Phosphatidylinositol 3-Kinase Controls Human Intestinal Epithelial Cell Differentiation by Promoting Adherens Junction Assembly and p38 MAPK Activation*

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The signaling pathways mediating human intestinal epithelial cell differentiation remain largely undefined. Phosphatidylinositol 3-kinase (PI3K) is an important modulator of extracellular signals, including those elicited by E-cadherin-mediated cell-cell adhesion, which plays an important role in maintenance of the structural and functional integrity of epithelia. In this study, we analyzed the involvement of PI3K in the differentiation of human intestinal epithelial cells. We showed that inhibition of PI3K signaling in Caco-2/15 cells repressed sucrose-isomaltase and villin protein expression. Morphological differentiation of enterocyte-like features in Caco-2/15 cells such as epithelial cell polarity and brush-border formation were strongly attenuated by PI3K inhibition. Immunofluorescence and immunoprecipitation experiments revealed that PI3K was recruited to and activated by E-cadherin-mediated cell-cell contacts in confluent Caco-2/15 cells, and this activation appears to be essential for the integrity of adherens junctions and association with the cytoskeleton. We provide evidence that the assembly of calcium-dependent adherens junctions led to a rapid and remarkable increase in the state of activation of Akt and p38 MAPK pathways and that this increase was blocked in the presence of anti-E-cadherin antibodies and PI3K inhibitor. Therefore, our results indicate that PI3K promotes assembly of adherens junctions, which, in turn, control p38 MAPK activation and enterocyte differentiation.

The epithelium of the small intestine is characterized by its rapid and constant renewal. This process involves cell generation and migration from the stem cell population located at the bottom of the crypt to the extrusion of terminally differentiated cells at the tip of the villus (1). Thus, the crypt is mainly composed of proliferative and poorly differentiated cells, whereas the villus is lined with functional absorptive, goblet, and endocrine cells (1). The molecular and cellular mechanisms responsible for the fine coordination between proliferation, migration, and differentiation along the crypt-villus axis are still largely unknown.

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E-cadherin-mediated cell-cell attachment plays an important role in the differentiation, polarization, and homeostasis of many epithelia (2–4). Cadherins are responsible for cell-cell adhesion through a calcium-dependent interaction of their extracellular domains. Their cytoplasmic tails are linked to the cytoskeleton through a complex of proteins that includes α-, β-, and γ-catenins. This link is involved in the strengthening of cell-cell adhesion and in the cohesion of epithelial tissues (5). The importance of cadherins in the renewal of the intestinal epithelium has been demonstrated in vivo in two mouse models. Overexpression of E-cadherin in the crypts of the small intestine reduces cell proliferation and migration (6). Conversely, expression of a dominant-negative N-cadherin leads to over-polarization, uncoordinated differentiation, and a Crohn’s disease phenotype (7).

The intracellular signaling pathways that transmit extracellular cues for epithelial differentiation along the crypt-villus axis of the intestine remain poorly defined. We recently reported that p38 MAPK1 plays a crucial role in intestinal epithelial cell differentiation by enhancing the transactivation capacity of CDX2 (8), an intestine-specific homeobox gene product well known for its broad effect on enterocyte differentiation (9). However, the upstream signaling pathways activating p38 MAPK in committed intestinal cells induced to differentiate remain to be defined. Interestingly, in vitro experiments have shown that the establishment of cell-cell contacts in intestinal cell cultures could be a critical step in initiating p38 MAPK action (8), cell cycle arrest (10, 11), and induction of the differentiation process (8, 12–15).

An important role for p38 MAPK in various mammalian cell differentiation processes has recently been proposed (16). For instance, differentiation of C2C12 and L8 myoblasts into myotubes has been found to be mediated by p38 activation (17, 18). Although this skeletal muscle differentiation requires phosphatidylinositol 3-kinase (PI3K), it is not yet clear whether PI3K and p38 MAPK act in a common pathway (19, 20). The PI3K family members are lipid kinases that phosphorylate phosphoinositides at position 3 of the inositol ring (21), acting as membrane anchors that locate and activate pleckstrin homology domain-containing effectors such as the well-characterized serine/threonine kinase Akt (22). Class I PI3Ks are generally composed of a p85 regulatory subunit and a p110 catalytic subunit (21). This class of PI3Ks can be activated by a

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; ZO-1, zona occludens-1; PBS, phosphate-buffered saline; GFP, green fluorescent protein; PIPES, 1,4-piperazinediethanesulfonic acid; APC, adenomatous polyposis coli.
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wide variety of extracellular stimuli, including those elicited by E-cadherin-mediated cell-cell adhesion (23).

