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The junction-associated protein, zonula occludens-1, localizes to the nucleus before the maturation and during the remodeling of cell–cell contacts

(tight junctions/intercellular junctions/membrane-associated guanylate kinase homologues/wound healing)

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ABSTRACT **The junction-associated protein zonula occludens-1 (ZO-1) is a member of a family of membrane-associated guanylate kinase homologues thought to be important in signal transduction at sites of cell–cell contact. We present evidence that under certain conditions of cell growth, ZO-1 can be detected in the nucleus. Two different antibodies against distinct portions of the ZO-1 polypeptide reveal nuclear staining in subconfluent, but not confluent, cell cultures. An exogenously expressed, epitope-tagged ZO-1 can also be detected in the nuclei of transfected cells. Nuclear accumulation can be stimulated at sites of wounding in cultured epithelial cells, and immunoperoxidase detection of ZO-1 in tissue sections of intestinal epithelial cells reveals nuclear labeling only along the outer tip of the villus. These results suggest that the nuclear localization of ZO-1 is inversely related to the extent and/or maturity of cell contact. Since cell–cell contacts are specialized sites for signaling pathways implicated in growth and differentiation, we suggest that the nuclear accumulation of ZO-1 may be relevant for its suggested role in membrane-associated guanylate kinase homologue signal transduction.**

Zonula occludens-1 (ZO-1) is a 210- to 225-kDa peripheral membrane protein of unknown function. It is found associated with the cytoplasmic surfaces of tight junctions (1, 2), cell–cell contacts of cultured nonepithelial brain astrocytes (3), and the intercalated disks (a modified adherens junction) of cardiac myocytes (4). Localization to these structures seems to require established cell–cell contacts, since treatments that prevent the homotypic interaction of E-cadherins (5, 6) or block downstream signaling from this interaction (7) inhibit recruitment of ZO-1 to the margin of cell–cell contact.

ZO-1 has recently been identified as a member of a family of putative signaling proteins called membrane-associated guanylate kinase homologues (MAGUKs; ref. 8). The founding member of this family is the product of the lethal (1) discs-large-1 (*dlg*) tumor suppressor gene of *Drosophila* (9). Loss-of-function alleles of *dlg* affect junction formation and result in the overgrowth of imaginal wing disc epithelia, suggesting that this junction associated protein may play a role in epithelial growth control. Other members of this family include: ZO-2, a second tight junction-associated protein (10); PSD-95/SAP-90 and SAP-97, which localize to synaptic junctions (11, 12); p55, which participates in erythrocyte membrane-cytoskeletal interactions (13); the lin 2 gene product of *Caenorhabditis elegans* (14); and a human homologue of dlg, hdlg (15).

MAGUK family members are generally associated with the plasma membrane and cytoskeletal elements at specialized sites of cell–cell contact. They manifest a conserved modular organization of domains that show homology to functionally defined signaling molecules: a src homology region 3 (SH3 domain), a region homologous to guanylate kinases, and an 80- to 90-aa GLGF motif (termed PDZ domains, for PSD-95/Discs-large/ZO-1; originally described by M. Kennedy, California Institute of Technology, Pasadena, CA). Recent evidence suggests that PDZ domains mediate interactions with certain plasma membrane proteins and may enable MAGUKs to crosslink certain transmembrane proteins to the cortical cytoskeleton (16, 17). Moreover, ZO-1 uniquely contains a region of proline-rich stretches, suggesting potential SH3 domain binding sites, as have been identified in several guanine nucleotide exchange factors, four consensus sites for tyrosine phosphorylation/SH-2 domain binding, alternative splicing domains, and a leucine zipper motif (reviewed in ref. 18). MAGUKs are currently thought to contribute to the structural organization of components mediating particular signal transduction events (19).

With such a compelling repertoire of signaling domains and the knowledge that loss-of-function mutations of one MAGUK family member (*dlg*) produces an overgrowth phenotype, it seems likely that ZO-1 plays a role in transducing signals from the margin of cell contact to downstream effectors. We present evidence that ZO-1 can accumulate in the cell's nucleus, in addition to sites of cell–cell contact. While the functional significance of the nuclear localization is presently unclear, we demonstrate that these distinct subcellular distributions of ZO-1 are exquisitely sensitive to the state of cell–cell contact.

MATERIALS AND METHODS

Cell Culture and Transfection. All cells (MDCK, MSV-MDCK, LLC-PK1, and CV-1) were maintained in a humidified incubator at 37°C under 10% CO_2 atmosphere in minimal essential medium (DMEM) that was supplemented with 10% fetal calf serum, 50 units of penicillin per ml, 50 μg of streptomycin per ml, and 2 mM L-glutamine. Transfections were carried out as described (20).

