitrocellulose. The nitrocellulose is incubated sequentially with an anti-ZO-1 mAb, a biotin-conjugated secondary antibody and ^{125}I-streptavidin. ZO-1 bands from the nitrocellulose are excised and ^{125}I counted as a measure of total protein. ^{32}P/^{125}I ratios provide an indication of the total amount of phosphate on ZO-1. The bottom graphs provide examples from one experiment and demonstrate the linear relationships between sample volume loaded onto gels and cpm for both ^{32}P and ^{125}I.

pattern compared to control cells (*compare* Figs. 2*C* with 3*B*). ZO-1 in these contact naive cells is found in a variety of configurations, including both diffuse and punctate cytoplasmic distributions, with localization of the protein at the cell surface in some areas (Fig. 3*B*). The canonical discrete linear localization of ZO-1 at sites of cell-cell contact is never observed under these conditions.

Nonepithelial Cells

The presence of ZO-1 in cells that do not form tight junctions has recently been reported [15, 16]. We chose

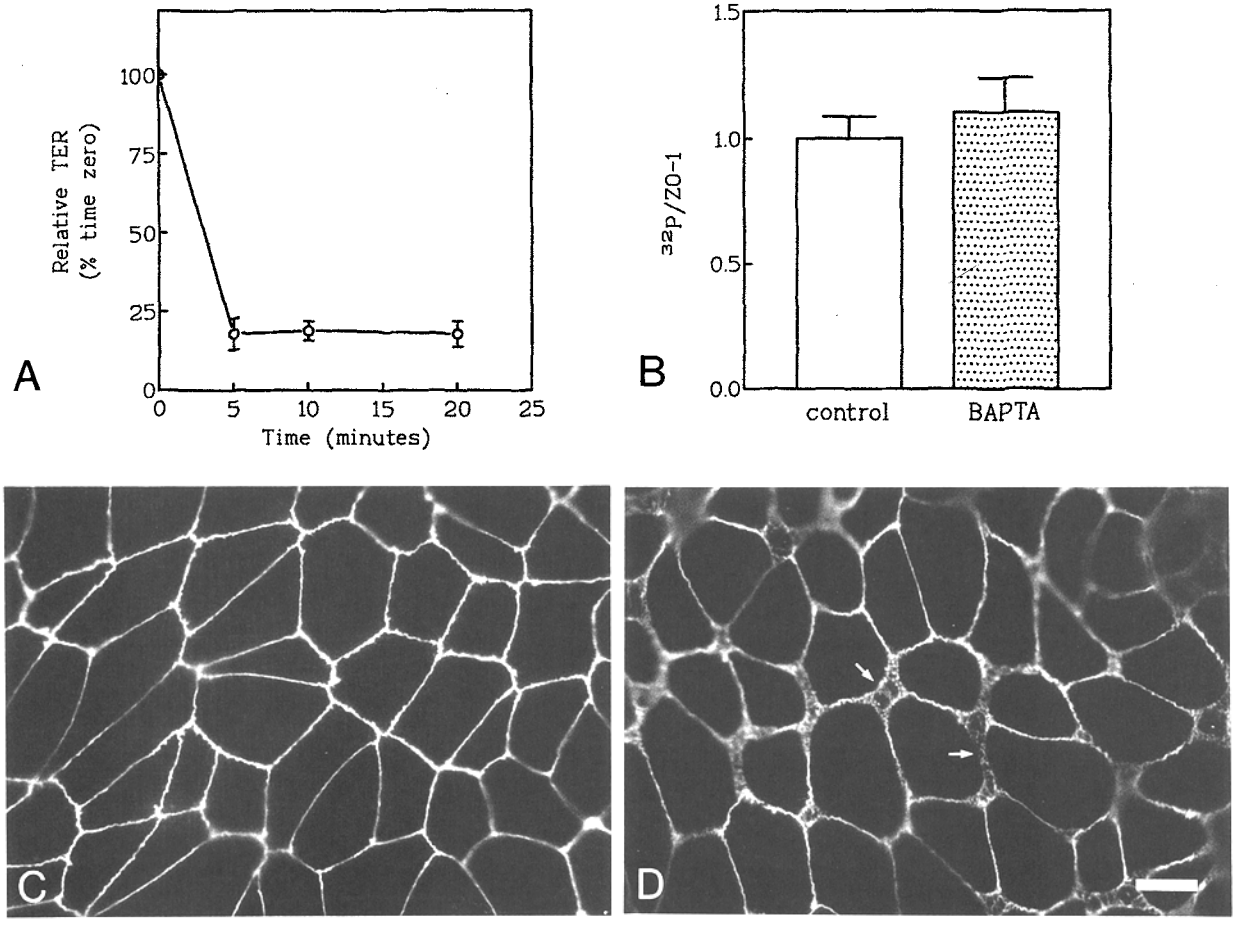

Fig. 2. Effect of calcium chelation on MDCK cell monolayers. (*A*) Following exposure to 4 mM BAPTA at $t = 0$, TER of MDCK cells drops to approximately 20% of initial values after 5 min. (*B*) Measurement of total phosphate content of ZO-1. ZO-1 recovered from control cells and cells treated with BAPTA for 5 min shows no significant difference in phosphorylation. Mean ± SEM: control, 1.00 ± 0.08; BAPTA-treated cells, 1.10 ± 0.14 ($n = 6$). (*C, D*) Immunofluorescent localization of ZO-1 in MDCK cells. (*C*) Control cells show discrete continuous ZO-1 staining concentrated around cell peripheries. (*D*) BAPTA-treated cells have begun to pull apart from one another (arrows), but ZO-1 remains tightly associated with the membrane in all areas. Bar, 10 μm.

to examine the phosphorylation state of ZO-1 in the S180 mouse sarcoma cell line and in the same cell line transfected with E-cadherin (S180L; [26]). No significant difference in total ZO-1 phosphorylation is detected between the S180 and the S180L cells; however, ZO-1 from both S180 and S180L cells contains significantly lower total phosphate than that from control MDCK cells (Fig. 4*A*).