In this study, the role and regulation of PI3K in intestinal epithelial cells were investigated. Using several approaches, we demonstrated that PI3K is necessary for the functional and morphological differentiation of intestinal epithelial cells. We also found that PI3K is recruited to and activated by E-cadherin-mediated cell-cell contacts in confluent Caco-2/15 cultures and that this activation appears to be essential for the integrity of the adherens junctions and the association of their components with the cytoskeleton. Finally, we have provided evidence that the assembly of the adherens junctions stimulates Akt and p38 MAPK in a PI3K-dependent manner.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP and the enhanced chemiluminescence immuno

nucodetection system (ECL) were obtained from Amersham Biosciences, Inc. (Baie d’Urfé, Québec, Canada). Antiserum that specifically recognizes p38 MAPK on Western blots (24) was a kind gift from Dr. J. Landry (Université Laval, Laval, Québec). Monoclonal antibody HSI-14 (25) against sucrase-isomaltase was kindly provided by Dr. A. Quaroni (Cornell University, Ithaca, NY). Monoclonal antibody CI110 recognizing the 55-kDa apoptotic fragment and the 113-kDa non-cleaved frag

ment of poly(ADP-ribose) polymerase was a kind gift from Dr. G. Poirier (Université Laval). Fluorescein isothiocyanate-labeled goat anti-rabbit IgG and rhodamine-labeled goat anti-mouse IgG were from Roche Molecular Biochemicals (Laval). Antibodies raised against villin, α-catenin and β-catenin were purchased from Transduction Laboratories (Mississauga, Ontario, Canada). Antibody recognizing the phosphorylated and active form of p38 MAPK was from Promega (Nepean, Ontario). Antibody directed against the PI3K p85 regulatory subunit was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Akt and anti-

phospho-Akt(Ser473) antibodies were purchased from Cell Signaling (Mississauga). Antibody directed against total actin was from Roche Molecular Biochemicals. The anti-ZO-1 antibody and the anti-E-cad

herin antibody used in antibody inhibition experiments were from Zymed Laboratories, Inc. (South San Francisco, CA). The specific inhibi

tor of PI3K (LY294002) was purchased from Calbiochem (Mississauga). All other materials were obtained from Sigma (Oakville, Ontario) unless otherwise stated.

Cell Culture—The Caco-2/15 cell line was obtained from Dr. A. Quaroni. The clone of the parent Caco-2 cells (HTB37; American Type Culture Collection, Manassas, VA) has been extensively characteri

zed elsewhere (13, 15, 26) and was originally selected for expressing the highest level of sucrase-isomaltase among 16 clones obtained by random cloning. This cell line was routinely cultured in plastic dishes in Dulbecco’s modified Eagle’s medium (Invitrogen, Burlington, Ontario) containing 10% fetal bovine serum, 4 mM glutamine, 20 mM HEPES, 50 units/ml penicillin, and 50 μg/ml streptomycin. Caco-2/15 cells were used between passages 53 and 78. Studies were performed on cultures used between passages 53 and 78. Studies were performed on cultures

PI3K (p110α) and a dominant-negative form of PI3K (Δp85) were ob

tained from Dr. Julian Downward (Imperial Cancer Research Fund, London, United Kingdom) and Dr. Masato Kasuga (Kobe University School of Medicine, Kobe, Japan). Green fluorescent protein (GFP) (CLONTECH, Palo Alto, CA) was subcloned into the expression vector pR-Luc (CLONTECH).

Transient Transfections—Newly confluent Caco-2/15 cells grown on glass coverslips in six-well plates were cotransfected by lipofection (LipofectAMINE 2000, Invitrogen) with 0.5 μg of pLHCHX-GFP and 0.5 μg of pCDNA containing or not the dominant-negative form of PI3K (Δp85). Two days following transfection, cells were fixed for GFP fluorescence and immunofluorescence with either anti-E-cadherin antibody or anti-total actin antibody.

Luciferase Assays—Confluent Caco-2/15 cells grown in 24-well plates were transfected by lipofection (LipofectAMINE 2000) as described previously (15) with 0.1 μg of sucrase-isomaltase/luciferase reporter. One day following transfection, cells were treated with 0, 1, 2, 5, 10, and 20 μM LY294002 for 2 h, and luciferase activity was measured. In other experiments, the sucrase-isomaltase reporter gene vector was cotransfected with 0.1 μg of pCDNA containing or not the dominant-negative (Δp85) or constitutively active (p110α) form of PI3K. Luciferase activity was measured 36 h after transfection. The pRL-SV40 Re

nilla luciferase vector was used as a control for transfection efficiency.

Electron Microscopy—Cell cultures were rinsed with PBS, prefixed for 15 min with a 1:1 mixture of culture medium (Dulbecco’s modified Eagle’s medium) and freshly prepared 2.5% glutaraldehyde in cado

dy buffer. Cells were then washed four times with ice-cold PBS, and then soluble proteins were ex

tracted on ice with cold lysis/cytoskeleton stabilization buffer (0.5 mM Tris (pH 7.5), 5 mM EDTA, 2.5 mM MgCl2). The cytoskeleton-associated proteins (insoluble fraction) were isolated by centrifugation at 13,000 g for 30 min. The soluble fraction (supernatant) was obtained by ultracentrifugation. The cytoskeleton fraction was then fixed for 30 min with 2.8% glutaraldehyde at room temperature. After two rinses, specimens were post-fixed for 60 min with 2% osmium tetroxide in cadydoy buffer. The cells were then dehydrated using increasing ethanol concentrations (40, 70, 90, 95, and 100%); three times each) and covered twice for 3 h with a thin layer of Araldite 502 resin (for ethanol substitution). Finally, the resin was allowed to polymerize at 60 °C for 48 h. The specimens were detached from the plastic vessels, inverted in embedding molds, covered with Araldite 502, and re incubated at 60 °C for 48 h. Thin sections were prepared using an ultramicrotome, contrasted with lead citrate and uranyl acetate, and observed in a blind fashion on a Jeol 100 CX transmission electron microscope. All reagents were purchased from Electron Microscopy Sciences (Cedarlane, Hornby, Ontario).