Plasmid Construction. The human cDNA for the α+ isoform of ZO-1 (8) was kindly provided by J. Anderson (Yale University) and subcloned into the mammalian expression vector pCB6 (kindly provided by M. Roth, University of Texas Southwestern Medical Center, Dallas; ref. 21). ZO-1 was tagged by incorporating an oligonucleotide encoding 13 C-

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Abbreviations: ZO-1, zonula occludens-1; MAGUK, membrane-associated guanylate kinase homologue; NLS, nuclear localization sequence.

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terminal amino acids of the Sendai virus L protein upstream from the stop codon (22).

Indirect Immunofluorescence. Cells were fixed for 25 min with freshly prepared 3.0% paraformaldehyde (wt/vol) in PBS (pH 7.4), supplemented with Ca^{2+}/Mg^{2+} and permeabilized with a PBS buffer containing 0.3% Triton X-100 and 0.3% BSA for 5 min. All antibody incubations and washes were carried out using 0.3% BSA in PBS. Cells were incubated for 1 hr at room temperature with either of the following primary antibodies: anti-ZO-1 rat monoclonal (40.76; kindly provided by M. Mooseker, Yale University) or anti-ZO-1 rabbit polyclonal (cat. no. 61–7300, lot no. 40921602; Zymed). For anti-ZO-1 rat monoclonal antibody, the ascites were HPLC-purified; the protein concentration of the IgG fraction was determined to be 0.2 mg/ml. We diluted this antibody 1:30. Anti-ZO-1 rabbit polyclonal antibody (1:300) was affinity-purified by the manufacturer from rabbit antiserum using the 69-kDa fusion protein against which the antibody was raised (23). This fusion protein corresponds to amino acids 463-1109 of the human ZO-1 cDNA (8). Anti-Sendai virus mAb (22) was a kind gift of J. Neubert (Max-Planck-Institut für Biochemie, Martinsried, Germany). Rabbit polyclonal antibody was raised against the 13-aa C terminus of the Sendai virus L protein. Texas Red-conjugated anti-rabbit IgG (Amersham) and fluorescein isothiocyanate-conjugated anti-rat IgG (Southern Biotechnology Associates) secondary antibodies were used for 1 hr at room temperature at a 1:100 dilution. Detergent extraction before fixation experiments were performed as described by Kreis (24). Immunoperoxidase experiments were performed on 10% phosphate-buffered, formalin-fixed, paraffin-embedded tissues according to standard protocols. Signal amplification was performed with a streptavidin/biotin system (Vector Laboratories). Anti-rabbit biotinylated IgG (catalog no. RPN.1004; Amersham) was used at 1:100; streptavidin–horseradish peroxidase (catalog no. ORO3L; Oncogene Science) was diluted 1:200 in PBS. Peroxidase activity was visualized using 3-amino-9-ethylcarbazole (Immunotech). *En face* immunofluorescence images were visualized using the Zeiss Axiophot microscope and photographed using Kodak T-MAX 3200 ASA and Ektachrome films (Eastman Kodak).

SDS/PAGE and Western Blotting. Procedures were carried out using standard methods (25, 26). Blots were incubated with either goat anti-mouse, anti-rabbit, or anti-rat secondary antibodies conjugated to horseradish peroxidase (working dilution 1:2000; Sigma). Labeled proteins were visualized with the enhanced chemiluminescence detection method (ECL; Amersham).

RESULTS

Localization of ZO-1 in Confluent and Nonconfluent Cell Cultures. The tight junction localizing protein ZO-1 characteristically forms a continuous band around the apices of well-differentiated, confluent, polarized epithelial cells in culture [Fig. 1 *A* (MDCK cells) and *C* (LLC-PK1 cells)]. However, under nonconfluent conditions, endogenous ZO-1 can localize to the nucleus in addition to the margin of cell–cell contact (Fig. 1 *B* and *D*). This staining corresponds to the structure labeled with the DNA-binding dye 4′,6-diamidino-2-phenylindole (DAPI; data not shown). Moreover, optical sectioning in the *xz* or *xy* direction using a confocal laser scanning microscope confirms that the staining is throughout the nucleus and not just localized to the nuclear membrane (data not shown). This nuclear staining is detected with two different antibodies that recognize distinct portions of the ZO-1 polypeptide; the rat monoclonal antibody R4O.76 recognizes the C-terminal region of ZO-1 (Fig. 1) while the rabbit polyclonal antibody 7445 was raised against a bacterial fusion protein encoding the guanylate kinase region (Figs. 2–5). Both antibodies recognize a single 220-kDa molecular species in