Examination of the localization of ZO-1 in S180 and S180L cells by immunofluorescence reveals the protein diffusely distributed along the inner surface of the plasma membrane in both cell types. This localization was observed at both free cell surfaces and sites of cell-cell contact. ZO-1 also appears to be concentrated in some of the lamellipodia extending from these cells (Fig. 4*B* and *C*). These regions of the cell are likely to be engaged in actin-based motile activity. Interestingly, the presence of E-cadherin in the S180L cells, previously shown to be localized to sites of cell-cell contact [26], does not substantially alter the localization of ZO-1, although an apparent greater degree of cell-cell contact in this cell type gave the cell sheets a more epithelioid morphology with correspondingly enhanced fluorescence at the cell periphery (Fig. 4*C*).

Primary cultures of type I astrocytes derived from neonatal rat brain express high levels of ZO-1 [15], although these cells have not been reported to form tight junctions [5, 21]. Comparison of the phosphate content of ZO-1 derived from astrocytes to that from MDCK cells shows no significant difference (Fig. 5*A*). Figure 5*B* shows the immunofluorescent distribution of ZO-1 in these primary astrocyte cultures. As we reported previously, although there is some punctate cytoplasmic staining, most of the ZO-1 staining is concentrated at the

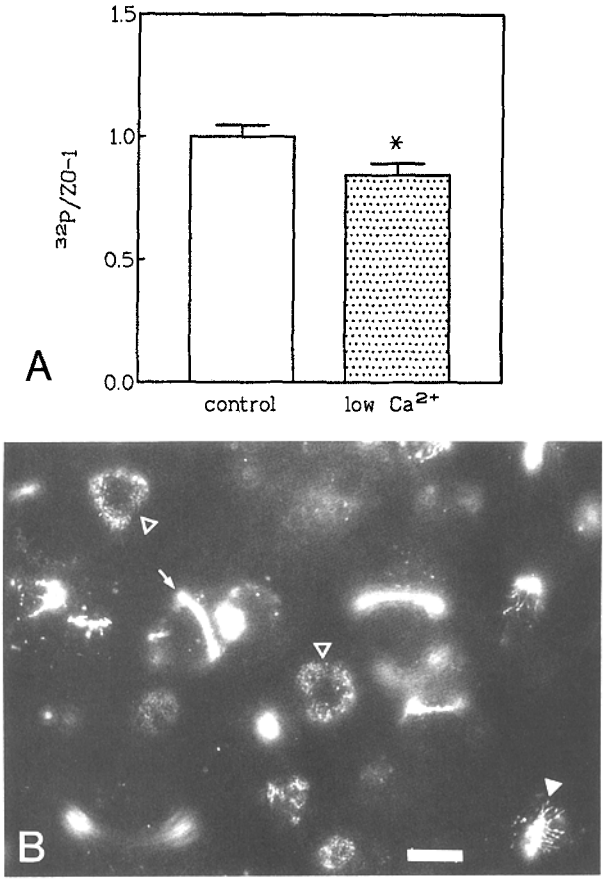

Fig. 3. Analysis of ZO-1 in MDCK cells maintained in low calcium. (*A*) Measurement of the total phosphate content of ZO-1. Analysis of ZO-1 recovered from cells grown in control medium (1.3 mM calcium) and LCM (~5 μM calcium) reveals a significantly ($*P < 0.03$) lower level of ZO-1 phosphorylation in the LCM cells. Mean ± SEM: control, 1.00 ± 0.03; LCM cells, 0.85 ± 0.04 ($n = 8$). (*B*) Immunofluorescent localization of ZO-1 in MDCK cells maintained in low calcium for three days. ZO-1 is found in a variety of localizations, including distinct bars (arrow) which sometimes show trailing lines of ZO-1 (filled triangle), and punctate labeling (open triangles). Diffuse staining of the cytoplasm is also visible within some cells. Bar, 10 μm.

