Polyamines regulate β-catenin tyrosine phosphorylation via Ca\(^{2+}\) during intestinal epithelial cell migration

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Guo, Xin, Jaladanki N. Rao, Lan Liu, Mort Rizvi, Douglas J. Turner, and Jian-Ying Wang. Polyamines regulate β-catenin tyrosine phosphorylation via Ca\(^{2+}\) during intestinal epithelial cell migration. Am J Physiol Cell Physiol 283: C722–C734, 2002. First published April 24, 2002; 10.1152/ajpcell.00054.2002.—Polyamines are essential for intestinal epithelial cell migration. Polyamines induce cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)), prevented induction of β-catenin phosphorylation, and decreased cell migration. Elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) induced by the Ca\(^{2+}\) ionophore ionomycin restored β-catenin phosphorylation and promoted migration in polyamine-deficient cells. Decreased β-catenin phosphorylation through the tyrosine kinase inhibitor herbimycin-A or genistein blocked cell migration, which was accompanied by reorganization of cytoskeletal proteins. These results indicate that β-catenin tyrosine phosphorylation plays a critical role in polyamine-dependent cell migration and that polyamines induce β-catenin tyrosine phosphorylation at least partially through [Ca\(^{2+}\)]\(_{\text{cyt}}\).

ornithine decarboxylase; cell adhesion; tyrosine kinase; α-catenin; intracellular calcium; restitution; intestinal epithelial cells

β-catenin is a versatile intracellular protein that mediates cell-cell adhesion and also actively participates in intracellular signal transduction (5, 52). On one hand, β-catenin directly associates with the highly conserved cytoplasmic domain of the cell adhesion protein E-cadherin, the member of a family of transmembrane receptors that are primarily found at the adhe-...
of cells. In contrast, decreased PY-β-catenin through ectopic expression of the PTP-LAR gene inhibits cell migration (28).

Studies from our laboratory (45–47) and others (3, 23, 26) have demonstrated that the cellular polyamines spermidine and spermine and their precursor putrescine are necessary for the stimulation of cell migration after wounding and play an important role in the maintenance of gastrointestinal mucosal integrity. In an in vitro model mimicking the early cell division-independent stage of intestinal epithelial restitution, cell migration is associated with a dramatic increase in polyamine synthesis (34, 47, 48). Depletion of cellular polyamines by inhibiting ornithine decarboxylase (ODC), the first rate-limiting enzyme in polyamine biosynthesis, with d,L-α-difluoro-methylornithine (DFMO) inhibits cell migration, which is completely prevented by exogenous polyamines. Although exact roles of polyamines at cellular and molecular levels are still unclear, increasing evidence indicates that polyamines modulate cell migration after wounding through multiple signaling pathways (22, 25, 54). In intestinal epithelial cells that do not express voltage-dependent Ca²⁺ channels (VDCC), we have recently reported (35, 49) that polyamines stimulate the expression of K⁺ channel genes, resulting in membrane hyperpolarization, and increase intracellular free Ca²⁺ concentration ([Ca²⁺]cyt). However, little is known about the effects of this elevated [Ca²⁺]cyt following activation of K⁺ channels except that it involves the activation of RhoA signaling (33).

The current study was designed to test the hypothesis that polyamines enhance cell migration during restitution by altering β-catenin tyrosine phosphorylation via a Ca²⁺-dependent process in intestinal epithelial cells (IEC-6 line). First, we examined the effects of cellular polyamines on the tyrosine phosphorylation of β-catenin during IEC-6 cell migration. Second, we determined whether manipulating [Ca²⁺]cyt, either by increase or decrease, would alter levels of β-catenin tyrosine phosphorylation in the presence or absence of cellular polyamines. Third, we determined whether inhibition of β-catenin tyrosine phosphorylation by treatment with the specific TK inhibitors genistein and herbimycin-A would decrease cell migration and alter cellular distribution of actin filaments after wounding. Some of these data have been published in abstract form (15).

MATERIALS AND METHODS

Chemicals and supplies. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and dialyzed fetal bovine serum (dFBS) were obtained from GIBCO-BRL (Gaithersburg, MD), and biochemicals were from Sigma (St. Louis, MO). The monoclonal antibodies against phosphotyrosine (PY-20), β-catenin, α-catenin, and E-cadherin were purchased from Transduction Laboratories (Lexington, KY). DFMO was purchased from Ilex Oncology (San Antonio, TX). Matrigel was from Collaborative Research (Bedford, MA). Rhodamine phalloidin and unlabeled phalloidin were obtained from Molecular Probes (Eugene, OR).

Cell cultures and general experimental protocols. The IEC-6 cell line was purchased from the American Type Culture Collection (ATCC) at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (32). IEC-6 cells originated from intestinal crypt cells, as judged by morphological and immunological criteria. They are nontumorigenic and retain the undifferentiated character of epithelial stem cells.

Stock cells were maintained in T-150 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate. Flasks were incubated at 37°C in a humidified atmosphere of 90% air-10% CO₂. Stock cells were subcultured once a week at 1:20; medium was changed three times weekly. The cells were restarted from original frozen stock every seven passages. Tests for mycoplasma were routinely negative, and passages 15–20 were used in the experiments. There were no significant changes of biological function and characterization from passages 15–20.