Isolation of Cytoskeleton-associated Proteins—First, the cells were washed twice with ice-cold PBS, and then soluble proteins were ex

tracted on ice with cold lysis/cytoskeleton stabilization buffer (0.5% Triton X-100, 50 mM NaCl, 10 mM PIPES (pH 6.8), 300 mM sucrose, and 3 mM MgCl2). The cytoskeleton-associated proteins (insoluble fraction) were isolated by centrifugation at 13,000 g for 30 min. The soluble fraction (supernatant) was obtained by ultracentrifugation. The cytoskeleton fraction was then fixed for 30 min with 2.8% glutaraldehyde at room temperature. After two rinses, specimens were post-fixed for 60 min with 2% osmium tetroxide in cadydoy buffer. The cells were then dehydrated using increasing ethanol concentrations (40, 70, 90, 95, and 100%); three times each) and covered twice for 3 h with a thin layer of Araldite 502 resin (for ethanol substitution). Finally, the resin was allowed to polymerize at 60 °C for 48 h. The specimens were detached from the plastic vessels, inverted in embedding molds, covered with Araldite 502, and re incubated at 60 °C for 48 h. Thin sections were prepared using an ultramicrotome, contrasted with lead citrate and uranyl acetate, and observed in a blind fashion on a Jeol 100 CX transmission electron microscope. All reagents were purchased from Electron Microscopy Sciences (Cedarlane, Hornby, Ontario).

Co-immunoprecipitation Experiments—Cells were washed twice with ice-cold PBS and lysed in chilled lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 7.5), 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml apro

tin, 0.1 mM orthovanadate, and 40 μM β-glycerophosphate). Cell lysates were then cleared of cellular debris by centrifugation. Primary antibodies were added to 600 μg of each cell lysate and incubated for 2 h at 4 °C under agitation. Forty μg of protein A-Sepharose (Amersham Biosciences, Inc.) were subsequently added for 1 h (4 °C under agita

tion). Immunocomplexes were then harvested by centrifugation and washed four times with ice-cold lysis buffer. Proteins were solubilized with Laemmli buffer and separated by SDS-PAGE.

Immunofluorescence Microscopy—Caco-2/15 cells grown on sterile glass coverslips were washed twice with ice-cold PBS. Cultures were then fixed with 30% methanol and 70% acetone for 15 min at −20 °C, permeabilized with a solution of 0.1% of Triton X-100 in PBS for 10 min, and blocked with PBS and 2% bovine serum albumin for 20 min at room temperature. Cells were finally immunostained for 1 h with the primary antibody and for 30 min with the secondary antibody. For F-actin staining, fixed cells were incubated with 1 μg/ml fluorescein isothiocya

nate-phalloidin for 30 min. For total actin staining, fixed cells were incubated with antibody raised against total actin and then with rhe
dophil-labeled goat anti-rabbit IgG. Negative controls (no primary antibody) were included in all experiments.

Expression Vectors and Reporter Constructs—The sucrase-isomaltase reporter gene vector (pGL2 reporter construct as described previously (9). The pRL-SV40 Renilla luciferase vector was from Promega. Expression vectors for a constitutively active form of PI3K (p110α) and a dominant-negative form of PI3K (Δp85) were ob

tained from Dr. Julian Downward (Imperial Cancer Research Fund, London, United Kingdom) and Dr. Masato Kasuga (Kobe University School of Medicine, Kobe, Japan). Green fluorescent protein (GFP) (CLONTECH, Palo Alto, CA) was subcloned into the expression vector pRL (CLONTECH).
and 40 mM β-glycerophosphate). p38 MAPK was immunoprecipitated from 400 μg of cell lysate. Immunocomplexes were then washed four times with ice-cold lysis buffer and three times with ice-cold kinase buffer (20 mM p-nitrophenyl phosphate, 10 mM MgCl₂, 1 mM dithiothreitol, and 30 mM HEPES (pH 7.4)) prior to the kinase assay. The kinase reactions were initiated by incubating the immunocomplexes at 30 °C in the presence of myelin basic protein and [γ-32P]ATP at 2 μg and 2 μCi assay, respectively. After 30 min, the reaction was stopped by adding Laemmli buffer. Radiolabeled substrates were separated from immunocomplexes by SDS-12.5% PAGE and autoradiographed. Incorporation of [32P] by myelin basic protein was linear over the course of the kinase assay.

