Polyamines are necessary for synthesis and stability of occludin protein in intestinal epithelial cells

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Polyamines are necessary for synthesis and stability of occludin protein in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 288: G1159–G1169, 2005. First published February 3, 2005; doi:10.1152/ajpgi.00407.2004.—Occludin is an integral membrane protein that forms the sealing element of tight junctions and is critical for epithelial barrier function. Polyamines are implicated in multiple signaling pathways driving different biological functions of intestinal epithelial cells (IEC). The present study determined whether polyamines are involved in expression of occludin and play a role in intestinal epithelial barrier function. Studies were conducted in stable Cdx2-transfected IEC-6 cells (IEC-Cdx2L1) associated with a highly differentiated phenotype. Polyamine depletion by α-difluoromethylornithine (DFMO) decreased levels of occludin protein but failed to affect expression of its mRNA. Other tight junction proteins, zonula occludens (ZO)-1, ZO-2, claudin-2, and claudin-3, were also decreased in polyamine-deficient cells. Decreased levels of tight junction proteins in DFMO-treated cells were associated with dysfunction of the epithelial barrier, which was overcome by exogenous polyamine spermidine. Decreased levels of occludin in polyamine-deficient cells was not due to the reduction of intracellular-free Ca2+ concentration ([Ca2+]i), because either increased or decreased [Ca2+]i did not alter levels of occludin in the presence or absence of polyamines. The level of newly synthesized occludin protein was decreased by ~70% following polyamine depletion, whereas its protein half-life was reduced from ~120 min in control cells to ~75 min in polyamine-deficient cells. These findings indicate that polyamines are necessary for the synthesis and stability of occludin protein and that polyamine depletion disrupts the epithelial barrier function, at least partially, by decreasing occludin.

epithelial barrier; paracellular permeability; cdx2 gene; claudin; zonula occludens-1; zonula occludens-2; ornithine decarboxylase; protein stability

EPITHELIAL CELLS LINING THE gastrointestinal mucosa form an important barrier to a wide array of noxious substances in the lumen. Integrity of normal function of the intestinal epithelial barrier depends on specialized structures involved in cell-cell contacts known as tight junctions and adherens junctions. The tight junction located at the apical region of epithelial lateral membrane provides the barrier that is selectively permeable to certain hydrophilic molecules, ions, and nutrients, whereas the adherens junction mediates strong cell-to-cell adhesions between adjacent epithelial cells and regulates the tight junction assembly and function (9, 32, 38, 50). The tight junction seals epithelial cells together in a way that prevents even small molecules from leaking between cells and also functionally separates the plasma membrane into an apical and a basolateral domain (9, 38, 41). Although the understanding about the architecture and function of the tight junction is far from complete, there is no doubt that tight junction is a multiprotein complex that is highly dynamic and tightly regulated by numerous factors.

Major transmembrane and cytosolic tight junction proteins in the mammalian epithelium include occludin, claudins, zonula occludens (ZO)-1, and ZO-2 (8, 12, 41, 54, 66). Occludin is an integral membrane protein specifically localized at tight junction complexes and required for normal tight junction physiology (14, 25, 64). Occludin has a tetranspanning membrane topology with two extracellular loops and three cytoplasmic domains, among which the extracellular loops are important for occludin localization (2, 11, 13, 27, 39, 64). Increasing evidence indicates that occludin is the protein that forms the actual sealing element of tight junctions and is involved in fence functions of the epithelial barrier (4, 10, 27, 34, 64, 65). For example, ectopic expression of wild-type occludin in Madin-Darby canine kidney cells increases the number of tight junction strands and promotes the epithelial barrier function (34). In contrast, inhibition of occludin activity by a dominant negative occludin mutant disrupts tight junction structures and results in dysfunction of the epithelial barrier (4). Claudins are another family of integral membrane proteins of tight junctions and can interact with occludin in a collaborating way to achieve the full function of tight junctions (8, 9, 13, 24). On the other hand, ZO-1 and ZO-2 are the cytoplasmic face of tight junctions and directly bind to the COOH terminus of intracellular domain of occludin, and the interaction between occludin and ZO-1 or ZO-2 protein is crucial for maintaining normal structure of the tight junctions and epithelial barrier function (10, 15, 24, 63).