The general protocol of the experiments and the methods used were similar to those described previously (33, 35). Briefly, IEC-6 cells were plated at 6 × 10⁴/cm² in DMEM plus 5% dFBS, 10 μg/ml insulin, and 50 μg/ml gentamicin. Cells were incubated in a humidified atmosphere at 37°C in a humidified atmosphere of 90% air-10% CO₂ (vol/vol) for 4 days, followed by a period of different experimental treatments.

In the first series of studies, we examined changes in tyrosine phosphorylation of β-catenin in migrating intestinal epithelial cells after wounding in the presence or absence of polyamines. Cells were grown in control cultures or cultures containing 5 mM DFMO or DFMO plus 5 μM spermidine for 4 days. PY-β-catenin levels were measured at various times after removal of part of the cell layer by immunoprecipitation and Western blot analysis. Cells were washed three times with ice-cold Dulbecco’s PBS (D-PBS), and different solutions were then added according the assays to be conducted.

In the second series of studies, we investigated whether increasing or decreasing cytosolic free Ca²⁺ ([Ca²⁺]cyt) altered tyrosine phosphorylation of β-catenin in normal and polyaniline-deficient IEC-6 cells after wounding. 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⁺. The free Ca²⁺ in the Ca²⁺-free medium is <0.002 mM. The PY-β-catenin levels and amounts of cell migration were measured at various times after treatment with ionomycin or the Ca²⁺-free medium in the presence or absence of polyamines.

In the third series of studies, we determined whether TK inhibitors genistein and herbimycin-A affected PY-β-catenin formation, F-actin distribution, and cell migration. Cells were grown in the presence or absence of DFMO or DFMO plus spermidine for 4 days and were then exposed to various concentrations of genistein or herbimycin-A immediately after wounding. Levels of PY-β-catenin and cell migration were measured at different times after wounding, and F-actin formation and distribution were assayed 6 h after treatments.

Cell lysis and immunoprecipitation. IEC-6 cell lysis and β-catenin immunoprecipitation from whole cell lysates were carried out according to the method of Müller et al. (28) with minor changes. Briefly, 90 min before cell lysis, sodium orthovanadate, a specific inhibitor of PTP, was added to cultures to inhibit PTP activity. Cells were washed with ice-cold D-PBS three times and then lysed in ice-cold lysis...
buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glyceral, 20 mM pyrophosphate, 1% Triton-X-100, 100 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.2 mM ammonium molybdate, and 2 mM sodium orthovanadate). Cells were sonicated for 20 s, whole cell lysates were centrifuged at 4°C, and the supernatant was stored at −80°C until used. The protein concentration was measured by the Bradford method (7). Equal amounts of protein (300 μg) for each sample were incubated with the specific antibody against β-catenin (2 μg) for 3 h at 4°C; protein G-PLUS-agarose was added, and incubation continued overnight at 4°C. The precipitates were washed five times with ice-cold D-PBS, and the beads were resuspended in SDS sample buffer for subsequent Western blotting analysis.

**Western blotting analysis.** The proteins from above the immunoprecipitation or the supernatant of whole cell lysates were boiled for 5 min and then subjected to electrophoresis on 7.5% acrylamide gel according to Laemmli (21). After the transfer of proteins onto nitrocellulose, the filters were incubated in nonfat dry milk in 1× TBST buffer (Tris-buffered saline containing Tween 20), except the anti-phosphotyrosine immunoblot, which was incubated in 2% BSA in TBST. Immunological evaluation was then performed for 1 h in 1% BSA-TBST buffer containing the specific antibody against phosphotyrosine, β-catenin, α-catenin, or E-cadherin protein. The filters were subsequently washed with 1× TBST and incubated for 1 h with the second antibody conjugated to peroxidase. After extensive washing with 1× TBST, the immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (NEL-100; NEN). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s.

**Measurement of [Ca²⁺]cyt.** Details of the digital imaging methods employed for measuring [Ca²⁺]cyt have been published (33). Briefly, IEC-6 cells were plated on 25-mm coverslips and incubated in culture medium containing 3.3 μM fura 2-AM for 30–40 min at room temperature (22–24°C). The fura 2-loaded cells were then superfused with standard bath solution for 20–30 min at 22–24°C to wash away extracellular dye and permit intracellular esterases to cleave cytosolic fura 2-AM into active fura 2. Fura 2 fluorescence (510-nm emission; 380- and 360-nm excitation) from the cells and background fluorescence were imaged by using a Nikon Diaphot microscope equipped for epifluorescence. Fluorescent images were obtained by using a microchannel plate image intensifier (Amperex XX1381; Opelco, Washington, DC) coupled by fiber optics to a Pulnix charge-coupled device video camera (Stanford Photonics, Stanford, CA).

Image acquisition and analysis were performed with a MetaMorph Imaging System (Universal Imaging). Video frames containing images of fura 2 fluorescence from cells and the corresponding background images (fluorescence from fields devoid of cells) were digitized at a resolution of 512 horizontal × 480 vertical pixels and eight bits by using a Matrix LC imaging board operating in an IBM-compatible computer. Images were acquired at a rate of one averaged image every 3 s when [Ca²⁺]cyt was changing and every 60 s when [Ca²⁺]cyt was relatively constant. [Ca²⁺]cyt was calculated from fura 2 fluorescence emission excited at 380 and 360 nm using the ratio method (31). In most experiments multiple cells (usually 10–15 cells) were imaged in a single field, and one arbitrarily chosen peripheral cytosolic area (4 × 6–4 × 6 pixels) from each cell was spatially averaged.

