Phosphatidylinositol 3-kinase mediates proliferative signals in intestinal epithelial cells

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Background and aims: Determination of intracellular signalling pathways that mediate intestinal epithelial proliferation is fundamental to the understanding of the integrity and function of the intestinal tract under normal and diseased conditions. The phosphoinositide 3-kinase (PI3K)/Akt pathway transduces signals initiated by growth factors and is involved in cell proliferation and differentiation. In this study, we assessed the role of PI3K/Akt in transduction of proliferative signals in intestinal epithelial cells.

Methods: A rat intestinal epithelial (RIE) cell line and human colorectal cancer HCA-7 and LS-174 cell lines served as *in vitro* models. The Balb/cJ mouse was the *in vivo* model.

Results: PI3K activation was critical for G1 cell cycle progression of intestinal epithelial cells. Ectopic expression of either active p110 α or Akt-1 increased RIE cell proliferation. *In vivo* experiments demonstrated that PI3K activation was closely associated with the proliferative activity of intestinal mucosa. Treatment of mice with PI3K inhibitors blocked induction of PI3K activity and attenuated intestinal mucosal proliferation associated with oral intake. Epidermal growth factor and transforming growth factor α stimulated PI3K activation which was required for growth factor induced expression of cyclin D1.

Conclusions: The PI3K/Akt pathway transduces mitogenic signals from growth factor receptors to the cell cycle machinery and plays a critical role in regulation of intestinal epithelial proliferation.

The intestinal epithelium is one of the most rapidly proliferating tissues in the body. Intestinal epithelial cells constitutively proliferate at a high rate to preserve the balance with continuous cell loss through mechanical attrition and terminal differentiation.^{1,2} The epidermal growth factor (EGF) family, including EGF and transforming growth factor α (TGF- α), and their receptor (EGFR) play critical roles in intestinal epithelial proliferation.^{1,3,4} Binding of the ligand to EGFR leads to activation of receptor associated tyrosine kinase that phosphorylates tyrosine residues of cellular signalling proteins.⁵ A number of signalling pathways are involved in transduction of signals from EGFR to the nucleus. Among them, the Ras/MAPK pathway, the phosphoinositide 3-kinase (PI3K)/Akt pathway, and the phospholipase C/protein kinase C pathway are important for regulation of cell growth, migration, differentiation, and apoptosis.^{5,6}

Class IA PI3Ks (PI3K α , PI3K β , and PI3K δ) that consist of an 85 kDa regulatory subunit and a catalytic 110 kDa subunit (p110 α , p110 β , and p110 δ) transmit signals from tyrosine kinase coupled receptors and play critical roles in mitogenic signalling.⁷ p110 α and p110 β are expressed in all tissues whereas p110 δ is predominantly expressed in leucocytes.⁸ p110 α is involved in EGF induced mitogenic responses;⁹ in contrast, p110 β is necessary for insulin induced mitogenic responses.¹⁰ The PI3K catalytic subunit phosphorylates the D3 hydroxyl group of phosphoinositides; products include PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. PI3K generated D3 phosphorylated phosphoinositides bind to the PH domain of both protein kinase B (Akt) and phosphoinositide dependent kinase-1 (PDK-1) and induce their translocation to the plasma membrane where PDK-1 phosphorylates and activates Akt kinase.^{7,11} Three isoforms of Akt (Akt1, Akt2, and Akt3) are encoded by three separate genes and possess conserved threonine and serine residues (T308 and S473 in Akt1) which are critical for Akt activation by PI3K.¹² Although all three isoforms of Akt can be activated by growth factor,¹³ Akt1 is the predominant isoform in most

tissues.¹⁴ We have demonstrated that ectopic expression of active Raf and Akt1 results in transformation of rat intestinal epithelial (RIE) cells, suggesting a critical role of this isoform of Akt in intestinal epithelial cell biology.¹⁵

The PI3K/Akt pathway is oncogenic and is involved in the proliferation of transformed intestinal epithelial cells.^{15,16} On the other hand, inhibition of PI3K activity resulted in differentiation of HT-29 and Caco-2 cells.¹⁷ These results suggest that proliferation of transformed intestinal epithelial cells requires constitutive activation of PI3K. To our knowledge, the role of PI3K/Akt in normal intestinal epithelial proliferation has not been extensively investigated. In the present study, the intracellular signalling pathways that mediate intestinal epithelial proliferation were elucidated. Using rat RIE cells, human HCA-7 and LS-174 cells, and a mouse model, we demonstrated that PI3K activity was essential for intestinal epithelial cell proliferation *in vitro* and *in vivo*. We also found that EGF and TGF- α treatment rapidly induced PI3K activity, which is required for cyclin D1 expression, suggesting that the PI3K/Akt pathway transduces proliferative signals between growth factor receptor and cell cycle machinery in intestinal epithelial cells.