The natural polyamines, spermidine and spermine and their precursor putrescine, are organic cations found in all eukaryotic cells and have distinct regulatory roles in intestinal epithelial cells (IECs) (35, 56). Polyamines modulate expression of various genes involved in mucosal growth, repair, and apoptosis (30, 36, 51, 59–61), and the control of cellular polyamines is thought to be a central convergence point for the multiple signaling pathways driving different epithelial cell functions. We (17, 18) have recently demonstrated that polyamines modulate intercellular junctions in normal IECs (IEC-6

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line) and that depletion of cellular polyamines decreases adherens junction proteins such as E-cadherin, β-catenin, and α-catenin. Because the IEC-6 cells are undifferentiated crypt-type epithelial cells lacking expression of some tight junctions (18, 45), an in vitro model using normal differentiated IECs is necessary for identifying the fundamental mechanisms by which epithelial barrier function is regulated under biological conditions. Our previous studies (47, 49) have shown that forced expression of the Cdx2 gene in IEC-6 cells induces the development of a differentiated phenotype. These differentiated Cdx2-transfected IEC-6 cells (IEC-Cdx2L1 cell line) exhibit multiple morphological features of villus-type enterocytes with well-developed tight junctions and appear to provide an excellent in vitro system for investigating intestinal epithelial barrier function. The present study was designed to test the hypothesis that polyamines are involved in expression of occludin in differentiated IEC-Cdx2L1 cells. Some of these data have been published previously in abstract form (19).

**MATERIALS AND METHODS**

**Chemicals and supplies.** Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and dialyzed FBS were obtained from GIBCO-BRL (Gaithersburg, MD), and biochemicals were from Sigma (St. Louis, MO). The affinity-purified rabbit polyclonal antibodies against occludin, claudin-2, claudin-3, ZO-1, and ZO-2 were purchased from Zymed Laboratories (San Francisco, CA). The monoclonal antibody against E-cadherin was purchased from Transduction Laboratories (Lexington, KY). α-Difluoromethylornithine (DFMO) was purchased from Ilex Oncology (San Antonio, TX). The 12-mm Transwell filters (0.4 μm pore size, clear polyester) were obtained from Costar (Cambridge, MA). Fluorescein-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Disposable culture ware was purchased from BD Falcon (Becton Dickinson, Franklin Lakes, NJ).

**Cell cultures and general experimental protocols.** The IEC-6 cell line at passage 13 was purchased from the American Type Culture Collection. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (45). IEC-6 cells originated from intestinal crypt cells, as judged by morphological and immunological criteria. They are nontransformed epithelial stem cells. The stable IEC-Cdx2L1 cells were developed and characterized by Suh and Traber (55) and were kind gifts from Dr. Peter G. Traber (Baylor College of Medicine, Houston, TX). The expression vector, the LaczSwitch System (Stratagene, La Jolla, CA), was used for directing the conditional expression of Cdx2, and isopropyl-β-D-thiogalactopyranoside (IPTG) served as an inducer for the gene expression. Stock stable IEC-Cdx2L1 cells were grown in DMEM as described in our previous publications (47, 49). Before experiments, cells were grown in DMEM containing 4 mM IPTG for 16 days to induce cell differentiation.

In the first series of studies, we determined whether manipulation of [Ca2+] cyt, either increased or decreased, altered occludin expression in the presence or absence of polyamines. Based on our previous studies (48, 62), the Ca2+ ionophore ionomycin (1 μM) was used to increase [Ca2+] cyt, whereas the Ca2+-free medium was employed to decrease [Ca2+] cyt. In the Ca2+-free medium, 1.8 mM CaCl2 was replaced by 1.8 mM MgCl2, and additional 0.1 mM EGTA was added to chelate the residual Ca2+. Free Ca2+ concentration in the Ca2+-free medium was <0.002 μM. Levels of mRNAs and proteins of occludin, ZO-1, and ZO-2 were measured at various times after treatment with the Ca2+-free medium or ionomycin in normal (without DFMO) and polyamine-deficient (with DFMO) differentiated IEC-Cdx2L1-cells.

In the third series of studies, we focused on experiments to investigate the effect of cellular polyamines on the protein synthesis and stability of occludin in differentiated IEC-Cdx2L1 cells. The level of newly synthesized occludin protein was measured by using [35S]-methionine-labeling technique, and the occludin stability was examined by determination of the protein half-life. Cells were grown in control cultures and cultures containing DFMO alone or DFMO plus spermidine for 6 days and then pulse-labeled with [35S]-methionine. To determine the half-life of occludin protein, cycloheximide (50 μg/ml) was added to cultures, and levels of occludin protein were assayed at different times after treatment with cycloheximide by Western blot analysis.