**Measurement of cell migration.** The migration assays were carried out as described in our earlier publications (35, 47). Cells were plated at 6 × 10⁴ cells/cm² in DMEM/dFBS on 60-mm dishes thinly coated with Matrigel according to the manufacturer’s instructions and were incubated as described for stock cultures. The cells were fed on day 2 and migration was tested on day 4. To initiate migration, the cell layer was scratched with a single-edged razor blade cut to ~27 mm in length. The scratch began at the diameter of the dish and extended over an area 7–10 mm wide. The migrating cells in six contiguous 0.1-mm squares were counted at ×100 magnification beginning at the scratch line and extending as far out as the cells had migrated. All experiments were carried out in triplicate, and the results were reported as the number of migrating cells per millimeter of scratch.

**β-Catenin and actin immunohistochemical staining.** β-Catenin staining was performed in IEC-6 cells according to the method described by Waterman-Storer et al. (50) with minor changes. Briefly, the cells were washed with D-PBS, fixed in 4% paraformaldehyde, and then incubated with the specific anti-β-catenin antibody used in Western blot analysis at 1:100 dilution overnight at 4°C. The cells were then washed three times and incubated with secondary antibody conjugated with FITC (1:100) for 2 h at room temperature. After the slides were rinsed three times, they were mounted and viewed through a Zeiss confocal microscope (model LSM410). Actin immunohistochemical staining was carried out as the method described by Vlietkind and Swierenga (43) with minor changes. Briefly, cells were washed with D-PBS and then with D-PBS without calcium and magnesium (D-PBS-Ca²⁺/Mg²⁺) and fixed for 10 min at room temperature in one part 37% formaldehyde plus nine parts PEM buffer (10 mM PIPES, 5 mM EGTA, and 2 mM MgCl₂, pH 6.8, containing 37% formaldehyde plus nine parts PEM buffer (10 mM PIPES, 5 mM EGTA, and 2 mM MgCl₂, pH 6.8, containing 37% formaldehyde plus nine parts PEM buffer). After being washed two times with D-PBS-Ca²⁺/Mg²⁺, the cells were covered with 0.2% Triton X-100 in PBS for 5 min. The fixed cells were rehydrated in D-PBS-Ca²⁺/Mg²⁺ for 30 min at room temperature and incubated for 30 min with 1% BSA in PBS (PBS/BSA) to reduced nonspecific background staining. The cells were then incubated with a 1:40 dilution of rhodamine-labeled phalloidin in PBS/BSA for 45 min at room temperature. After three washes, slides were mounted and examined in a confocal microscope.

**Statistical analysis.** All data are expressed as means ± SE from six dishes. Autoradiographic and immunofluorescence labeling results were repeated three times. The significance of the difference between means was determined by ANOVA. The level of significance was determined by using Dunnett’s multiple range test (18).

**RESULTS**

**Effect of polyamines on total β-catenin expression and β-catenin tyrosine phosphorylation.** To study the involvement of cellular polyamines in the regulation of β-catenin in normal intestinal epithelial cells, we examined total β-catenin protein expression, tyrosine phosphorylation of β-catenin, and cellular distribution of β-catenin after polyamine depletion in intact IEC-6 cells (nonwounding). Cells were grown for 4 and 6 days in the presence or absence of DFMO, the highly specific inhibitor of ODC. Our previous studies have shown that exposure of IEC-6 cells to DFMO for 4 and 6 days almost completely depletes cellular polyamines. The levels of putrescine and spermidine were undetectable at 4 and 6 days after DFMO treatment, whereas spermine was decreased by >60% in DFMO-treated cells (35, 48). The data in Fig. 1A show that polyamine depletion by DFMO slightly decreased basal levels of β-catenin.
protein expression in IEC-6 cells. The levels of total β-catenin protein were decreased by ~20% at day 4
and ~25% at day 6 in DFMO-treated cells, which was completely prevented by spermidine given together
with DFMO (Fig. 1Aa). Polyamine depletion did not significantly alter tyrosine phosphorylation of β-catenin
in intact IEC-6 cells. There were no significant differences in levels of PY-β-catenin between control
cells and polyamine-deficient cells treated with or without exogenous spermidine (Fig. 1Ab). For immu
noprecipitation, pretreatment of the cells with 1 mM sodium orthovanadate, a specific inhibitor of PTPs,
was necessary to detect tyrosine phosphorylation through prevention of the degradation of PY-β-catenin
(28). In addition, the localization of β-catenin in intact IEC-6 cells remained unchanged after polyamine de
pletion (Fig. 1B). β-Catenin was located along the entire cell-cell contact region of adjacent cells regardless
of the presence or absence of cellular polyamines. There was no significant immunostaining of β-catenin
in the cytoplasm and nucleus of IEC-6 cells from all three groups.