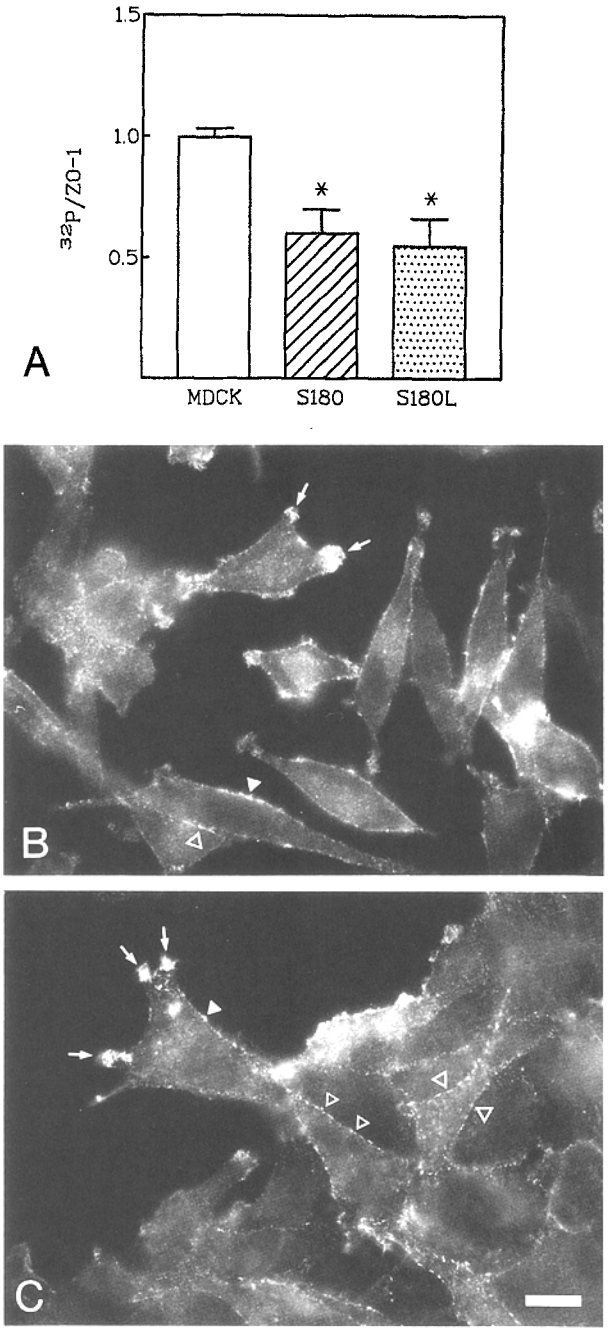

Fig. 4. Analysis of ZO-1 in S180 and S180L cells. (*A*) Measurement of the total phosphate content of ZO-1. ZO-1 from S180 and S180L cells shows significantly ($*P < 0.01$) lower levels of phosphorylation than that from control MDCK cells. No significant difference was observed between S180 and S180L cells. Mean ± SEM: MDCK cells, 1.00 ± 0.03; S180 cells, 0.60 ± 0.10; S180L cells, 0.55 ± 0.11 ($n = 8$). (*B, C*) Immunofluorescent localization of ZO-1 in S180 and S180L cells. (*B*) S180 cells show a fusiform morphology. (*C*) S180L cells pack together more tightly (lower right) and develop extensive areas of cell-cell contact. One cell can be seen extending into an open area at the edge of a cluster of cells. In both cell types, ZO-1 is distributed along free cell surfaces (filled triangles) and at sites where cells overlap each other (open triangles). It is also found concentrated in lamellipodia (arrows). Bar, 10 μm.

cell surface where cells make contact with their neighbors.

Phosphopeptide Analysis

Immunoprecipitation of ZO-1 from MDCK cells, S180 cells, and astrocytes demonstrated that ZO-1 is phosphorylated in all three cell types under the conditions examined (Fig. 6*A*). ZO-1 has been shown to be phosphorylated exclusively at serine residues [1], and reports of the full-length ZO-1 cDNA sequence [16, 38] demonstrate that there are 177 serine residues in the protein,