**RT-PCR.** Total cellular RNA was isolated by using RNAeasy Mini Kit (Qiagen, Valencia, CA). Equal amounts of total RNA (2 μg) were transcribed to synthesize single-strand cDNA with a RT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA). The specific sense and antisense primers for occludin included 5′-TTG GCA GAG AGG CTA TGG-3′ and 5′-ACC CAC TCT TCA ACA TTG GG-3′ and the expected size of occludin fragments was 623 bp. The specific sense and antisense primers for ZO-1 included 5′-GCCCTCTGAGTTAAG-CAT-3′ and 5′-AAGAGCTGCGTATTTTAA-3′, and the expected size of ZO-1 fragments was 249 bp. The specific sense and antisense primers for ZO-2 included 5′-CGGTGAGACCCGCGATCT-3′ and 5′-GAGTGAAGCCTCAGGATG-3′, and the expected size of ZO-2 fragments was 461 bp. The specific sense and antisense primers for claudin-2 included 5′-TCAACTGTTGGCCTAC-TCC-3′ and 5′-GGTGTCGCCACAC-TCCAT-3′, and the expected size of claudin-2 was 154 bp. The specific sense and antisense primers for claudin-3 included 5′-CATCCCTGCTGGCCGCT-TCG-3′ and 5′-CCTGTAGATGTTGGCGGCAC-3′, and the expected size of claudin-3 was 174 bp. These particular sequences are chosen based on specificity established by previous publications (18, 53). RT-PCR was performed as described in our earlier publications (18, 49). To ensure that we were working within the linear phase of each amplification reaction, aliquots of individual PCR reactions were removed at two- to three-cycle intervals, electrophoresed on 1% agarose gels, and stained with ethidium bromide. To quantify the PCR products (the amounts of mRNA) of occludin, ZO-1, ZO-2, claudin-2, and claudin-3, an invariant mRNA of β-actin was used as an internal control. The optical density (OD) values for each band on the gel were measured by a Gel Documentation System (UVP, Upland, CA) and their signals were normalized to the OD values in the β-actin signals.

**Western blot analysis.** Cell samples, dissolved in ice-cold RIPA-buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethyl-sulfonyl fluoride, 20 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 mM sodium orthovanadate) were sonicated and centrifuged at 14,000 rpm for 15 min at 4°C. The protein concentration of the supernatant was measured by the methods described by Bradford (7), and each lane was loaded with 20 μg of protein equivalent. The supernatant was boiled for 5 min and then subjected to electrophoresis on 7.5% acrylamide gels. Briefly, after the transfer of protein onto nitrocellulose filters, the filters were incubated for 1 h in 5% nonfat dry milk in 1× TBS-T buffer (Tris-buffered saline, pH 7.4, with 0.1%
**RESULTS**

**Tight junction expression and epithelial barrier function in differentiated IECs.** As reported in our previous studies (47, 49) and others (55), the stable IEC-Cdx2L1 cells grown in the presence of 4 mM IPTG for 16 days were associated with a significant development of differentiated phenotype. These differentiated IEC-Cdx2L1 cells were polarized, showed lateral membrane interdigitations and microvilli at the apical pole, and also expressed brush-border enzymes such as sucrase-isomaltase (data not shown). These differentiated IEC-Cdx2L1 cells highly expressed tight junction proteins occludin, ZO-1, ZO-2, claudin-2, and claudin-3 (Fig. 1A). Basal levels of claudin-1 and claudin-4 proteins were too low to be detectable by Western blot analysis in differentiated Cdx2L1 cells (data not shown). In contrast, undifferentiated parental IEC-6 cells did not express occludin and claudins, although they highly expressed ZO-1 and ZO-2 proteins (Fig. 1C).

Consistently, barrier function in differentiated IEC-Cdx2L1 cells was improved significantly as indicated by TEER across the cell monolayer and paracellular tracer flux. Differentiated IEC-Cdx2L1 cells exhibited a higher steady-state level of TEER and lower basal paracellular permeability than parental IEC-6 cells. The value of TEER in differentiated IEC-Cdx2L1 cells was ~1.8-fold greater than that of parental IEC-6 cells (from 23.1 ± 0.6 to 41.4 ± 1.2 Ω·cm², n = 18, P < 0.05), whereas levels of paracellular flux of two widely accepted membrane impermeable tracers, ³H-inulin and ¹⁴C-mannitol, were decreased by ~80% and ~30% in differentiated IEC-Cdx2L1 cells, respectively. In addition, enhanced barrier function in differentiated IEC-Cdx2L1 cells is not due simply to clonal variation, because identical results were observed when another independently transfected clone, IEC-Cdx2L2, was analyzed (data not shown). Increased barrier function in differentiated IEC-Cdx2L1 cells also is not due to the effects of IPTG. There were no significant differences in levels of TEER and paracellular permeability between nontransfected parental IEC-6 cells and cells transfected with the empty vector containing no Cdx2 cDNA but maintained in the medium containing IPTG for 16 days (data not shown). Treatment with IPTG for 16 days also did not affect levels of tight junction proteins nor epithelial barrier function in nontransfected parental IEC-6 cells. Furthermore, levels of various tight junction proteins and barrier function in Cdx2-transfected IEC-6 cells before treatment with IPTG to induce differentiation were identical to those of nontransfected parental IEC-6 cells (data not shown). These results suggest that stable IEC-Cdx2L1 cells with a differentiated phenotype are a better system to characterize epithelial barrier events in vitro.