Inhibition of E-cadherin-mediated Cell-Cell Contacts—Day 2 post-confluent Caco-2/15 cells were serum-starved for 16 h in Dulbecco’s modified Eagle’s medium supplemented with 20 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin, and 4 mM glutamine. The adherens junctions were then disrupted by treatment with 4 mM EDTA for 30 min at 37 °C. Intercellular contacts were subsequently allowed to re-establish by restoration of the extracellular calcium concentration by replacing the EDTA-containing medium with fresh medium (1.8 mM CaCl₂) (23, 29). In some experiments, the fresh medium contained 100 μg/ml anti-E-cadherin antibody or mouse IgG purified from nonimmune serum. After selected time intervals of calcium restoration, cells were washed twice with ice-cold PBS and lysed to detect phospho-Akt and to assay p38 MAPK activity or were fixed for immunofluorescence.

RESULTS

PI3K Activity Is Essential for Differentiation of Intestinal Epithelial Cells—To investigate the role of PI3K in intestinal epithelial cell differentiation, we evaluated the impact of its inhibition on the spontaneous enterocyte differentiation of the human colon cancer cell line Caco-2/15. This cell line provides a unique and well characterized model for the study of intestinal epithelial differentiation because these cells undergo differentiation to a small bowel-like phenotype with microvilli, dome formation, and the expression of sucrase-isomaltase several days after reaching confluence (12–15). To block PI3K signaling, we used LY294002, a compound that acts as a competitive inhibitor of the adenosine triphosphate-binding site of PI3K and has been shown to cause specific inhibition with an IC₅₀ of 1.4–5 μM in intact cells (30). Daily addition of 10 μM LY294002 beginning at confluence strongly attenuated the expression levels of two enterocytic differentiation markers, viz. sucrase-isomaltase and villin, compared with untreated cells at days 3, 6, and 12 post-confluence (Fig. 1A). Loss of PI3K activity did not interfere with overall protein expression, as shown by similar actin levels in LY294002-treated and untreated cells (Fig. 1A). To ascertain that the loss of differentiation marker expression was not a consequence of increased apoptosis, we evaluated the expression of poly(ADP-ribose) polymerase, a well known caspase-3 substrate (31), in cells treated with LY294002. As shown in Fig. 1A, chronic treatment of confluent Caco-2/15 cells with the PI3K inhibitor had no effect on poly(ADP-ribose) polymerase cleavage, suggesting that persistent inhibition of PI3K did not affect Caco-2/15 cell survival.

The role of PI3K in sucrase-isomaltase expression was further investigated by transient transfection of newly confluent Caco-2/15 cells with a luciferase reporter gene under the control of the human sucrase-isomaltase promoter. As shown in Fig. 1B, sucrase-isomaltase gene expression was inhibited in a dose-dependent manner by the PI3K inhibitor LY294002, with a maximal effect observed at 20 μM (91% inhibition). Furthermore, overexpression of a dominant-negative form of the regulatory p85 subunit (Δp85) reduced sucrase-isomaltase gene expression by 50%. Conversely, overexpression of a constitutively active p110 subunit (p110<sup>ac</sup>) slightly enhanced sucrase-isomaltase gene expression.

Caco-2/15 cell cultures were characterized by transmission electron microscopy day 14 post-confluence. As shown in Fig. 2 (panels 1, 3, and 5), post-confluent Caco-2/15 cells exhibited ultrastructural characteristics similar to those found in the intact villus epithelium, including well organized brush borders, terminal webs at the luminal aspect of absorptive cells, and typical junctional complexes. Interestingly, treatment of confluent Caco-2/15 cells with the PI3K inhibitor remarkably affected cell polarization and brush-border formation. Indeed, LY294002-treated cells exhibited a less polarized and flat phenotype compared with untreated cells (Fig. 2, panels 1 and 2). The morphology of the brush border was altered markedly, as visualized by a reduction in the number of microvilli (Fig. 2, panels 3 and 4). Interestingly, poorly defined apical junctional complexes were observed in LY294002-treated cells (Fig. 2, panels 5 and 6). Taken together, these results indicate that PI3K activity is necessary for functional and morphological differentiation of intestinal epithelial cells.

PI3K Transiently Controls Adherens Junction Integrity—Cell-cell adhesion plays a crucial role in the polarization and differentiation of epithelial cells (2–4). Our observation that LY294002-treated cells exhibited a less polarized and differentiated phenotype as well as poorly defined apical junctions prompted us to investigate whether PI3K might control the assembly of adherens and tight junctions in Caco-2/15 cells. We
performed E-cadherin and ZO-1 staining in a two-step experiment in which tight and adherens junctions of day 2 post-confluent Caco-2/15 cells were disrupted by chelating extracellular calcium and subsequently allowed to re-establish by restoration of extracellular calcium (23, 29) in the presence or absence of LY294002. The untreated cells showed typical honeycomb E-cadherin and ZO-1 staining (Fig. 3A, panels 1 and 5). After a 30-min EGTA treatment, cells became rounded, whereas E-cadherin and ZO-1 staining formed a diffuse ring at the cell periphery (Fig. 3A, panels 2 and 6). Following calcium restoration, E-cadherin and ZO-1 redistributed at the sites of cell-cell contact, and the cells reacquired their epithelial shape (Fig. 3A, panels 3 and 7), suggesting that tight and adherens junctions were almost completely reformed. However, in LY294002-treated cells, most of the immuno-reactive E-cadherin remained diffusely distributed (Fig. 3A, panel 4). Interestingly, similar results were also noted in Caco-2/15 cells transiently transfected with the dominant-negative mutant of p85 (Fig. 3B), indicating that PI3K inhibition interfered with the assembly of adherens junctions. This phenomenon appears to be specific for adherens junctions because ZO-1 redistributed at the sites of cell-cell contact even in the presence of LY294002 (Fig. 3A, panel 8).