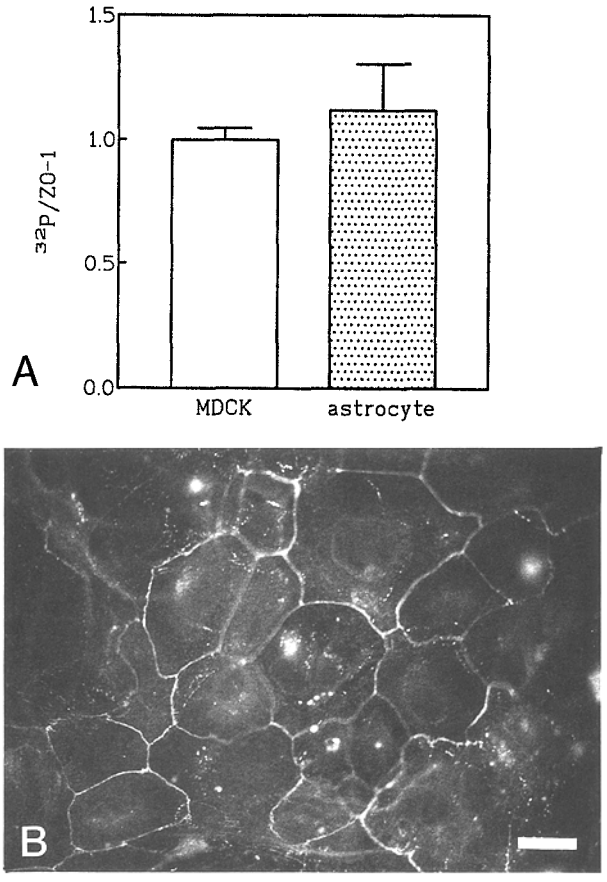

Fig. 5. Analysis of ZO-1 in primary cultures of neonatal rat astrocytes. (*A*) Measurement of the total phosphate content levels of ZO-1. ZO-1 from astrocytes shows no significant difference in phosphorylation relative to MDCK controls. Mean ± SEM: MDCK cells, 1.00 ± 0.04; astrocytes, 1.12 ± 0.18 (n = 11). (*B*) Immunofluorescent localization of ZO-1 in astrocyte cultures. Although some punctate staining is observed in the cell cytoplasm, ZO-1 appears to be tightly associated with the cell surface at sites of cell-cell contact. Bar, 25 μm.

although it is likely that only a subset of these are phosphorylated. In an initial effort to identify which of these sites may be important in the ZO-1 phosphorylation differences observed, one-dimensional proteolytic digests of ^{32}P-labeled ZO-1 from control MDCK cells, MDCK cells grown in LCM, and S180 cells, were performed. Both LCM MDCK and S180 cells have lower levels of total ZO-1 phosphorylation when compared to MDCK cells under normal growth conditions (Figs. 3 and 4).

Immunoprecipitated and electrophoretically purified ZO-1 was digested with a range of V8 protease concentrations, and the resultant fragments separated on an 8–18% gradient gel (Fig. 6*B, C*). A large number of labeled phosphopeptides are generated, and differences in banding patterns are discerned. ZO-1 derived from control MDCK cells shows bands at 67 and 12 kD that were absent or significantly reduced in ZO-1 derived from either MDCK cells maintained in LCM or S180 cells. These bands were identified on a number of separate digests, and were consistently found only in ZO-1 derived from MDCK cells in normal calcium. Differences in the relative intensities of other phosphopeptide bands are also apparent in comparing the digests from control MDCK cells with those from LCM MDCK and S180 cells. It is unlikely that the observed differences in these phosphopeptides are due to varying degrees of ZO-1 digestion between samples, as a wide range of stages of proteolytic degradation was examined. The large array of phosphopeptides present suggests that a significant proportion of the phosphorylation of ZO-1 does not change under the conditions examined.

Discussion

A large body of evidence indicates that regulation of protein phosphorylation plays a role in the control of tight junction permeability [2, 10, 11, 18, 28, 31]. Citi [8] recently made the observation that the opening of tight junctions that results from calcium chelation is suppressed by protein kinase inhibitors. The kinase inhibitor H7 has also been reported to slow the resealing of tight junctions in cells switched from low to normal calcium [30]. Given that the response of epithelial cells to calcium removal is likely to be initiated by calcium-dependent cell adhesion molecules (cadherins), these results suggested that phosphorylation of some component in a pathway from cadherins to tight junctions is important for this response. To date, no information is available about which, if any, of the known tight junctional components may play a role in this regard. We have investigated the phosphorylation state and distribution of ZO-1 in epithelial cells under two conditions of extracellular calcium level modulation, and also in two cell types which do not form tight junctions but do contain ZO-1. Our results demonstrate that the phosphorylation state of ZO-1 changes, but these changes correlate with the distribution of ZO-1 within cells rather than with the expression of E-cadherin.