**Changes in expression of tight junctions following polyamine depletion.** Our previous studies (18) have demonstrated that polyamines are necessary for expression of adherens junction proteins in undifferentiated parental IEC-6 cells. Be-
cause tight junctions are not well developed in parental IEC-6 cells, differentiated IEC-Cdx2L1 cells were used in the present study. To determine the role of cellular polyamines in the regulation of tight junction expression, differentiated IEC-Cdx2L1 cells were cultured in the DMEM containing DFMO, a specific inhibitor of polyamine synthesis, for 4, 6, and 8 days. Consistent with our previous publications (47, 49), exposure to a specific inhibitor of polyamine synthesis, for 4, 6, and 8 days. Cdx2L1 cells were cultured in the DMEM containing DFMO, regulation of tight junction expression, differentiated IEC-Cdx2L1 cells were used in the present study. To determine the role of cellular polyamines in the study. To determine the role of cellular polyamines in the regulation of tight junction expression, differentiated IEC-Cdx2L1 cells were cultured in the DMEM containing DFMO, a specific inhibitor of polyamine synthesis, for 4, 6, and 8 days.

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Results presented in Fig. 1 show that depletion of cellular polyamines by treatment with DFMO decreased protein levels of tight junctions occludin, ZO-1, ZO-2, claudin-2, and claudin-3 in differentiated IEC-Cdx2L1 cells. The levels of occludin protein in the cells exposed to DFMO for 4, 6, and 8 days were decreased by ~80% (Fig. 1A, top, and B). Although there was no inhibition of ZO-1 and ZO-2 expression in undifferentiated parental IEC-6 cells treated with DFMO (Fig. 1C), levels of ZO-1 and ZO-2 proteins in differentiated IEC-Cdx2L1 cells decreased significantly after polyamine depletion. Levels of ZO-1 and ZO-2 proteins in differentiated IEC-Cdx2L1 cells exposed to DFMO for 4, 6, and 8 days were decreased by ~55% and ~40%, respectively. Treatment with DFMO for 4 days did not alter expression of claudin-2, but its levels were decreased by ~50% on day 6 and by ~80% on day 8, respectively. Changes in claudin-3 expression were similar to those observed in claudin-2 following polyamine depletion, and its protein levels were decreased by ~50% in cells exposed to DFMO for 6 and 8 days. In the presence of DFMO, decreased levels of occludin, ZO-1, ZO-2, claudin-2 and claudin-3 proteins were completely abolished by addition of exogenous spermidine (5 μM). Putrescine (10 μM) had an effect equal to spermidine on levels of tight junctions when it was added to cultures that contained DFMO (data not shown). On the other hand, the steady-state levels of occludin, ZO-1, and ZO-2 proteins were not affected by the addition of exogenous spermidine (5 μM) in cells grown without DFMO (data not shown); and the similar effect of spermidine on basal levels of other proteins, such as p53 and c-Myc, has been reported in our previous publications (30, 42).

To extend these positive findings that polyamine depletion decreased levels of intercellular junction proteins, immunofluorescence staining was performed to determine the cellular distribution of occludin, ZO-1, and E-cadherin in differentiated IEC-Cdx2L1 cells. In control cells (Fig. 2A), immunoreactivities for occludin, ZO-1, and E-cadherin proteins were primarily distributed in the apical regions of the cell membrane. In contrast, in cells treated with DFMO, these proteins were largely absent from the apical region, with most of the signal localized to the basal region of the cell. These results indicate that polyamine depletion leads to a redistribution of intercellular junction proteins, which may be indicative of altered cell-cell interactions and tight junction assembly.

**Fig. 1.** Changes in expression of occludin, zonula occludens (ZO)-1, ZO-2, claudin-2, and claudin-3 proteins in control differentiated Cdx2-transfected IEC-6 cells (IEC-Cdx2L1) and cells treated with either α-difluoromethylornithine (DFMO) alone or DFMO + spermidine (SPD) are shown. Before experiments, stable IEC-Cdx2L1 cells were grown in DMEM containing 5% FBS and 4 mM isopropl β-D-thiogalactopyranoside (IPTG), the inducer for expression of the Cdx2 gene, for 16 days to induce cell differentiation. These differentiated IEC-Cdx2L1 cells were grown in DMEM containing 5% dialedyzed FBS in the presence or absence of DFMO (5 mM) or DFMO plus SPD (5 μM) for 4, 6, and 8 days. Whole cell lysates were harvested for Western blot analysis. A: representative immunoblots of Western analysis. Twenty micrograms of total protein were applied to each lane and subjected to electrophoresis on 7.5% acrylamide gel. Immunoblots were hybridized with the antibodies specific for occludin (~65 kDa), ZO-1 (~225 kDa), ZO-2 (~160 kDa), claudin-2 (~22 kDa), and claudin-3 (~24 kDa) as described in MATERIALS AND METHODS. After the blot was stripped, actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. B: quantitative analysis derived from densitometric analysis of immunoblots of occludin from cells described in A. Values are means ± SE of data from 3 separate experiments; relative levels of proteins were corrected for loading as measured by densitometry of actin. *P < 0.05 compared with the corresponding control and DFMO + SPD. C: representative immunoblots of Western analysis for ZO-1 and ZO-2 proteins in parental IEC-6 cells exposed to DFMO or DFMO plus SPD for 4, 6, and 8 days. Three experiments were performed that showed similar results.
Membrane-impermeable tracer molecules, $^3$H-inulin and $^{14}$C-mannitol, were added to the insert medium, and the entire basal medium was collected 2 h thereafter for paracellular trace flux assays. Values are means ± SE of data from 8 samples. $^*P < 0.05$ compared with control and DFMO + SPD.