Effect of cellular polyamines on tyrosine phosphorylation of β-catenin during cell migration after wounding. To determine changes in tyrosine phosphorylation of β-catenin during intestinal epithelial cell migration, we collected whole cell lysates at various times after wounding (removal of part of the monolayer), and they were immunoprecipitated with the specific anti-β-catenin antibody. Levels of PY-β-catenin in the precipitates were analyzed by Western blotting with an anti-phosphotyrosine antibody. As shown in Fig. 2A (top), PY-β-catenin levels were significantly increased at 2 h and peaked 6–8 h after wounding in control cells (without DFMO). Maximum increases in PY-β-catenin levels were approximately three times the prewounding control level [Fig. 2, A (left) and Bb]. This induction of tyrosine phosphorylation of β-catenin during intestinal epithelial cell migration absolutely requires polyamines because depletion of cellular polyamines by DFMO significantly inhibited the formation of PY-β-catenin (Fig. 2A, middle). In DFMO-treated cells, there were slight increases in PY-β-catenin after wounding. Spermidine at a dose of 5 μM given together with DFMO prevented the decreased PY-β-catenin in migrating IEC-6 cells. The levels of PY-β-catenin in cells treated with DFMO plus spermidine were indistinguishable from those of control cells after wounding (Fig. 2A, left vs. right). On the other hand, there were no significant changes in levels of total β-catenin and E-cadherin proteins after wounding regardless of the presence or absence of cellular polyamines [Fig. 2, A (middle and bottom), Bb, and Bc]. These findings indicate that intestinal epithelial cell migration after wounding is associated with a significant increase in tyrosine phosphorylation of β-catenin, which is highly regulated by cellular polyamines.

Changes in PY-β-catenin binding activity in migrating cells. To examine the consequences of the β-catenin phosphorylation during cell migration, we immunoprecipitated the cadherin-catenin complex at various
times after wounding by the specific antibody against β-catenin, and the binding activity of β-catenin was analyzed by determining the levels of α-catenin or E-cadherin in this complex. Figure 3 clearly shows that increased PY-β-catenin in the cadherin-catenin complex reduced its binding activity to α-catenin but not to E-cadherin in migrating IEC-6 cells. Although levels of total α-catenin protein (Fig. 3Ab) were unaffected after wounding in IEC-6 cells, the binding activity of β-catenin to α-catenin decreased with the increase in PY-β-catenin. Significant decrease in the binding activity to α-catenin occurred at 2 h, the time when the marked increase in PY-β-catenin was observed after wounding. The maximum inhibition occurred at 6 h after wounding, and the β-catenin binding activity was decreased by ~70% (Fig. 3B). We also examined the binding activity of β-catenin to α-catenin during migration in polyamine-deficient cells and demonstrated that, consistent with the effect on tyrosine phosphorylation of β-catenin, the binding activity of β-catenin in DFMO-treated cells remained unchanged after wounding (data not shown).

Effect of [Ca^{2+}]_{cyt} on β-catenin tyrosine phosphorylation during cell migration. Our previous studies (35, 49) have demonstrated that cellular polyamines regulate [Ca^{2+}]_{cyt} by governing the driving force for Ca^{2+} influx via controlling activity of voltage-gated K^+ (Kv) channels in IEC-6 cells that do not express VDCC. Depletion of cellular polyamines by DFMO inhibited Kv channel expression and resulted in membrane depolarization (data not shown), which was associated with a decrease in [Ca^{2+}]_{cyt} (Fig. 4A). [Ca^{2+}]_{cyt} in DFMO-treated cells was ~50% of normal values (without DFMO), and addition of spermidine to the cultures containing DFMO restored [Ca^{2+}]_{cyt} to normal levels. In addition, exposure of both control and polyamine-deficient cells to the Ca^{2+} ionophore ionomycin reversibly increased [Ca^{2+}]_{cyt} by promoting Ca^{2+} influx (Fig. 4B). [Ca^{2+}]_{cyt} in control cells was increased after the addition of 1 μM ionomycin for 5 min and rapidly returned to basal levels when ionomycin was washed out (Fig. 4Ba). Exposure of polyamine-deficient cells to ionomycin also significantly increased [Ca^{2+}]_{cyt}, but the peak of ionomycin-induced Ca^{2+} influx was reduced compared with that observed in control cells. This reduced response of DFMO-treated cells to ionomycin resulted from a decrease in the Ca^{2+}-driving force for Ca^{2+} influx, because polyamine depletion inhibited Kv channel expression and resulted in membrane depolarization (33, 35).
exposed to the Ca\(^{2+}\)-free medium immediately after wounding (Fig. 5Aa, left vs. middle). There was no significant increase in PY-\(\beta\)-catenin during the entire period of cell migration. Some small protein fragmentation was regularly detected in cells exposed to the Ca\(^{2+}\)-free medium for 4 and 6 h (Fig. 5Aa, middle). Although the etiology of this is still unclear, these small proteins might result from degradation of \(\beta\)-catenin or other cell-adhesion proteins. In contrast, increasing \([\text{Ca}\(^{2+}\)]_{\text{cyt}}\) through use of ionomycin significantly increased levels of PY-\(\beta\)-catenin (Fig. 5Aa, left vs. right). Maximum increases in PY-\(\beta\)-catenin levels occurred at 4 and 6 h after the incubation with ionomycin and presented ~25% additional increase compared with those of cells treated with normal DMEM medium (Fig. 5Aa). In DFMO-treated cells, removal of extracellular Ca\(^{2+}\) from the cultural medium further decreased PY-\(\beta\)-catenin levels [Fig. 5, Ab (left vs. middle) and Bb]. Although the basal level of PY-\(\beta\)-catenin in DFMO-treated cells was low, the response of tyrosine phosphorylation of \(\beta\)-catenin to ionomycin was similar to that observed in control cells during cell migration (Fig. 5, Ab and Bb). Neither the Ca\(^{2+}\)-free medium nor ionomycin altered levels of total \(\beta\)-catenin in control and DFMO-treated cells (Fig. 5, Aa and Ab, bottom). These data indicate that \(\beta\)-catenin tyrosine