Previous results demonstrated that ZO-1 is phosphorylated at serine residues under normal conditions. Analysis of the ZO-1 cDNA sequence [16, 38] reveals 177 serine residues in the protein, 33 of which fit into a consensus sequence for casein kinase II [33] and 34 into the consensus sequence for protein kinase C [19]. The broad display of phosphopeptides in the proteolytic digests (Fig. 6) suggests that a significant proportion of these residues are phosphorylated in ZO-1. The technique that we use to detect changes in ZO-1 phosphorylation (Fig. 1) is very reproducible, but measures total phosphate content of the protein and therefore would

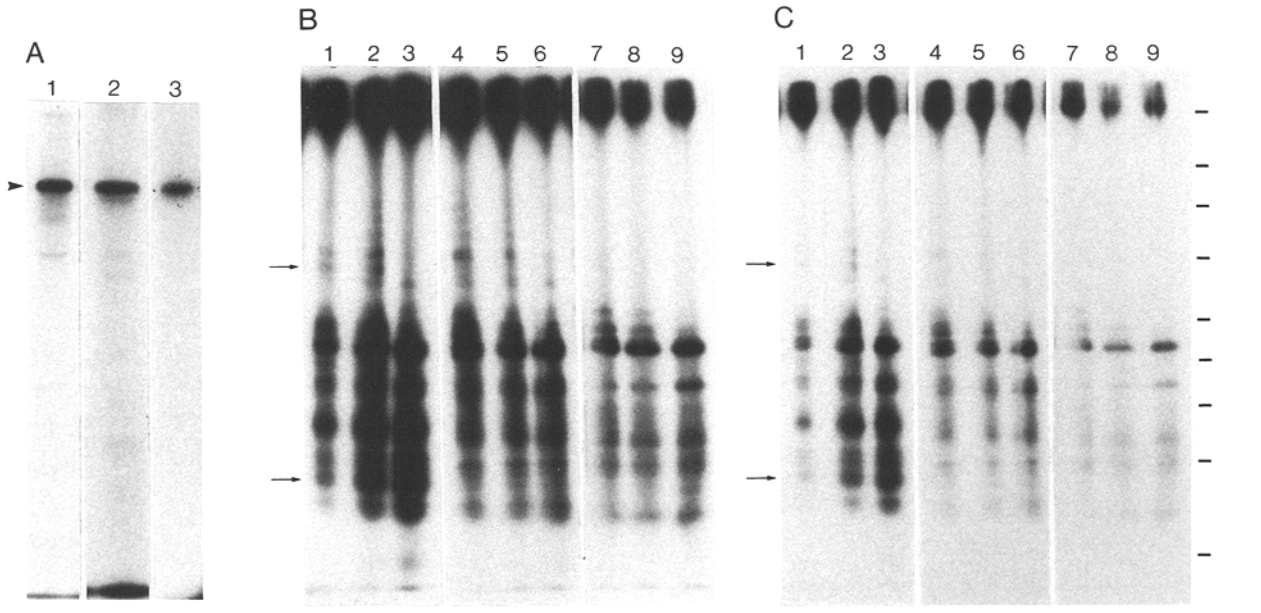

Fig. 6. One-dimensional proteolytic digest analysis of ^{32}P-labeled ZO-1 from control and LCM-maintained MDCK cells and from S180 cells. (*A*) Autoradiograph of ZO-1 immunoprecipitated from ^{32}P-labeled MDCK cells (lane *1*), S180 cells (lane *2*), and astrocytes (lane *3*) shows that ZO-1 is phosphorylated in all three cell types under these conditions. (*B, C*) Two autoradiographic exposures of a digest of ZO-1 from control MDCK cells (lanes *1, 2, 3*), MDCK cells maintained in LCM for three days (lanes *4, 5, 6*), and S180 cells (lanes *7, 8, 9*). Amounts of V8 protease used: lanes *1, 4,* and *7,* 0.05 μg; lanes *2, 5,* and *8,* 0.1 μg; lanes *3, 5,* and *9,* 0.5 μg. Arrows mark positions of 12 and 67 kD phosphopeptides present only in control MDCK cells. Molecular mass standards marked by bars at far right, in kD: 200, 116, 97, 66, 45, 31, 21.5, 14.5, 6.5.

have reduced sensitivity to changes in the phosphorylation of a small number of residues.

The observation that ZO-1 in a low resistance strain of MDCK cells is more highly phosphorylated than that from a high resistance strain [37] raises the possibility that phosphorylation of this protein is involved in the regulation of tight junction permeability. The chelation of extracellular calcium with BAPTA causes a rapid opening of the tight junction in MDCK cells; however, this junctional opening is not reflected by any significant difference in the degree of total ZO-1 phosphorylation (Fig. 2). These results suggest that ZO-1 is not the target of the inhibitable kinase(s) implicated by the work of Citi [8]. It should be noted that the rapid removal of calcium by BAPTA may act on the junction in a manner that bypasses the normal tight junction regulatory machinery. In what may be a more physiologically relevant paradigm, the large increase in TER resulting from glucocorticoid treatment of the mammary epithelial cell line 31EG4 [39] is also not accompanied by a change in the total phosphate content of ZO-1 or in the phosphopeptide digest pattern of ZO-1 (K.L. Singer et al., *manuscript submitted*). Hence, it would appear that there is no clear relationship between TER and ZO-1 phosphorylation, at least in the paradigms examined to date. However, we cannot rule out the possibility that phosphorylation of ZO-1 may be involved in an alternate pathway(s) regulating junctional permeability.