Fig. 3. Changes in epithelial barrier function in differentiated IEC-Cdx2L1 cells described in Fig. 1. A: changes in transepithelial electrical resistance (TEER) in cells exposed to DFMO or DFMO + SPD. Cells were grown in control cultures or cultures containing 5 mM DFMO alone or DFMO plus 5 μM SPD for 6 days. TEER assays were performed on 12-mm Transwell filters as described in MATERIALS AND METHODS. Values are means ± SE of data from 8 samples. B: $^3$H-inulin permeability. C: $^{14}$C-mannitol permeability after cells were grown in control cultures or cultures containing DFMO or DFMO plus SPD for 4 days and were then trypsinized, plated at confluent density on the insert, and maintained under the same culture conditions for additional 48 h. Membrane-impermeable tracer molecules, $^3$H-inulin and $^{14}$C-mannitol, were added to the insert medium, and the entire basal medium was collected 2 h thereafter for paracellular trace flux assays. Values are means ± SE of data from 8 samples. $^*P < 0.05$ compared with control and DFMO + SPD.

Fig. 2. Cellular distribution of occludin, ZO-1, and E-cadherin (E-cad) proteins in differentiated IEC-Cdx2L1 cells described in Fig. 1. Cells were plated in a 4-well chamber slide, grown in DMEM containing 5% dialyzed FBS in the presence or absence of DFMO or DFMO + SPD for 6 days, and then fixed for immunostaining. Cells were permeabilized and incubated with the specific antibody against occludin, ZO-1, or E-cad, and then with anti-IgG conjugated with FITC. Slides were viewed through a Zeiss confocal microscope. A: control. B: cells treated with DFMO alone for 6 days. C: cells treated with DFMO + SPD for 6 days. Original magnification: ×1,000. Three experiments were performed that showed similar results.

Effect of polyamine depletion on expression of tight junction mRNAs. To define levels where polyamines are implicated in expression of tight junctions, changes in mRNAs of occludin, ZO-1, ZO-2, claudin-2, and claudin-3 were examined in the presence or absence of cellular polyamines. As shown in Fig. 4, depletion of cellular polyamines by DFMO did not inhibit expression of occludin, ZO-1, and ZO-2 mRNAs, although it significantly decreased levels of their proteins (Fig. 1). There were no significant changes in mRNA levels of occludin, ZO-1, and ZO-2, regardless of treatment with DFMO alone or DFMO plus spermidine for 4 and 6 days (Fig. 4). On the other hand, polyamine depletion inhibited expression of claudin-2...
and claudin-3 mRNAs, which was completely prevented by exogenous spermidine (Fig. 4, bottom). These findings suggest that polyamines are involved in expression of various tight junction proteins through different signaling pathways in IECs.

Effect of [Ca\(^{2+}\)]\(_{cyt}\) on occludin expression. To keep this study focused, we specifically elucidated the mechanism by which polyamines modulate occludin in IECs. Our previous studies have demonstrated that cellular polyamines regulate [Ca\(^{2+}\)]\(_{cyt}\) concentration by governing the driving force for Ca\(^{2+}\) influx via controlling activity of voltage-gated K\(^{+}\) (K\(_{V}\)) channels in IECs and that depletion of cellular polyamines inhibited K\(_{V}\) channel expression and resulted in membrane depolarization, which was associated with a decrease in [Ca\(^{2+}\)]\(_{cyt}\) (17, 48, 49, 62). In fact, [Ca\(^{2+}\)]\(_{cyt}\) in differentiated IEC-Cdx2L1 cells exposed to DFMO for 6 days was decreased by \(-35\%\) (from \(139 \pm 6\) nM in control cells to \(91 \pm 3\) nM in DFMO-treated cells; \(n = 28, P < 0.05\)). Spermidine, given together with DFMO, restored [Ca\(^{2+}\)]\(_{cyt}\) to normal levels.