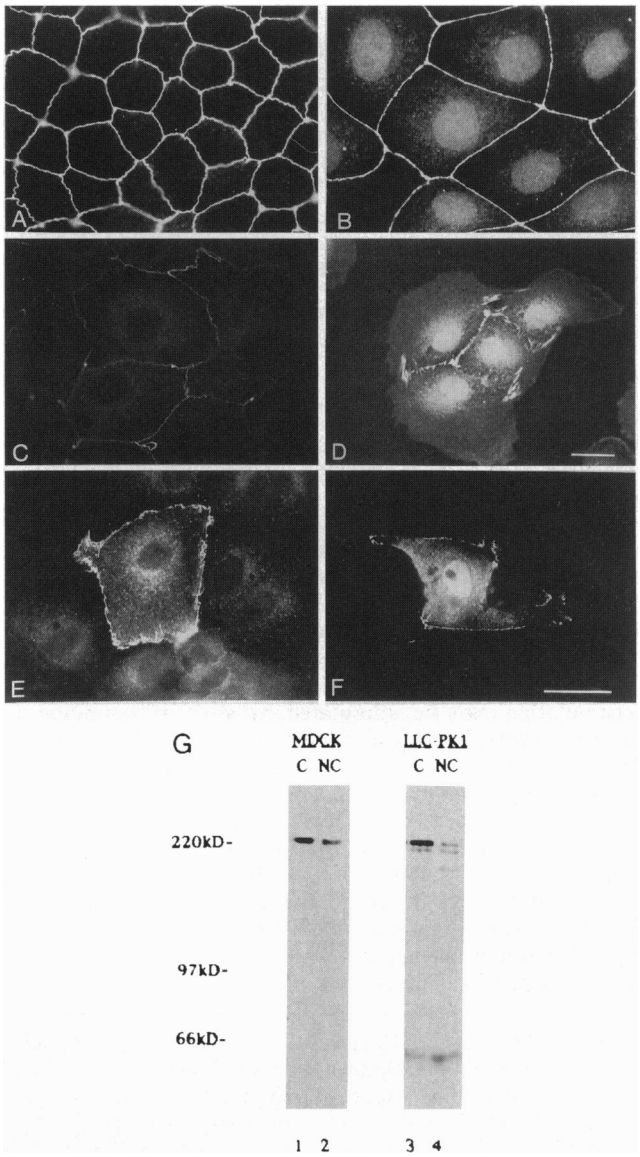

FIG. 1. Subcellular distribution of endogenous and epitope-tagged ZO-1 in confluent and subconfluent polarized epithelial cell cultures. MDCK and LLC-PK1 cells were plated at confluent and subconfluent densities on glass coverslips before being fixed and permeabilized for immunofluorescence analysis using the anti-ZO-1 rat monoclonal antibody R4O.76 as described. (*A*) MDCK cells plated and maintained under confluent conditions for at least 4 days; note that ZO-1 localizes exclusively to the apical margin of cell–cell contact. (*B*) MDCK cells plated under subconfluent conditions (cell islands of ≈10 cells) and prepared for immunofluorescence analysis between 24 and 48 hr after seeding. Note ZO-1 labeling of nuclei in addition to points of cell–cell contact. (*C* and *D*) LLC-PK1 cells plated at confluent (*C*) and subconfluent (*D*) densities. (*E* and *F*) LLC-PK1 cells transfected with an epitope-tagged human ZO-1 cDNA. (*E*) Transfected cell stained with the anti-Sendai monoclonal antibody. Note that the tagged protein is capable of being localized to the cell margin at points of cell–cell contact. (*F*). Transfected cell detected with an affinity purified anti-Sendai polyclonal antibody. Note that in this transfected cell, epitope tagged ZO-1 localizes to both the nucleus and the margin of cell contact. Bar lengths in D and F measure 10 and 20 μm, respectively. (*G*) Antibody specificity (antibody 7445) and relative amounts of ZO-1 in confluent (C) and nonconfluent (NC) cell cultures. Note that there is less ZO-1 per μg of total protein from nonconfluent versus confluent cultures.

MDCK cells by SDS/PAGE and Western analysis (Fig. 1*G*, lanes 1 and 2). ZO-1 migrates as a doublet in LLC-PK1 cells (lanes 3 and 4) and represents the two isoforms ($\alpha+/\alpha-$)