To determine the possibility that polyamines regulate tyrosine phosphorylation of \(\beta\)-catenin through a process involving \([\text{Ca}\(^{2+}\)]_{\text{cyt}}\), we examined whether decreased or increased \([\text{Ca}\(^{2+}\)]_{\text{cyt}}\) altered PY-\(\beta\)-catenin levels during cell migration in the presence or absence of cellular polyamines. In control cells (without DFMO), the induction of PY-\(\beta\)-catenin levels during cell migration cells was completely prevented when cells were exposed to the Ca\(^{2+}\)-free medium immediately after wounding (Fig. 5Aa, left vs. middle). There was no significant increase in PY-\(\beta\)-catenin during the entire period of cell migration. Some small protein fragmentation was regularly detected in cells exposed to the Ca\(^{2+}\)-free medium for 4 and 6 h (Fig. 5Aa, middle). Although the etiology of this is still unclear, these small proteins might result from degradation of \(\beta\)-catenin or other cell-adhesion proteins. In contrast, increasing \([\text{Ca}\(^{2+}\)]_{\text{cyt}}\) through use of ionomycin significantly increased levels of PY-\(\beta\)-catenin (Fig. 5Aa, left vs. right). Maximum increases in PY-\(\beta\)-catenin levels occurred at 4 and 6 h after the incubation with ionomycin and presented ~25% additional increase compared with those of cells treated with normal DMEM medium (Fig. 5Aa). In DFMO-treated cells, removal of extracellular Ca\(^{2+}\) from the cultural medium further decreased PY-\(\beta\)-catenin levels [Fig. 5, Ab (left vs. middle) and Bb]. Although the basal level of PY-\(\beta\)-catenin in DFMO-treated cells was low, the response of tyrosine phosphorylation of \(\beta\)-catenin to ionomycin was similar to that observed in control cells during cell migration (Fig. 5, Ab and Bb). Neither the Ca\(^{2+}\)-free medium nor ionomycin altered levels of total \(\beta\)-catenin in control and DFMO-treated cells (Fig. 5, Aa and Ab, bottom). These data indicate that \(\beta\)-catenin tyrosine

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**Fig. 3.** Changes in the binding activity of \(\beta\)-catenin to \(\alpha\)-catenin and E-cadherin in migrating IEC-6 cells after wounding. A: representative autoradiograms of Western blots for \(\beta\)-catenin binding activity (a) and total \(\alpha\)-catenin (b). Cells were grown in control DMEM for 4 days, and cell lysates were collected at indicated time points after wounding. After lysis the catenin/cadherin complex was immunoprecipitated by a specific antibody against \(\beta\)-catenin, and precipitates were separated by electrophoresis on a 7.5% acrylamide gel. Binding activity of \(\beta\)-catenin was analyzed by determining the levels of \(\alpha\)-catenin or E-cadherin in this complex by Western blotting with the antibody against \(\alpha\)-catenin or E-cadherin. Total \(\alpha\)-catenin protein levels during migration after wounding were analyzed by Western blotting with anti-\(\alpha\)-catenin antibody. B: quantitative analysis of \(\alpha\)-catenin, \(\beta\)-catenin, and E-cadherin (a) and total \(\alpha\)-catenin (b) by densitometry from bands described in A. Values are means ± SE from 3 separate experiments. *\(P < 0.05\) compared with groups at 0 h.

**Fig. 4.** Effects of polyamine depletion by DFMO and the Ca\(^{2+}\) ionophore ionomycin on cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) in IEC-6 cells. A: summarized data showing [Ca\(^{2+}\)]\(_{\text{cyt}}\) measured in peripheral areas of cells grown in control cultures and in cultures containing 5 mM DFMO with or without 5 \(\mu\)M SPD for 4 days. Data are means ± SE; \(n = 20\). *\(P < 0.05\) compared with controls. B: representative records of [Ca\(^{2+}\)]\(_{\text{cyt}}\) in control (a) and DFMO-treated cells (b) before, during, and after application of ionomycin. After cells were grown in the presence or absence of DFMO for 4 days, 1 \(\mu\)M ionomycin was added into the media, and [Ca\(^{2+}\)]\(_{\text{cyt}}\) was continuously monitored for 5 min after the administration of ionomycin. Values are means from 10 cells, and the experiment was repeated 3 times with similar results.
phosphorylation is Ca\(^{2+}\) dependent in intestinal epithelial cells and that polyamines regulate PY-\(\beta\)-catenin levels, at least partially, through [Ca\(^{2+}\)]\(_{\text{cyt}}\) during cell migration after wounding.