To test the possibility that polyamines modulate occludin expression by altering [Ca\(^{2+}\)]\(_{cyt}\), the following two studies were performed in differentiated IEC-Cdx2L1 cells. First, we determined whether the decreased [Ca\(^{2+}\)]\(_{cyt}\) caused by removal of extracellular Ca\(^{2+}\) inhibits expression of occludin in control cells (without DFMO). As shown in Fig. 5, exposure to the Ca\(^{2+}\)-free medium for 6 h did not alter expression of occludin. There were no significant differences in levels of occludin protein and mRNA between control cells and cells exposed to the Ca\(^{2+}\)-free medium for 2, 4, and 6 h. In addition, decreased [Ca\(^{2+}\)]\(_{cyt}\) by exposure to the Ca\(^{2+}\)-free medium also had no effect on expression of ZO-1 and ZO-2 proteins. However, expression of E-cadherin protein, which is a calcium-dependent protein, was dramatically decreased after exposure to the Ca\(^{2+}\)-free medium (Fig. 5A, bottom, and B). Levels of E-cadherin protein were decreased by \(-60\%\) at 2 h, by \(-85\%\) at 4 h, and by \(-90\%\) at 6 h after exposure to the Ca\(^{2+}\)-free medium.

Second, we examined whether increased [Ca\(^{2+}\)]\(_{cyt}\) by the Ca\(^{2+}\) ionophore, ionomycin, induced expression of occludin in control and polyamine-deficient IEC-Cdx2L1 cells. Consistent with our previous studies (48, 49), exposure to 1 \(\mu\)M ionomycin dramatically increased [Ca\(^{2+}\)]\(_{cyt}\) in control and DFMO-treated cells (data not shown). Results presented in Fig. 6 show that increased [Ca\(^{2+}\)]\(_{cyt}\) by ionomycin did not increase levels of occludin protein in control and polyamine-deficient cells. Consistently, there were no significant changes in ZO-1 and ZO-2 proteins after exposure to ionomycin. In addition, neither the Ca\(^{2+}\)-free medium nor ionomycin affected cell attachment and cell viability nor induced apoptosis in control and DFMO-treated cells as measured by Trypan blue staining and Annexin-V staining for apoptotic cells (data not shown). These results indicate that polyamines regulate expression of occludin protein through a mechanism that is independent of [Ca\(^{2+}\)]\(_{cyt}\) in differentiated IEC-Cdx2L1 cells.

Effect of polyamine depletion on occludin protein synthesis and stability. To determine whether polyamines regulate occludin at the translation level, the level of newly synthesized occludin protein was examined in this study. As shown in Fig. 7, polyamine depletion by DFMO significantly decreased the occludin protein synthesis in differentiated IEC-Cdx2L1 cells. The level of newly synthesized occludin protein was decreased by \(-70\%\) in cells exposed to DFMO for 6 days. To determine the effect of polyamines on the stability of occludin, the half-life of occludin protein was examined in the presence or absence of cellular polyamines. The method used in this study was validated by pulse-chase analysis as described in our previous work (18). Results presented in Fig. 8 show that an increase in occludin protein degradation also contributes to the decreased levels of occludin in polyamine-deficient cells. In control cells (without DFMO), the levels of occludin protein declined gradually after protein synthesis was inhibited by administration of cycloheximide, with a half life of \(-120\) min (Fig. 8, Aa and B). In DFMO-treated cells, the stability of occludin protein decreased significantly compared with that
observed in control cells (Fig. 8A, a vs. b). The half-life of occludin protein in polyamine-deficient cells was ~75 min (Fig. 8B). Spermidine given together with DFMO not only totally overcame the decrease in occludin protein synthesis but also completely prevented the instability of occludin protein as well. The level of newly synthesized occludin protein and its half-life in cells treated with DFMO plus spermidine was similar to those observed in controls (Fig. 8A, a vs. c). These results clearly indicate that polyamines are implicated in regulation of occludin protein synthesis and stability in IECs.

**DISCUSSION**

An increasing body of evidence indicates that cellular polyamines play a critical role in maintenance of the intestinal epithelial integrity, but few specific functions of polyamines at cellular and molecular levels are defined. We (18) have recently reported that polyamines are implicated in regulation of the intestinal epithelial barrier function and that depletion of cellular polyamines increases epithelial paracellular permeability at least partially by inhibiting expression of adherens junctions in undifferentiated parental IEC-6 cells. The present studies further confirm our previous observations by demonstrating that polyamines are crucial for expression of adherens junctions in differentiated IEC-Cdx2L1 cells. The most significant of the new findings reported in this study, however, is that polyamines are required for normal function of tight junctions and that polyamines regulate expression of various tight junction proteins through different mechanisms. Polyamines regul-