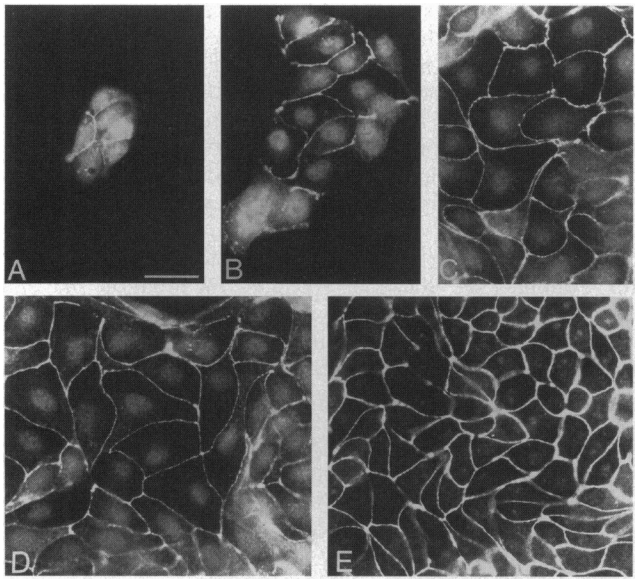

FIG. 2. ZO-1 is accumulated in the nucleus 24 hr after cell plating. MDCK cells were trypsinized and seeded at subconfluent density on glass coverslips. Cells were prepared for immunofluorescence analysis 12 (*A*), 24 (*B*), 36 (*C*), 48 (*D*), and 72 (*E*) hr after plating and stained with the anti-ZO-1 polyclonal antibody 7445. Note that nuclear accumulation is detected ≈24 hr postplating and persists until cells become increasingly confluent. (Bar = 20 μm.)

known to be expressed in this cell type (23). It is interesting to note that nuclear staining is consistently detected under conditions where cells actually contain less ZO-1 per microgram of total cellular protein (Fig. 1*G*, compare lanes 1 with 2 and lanes 3 with 4). This would argue against the idea that nuclear localization is a consequence of high levels of endogenous ZO-1 expression.

To provide further evidence that it is ZO-1, and not simply a cross-reactive molecular species, that can localize to the

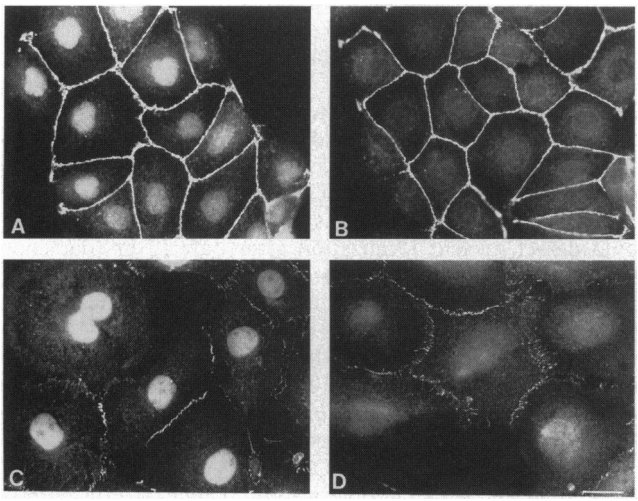

FIG. 3. Nuclear ZO-1 is Triton X 100-extractable. MDCK cells (*A* and *B*) and CV-1 cells (*C* and *D*) were seeded at nonconfluent densities 48 hr before being prepared for immunofluorescence analysis. The cells in *B* and *D* were extracted (0.25% Triton X-100 for 5 min) before being fixed in paraformaldehyde. The cells in *A* and *C* were fixed directly in paraformaldehyde. Note that nuclear staining with anti-ZO-1 polyclonal antibody 7445 is clearly visible in fixed cells (*A* and *C*), but not in cells that were Triton X-100-extracted before fixation (*B* and *D*). Also note that ZO-1 localized to cell–cell contacts appears to be largely Triton X-100-inextractable. Methanol-fixed cells give an immunofluorescence pattern identical to that seen in *B* and *D*.