Association of observed changes in PY-\(\beta\)-catenin and rates of cell migration. Consistent with the inhibitory effect on tyrosine phosphorylation of \(\beta\)-catenin, polyamine depletion by DFMO also significantly inhibited cell migration in IEC-6 cells, which was completely prevented by spermidine given together with DFMO (Fig. 6A). Furthermore, removal of extracellular Ca\(^{2+}\) from the cultural medium decreased cell migration in control (Fig. 6Ba) and DFMO-treated cells (Fig. 6Bb), whereas [Ca\(^{2+}\)]\(_{\text{cyt}}\) increased by ionomycin promoted cell migration. Rates of cell migration were decreased by \(\sim 80\%\) when the Ca\(^{2+}\)-free medium was given immediately after wounding in both two groups. Exposure of IEC-6 cells to the Ca\(^{2+}\)-free medium for 6 h did not alter cell attachment and cell viability (data not shown). In cells exposed to ionomycin, the rate of cell migration was increased by \(\sim 20\%\) in controls and \(\sim 40\%\) in DFMO-treated cells, respectively. These results indicate that changes in \(\beta\)-catenin tyrosine phosphorylation are accompanied by the rates of intestinal epithelial cell migration.

Effects of inhibition of \(\beta\)-catenin tyrosine phosphorylation by TK inhibitors on cell migration. To study the role of induced \(\beta\)-catenin tyrosine phosphorylation in the process of cell migration after wounding, we carried out two types of experiments by using the potent and specific inhibitors of TKs, genistein (1) and herbimycin-A (12). In the first study, we examined whether inhibition of tyrosine kinase activity by genistein or herbimycin-A altered the levels of PY-\(\beta\)-catenin and cell migration in control cells (without DFMO). Figure 7 clearly shows that exposure to genistein and herbimycin-A during cell migration decreased levels of PY-\(\beta\)-catenin but had no effect on total \(\beta\)-catenin protein in

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IEC-6 cells. When various doses of genistein were tested, β-catenin tyrosine phosphorylation was inhibited dose-dependently, with concentrations ranging from 25 to 100 μM. Maximum inhibition of PY-β-catenin occurred at 100 μM, where the levels of PY-β-catenin were decreased by ~80% (Fig. 7B, left). In cells treated with herbimycin-A, PY-β-catenin levels were decreased by ~45, ~60, and ~80% at doses of 0.5, 1, and 2 μg/ml, respectively (Fig. 7B, right). Consistent with the effect on β-catenin phosphorylation, exposure to genistein or herbimycin-A also dose-dependently inhibited cell migration in control cells (Fig. 8). When various doses of genistein were given immediately after wounding, rates of cell migration were decreased by ~25, ~40, and ~70% at 25, 50, and 100 μM, respectively. Similarly, cell migration was inhibited by ~30, ~40, and ~55% when herbimycin-A was given at doses of 0.5, 1, and 2 μg/ml during the period of cell migration. There was no apparent loss of cell viability in cells treated with genistein or herbimycin-A (Fig. 8B).

In the second study, we examined effects of inhibition of TK activity on the restoration of β-catenin tyrosine phosphorylation and cell migration by increasing [Ca²⁺]cyt in polyamine-deficient cells. As shown in Fig. 9, both PY-β-catenin levels and rates of cell migration were significantly decreased in DFMO-treated cells. When 1 μM ionomycin was given immediately after wounding, it not only restored PY-β-catenin levels to near normal in polyamine-deficient cells but also promoted cell migration. Treatment with either genistein (100 μM) or herbimycin-A (2 μg/ml) during the period of cell migration prevented the restoration of both PY-β-catenin and cell migration by ionomycin in DFMO-treated cells. There were no significant differences in levels of PY-β-catenin and cell migration between DFMO-treated cells and DFMO-treated cells exposed to ionomycin plus genistein or herbimycin-A. These results indicate that induced tyrosine phosphorylation of β-catenin following elevation of [Ca²⁺]cyt plays a critical role in polyamine-dependent intestinal epithelial cell migration after wounding.

Effects of [Ca²⁺]cyt and PY-β-catenin on distribution of actin filaments. In this study, we examined whether increasing or decreasing PY-β-catenin levels altered cellular distribution of actin filaments after wounding. In control migrating cells, long stress fibers could be seen traversing the cell, a network of cross-linked actin fibers of actin cortex just inside the plasma membrane (Fig. 10A). Some very fine short actin fibers outside the actin cortex extended into lamellipodia, sometimes with a bright outer edge. Exposure of control cells to the Ca²⁺-free medium during migration greatly reduced long stress fibers, and in some cells they appeared to be absent (Fig. 10A, a vs. b). There was a pronounced actin cortex around the entire cells that could be seen over the entire monolayer. Decreased PY-β-catenin by genistein, herbimycin-A or polyamine depletion with DFMO also affected cellular organization of actin filaments (Fig. 10A, c, d, and e). The features of distribution of actin stress fibers in all these three groups were similar to those observed in cells exposed to the Ca²⁺-free medium (Fig. 10A, b vs. c, d, or e). The number of long stress fibers was remarkably reduced, but the heavy actin cortex was observed around the whole cell in every experiment. Increased [Ca²⁺]cyt by exposure of polyamine-deficient cells to ionomycin not only promoted tyrosine phosphorylation of β-catenin (Fig. 9, A and B) but also restored the distribution of actin filaments to near normal (Fig. 10A, e vs. f). The organization of actin stress fibers in cells treated with DFMO but exposed to ionomycin after wounding was indistinguishable from that of control cells (Fig. 10A, a vs. f). On the other hand, the quantity of actin protein in the different treatment groups, as measured by Western blotting analysis,
showed no significant difference between them (Fig. 10B). These data indicate that PY-β-catenin affects the regulation of actin distribution but has no effect on actin protein formation.