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**Fig. 5.** Effect of decreased intracellular Ca\(^{2+}\) concentration by exposure to the Ca\(^{2+}\)-free medium on expression of occludin, ZO-1, ZO-2, and E-cadherin in normal differentiated IEC-Cdx2L1 cells. A: representative immunoblots of Western analysis for occludin, ZO-1, ZO-2, and E-cadherin (~120 kDa) proteins. Cells were grown in the standard DMEM for 6 days and then incubated with the Ca\(^{2+}\)-free medium. Whole cell lysates were harvested at indicated times after incubation with Ca\(^{2+}\)-free medium, and protein levels of occludin, ZO-1, ZO-2, and E-cadherin were measured by Western blotting analysis using various specific antibodies. After the blot was stripped, actin immunoblotting was performed as an internal control for equal loading. B: quantitative analysis derived from densitometric analysis of immunoblots from cells described in A. Values are means ± SE of data from 3 separate experiments. C: changes in occludin mRNA in cells described in A. Total cellular RNA was harvested at various times after exposure to Ca\(^{2+}\)-free medium, and occludin mRNA levels were assayed by RT-PCR analysis. Equal loading was monitored by measurement of β-actin mRNA. Three separate experiments were performed that showed similar results.

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**Fig. 6.** Effect of increased intracellular Ca\(^{2+}\) concentration by treatment with the Ca\(^{2+}\) ionophore, ionomycin (Iono), on expression of occludin, ZO-1, and ZO-2 proteins in normal and polyamine-deficient IEC-Cdx2L1 cells. A: representative immunoblots of Western analysis for occludin, ZO-1, and ZO-2 proteins. Differentiated IEC-Cdx2L1 cells were grown in control cultures or cultures containing DFMO (5 mM) for 6 days and then exposed to 1 μM ionomycin for 6 h. Whole cell lysates were harvested and levels of occludin, ZO-1, and ZO-2 proteins were assayed by Western blot analysis. Actin immunoblotting was performed as internal control for equal loading. B: quantitative analysis derived from densitometric analysis of immunoblots from cells described in A. Values are means ± SE of data from 3 separate experiments. *P < 0.05 compared with the corresponding control groups.
late occludin primarily by altering its protein synthesis and stability rather than its mRNA. These findings provide, for the first time, new evidence showing that cellular polyamines are necessary for normal function of the intestinal epithelial barrier in association with their ability to regulate levels of tight junction proteins, especially occludin.

The requirement of polyamines for expression of tight junction proteins is specific in differentiated IECs, because depletion of cellular polyamines fails to inhibit levels of ZO-1 and ZO-2 proteins in parental IEC-6 cells (Fig. 1C). Although the exact reasons for the different responses of ZO-1 and ZO-2 expression to polyamines in parental IEC-6 cells and differentiated IEC-Cdx2L1 cells remain unclear, it may be related to the following facts and possibilities. First, parental IEC-6 cells originate from intestinal crypts and retain the undifferentiated character of epithelial crypt cells (45). In contrast, stable IEC-Cdx2L1 cells have multiple morphological and molecular characteristics of differentiated phenotype and represent villus-type enterocytes (47, 49, 55). Second, polyamines may have different regulatory effects when these compounds are presented in the villus in general. Our previous studies (47, 60) and others (35) have shown that polyamines in the crypt are absolutely required for epithelial cell proliferation, but roles of induced polyamines in the villus are still unknown. Third, tight junctions are not well developed in undifferentiated parental IEC-6 cells. For example, expression of occludin and claudin-2 and -3 was observed only in differentiated IEC-Cdx2L1 cells, although both parental IEC-6 cells and differentiated IEC-Cdx2L1 cells expressed ZO-1 and ZO-2 proteins. It is possible that expression of the premature tight junctions in parental IEC-6 cells is regulated by a distinct mechanism insensitive to cellular polyamines. On the other hand, polyamines are necessary for expression of adherens junctions in both parental IEC-6 cells and differentiated IEC-Cdx2L1 cells and depletion of cellular polyamines decreases levels of E-cadherin, β-catenin, and α-catenin proteins.

The findings reported here indicate that polyamines are implicated in different levels in regulation of various tight junctions. Polyamine depletion decreased levels of occludin, ZO-1, and ZO-2 proteins without affecting their mRNAs, but...
inhibited expression of both mRNAs and proteins of claudin-2 and claudin-3 (Figs. 1 and 4). In addition, we have recently reported that polyamines regulate expression of the adherens junction protein E-cadherin at the transcriptional level and that depletion of cellular polyamines decreases E-cadherin mRNA and protein primarily through inhibition of transcription of the E-cadherin gene (18). These different mechanisms involved in regulation of adherens junctions and tight junctions by polyamines are not surprising, because polyamines have been involved in multiple signaling pathways in the expression of various genes in IECs. It has been shown that polyamines modulate transcription, but not posttranscription, of c-myc and c-jun genes in IEC-6 cells (42). In contrast, polyamines regulate the stability of mRNAs and proteins of p53 (30) and JunD (29) without affecting the transcriptional rates of these two genes.