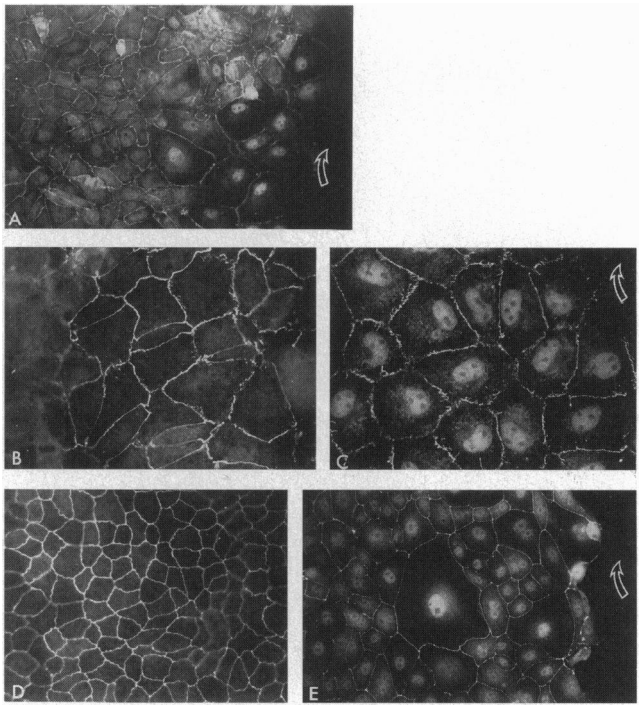

FIG. 4. Conditions that alter the nuclear accumulation of ZO-1. ZO-1 localizations were examined in wounded monolayers of both CV-1 and MDCK cells. CV-1 (*A–C*) and MDCK (*D* and *E*) monolayers were slashed with a 200-μl Pipetman tip to create a discreet 2- to 4-mm "wound." Wounded monolayers were then returned to the incubator for 24 hr before being prepared for immunofluorescence analysis using the anti-ZO-1 polyclonal antibody (7445). (*A*) Low magnification (×40) view of wounded CV-1 monolayer. Open arrow shows direction of the wound. Note that only cells proximal to the wound show nuclear labeling. (*B* and *C*) The same monolayer as in *A*, but a different region of the wound, is shown at higher magnification (×63); *B* is distal and *C* is proximal to the wound. (*D* and *E*) Wounded MDCK monolayer (×40): *D* is distal and *E* is proximal to the wound site.

nucleus, LLC-PK1 cells were transiently transfected with a cDNA encoding a C-terminal epitope tagged form of ZO-1 (α+ isoform). In transfected cells, the tagged protein is incorporated into the margin of cell–cell contact, like the endogenous ZO-1 (Fig. 1*E*), suggesting that the placement of the epitope tag did not grossly affect ZO-1's ability to localize to its site of ultimate functional residence (data not shown). In some transfected cells, exogenous ZO-1 can be localized to the nucleus in addition to sites of cell–cell contact (Fig. 1*F*). Interestingly, nuclear labeling observed with the epitope tagged ZO-1 was more often detected in less columnar cells, which often lay in close proximity to an opening in the monolayer. We suggest, therefore, that consistent with endogenous ZO-1 localizations in subconfluent and confluent cells (Fig. 1 *A–D*), epitope-tagged ZO-1 distributions may be dependent on the maturity of the cell monolayer (Fig. 1 *E* and *F*).

Time Course of ZO-1 Nuclear Accumulation. Clear accumulation of ZO-1 in the nucleus is consistently observed ≈24 hr after cell plating (Fig. 2*B*). While cells that are analyzed at earlier time points appear to contain nuclear ZO-1 (as assessed by confocal analysis; data not shown), this signal is largely obscured by a diffuse cytoplasmic pool (Fig. 2*A*). Nuclear labeling persists as the cells continue to cover the free spaces on the tissue culture dish (48–56 hr, Fig. 2 *C* and *D*) and gradually diminishes in intensity as the cells reach confluency (72 hr; Fig. 2*E*).

Differential Extractibility of Nuclear and Junction-Associated ZO-1 Pools. Since ZO-1 is a member of a family of proteins that are thought to participate in the dynamic link between specialized plasma membrane domains and the un-