MDCK cells maintained in low calcium medium for three days provide a paradigm where intercellular contacts, including tight junctions, are absent, and ZO-1 undergoes a drastic redistribution [35]. Under these conditions, we observe a small but reproducible and significant decrease in the total phosphate content of ZO-1 (Fig. 3). We also observe significantly lower levels of phosphorylation on ZO-1 derived from the nonepithelial, non-tight junction-forming S180 and S180L cells (Fig. 4), suggesting that a higher phosphate content of ZO-1 associated with the tight junction may be significant. However, astrocytes, another cell type which are not known to form tight junctions, show no difference in ZO-1 phosphorylation when compared to control MDCK cells (Fig. 5).

The immunofluorescent localization of ZO-1 varies in the cells and conditions examined. In cases where ZO-1 is concentrated at cell-cell contact sites, no differences in ZO-1 phosphorylation are observed. The normal discrete and continuous staining pattern observed in control cells is minimally disrupted in MDCK cells treated with BAPTA: ZO-1 remains tightly associated with the plasma membrane, presumably at the tight junction, even as cells begin to separate from their neighbors (Fig. 2). Astrocytes, which also show no

difference in the phosphorylation state of the protein when compared to control MDCK cells, also primarily display concentration of ZO-1 at the plasma membrane at sites of intercellular contact (Fig. 5). Finally, the 31EG4 mammary epithelial cells, which show a glucocorticoid-induced increase in TER and no change in total ZO-1 phosphorylation, also display a ZO-1 staining pattern similar to MDCK controls before and after glucocorticoid treatment (K.L. Singer et al., *manuscript submitted*). Alternatively, in cells where lower levels of total ZO-1 phosphorylation are observed relative to MDCK cell controls, ZO-1 is visualized in atypical locations. MDCK cells maintained in LCM show more diffuse ZO-1 staining both within the cytoplasm and associated with the cell surface (Fig. 3). Punctate staining is also observed throughout the cell. Similarly, S180 and S180L cells, which show the lowest overall level of ZO-1 phosphorylation, show diffuse ZO-1 labeling of the entire cell surface, and concentration of the protein in the cytoplasm associated with lamellipodia (Fig. 4). Taken together, these results suggest that altered levels of ZO-1 phosphorylation may be related to the location of the protein within the cell; only when concentrated at sites of cell-cell contact is ZO-1 fully phosphorylated. However, further investigation is clearly necessary to determine whether differences in the phosphorylation of specific sites on ZO-1 is a result of or a signal for localization changes.

Comparison of the one-dimensional proteolytic digest patterns of ^{32}P-labeled ZO-1 from those cells in which we see differences in total ZO-1 phosphorylation indicates that changes in the phosphorylation of specific peptides can be detected under these conditions (Fig. 6). It is evident that ZO-1 contains numerous phosphorylated residues, resulting in the large number of labeled phosphopeptides obtained. The digest patterns obtained from all three cell types are very similar. However, at least two peptides that were phosphorylated in the control cells are absent or significantly reduced in MDCK cells maintained in LCM and in S180 cells, consistent with lower total ZO-1 phosphorylation. The high degree of similarity in the banding patterns obtained from S180 cells and MDCK cells maintained in LCM indicates that the additional phosphopeptides found in control MDCK cells are unlikely to be due to nonspecific kinase activity. Isolation and characterization of these differentially labeled phosphopeptides may provide information on what sites within ZO-1 are important in phosphate exchange.

It has been suggested that ZO-1 localizes to cadherin-based junctions when no tight junction is present [16]. We compared the localization of ZO-1 in S180 and S180L cells, and found that the expression of E-cadherin in S180L cells was not sufficient to cause a redistribution of ZO-1, although S180L cells have been reported to form adherens-type junctions in response to the expression of E-cadherin [26]. This result suggests that factors other than cadherins are required to obtain localization of ZO-1 at adherens junctions.

Acute opening of the tight junction by calcium chelation, believed to be mediated by cadherins, had no immediate effect on ZO-1 distribution or its phosphorylation state. Only after extended periods in LCM, conditions where the tight junction is completely disassembled, does the phosphorylation state of ZO-1 change. In addition, expression of E-cadherin in S180L cells had no apparent effect on ZO-1 localization or its phosphorylation state. Taken together, these results suggest that ZO-1 distribution and phosphorylation are not directly effected by E-cadherin. Although these findings do not preclude a role for E-cadherin in the localization of ZO-1 at tight or adherens junctions, they suggest that the relationship may be indirect.