MATERIALS AND METHODS

Materials

LY 294002, wortmannin, and PD-98059 were purchased from Calbiochem (La Jolla, California, USA). The antiphosphorylated Akt antibody was purchased from Cell Signaling (Beverly, Massachusetts, USA). Antiactive ERK1/2 antibodies

Abbreviations: PI3K, phosphoinositide 3-kinase; RIE, rat intestinal epithelial; PCNA, proliferating cell nuclear antigen; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF- α , transforming growth factor α ; PDK-1, phosphoinositide dependent kinase-1; DMSO, dimethyl sulphoxide; PBS, phosphate buffered saline; FBS, fetal bovine serum; IPTG, isopropyl-1-thio- β -D-galactopyranoside; MEK, MAPK/ERK kinase; Cdk, cyclin dependent kinase

were from Promega (Madison, Wisconsin, USA) and Santa Cruz Biotechnology (Santa Cruz, California, USA). Anticyclin D1 antibody was purchased from Upstate Biotechnology (Lake Placid, New York, USA). The antibody for proliferating cell nuclear antigen (PCNA) immunostaining was purchased from Santa Cruz. EGF and TGF- α were purchased from Sigma (St Louis, Missouri, USA).

Animal experiments

All animals were treated in a manner which complied with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of UTMB. Three month old Balb/cJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and were housed in an animal holding room under controlled light, temperature, and humidity. For diet controlled intestinal epithelial proliferation, mice were housed in wire bottomed cages and received clear liquid Pedialyte (Ross, Columbus, Ohio, USA) with 5% glucose for the indicated times. Pelleted Prolab 2500 rodent diet (PMI Nutrition International, LLC, Brentwood, Missouri, USA) was then resumed ad libitum. LY-294002 was dissolved in 50% dimethyl sulphoxide (DMSO) in phosphate buffered saline (PBS) and was administered by intraperitoneal injection (10 mg/kg body weight).^{18, 19} Wortmannin was dissolved in 4% methanol in saline and given (1.5 mg/kg body weight) by orogastric gavage.^{17, 19} Mucosa of the jejunum (1 cm distal to the ligament of Treitz) and ileum (3 cm proximal to the ileocecal valve) was scraped for preparation of protein lysates. Full thickness intestine was fixed in 4% paraformaldehyde for immunohistochemistry.

Cell culture

Well differentiated human colon cancer cell line, LS-174, was purchased from ATCC (Manassas, Virginia, USA). Differentiated human rectal cancer HCA-7 cells were a gift from Dr S Kirkland (University of London, London, UK). Both cell lines were maintained in McCoy's 5A medium containing 10% fetal bovine serum (FBS). RIE cells were a gift from Dr KD Brown (Cambridge, UK) and were grown in Dulbecco's modified Eagle's medium containing 10% FBS. For cell growth study, cells were seeded in 12 well plates (10^4 cells/well) and treated for the indicated times. Cells were trypsinised and thoroughly mixed in PBS prior to counting. Four samples from each well were counted using a haemocytometer.

[³H]-Thymidine incorporation

DNA synthesis was estimated by [³H]-thymidine incorporation, as previously described.²⁰ Cell number from an extra well that was subjected to similar treatment was counted and used to standardise [³H]-thymidine counts. Results were expressed as cpm per 50 000 cells.

Flow cytometry

Cells were fixed in 70% ETOH, treated in 1 ml 0.1% RNase (Sigma), and stained with propidium iodide (Sigma). DNA was analysed by a flow cytometer; cell cycle profile was expressed as percentage of cells in each cell cycle stage.

Immunoblot analysis

Cells or intestinal mucosa were lysed for 30 minutes in lysis buffer (50 mM glycerol phosphate, 10 mM Hepes, pH 7.4, 1% Triton X-100, 70 mM NaCl, 1 mM Na₃VO₄, 25 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 μ M phenylmethanesulphonyl fluoride) and sonicated for 20 seconds. Clarified whole cell lysates were denatured and fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes (Bio-Rad, Hercules,

California, USA) which were incubated with the antibodies indicated and developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, Illinois, USA).