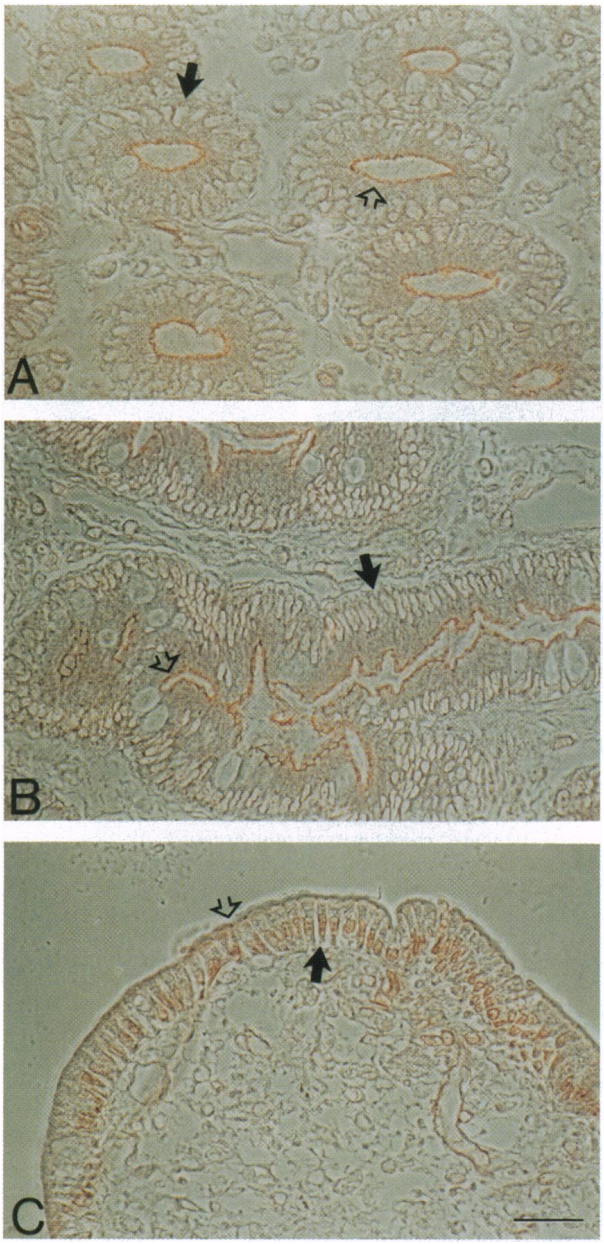

FIG. 5. Nuclear ZO-1 can be detected in intestinal epithelial cells. Paraffin-embedded tissue sections from dog intestine were labeled with anti-ZO-1 polyclonal antibody (7445) using the immunoperoxidase method. ZO-1-specific labeling was examined along the intestinal epithelium lining the crypt–villus axis. (*A*) Transverse section of intestinal crypts. (*B*) Longitudinal section along crypt–villus junction. (*C*) Longitudinal section of the outer region of the villus epithelium. Open arrows point to ZO-1 localizing to junctions; closed arrows point to nuclei. Note that nuclear ZO-1 is only detected at the villus tip. (Bar = 50 μm.)

derlying cortical cytoskeleton, we wondered whether nuclear ZO-1 is similarly involved in the formation of an extensively cross-linked meshwork of protein–protein interactions. To address this, we chose to explore the relative extractability of both nuclear and junction-associated pools of ZO-1. Nonconfluent MDCK and CV-1 cells were either fixed directly in paraformaldehyde or extracted in a bath of 0.25% Triton X-100 for 5 min before being fixed. We find that the nuclear staining is completely Triton X-100-extractable, while the fraction of ZO-1 present at the junction appears largely inextractable. We suggest, therefore, that the nuclear and junction-associated pools of ZO-1 may participate in structurally and functionally distinct molecular assemblies.

Cell fractionation studies were also undertaken to show that a protein exhibiting the same molecular mobility as ZO-1 could be extracted from a nuclear fraction of subconfluent, but not confluent cells. However, we were unable to estimate the enrichment of ZO-1 in nuclear versus cytoplasmic pools with standard cell fractionation protocols. It is well-known that even the most gentle insult to a cell can cause generalized leaking of nuclear constituents into the cytoplasm and that a number of proteins that clearly localize to the nucleus by immunofluorescence analysis fail to cofractionate with nuclei (for example, see ref. 27). Nuclear fractionation may be especially problematic for studying a protein like ZO-1, whose localization to plasma membrane, cytosolic, and nuclear compartments is highly sensitive to the quality of cell–cell contacts, necessarily modified during any standard fractionation protocol. Moreover, our finding that the nuclear ZO-1 staining is more easily extracted than the junction-associated staining (Fig. 3 and above) further argues against the feasibility of a fractionation study.

Conditions That Affect the Nuclear Accumulation of ZO-1. We wished to find conditions where ZO-1 localization to the nucleus would be stimulated or inhibited. Since subconfluent, but not confluent, cell cultures revealed nuclear ZO-1 staining, we wondered if removing cell–cell contacts by wounding a confluent monolayer could be sufficient to stimulate nuclear accumulation of ZO-1. Fig. 4 shows that in both MDCK cells and CV-1 cells, nuclear localization can be stimulated along the site of wounding (marked with an open arrow). Nuclear staining can often be observed many cell diameters away from the initial wound site and appears to correlate with a zone of cells that must remodel their cell–cell contacts, migrate, and close the wound. Nuclear accumulation was not obvious until ≈24 hr after wounding the monolayer, consistent with the time course observed when cells are trypsinized and freshly plated at subconfluent density (Fig. 2*B*). Moreover, as with the time course in Fig. 2, the nuclear labeling persisted across the zone of wound closure and gradually diminished as cells reached confluent density (56 hr was sufficient time to close a 2-mm wound). We chose to analyze different conditions in which cell–cell contact and proliferative properties would be altered. The ability of several chemical and/or pharmacologic agents to stimulate or inhibit the nuclear localization were tested, but no effects were observed. The phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), phosphatase inhibitor, orthovanadate, EGTA, and serum starvation conditions all failed to enhance nuclear accumulation in subconfluent or confluent monolayers. Hepatocyte growth factor/scatter factor changed the kinetics of this nuclear accumulation, as we could detect nuclear localization after only 12 hr (data not shown). However, hepatocyte growth factor/scatter factor did not appear to increase the amount of ZO-1 accumulated within the nucleus.