Previous studies have shown that the E-cadherin and β-catenin associated with functional adherens junctions are indirectly linked to the cytoskeleton (32). Thus, they cannot be extracted with a solution of 0.5% of the nonionic detergent Triton X-100, but are found in the insoluble fraction. Inhibition of PI3K resulted in a significant reduction in the proportion of E-cadherin and β-catenin associated with the cytoskeleton after 16–72 h of treatment in newly confluent Caco-2/15 cells (Fig. 4A, upper panels). Total amounts of E-cadherin or β-catenin proteins remained comparable. Interestingly, inhibition of PI3K activity by LY294002 or by ectopic expression of the dominant-negative mutant of p85 in newly confluent cells also led to a partial disruption of F-actin at the periphery of the cytoplasmic membrane (Fig. 4B, right panel) and to a drastic redistribution of total actin (Fig. 4C, right panel), respectively; expression of GFP alone, however, did not influence actin distribution (data not shown). It was also recently reported that the regulation of adherens junction assembly/disassembly is dependent upon cellular context and junction maturation (33, 34). To test the hypothesis that PI3K activity is also important for the maintenance of mature adherens junctions in differentiated cells, we performed Triton X-100 extraction on day 9 post-confluent cells. In this situation, PI3K inhibition did not interfere with the association of E-cadherin or β-catenin with the cytoskeleton even after 72 h of treatment (Fig. 4A, lower panels). Overall, the results indicate that PI3K regulates E-cadherin and β-catenin association with the cytoskeleton at the time cells reach confluence, suggesting that PI3K activity is involved in the assembly of E-cadherin-mediated cell-cell contact rather than in the maintenance of mature adherens junctions.

PI3K Is Recruited to Adherens Junctions in Confluent Caco-2/15 Cells—To explore the possibility that PI3K is recruited in response to E-cadherin engagement and acts locally to control adherens junction formation in intestinal epithelial cells, sub-confluent (day −2), confluent (day 0), and post-confluent (day 3) Caco-2/15 cells were double-labeled with antibodies to E-cadherin and the p85 regulatory subunit of PI3K. As illustrated in Fig. 5A (panel 1), expression of E-cadherin in subconfluent cells was detected at the sites of cell-cell contact, yet a significant amount of E-cadherin was diffusely distributed throughout the cytoplasm, which is a typical feature of immature junctions. In the same cells, p85 staining was restricted to the cytoplasm (Fig. 5A, panel 2), and no significant co-localization of E-cadherin and p85 was observed (panel 3). In newly confluent cells, E-cadherin accumulated at cell-cell interfaces in a typical honeycomb pattern characteristic of specialized and functional adherens junctions (Fig. 5A, panel 4). In the same cells, p85 was still partially localized in the cytoplasm, but was also clearly visible at the sites of cell-cell contact (Fig. 5A, panel 5, see arrowheads) with significant co-localization of E-cadherin and p85 (panel 6, see arrowheads). In day 3 post-confluent cells, p85 and E-cadherin were localized homogeneously at

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**Fig. 2.** PI3K inhibition alters morphological differentiation of human intestinal epithelial cells. Caco-2/15 cells were treated from days 0 to 14 post-confluence with or without 10 µM LY294002. Cells were fixed in glutaraldehyde and osmium tetroxide before epoxy embedding for electronic microscopy analysis. Bars = 5 µm (panels 1 and 2), 500 nm (panels 3 and 4), and 200 nm (panels 5 and 6).

**Fig. 3.** PI3K inhibition interferes with adherens junction assembly. A, day 1 post-confluent Caco-2/15 cell monolayers were serum-starved for 16 h and then treated with 4 mM EGTA for 30 min with (+) or without (−) 10 µM LY294002. The EGTA-containing medium was replaced with fresh calcium-containing medium for 30 min with or without the inhibitor. Cells were fixed for immunofluorescence and co-stained for E-cadherin and ZO-1 proteins. B, confluent Caco-2/15 cells (day 0) were cotransfected with 0.5 µg of pLHCX-GFP plasmid and 0.5 µg of pcDNA3 containing or not the dominant-negative form of PI3K (pAp85). One day following transfection, cells were serum-starved for 16 h and treated with 4 mM EGTA for 30 min. The EGTA-containing medium was then replaced with fresh calcium-containing medium for 30 min. Cells were fixed for GFP fluorescence and immunofluorescence (IF) with anti-E-cadherin antibody. Bar = 10 µm.
the cell-cell interfaces (Fig. 5A, panels 7 and 8), and the superimposition of both stainings showed a strong co-localization of these proteins (panel 9). We further studied the dynamics of E-cadherin/p85 interactions by co-immunoprecipitation assays. Fig. 5B shows that E-cadherin/p85 association in Caco-2/15 cells was significantly enhanced when the cells reached confluence (day 0) and further increased at day 3 post-confluence. Taken together, these results suggest that PI3K is recruited to E-cadherin-mediated cell-cell contacts at the time Caco-2/15 cells reach confluence.