Exotic expression of PI3K

To express p110 α or Akt1 in RIE cells, an inducible expression system, LacSwitch (Stratagene, La Jolla, California, USA) was employed.^{21, 22} Myristoylated p110 α (Upstate Biotechnology) or HA tagged myristoylated Akt1 (a gift from Dr PN Tsichlis, Thomas Jefferson University) was inserted into the eukaryotic Lac operator containing vector, pOPRSV1/MCS. These vectors were transfected into RIE cells that were previously transfected with a eukaryotic Lac repressor-expressing vector, pCMVLacI. Double transfected RIE cells were selected with hygromycin (150 μ g/ml) and geneticin (600 μ g/ml) for two weeks. Expression of inserted genes was repressed until an inducer (IPTG; isopropyl-1-thio- β -D-galactopyranoside) was added.

A dominant negative mutant of the PI3K regulatory subunit, Δ p85 α (a gift from Dr B Vanhaesebroeck, Ludwig Institute for Cancer Research) which lacks the binding site for the catalytic subunit p110 α ²³ was inserted into the LZRS retroviral system (a gift from Dr Gary Nolan, Stanford University). The construct, designated LZRS- Δ p85 α , was transfected into Phoenix packaging cells (ATCC). RIE cells were infected with fresh viral supernatants that contained either empty vector or LZRS- Δ p85 α , selected with 4 mg/ml puromycin for 10 days, and referred to as RIE/LZRS and RIE/ Δ p85 α cells, respectively.

Immunohistochemistry

Intestine was fixed in 4% paraformaldehyde, paraffin embedded, and sectioned. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide for 20 minutes at room temperature. After the sections were blocked in 1.5% normal serum in PBS for one hour, primary antibody was added to the sections and incubated overnight at 4°C. The sections were then incubated with biotinylated secondary antibody and ABC-AP reagent (Vectorstain ABC-AP kit, Vector Laboratories, Burlingame, California, USA). Peroxidase activity was demonstrated by applying 3,3'-diaminobenzidine containing 0.02% of hydrogen peroxide for 10 minutes. Sections were counterstained with toluidine blue O.

Statistical analysis

Results are expressed as means (SD). All statistical analyses were performed on a personal computer with the StatView 5.0.1 software (SAS Institute Inc. Cary, North Carolina, USA). Analyses between multiple groups were determined by analysis of variance (ANOVA). Post hoc ANOVA tests were determined by Fisher's protected least significant difference. Analyses between two groups were carried out using the unpaired Student's *t* test. Differences with a *p* value <0.05 were considered statistically significant.

RESULTS

Inhibition of PI3K activity inhibits RIE cell proliferation

To investigate the role of PI3K in intestinal epithelial proliferation, we studied the effect of inhibition of PI3K activity on proliferation of RIE cells which were derived from normal rat small intestinal crypt cells and display properties of non-transformed epithelial cells.²⁴ Treatment with a selective PI3K inhibitor, LY-294002 (20 μ M), reduced RIE cell growth by 80% (fig 1A) whereas a selective MAPK/ERK kinase (MEK) inhibitor, PD-98059, inhibited cell growth by ~20%. [³H]-Thymidine incorporation assay demonstrated that LY-294002 inhibited DNA synthesis in RIE cells in a concentration dependent fashion (fig 1B). Treatment with

Figure 1 The role of phosphoinositide 3-kinase (PI3K) activity in rat intestinal epithelial (RIE) cell proliferation. (A) RIE cell growth. Cells (10^4) were seeded in 12 well plates and treated with 20 μ M LY-294002 (LY), 25 μ M PD-98059 (PD), or LY-294002 plus PD-98059 for four days. Cells were then trypsinised and mixed in phosphate buffered saline. Cell numbers from four samples of each well were counted. Values are means (SD) of cell number/well from triplicate wells. * $p < 0.05$. Results are representative of three separate experiments. (B) [3 H]-thymidine incorporation in RIE cells. Cells were treated with LY-294002 or PD-98059 at the indicated concentrations for 24 hours. [3 H]-Thymidine incorporation was measured after a three hour pulse. Results are means (SD) of cpm/50 000 cells from quadruplicate wells. * $p < 0.05$. This experiment was independently repeated three times. (C) Cell cycle profile. RIE cells were treated with dimethyl sulphoxide, LY-294002 (20 μ M), PD-98059 (50 μ M), or PD-98059 plus LY-294002 for 48 hours. DNA was analysed by a flow cytometer; percentage of cells in G0/G1 phase and S phase are shown. Values are means (SD) from three experiments. * $p < 0.05$.