Nuclear ZO-1 is Detected Along a Distinct Region of the Intestinal Crypt–Villus Axis. To determine if the nuclear accumulation of ZO-1 occurs *in situ*, we chose to examine a tissue in which cell–cell contacts are formed and lost as a part of a normal cellular life cycle. One such tissue is the intestine, where epithelial cells undergo a well-documented differentiation program. Along the crypt–villus axis, intestinal cells can be divided into regions where cells are proliferating, migrating, differentiating, and undergoing programmed cell death (28). Fig. 5 shows that while ZO-1 tight junction staining can be detected in crypt (Fig. 5*A*), crypt–villus (Fig. 5*B*), and outer villus epithelial cells (Fig. 5*C*; indicated by open arrows), nuclear ZO-1 can only be detected in cells along, approximately, the outer one-fourth of the villus (Fig. 5*C*; indicated by closed arrows). This represents the region of the villus where cells are preparing to die and exfoliate.

DISCUSSION

Nuclear Localization of ZO-1. In this study, we demonstrate that ZO-1 can be detected in the nucleus under several different experimental conditions. Nuclear labeling was detected by indirect immunofluorescence in three different cell lines (MDCK, LLC-PK1, and CV-1) using two different antibodies against distinct portions of the ZO-1 polypeptide (Figs. 1 and 4). Transient expression of an exogenous, epitope-tagged ZO-1 polypeptide could also be detected in transfected cell nuclei using two distinct epitope tag-specific antibodies. Thus, since this pattern was detected with four different antibodies, we think it unlikely that the nuclear staining represents nonspecific or cross-reactive interactions. Moreover, the nuclear localization appears to correlate with biologically significant events in the maturation of a cell monolayer: nuclear accumulation follows a characteristic time course (Fig. 2), can be stimulated (during the remodeling of cell–cell contacts accompanying wound healing; Fig. 4), and is detected in a tissue whose epithelial cells undergo a loss of cell–cell contact as they execute their normal postmitotic program (Fig. 5).

The nuclear pool of ZO-1 does not appear to be $\alpha+/\alpha-$ isoform specific as the endogenous $\alpha-$ isoform (expressed exclusively in MDCK cells; Fig. 1 *A* and *B*) and the transiently transfected $\alpha+$ isoform can both be localized to the nucleus (Fig. 1 *E* and *F*). It also seems unlikely that the nuclear localization is due to diffusion followed by nonspecific trapping within the nucleus, because ZO-1 is >200 kDa in molecular mass. Proteins of this size would likely require an active transport process through the nuclear pore complex (29). Sequence analysis of ZO-1 reveals two lysine-rich stretches of amino acids, which might serve as putative nuclear localization sequences (NLSs): 95-RRKKK and 490-KKKDVYRR. We have not yet tested whether these sequences are responsible for the nuclear enrichment of ZO-1. Neither of these sequences is identical to simian virus 40 or nucleoplasmin NLSs (29), but the first sequence is very similar to the NLS-containing pentapeptide recently described in a fission yeast DNA polymerase (RKRKK; ref. 30) and a RKRKR NLS recently described for the T-cell protein tyrosine phosphatase p45 (27). It is of interest to note that expression of a cDNA encoding only the first 200 aa of ZO-1 (and including one of these putative NLS, lysine-rich regions) fails to localize to sites of cell contact and accumulates exclusively within the nucleus (A.S.N., A.S.F., and J. Anderson, unpublished data). However, it should be pointed out that ZO-1 need not have an NLS of its own, since many nuclear localizing proteins interact with NLS-containing proteins and gain entry to the nucleus through a "piggyback" mechanism (31, 32). Whether ZO-1 can direct its own nuclear entry or requires an associated partner remains to be determined.

Nuclear Accumulation of ZO-1 is Related to Changes in Cell–Cell Contact. We observed that the nuclear labeling was more robust under nonconfluent rather than confluent conditions. Correlation of this phenomenon with confluency was not peculiar to just one cell type, but rather was generally observed in the three different lines examined: two polarized (MDCK, LLC-PK1) and one nonpolarized cell line (CV-1 cells).

Since the nuclear staining was often more pronounced along the cell-free edge of an island of cells or within a cell lacking neighbors, we hypothesized that the signal for nuclear accumulation might be the loss of or reduction in cell–cell contact, suggesting that the nuclear localization might be inversely related to the extent and/or maturity of cell–cell contacts. This hypothesis is supported by the observation that nuclear accumulation could be stimulated at the sites where a free edge had been generated. The time course for nuclear accumulation after wounding (Fig. 4) was identical to that seen after seeding cells at subconfluent density (24 hr; Fig. 2), suggesting that the two phenomena are related and that the signaling responsible for nuclear accumulation might be similar.