The concentration of ZO-1 in the lamellipodia of S180 and S180L cells is particularly interesting in light of reports that ZO-1 binds spectrin [17]. It may be that ZO-1 functions in some capacity in modulation of the actin cytoskeleton in these regions of cell motility. Our findings are consistent with observations that ZO-1 is found at the actin-based "purse string" contraction belt during epithelial wound repair [3], and at actin-based adherens junctions [16]. It is also clear from the S180 and S180L immunofluorescent images that cell-cell contact is not required for ZO-1 to move to the cell surface in these cells, as previously believed [35].

In summary, we report here a comparison of the phosphorylation state of ZO-1 found in varying cellular distributions, and provide evidence that ZO-1 can undergo specific changes in phosphate content. In addition, we demonstrate that localization of ZO-1 at the cell surface is not dependent on cell-cell contact, and that expression of E-cadherin alone within cells does not cause a redistribution of ZO-1 or a substantial change in the phosphorylation state of the molecule. Finally, ZO-1 can be concentrated in regions where actin filaments are engaged in motility, suggesting a direct or indirect association between ZO-1 and this cytoskeletal system.

We would like to thank Cheryl Richards for her help with the cell culture and immunohistochemistry; David Begg, Gary Firestone, Vik Maraj, Manijeh Pasdar and Colin Rasmussen for helpful discussions; Jaclyn Peebles and Greg Morrison for help with graphics and photography; and Grace Martin and Bob Campenot for rat tail collagen. We are grateful to all the members of our laboratories for their friendship, advice and support. This work was supported by an Establishment Award to B.R.S. from the Alberta Heritage Foundation for Medical Research and grants to B.R.S. from the Kidney Foundation of Canada and the Medical Research Council of Canada. A.H. is funded by a Studentship from the AHFMR. K.L.S. was supported by a grant from the National Institutes of Health (DK-42799) to Gary L. Firestone. B.R.S. is a Medical Research Council of Canada and AHFMR Scholar.