**PI3K Inhibition Interferes with Activation of Akt and p38 MAPK**—We have previously demonstrated that p38α, an isoform of the p38 MAPK family, controls expression of some intestine-specific genes, viz. villin and sucrase-isomaltase (8). To investigate whether PI3K and p38α MAPK act in a common

**Fig. 5. E-cadherin engagement recruits PI3K p85 to the site of cell-cell contact.** A, subconfluent, confluent (day 0), and day 3 post-confluent Caco-2/15 cells were fixed with methanol/acetic acid and permeabilized with a solution of 0.1% Triton X-100 for immunofluorescence and co-staining for PI3K p85 and E-cadherin proteins. Representative results of *in situ* indirect immunofluorescence from three independent experiments are shown. Bar = 10 μm. B, E-cadherin and PI3K p85 were immunoprecipitated (IP) from 600 μg of lysates from subconfluent, confluent (day 0), and day 3 post-confluent Caco-2/15 cells. Proteins of the immunoprecipitates were solubilized in Laemmli buffer and separated by SDS-PAGE. Proteins were analyzed by Western blotting (WB) to determine the amount of PI3K p85 and E-cadherin in the immunoprecipitates. The blots shown are representative of three independent experiments. α-E-cadherin, anti-E-cadherin antibody.

or parallel pathway in intestinal epithelial cells, we evaluated the expression and kinase activity of p38α in LY294002-treated Caco-2/15 cells. To determine the kinetics of PI3K inhibition by LY294002 in Caco-2/15 cells, we first analyzed the phosphorylation of Akt at Ser473, which is strongly dependent upon PI3K activity (22). As shown in Fig. 6 (upper panels), addition of LY294002 to newly confluent Caco-2/15 cells (day 0) did not influence Akt abundance, but strongly repressed Akt phosphorylation throughout the treatment. In contrast, p38α MAPK activity was inhibited only after 24, 48, and 72 h of treatment, whereas p38α protein expression remained unaffected by the PI3K inhibitor. This observation indicates that PI3K controls p38α MAPK activity in intestinal epithelial cells. In contrast to Akt, however, p38α MAPK does not seem to be a direct downstream effector of PI3K signaling in intestinal epithelial cells.

As demonstrated in Fig. 4A, newly confluent Caco-2/15 cells were more sensitive to PI3K inhibition than day 9 post-confluent cells (differentiated cells). Therefore, we evaluated the effect of LY294002 on p38α activity in late post-confluent cells. Treatment of day 9 post-confluent Caco-2/15 cells with LY294002 did not influence p38α activity even after 72 h of treatment (Fig. 6, lower panels), whereas Akt phosphorylation...
FIG. 6. PI3K inhibition interferes with Akt phosphorylation and p38 MAPK activity in newly confluent Caco-2/15 cells. A, upper panel, newly confluent (day 0) Caco-2/15 cells were treated with (+) or without (−) 10 μM LY294002 for 3–72 h, and cells were lysed at the end of the treatment. Lower panel, post-confluent differentiated Caco-2/15 cells were treated for 3–72 h with or without 10 μM LY294002, and cells were lysed at the same time at day 0 post-confluence. Cell lysates (400 μg) were immunoprecipitated with an antibody to p38α MAPK. The levels of immunoprecipitated p38α were analyzed by Western blotting, and the kinase activity of p38 was demonstrated by the phosphorylation of myelin basic protein (phospho-MBP). The phosphorylation of Akt was analyzed with an antibody that specifically recognizes Akt phosphorylated at Ser473 (phospho-Akt). The membrane was then reprobed with an antibody that recognizes Akt regardless of its phosphorylation state (total Akt). The results are representative of three independent experiments.

E-cadherin Engagement Leads to p38α and Akt Activation—We demonstrated that the kinetics of adherens junction disruption and p38α inactivation following LY294002 treatment in newly confluent Caco-2/15 cells were quite similar (Figs. 4A and 6, upper panels). This observation prompted us to investigate whether PI3K could indirectly control p38α MAPK activity by promoting E-cadherin–dependent cell-cell interactions. Hence, we monitored p38α activation in a calcium switch experiment (23, 29) in the presence or absence of LY294002. Removal of calcium from the culture medium of newly confluent Caco-2/15 cells resulted in disruption of the junctions (Fig. 3A) and clearly decreased Akt phosphorylation and p38 MAPK activity (Fig. 7A, second lane). Interestingly, Akt phosphorylation returned to the control level 60 min after calcium restoration, whereas p38α MAPK activity was almost completely re-established to the control level 5 min after calcium restoration, with an over-stimulation observed after 15 and 60 min (Fig. 7A, third through fifth lanes). PI3K inhibition abolished Akt phosphorylation and strongly attenuated p38α MAPK reactivation (Fig. 7A, sixth through eighth lanes). To verify that the modulation of Akt phosphorylation and p38α MAPK activation was actually due to adherens junction breakdown/reformation and not to experimental procedure, we blocked E-cadherin engagement during calcium restoration with an antibody known to block E-cadherin-mediated cell-cell interaction (35). As illustrated in Fig. 7B, the use of E-cadherin-blocking antibodies efficiently prevented Akt phosphorylation and p38α reactivation (monitored by Western blotting with an antibody recognizing the phosphorylated and active form of p38 MAPK) after 30 min of calcium restoration, indicating that E-cadherin engagement was necessary for Akt and p38α MAPK activation. Collectively, these results indicate that E-cadherin engagement stimulates p38α and Akt pathways through a PI3K-dependent mechanism.
DISCUSSION