**DISCUSSION**

Epithelial cell migration is a primary process during rapid mucosal restitution after superficial wounds in the gastrointestinal tract and requires precise control. Although cellular polyamines are absolutely required for this process, few specific functions of polyamines in epithelial cell migration have been defined. The dynamic link between the cadherin-catenin complex and the actin filament networks is essential for tight cell-cell contacts (2, 50), but the disruption of this association is crucial for the process through which epithelial cells are rapidly disassembled during restitution. Several studies have shown that disassembly of adhesion junctions is frequently associated to augmented tyrosine phosphorylation of proteins presented in the complex (2, 4, 20, 39, 44). Among these proteins, the most relevant changes have been detected in β-catenin. The results presented here provide, to our knowledge, the first demonstration that normal intestinal epithelial cell migration after wounding is accompanied by a significant increase in tyrosine phosphorylation of β-catenin. Cellular polyamines highly regulate this process by controlling [Ca²⁺]_cyt. These results suggest that tyrosine phosphorylation of β-catenin is involved in rapid early mucosal restitution and that polyamines are required for the stimulation of epithelial cell migration after wounding through, at least partially, alteration of cell-cell adhesion.

The findings reported here indicate that tyrosine phosphorylation of β-catenin reduces its interaction with α-catenin during intestinal epithelial cell migration. This specific change in the binding activity of PY-β-catenin to α-catenin is cell type-dependent because induction of PY-β-catenin in NBT-II cells has been shown to decrease its binding activity to both α-catenin and E-cadherin (28). The integrity of the cadherin-catenin complex is essential for strong cell-cell adhesion; therefore, this reduced interaction may lead to an overall decrease in intercellular contacts, thus promoting cell disassembly and consequent spreading during restitution. E-cadherin is the predominant cadherin in epithelial tissue and is responsible for the correct establishment and maintenance of adherens junctions (28, 39, 44). The adhesive function of E-cadherin requires the attachment to the actin cytoskeleton, an association mediated by catenins. β-Catenin is considered to be a tyrosine phosphorylation-sensitive component of the adhesion complexes, and the activation of tyrosine phosphorylation of β-catenin disrupts adherens junctions and dissociates E-cadherin from the cytoskeleton (4, 28, 39, 44, 53). Although the exact mechanisms involved are still unclear, different possibilities are proposed to explain the
adhesive changes of cadherin-catenin complex in response to β-catenin tyrosine phosphorylation (5, 39, 50). First, alteration in affinity of PY-β-catenin to α-catenin leads to a decreased stability of cadherin-catenin complex during epithelial cell migration. Second, conformational changes in the cadherin-catenin complex following tyrosine phosphorylation of β-catenin result in the disruption of linkage within cadherin and the cytoskeleton. Finally, recruitment of unknown proteins or factors to the cadherin-catenin complex enhances the dissociation of the cadherin-catenin from the cytoskeleton.

Cellular polyamines regulate tyrosine phosphorylation of β-catenin through Ca²⁺ in intestinal epithelial cells. Although depletion of cellular polyamines by DFMO did not affect basal level and cellular distribution of β-catenin protein in unwounded intestinal epithelial cells, it almost completely prevented the induction of tyrosine phosphorylation of β-catenin during epithelial cell migration after wounding (Fig. 2).
cells immediately exposed to Ca2+

treated cells. Images of cellular distribution of F-actin in control and DFMO-

or absence of DFMO for 4 days and herbimycin-A on F-actin distribution and formation in IEC-6 cells.

/H9262 ionomycin (1

F-actin staining. Cells were permeabilized and stained with rhodam-


cin-A (2

wounding.

/H9262 6 h in control cells immediately exposed to genistein (100 

m after wounding. Cells were grown in the presence of DFMO for 4 days and fixed 6 h after wounding for F-actin staining. Cells were permeabilized and stained with rhodamine-labeled phalloidin as described in MATERIALS AND METHODS. Original magnification, ×1,000. B: representative autoradiograms of Western blots for actin protein in cells described in A. Whole cell lysates (20 

were subjected to electrophoresis on a 7.5% acryl-

amide gel, and actin protein (42 kDa) was determined by Western immunoblotting analysis. Three experiments were performed that showed similar results.