10 μ M LY-294002 for 24 hours almost completely blocked DNA synthesis in RIE cells. Flow cytometry revealed that inhibition of PI3K activity by LY-294002 resulted in accumulation of RIE cells in the G1 phase of the cell cycle and that PD-98059 did not significantly alter the cell cycle profile (fig 1C).

PI3K activity is essential for RIE cell proliferation

As both PI3K α and Akt1 are involved in EGF induced mitogenic signalling,^{9, 13} we examined their roles in RIE cell proliferation. RIE cells were infected with a well characterised dominant negative mutant of a major PI3K regulatory subunit, Δ p85 α , which lacks the binding site for the catalytic subunit, p110 α , and inhibits activation of PI3K.²³ Ectopic

expression of Δ p85 α significantly reduced levels of pAkt and cyclin D1 (fig 2A). DNA synthesis was decreased by 50% in Δ p85 α expressing RIE cells compared with cells that were infected with vector (LZRS) only. Furthermore, we established a stably transfected RIE cell line in which expression of myristoylated p110 α (myr-p110 α) can be induced by IPTG.¹⁵ Levels of phosphorylated Akt (pAkt) which reflect PI3K activity¹¹ increased following induction of myr-p110 α ; the rate of DNA synthesis increased also (fig 2B). A stably transfected RIE cell line, in which HA tagged myristoylated Akt1 (HA-Akt-myr) can be induced by IPTG, was generated. Induction of Akt-myr which was noted by expression of HA significantly increased DNA synthesis (fig 2C). Taken together, these data suggest that the PI3K α isoform plays a

Figure 2 Ectopic expression of phosphoinositide 3-kinase (PI3K) in rat intestinal epithelial (RIE) cells. (A) Overexpression of dominant negative p85 α . RIE cells were infected with LZRS viral vector or LZRS containing Δ p85 α cDNA, as described in the methods section. Total cell lysates were harvested and analysed for levels of pAkt and cyclin D1 (CcnD1) proteins. [3 H]-Thymidine incorporation assay was performed in RIE/LZRS and RIE/ Δ p85 α cells. Results are expressed as cpm/50 000 cells. Values are means (SD) of relative cpm (versus LZRS control) from three experiments. * $p < 0.05$. (B) Conditional expression of p110 α . Stably transfected RIE-myr-p110 α cell line was established, as described in the methods section. Expression of myr-p110 α was induced by addition of 5 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG). Levels of pAkt were determined by western analysis at the indicated time points. [3 H]-Thymidine incorporation was assessed after the cells were treated with vehicle (CTR) or IPTG for 48 hours. Values are means (SD) of relative cpm (versus control) from three experiments. * $p < 0.05$. (C) Conditional expression of active Akt1. Stably transfected RIE-iHA-Akt-myr cells were treated with IPTG and induction of HA-Akt was measured by western analysis using anti-HA antibody. [3 H]-Thymidine incorporation was assessed after the cells were treated with vehicle (CTR) or IPTG for 48 hours. Values are means (SD) of relative cpm (versus control) from three experiments. * $p < 0.05$.

Figure 3 Phosphoinositide 3-kinase (PI3K) activity in proliferating mouse intestinal epithelium. (A) Proliferating indices. Balb/cJ mice were subjected to a normal diet (C), 72 hours of a liquid diet (L), or resumption of solids for 16 hours (S). The jejunum was immunostained for proliferating cell nuclear antigen (PCNA). PCNA positive cells were counted manually. Bars are means (SD) of proliferating cells/crypt from 90 representative crypts of three mouse intestines (30 crypts/mouse). * $p < 0.05$. (B) Western analysis for pAkt, pERK, and PCNA. Balb/c mice were subjected to a normal diet (C), 72 hours of a liquid diet (L), or resumption of solids for the indicated times. pAkt, pERK, and PCNA proteins in ileal mucosa were determined by western analysis. This experiment was independently repeated three times.

critical role in intestinal epithelial proliferation and Akt is an important downstream effector for this action of PI3K.