References

1. Anderson, J.M., Stevenson, B.R., Jesaitis, L.A., Goodenough,

D.A., Mooseker, M.S. 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

2. Balda, M.S., Gonzalez-Mariscal, L., Contreras, R.G., Macias-Silva, M., Torres-Marquez, M.E., Garcia Sainz, J.A., Cereijido, M. 1991. Assembly and sealing of tight junctions: Possible participation of G-proteins, phospholipase C, protein kinase C and calmodulin. *J. Membrane Biol.* **122:**193–202
3. Bement, W.M., Forscher, P., Mooseker, M.S. 1993. A novel cytoskeletal structure involved in purse string wound closure and cell polarity maintenance. *J. Cell Biol.* **121:**565–578
4. Bentzel, C.H., Hainau, B., Ho, S., Hui, S.W., Edelman, A., Anagnostopoulos, T., Benedetti, E.L. 1980. Cytoplasmic regulation of tight junction permeability: effect of plant cytokinins. *Am. J. Physiol.* **239:**C75–C89
5. Brightman, M.W., Reese, T.S. 1969. Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* **40:**648–677
6. Cereijido, M., Robbins, E.S., Dolan, W.J., Rotunno, C.A., Sabatini, D.D. 1978. Polarized monolayers formed by epithelial cells on permeable and translucent support. *J. Cell Biol.* **77:**853–880
7. Citi, S. 1993. The molecular organization of tight junctions. *J. Cell Biol.* **121:**485–489
8. Citi, S. 1992. Protein kinase inhibitors prevent junction dissociation induced by low extracellular calcium in MDCK cells. *J. Cell Biol.* **117:**169–178
9. Cleveland, D.W., Fischer, S.G., Kirschner, M.W., Laemmli, U.K. 1977. Peptide mapping by limited proteolysis in sodium dodecylsulfate and analysis by gel electrofocusing. *J. Biol. Chem.* **252:**1102–1106
10. Duffey, M.E., Hainau, B., Ho, S., Bentzel, C.J. 1981. Regulation of epithelial tight junction permeability by cyclic AMP. *Nature* **294:**451–453
11. Ellis B., Schneeberger, E.E., Rabito, C.A. 1992. Cellular variability in the development of tight junctions after activation of protein kinase C. *Am. J. Physiol.* **263:**F293–F300
12. Gumbiner, B. 1990. Generation and maintenance of epithelial cell polarity. *Curr. Op. Cell Biol.* **2:**881–887
13. Gumbiner, B., Lowenkopf, T., Apatira, D. 1991. Identification of a 160-kD polypeptide that binds to the tight junction protein ZO-1. *Proc. Natl. Acad. Sci. USA* **88:**3460–3464
14. Harlow, E., Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Publications, Cold Spring Harbor, NY
15. Howarth, A.G., Hughes, M.R., Stevenson, B.R. 1992. Detection of the tight junction-associated protein ZO-1 in astrocytes and other nonepithelial cell types. *Am. J. Physiol.* **262:**C461–C469
16. Itoh, M., Nagafuchi, A., Yonemura, S., Kitani-Yasuda, T., Tsukita, S., Tsukita, S. 1993. The 220-kD protein colocalizing with cadherins in non-epithelial cell is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J. Cell Biol.* **121:**491–502
17. Itoh, M.S., Yonemura, A., Nagafuchi, S., Tsukita, S., Tsukita, S. 1991. A 220-kD undercoat-constitutive protein: its specific localization at cadherin-based cell-cell adhesion sites. *J. Cell Biol.* **115:**1449–1462
18. Jacobson, H.R. 1979. Altered permeability in the proximal tubule response to cyclic cAMP. *Am. J. Physiol.* **236:**F71–F79
19. Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., Nishizuka, Y. 1985. Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **260:**12492–12499
20. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685
21. Landis, D.M.D., Weinstein, L.A., Skordeles, C.J. 1990. Serum influences the differentiation of membrane structure in cultured astrocytes. *Glia* **3:**212–221
22. Lillien, L.E., Sendtner, M., Rohrer, H., Hughes, S.M., Raff, M.C. 1988. Type-2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes. *Neuron* **1:**485–494
23. Madara, J.L. 1987. Intestinal absorptive cell tight junctions are linked to cytoskeleton. *Am. J. Physiol.* **253:**C171–C175
24. Madara, J.L., Moore, R., Carlson, S. 1987. Alteration of intestinal tight junction structure and permeability by cytoskeletal contraction. *Am. J. Physiol.* **253:**C854–C861
25. Martinez-Palomo, A., Meza, I., Beaty, G., Cereijido, M. 1980. Experimental modulation of occluding junctions in a cultured transporting epithelium. *J. Cell Biol.* **87:**736–745
26. Mege, R.M., Matsuzaki, F., Gallin, W.J., Goldberg, J.I., Cunningham, B.A., Edelman, G.M. 1988. Construction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* **85:**7274–7278
27. Meza, I., Ibarra, G., Sabenero, M., Martinez-Palomo, A., Cereijido, M. 1980. Occluding junctions and cytoskeletal components in a cultured transporting epithelium. *J. Cell Biol.* **87:**746–754
28. Mullin, J.M., Snock, K.V., Shurina, R.D., Noe, J., George, K., Misner, L., Imaizumi, S., O'Brien, T.G. 1992. Effects of acute vs. chronic phorbol ester exposure on transepithelial permeability and epithelial morphology. *J. Cell. Physiol.* **152:**35–47
29. Nelson, W.J., Veshnock, P.J. 1987. Modulation of fodrin (membrane skeleton) stability by cell-cell contact in Madin-Darby canine kidney cells. *J. Cell Biol.* **104:**1527–1537
30. Nigam, S.K., Denisenko, N., Rodriguez-Boulan, E., Citi, S. 1991. The role of phosphorylation in development of tight junctions in cultured renal epithelial (MDCK) cells. *Biochem. Biophys. Res. Commun.* **181:**548–553
31. Ojakian, G.K. 1981. Tumor promoters-induced changes in permeability of epithelial tight junctions. *Cell* **23:**95–103
32. Pasdar, M., Nelson, W.J. 1989. Regulation of desmosome assembly in epithelial cells: kinetics of synthesis, transport, and stabilization of desmoglein I, a major protein of the membrane core domain. *J. Cell Biol.* **109:**163–178
33. Pinna, L.A. 1990. Casein kinase 2: an 'eminence grise' in cellular regulation? *Biochim. Biophys. Acta* **1054:**267–284
34. Sedar, A.W., Forte, J.G. 1964. Effects of calcium depletion on the junctional complex between oxyntic cells of gastric mucosa. *J. Cell Biol.* **22:**173–188
35. Siliciano, J.D., Goodenough, D.A. 1988. Localization of the tight junction protein, ZO-1, is modulated by extracellular calcium and cell-cell contact in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* **107:**2389–2399
36. Stevenson, B.R., Anderson, J.M., Bullivant, S. 1988. The epithelial tight junction: Structure, function, and preliminary biochemical characterization. *Mol. Cell. Biochem.* **83:**129–145
37. Stevenson, B.R., Anderson, J.M., Braun, I.D., Mooseker, M.S. 1989. Phosphorylation of the tight junction protein ZO-1 in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. *Biochem. J.* **263:**597–599
38. Willott, E., Balda, M.S., Fanning, A.S., Jameson, B., Van Itallie, C., Anderson, J.M. 1993. The tight junction protein ZO-1 is homologous to the *Drosophila* discs-large tumor suppressor protein of septate junctions. *Proc. Natl. Acad. Sci. USA* **90:**7834–7838
39. Zettl, K.S., Sjaastad, M.D., Riskin, P.M., Parry, G., Machen, T.E., Firestone, G.L. 1992. Glucocorticoid-induced formation of tight junctions in mouse mammary epithelial cells in vitro. *Proc. Natl. Acad. Sci. USA* **89:**9069–9073