Fig. 10. Effects of Ca2+ and tyrosine kinase inhibitors genistein and herbimycin-A on F-actin distribution and formation in IEC-6 cells. A: images of cellular distribution of F-actin in control and DFMO-treated cells. a: 6 h after wounding in control cells. b: 6 h in control cells immediately exposed to Ca2+-free medium after wounding. c: 6 h in control cells immediately exposed to genistein (100 

m after wounding. d: 6 h in control cells immediately exposed to herbimycin-A (2 

ml) after wounding. e: 6 h after wounding in DFMO-treated cells. f: 6 h in DFMO-treated cells immediately exposed to ionomycin (1 

m) after wounding. Cells were grown in the presence or absence of DFMO for 4 days and fixed 6 h after wounding for F-actin staining. Cells were permeabilized and stained with rhodamine-labeled phalloidin as described in MATERIALS AND METHODS. Original magnification, ×1,000. B: representative autoradiograms of Western blots for actin protein in cells described in A. Whole cell lysates (20 

were subjected to electrophoresis on a 7.5% acryl-

amide gel, and actin protein (42 kDa) was determined by Western immunoblotting analysis. Three experiments were performed that showed similar results.

inhibitory effect of polyamine depletion on β-catenin tyrosine phosphorylation is consistent with data from others (36, 40) who have reported that polyamine-deficient IEC-6 cells have a general decrease in tyrosine phosphorylation of focal adhesion kinase (FAK), which is associated with the inhibition of both FAK activity and cell attachment. The results presented in this report, however, have further demonstrated that polyamines regulate tyrosine phosphorylation through a process dependent on [Ca2+]cyt in intestinal epithelial cells. As noted in Fig. 5, reduction of [Ca2+]cyt through polyamine depletion or the removal of extra-

cellular Ca2+ inhibited tyrosine phosphorylation of

β-catenin during cell migration, whereas elevation of [Ca2+]cyt by the Ca2+-ionophore ionomycin not only increased PY-β-catenin levels in control cells but also restored the tyrosine phosphorylation to near normal in polyamine-deficient cells.

Our previous studies have demonstrated that elevated [Ca2+]cyt is a major mediator for the stimulation of intestinal epithelial cell migration following an increase in cellular polyamines (33, 35, 49). Cytoplasmic free Ca2+ is an important intracellular second messenger that modulates a large number of physiological functions (6, 8, 9). [Ca2+]cyt is controlled by Ca2+ influx through Ca2+-permeable channels in the plasma membrane and Ca2+ release from internal Ca2+ stores (8, 31). Ca2+ influx depends on the Ca2+ driving force (i.e., the electrochemical gradient across the plasma membrane), which is predominantly regulated by membrane potential (E_m) while the Ca2+ concentration gradient is constant (10, 11, 13). Polyamines regulate [Ca2+]cyt concentration primarily by governing E_m through control of Kv channel expression in intestinal epithelial cells. Polyamine depletion decreases Kv channel expression and voltage-gated K+ currents, leading to membrane depolarization and decrease in [Ca2+]cyt through reduction of the driving force for Ca2+ influx (35, 49). We have recently demonstrated that small GTPase RhoA is a downstream target of elevated [Ca2+]cyt following activation of K+ channels by increased cellular polyamines and that Ca2+-activated RhoA activity increases stress fiber formation in migrating cells during restitution (33). The current studies provide additional new information that tyrosine phosphorylation of β-catenin is also implicated in the signaling pathway of Ca2+-mediated intestinal epithelial cell migration following increased poly-

amines after wounding.

Activation of β-catenin tyrosine phosphorylation due to elevated [Ca2+]cyt plays a critical role in polyamine-dependent cell migration during early epithelial restitution. Decreased PY-β-catenin levels caused by treatment with the specific TK inhibitors genistein and herbimycin-A inhibited normal cell migration (without DFMO) (Figs. 7 and 8). Consistent with our current observations, it has been shown that induction of the tyrosine phosphorylation of β-catenin by transfection of the cytoplasmic tyrosine kinases such as v-src gene (17, 24) or treatment with growth factors (19, 28, 42) causes unstable cell-cell adhesion and promotes cell migration. In contrast, decreased PY-β-catenin by transfection of either a dominant negative src mutant or the PTP-LAR gene inhibits cell migration (28, 38). An interesting and extended finding obtained in the current study is that decreased PY-β-catenin also pre-

vents the restoration of cell migration by increasing [Ca2+]cyt with ionomycin in polyamine-deficient cells (Fig. 9). Taken together, the current results and our previous findings (33, 35, 49) strongly support the contention that polyamines stimulate intestinal epithelial cell migration after wounding, at least partially, by altering cell-cell adhesion through β-catenin tyrosine phosphorylation mediated by [Ca2+]cyt.
Our results also show that tyrosine phosphorylation of β-catenin is involved in the regulation of cellular distribution of the cytoskeleton during epithelial cell migration. Decreases in tyrosine phosphorylation of β-catenin by genestein or herbimycin-A resulted in reorganization of actin filaments. The numbers of long stress fibers were greatly reduced, and the heavy actin cortex around the whole cell was observed (Fig. 10). Although the exact mechanisms involved are obscure, it is likely that the regulatory effect of β-catenin on cellular distribution of actin filaments is independent of its role at adherens junctions. It has been shown that β-catenin is an intracellular mediator and interacts with different proteins and transcription factors to perform various distinct signaling functions (5, 27, 52).


REFERENCES


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