Little is known regarding the mechanisms involved in the regulation of cell growth and differentiation in the human intestinal epithelium. The data presented in this report suggest that PI3K plays a crucial role in the control of differentiation events in this tissue. Indeed, we have demonstrated for the first time that PI3K is part of a signaling pathway necessary for functional and morphological differentiation of intestinal epithelial cells. Inhibition of PI3K decreased the expression of enterocyte markers, viz. sucrase-isomaltase and villin, and reduced cell polarization and brush-border formation. Furthermore, we demonstrated that E-cadherin engagement triggers the recruitment of PI3K and activates its signaling at the sites of cell-cell contact, and this activation appears to be essential for the assembly of adherens junctions and the association of their components with the cytoskeleton. Finally, we have provided evidence that one of the molecular events resulting from E-cadherin-mediated cellular aggregation is the activation of Akt and p38 MAPK in a PI3K-dependent manner.

A key question in intestinal development is what triggers the differentiation process. In this regard, it has been demonstrated that cell-cell junction systems, particularly adherens junctions, play an important role in the control of cell differentiation during intestinal ontogeny as well as during the continuous epithelial cell renewal in the mature organ. For instance, studies with E-cadherin knockout mice have revealed that E-cadherin-mediated cell adhesion is essential for the compaction of mesenchymal cells and their transition to a polarized epithelium (3, 36). In a chimeric transgenic animal model, expression of a dominant-negative N/E-cadherin mutant in villous enterocytes resulted in perturbation of cell-cell adhesion associated with an increased enterocyte migration rate along the crypt-villus axis, loss of the differentiated polarized phenotype, and increased apoptosis (7). However, signaling components that relay the signal from adherens junction proteins to the nuclear targets for the control of intestine-specific gene expression remain elusive. Our observations showing that PI3K was recruited to and activated by E-cadherin-mediated cell-cell contacts in intestinal epithelial cells suggest that it may be one of these signaling components. Such stimulation of PI3K by E-cadherin-mediated cell-cell contacts has recently been reported in other epithelial cell types (23, 37). Furthermore, inhibition of PI3K activity by ectopic expression of a dominant-negative form of the regulatory p85 subunit (Δp85) or by use of the LY294002 inhibitor repressed the expression of intestine-specific genes and delayed functional and morphological epithelial differentiation. Inhibition of PI3K was also found to alter adherens junction integrity in newly confluent monolayers of Caco-2/15 cells by reducing the amount of cytoskeleton-associated E-cadherin and β-catenin at the site of cell-cell contact. Thus, we believe that E-cadherin-dependent PI3K activation acts as an intermediate in the formation of adherens junctions, suggesting a bidirectional regulation between PI3K activity and adherens junction assembly. Such bidirectional regulation has been recently described for E-cadherin and Rac/Cdc42 (38–40). Taken together, these data indicate that PI3K plays an important role in regulating the integrity of adherens junctions, which in turn seems to be crucial for the efficient differentiation of intestinal epithelial cells. The recent demonstration that PI3K is involved in three-dimensional morphogenesis and tissue-specific differentiation in the mammary gland (28) strengthens this hypothesis.

The mechanism by which PI3K inhibition impairs adherens junctions is unknown. In mammary epithelial cells, this response appears to be mediated by changes at the level of the actin cytoskeleton (28). In this regard, our data indeed suggest that PI3K activity regulates the recruitment of F-actin at the site of cell-cell contact. It is known that phosphatidylinositol 3,4,5-triphosphate, a lipid product of PI3K, can recruit and activate the GTP exchange factor for Rac, which is required for adherens junction formation in Madin-Darby canine kidney cells and keratinocytes (41, 42), whereas Rac promotes the recruitment of F-actin to these junctions (42). These observations raise the possibility that a PI3K/Rac signaling pathway controls the integrity of adherens junctions in intestinal epithelial cells. However, in well polarized Caco-2/15 cells with mature intercellular junctions, we have observed that PI3K inhibition no longer interfered with the association of E-cadherin and β-catenin with the cytoskeleton. It is noteworthy that PI3K inhibition has no effect on the expression of differentiation markers such as sucrase-isomaltase and villin in well polarized Caco-2/15 cells.2 PI3K may thus act locally to control the early establishment of E-cadherin-mediated cell-cell contact and the initiation of the differentiation program rather than their maintenance. These observations are consistent with the fact that properties of E-cadherin-mediated cell-cell contact appear to be modulated by junction maturation (33). For instance, the regulation of E-cadherin function by Rac is progressively lost as the E-cadherin junction matures (33, 34).