It is of physiological significance that cellular polyamines regulate expression of tight junctions in IECs. Results presented in Fig. 1 show that polyamine depletion dramatically decreased levels of occludin, ZO-1, and ZO-2 proteins, but the epithelial barrier function was only inhibited by ~30% in DFMO-treated cells as indicated by a decrease in TEER and increase in paracellular permeability (Fig. 3). Although the exact reasons causing the differences are unclear, these findings suggest that 1) normal epithelial barrier function depends on multiple tight junction proteins; and 2) decreased levels of occludin, ZO-1, and ZO-2 proteins following polyamine depletion are associated with functional compensation of other tight junction or adherens junction proteins. Under normal conditions, the epithelial cells contain high levels of polyamines, which is dynamically regulated by polyamine biosynthesis, uptake, and degradation (35, 56). Cellular levels of polyamines are changed rapidly, either increased or decreased, in response to various physiological and pathogenic stimuli, leading to the activation or inactivation of different cellular signaling pathways. On the other hand, tight junctions form a physical fence to the diffusion of macromolecules through the paracellular space and also are involved in various physiological processes, such as neutrophil transmigration across an endothelium (37), epithelial cell division (1), and extrusion (5). Disruption of tight junction function occurs commonly in various pathological conditions such as inflammatory bowel disease, intestinal infections, cancers, and critical surgical stresses (3, 16, 21–23, 26, 46). To date, many signaling pathways, including tyrosine kinases, Ca2+, protein kinase C, and phospholipase C-γ, have been implicated in the regulation of tight junction permeability in epithelial cells (28, 33, 40, 50, 52, 57). The present studies provide a strong evidence for a role of cellular polyamines in the control of intestinal epithelial tight junctions.

The regulatory effect of polyamines on occludin is not due to [Ca2+]cyt, because either increasing or decreasing [Ca2+]cyt did not alter levels of occludin protein in the presence or absence of polyamines. It has been reported that polyamines regulate [Ca2+]cyt concentration by governing membrane potential through control of KV channels and that the elevated [Ca2+]cyt is a major mediator for distinct biological functions of polyamines (48, 49, 62). Polyamine depletion inhibits KV channel expression and causes membrane depolarization, leading to a decrease in [Ca2+]cyt (48, 62). We have recently found that polyamines are essential for E-cadherin gene expression, acting at least partially through [Ca2+]cyt (18). Therefore, it was logical and reasonable to consider the possibility that polyamines regulate occludin by altering [Ca2+]cyt in this study. However, as noted in Figs. 5 and 6, polyamines are necessary for occludin protein expression through a mechanism that is independent of [Ca2+]cyt in differentiated IEC-Cdx2L1 cells.

Results presented in Figs. 7 and 8 clearly show that polyamines regulate expression of occludin primarily by controlling its protein synthesis and stability. Depletion of cellular polyamines by DFMO not only inhibited the level of newly synthesized occludin protein but also decreased its protein stability. Because the decreases in both occludin protein synthesis and its half-life in DFMO-treated cells are completely prevented by exogenous spermidine, the decrease in occludin expression at translation and posttranslation levels must be related to polyamine depletion rather than to the nonspecific effect of DFMO. In addition, the possibility that polyamines regulate occludin expression at the translational level is further supported by the results showing that cycloheximide decreases the spermidine-mediated prevention of DFMO effect on occludin protein synthesis (Fig. 8). Although the exact mechanisms by which polyamines regulate translation and posttranslation of occludin remain unknown, they are possibly related to the specific molecular structure of polyamines. At physiological pH, putrescine, spermidine, and spermine possess two, three, and four positive charges, respectively (56). These compounds are shown to bind to negatively charged macromolecules such as DNA, RNA, and proteins to influence the sequence-specific DNA-, RNA- or protein-protein interactions, which alter gene transcription and translation and the stability of mRNAs and proteins (6, 43, 44, 56). Clearly, further studies are needed to define and characterize the specific regions or domains of occludin, which mediate or are involved in the regulatory effects of polyamines.

In summary, these results indicate that polyamines are required for expression of tight junctions in differentiated IECs. Polyamines regulate expression of various tight junction proteins through distinct cellular signaling pathways. Although the inhibitory effect of polyamine depletion on expression of occludin protein is independent of intracellular Ca2+, results presented here clearly indicate that reduced levels of occludin in polyamine-deficient cells result primarily from decreases in its protein synthesis and stability. These findings suggest that cellular polyamines are the biological regulators for tight junction expression and play an important role in the maintenance of intestinal epithelial barrier integrity under physiological conditions.

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