The localization of ZO-1 to the outer fourth of villus intestinal epithelial cells also supports the hypothesis that nuclear localization is coincident with a change in cell–cell contacts. It is known that cells in this region of the epithelium are preparing for programmed cell death (28) and that tight junctions in particular are undergoing dynamic alterations (33). More specifically, at sites of cellular extrusion, the tight junctions of the nonextruding cells appear to move down the lateral margin of the extruding cell as it extends into the lumen. In this manner, the nonextruding cells "zipper" the epithelium closed thereby preventing transepithelial leaks. It will be of great interest to determine the extent to which nuclear localization of ZO-1 may be related to such changes in tight junction structure accompanying cell death and extrusion events occurring at the villus tip.

Because subconfluent cells are presumably dividing more frequently than confluent cells, it was formally possible that the nuclear labeling could be due to trapping of a cytoplasmic pool of ZO-1 within a reforming nuclear compartment, rather than through a specific nuclear transport mechanism. This seems unlikely for two reasons: (*i*) ZO-1 is detected in nuclei of the fully differentiated population of enterocytes lining the outer fourth of the intestinal villus, but is not detected in nuclei from the proliferative region located in villus crypts (Fig. 5 *A* and *C*) and (*ii*) ZO-1 fails to accumulate within the nuclei of a rapidly dividing, virally transformed MSV-MDCK line (data not shown). Furthermore, our failure to correlate nuclear ZO-1 with proliferating cells argues that the nuclear accumulation observed at subconfluency is more a reflection of the state of cell–cell contacts than the proliferative state of the cell.

While it is formally possible that the specific labeling of subconfluent cell nuclei could be due to enhanced antibody accessibility of these nuclei or to preferential masking of ZO-1 epitopes in confluent cell nuclei, we believe that these scenarios are unlikely since the observation has been confirmed with four different antibodies: two ZO-1-specific antibodies that recognize distinct portions of the polypeptide, and two epitope tag-specific antibodies. Furthermore, we have found that an antibody directed against a constitutive nuclear component gives qualitatively similar labeling patterns under both cell density conditions (data not shown).

ZO-1 is a member of a family of MAGUK homologues whose founding member, *dlg*, exhibits tumor suppressor function in the *Drosophila* (9), and therefore has been implicated as a participant in signal transduction from sites of cell–cell contact. It is widely appreciated that the specialized structures at cell contacts (i.e., tight junctions, adherens junctions, and desmosomes) are focal points for cell–cell signaling pathways implicated in growth and differentiation (34, 19). This study demonstrates that ZO-1 can accumulate within a cell's nucleus and that this localization may be inversely related to the extent and/or maturity of cell–cell contact. It is possible that a signal, accompanying a reduction in cell–cell contacts, might be transduced through ZO-1, and that ZO-1 itself might regulate nuclear processes associated with a change in the growth or differentiation status of the cell.

A number of proteins that localize to specialized cell–cell contacts have recently been shown to exhibit transient nuclear localizations. For example, the focal adhesion localizing protein, zyxin, has been localized to nuclei (D. Nix and M. Beckerle, personal communication), and through the action of its LIM domains, has been implicated in protein–protein interactions associated with cellular differentiation (35). The cytoplasmic portion of the Notch receptor has been shown to localize to the nucleus when exogenously expressed (36–38). Notch receptor signaling is believed to regulate the compe-

tence of different cell types to respond to differentiation cues throughout development (39). Moreover, the adherens junction associated protein, β-catenin, has been detected in the nucleus (40). β-catenin (armadillo) is a component of the Wingless/Wnt signaling pathway, which is important for cell fate determination and segment polarity in *Drosophila* and *Xenopus* (41). Recently it has been shown that under very specific conditions, β-catenin and ZO-1 can form an immunoprecipitable complex (42). It will be of great interest to explore the functional consequences of such an association and the extent to which ZO-1 may participate in β-catenin signaling. It is also of interest to note that the dependence of ZO-1 localizations on confluency is not only unique to ZO-1 but has recently been described for the von Hippel-Lindau tumor suppressor gene product (43).

In the above examples of nuclear signaling mediated by plasma membrane-associated proteins, signal transduction mechanisms may not rely upon a cascade of multiple interacting cytosolic components, as has been documented extensively for the growth factor/receptor tyrosine kinase pathways. We propose that signaling events initiated at the cell surface by membrane proteins concentrated in specialized regions of the plasma membrane, such as the junctional complexes, may use a more direct mechanism. In this model, cell surface interactions would promote the dissociation of membrane-associated signaling complexes sequestered at the cytoplasmic faces of the plasma membrane allowing their subsequent translocation into the nucleus.

Note Added in Proof. Recent studies by A. Azim and A. Chishti (St. Elizabeth's Medical Center, Boston; personal communication) provide evidence that another MAGUK family member, hDlg, may localize to cell nuclei in addition to sites along the lateral membrane. The extent to which this nuclear/cell surface localization phenomenon may be a general property of MAGUKs remains to be investigated.

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