In addition to its participation in E-cadherin-mediated epithelial cell adhesion, β-catenin is a key player in the APC/Wnt signaling pathway. In normal colonic epithelial cells, APC, in combination with glycogen-synthase kinase-3β and axin, regulates free cytoplasmic β-catenin levels by binding to and targeting β-catenin for degradation by ubiquitination-dependent proteolysis. This regulates the availability of free β-catenin for binding with the T-cell factor/lymphoid enhancer family of transcription factors (43). In the absence of a Wnt signal, APC promotes the degradation of cytoplasmic β-catenin, whereas in the presence of a Wnt signal, β-catenin accumulates in the cytoplasm, translocates to the nucleus, and coordinates with the T-cell factor/lymphoid enhancer to activate gene transcription (44). Our observation that the inhibition of PI3K led to a decreased association of E-cadherin and β-catenin with the cytoskeleton fraction could suggest a possible regulation of the APC/Wnt/β-catenin signaling pathway by PI3K. However, inhibition of PI3K activity by ectopic expression of a dominant-negative form of p85 (Δp85) or by use of the LY294002 inhibitor did not enhance pTOPFLASH activity, a T-cell factor promoter/luciferase reporter plasmid that directly assays β-catenin/T-cell factor activity (45) (data not shown). Furthermore, our data shown in Fig. 4A clearly indicate that the total cellular amounts of β-catenin protein remained comparable following PI3K inhibition, suggesting that β-catenin was not targeted for degradation in the proteasome. Taken together, these data suggest that PI3K does not regulate the Wnt/β-catenin signaling pathway in newly confluent Caco-2/15 cells. However, future studies will be needed to further clarify the involvement of PI3K in the regulation of this pathway in intestinal epithelial cells.

Another interesting finding from this work is the demonstration that E-cadherin engagement led to a PI3K-dependent activation of Akt. Akt stimulation has been involved in skeletal muscle differentiation (46), which also depends on PI3K (19, 20). Such a contribution of Akt to intestinal epithelial cell differentiation would be interesting to explore. However, Akt has been implicated in the control of cell survival and thereby could mediate the protective action of E-cadherin-mediated cell-cell contacts against apoptosis (6, 37, 47).

Our study also provides evidence for the first time that

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2 P. Laprise and N. Rivard, unpublished data.
E-cadherin-mediated cell-cell contact triggers p38 MAPK cascade activation. The p38a MAPK pathway was demonstrated previously to be an important modulator of enterocyte differentiation (8). Thus, it seems that PI3K and p38a MAPK act in a common pathway in intestinal epithelial cells toward the regulation of their differentiation. We provided several indications that PI3K-dependent activation of p38a MAPK relies on the ability of PI3K to promote the assembly and integrity of adherens junctions. First, a good correlation of the kinetics of adherens junction disruption with a decrease in p38a MAPK activity was observed following PI3K inhibition. Second, in well polarized cells, PI3K inhibition did not alter adherens junction integrity or affect p38a activity. And third, PI3K inhibition, which inhibited adherens junction but not tight junction assembly, also attenuated E-cadherin-dependent activation of p38a. Therefore, the ability of PI3K to control adherens junction formation and p38a activity, known to control intestine-specific gene transcription (8), confers to this kinase a central role in the promotion of intestinal epithelial cell differentiation. However, the mechanism relating PI3K to adherens junction integrity and p38a MAPK activation remains to be elucidated. The complexity of the PI3K-dependent pathways is further emphasized by the recent work of Wang et al. (48), who showed that PI3K inhibition through overexpression of PTEN (which directly dephosphorylates the D3 phosphate group of the lipid products of PI3K) or wortmannin treatment results in an increase in alkaline phosphatase and sucrase-isomaltase enzymatic activities, suggesting a regulatory effect of this pathway on intestinal cell differentiation. Although it cannot be excluded that these contradictory effects are the result of the use of distinct cell lines, and they are not without precedent (15, 49), the fact that PTEN effects were observed mostly in the presence of butyrate is a good indication that alternative pathways may exist.

In conclusion, migration of intestinal epithelial cells from the crypts to the villus tips involves programming for proliferation-and differentiation-related events. A key question in intestinal development is what triggers cell cycle arrest and the differentiation process. In vitro cell culture experiments have shown that cell-cell contact can trigger differentiation and therefore substitute the in vivo signal. The data presented herein indicate that PI3K signaling plays an important role in initiating intestinal epithelial cell differentiation. In this model (Fig. 8), E-cadherin engagement recruits and activates PI3K signaling. This activation promotes the assembly of adherens junction components with the cytoskeleton, which in turn activates the p38 MAPK cascade, enhancing the transcriptional capacity of CDX2. Rac is a likely candidate as a signaling intermediate because, as reported in other cell types, it could be a downstream effector of PI3K (41) and an upstream activator of the p38 MAPK module (50). However, regardless of the exact mechanism, the ability of PI3K to control adherens junction formation and p38 MAPK activity confers to PI3K a central role in the promotion of intestinal epithelial cell differentiation.

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