PI3K/Akt activation is associated with intestinal mucosal proliferation

Luminal contents significantly regulate proliferation of intestinal mucosa.^{25,26} To delineate the role of PI3K in intestinal mucosal proliferation *in vivo*, Balb/cJ mice were subjected to a liquid diet for 72 hours. Disuse of the gut resulted in a significant reduction in the number of proliferating cells per crypt; proliferating indices returned to control levels by 16 hours after resumption of a solid diet (fig 3A). A liquid diet reduced levels of pAkt; resumption of solid food rapidly induced pAkt, suggesting regulation of PI3K activity by luminal contents (fig 3B). PCNA is synthesised in the S phase of the cell cycle and serves as an excellent marker for proliferating cells. Levels of PCNA and pAkt were concurrently regulated in mouse intestinal epithelium. In contrast, levels of phosphorylated ERK1/2 (pERK1/2) were increased with a liquid diet and did not correlate with the activity of intestinal epithelial proliferation.

PI3K activity is critical for intestinal mucosal proliferation

LY-294002 and wortmannin are selective inhibitors of PI3K²⁷ and can be used to block PI3K activity *in vivo*.^{17–19} To determine whether induction of PI3K activity is essential for intestinal mucosal proliferation, a relatively low dose of LY-294002 (10 mg/kg intraperitoneally) was used *in vivo*. Treatment with LY-294002 completely blocked solid food induced PI3K activation and attenuated induction of PCNA (fig 4A). Orogastric administration of wortmannin also blocked solid diet induced pAkt activation and PCNA expression (fig 4B). Immunohistochemistry demonstrated that a 72 hour liquid diet significantly reduced the immunoreactivity of PCNA in crypts of jejunal mucosa (fig 4C). Refeeding for two hours resulted in positive staining of PCNA in a large portion of crypt cells. Administration of LY-294002 almost completely abolished diet induced expression of PCNA.

PI3K in human intestinal epithelial cell proliferation

We next elucidated the role of PI3K in the proliferation of human intestinal epithelial cells. Well differentiated human

Figure 4 Inhibition of phosphoinositide 3-kinase (PI3K) activity in the liquid diet/solid diet mouse model. (A) LY-294002 treatment. Balb/cJ mice were subjected to a normal diet (C), 72 hours of a liquid diet (L), or two hours resumption of solids with (+) or without (–) LY-294002 treatment. LY-294002 (10 mg/kg body weight) was administered intraperitoneally 15 minutes prior to resumption of a solid diet. Levels of pAkt and proliferating cell nuclear antigen (PCNA) in the jejunal mucosa were determined by western analysis. This experiment was repeated twice (total of six mice per time point). (B) Wortmannin treatment. Balb/c mice were subjected to a normal diet (C), 72 hours of a liquid diet (L), or two hours resumption of a solid diet with (+) or without (–) wortmannin (W) treatment. Wortmannin (1.5 mg/kg body weight) was administered by orogastric gavage 30 minutes prior to refeeding. Levels of pAkt and PCNA in the ileal mucosa were determined by western analysis. This experiment was repeated twice (total of four mice per time point). (C) PCNA immunostaining. The immunoreactivity of PCNA was demonstrated in the jejunal mucosa ($\times 400$) from mice treated with: (a) control diet, (b) 72 hour liquid diet, (c) two hour resumption of solid diet, and (d) two hour resumption of a solid diet with LY-294002 treatment. Arrows indicate representatives of positively stained cells. Bars are means (SD) of proliferating cells/crypt from 90 representative crypts of three mouse intestines (30 crypts/mouse). * $p < 0.05$.

colon cancer cell lines that continuously proliferate and undergo differentiation under certain circumstances have provided important experimental models for human intestinal stem cells.²⁸ In the present study, we employed two well differentiated colorectal cancer cell lines, HCA-7 and LS-174.^{29,30} Both HCA-7 and LS-174 cells proliferate in tissue culture and differentiate when grown in extracellular matrix components (Matrigel) forming crypt-like structures (fig 5A). Positive alcian blue staining indicated that the LS-174 colonies contained colonic type mucin. Growth of these cells was largely dependent on PI3K activity (fig 5B). Inhibition of PI3K activity with LY-294002 (20 μ M) resulted in a strong reduction of DNA synthesis whereas inhibition of MEK activity by treatment with PD-98059 exerted a modest effect on DNA synthesis (fig 5C). Cell cycle analysis demonstrated that treatment with LY-294002 accumulated both LS-174 and HCA-7 cells in the G1 phase of the cell cycle (fig 5D). In contrast, PD-98059 did not significantly alter the cell cycle profile in these cells. In agreement with results from RIE cells and mouse intestinal mucosa, the PI3K pathway appears to be essential for human intestinal epithelial proliferation.

PI3K/Akt pathway mediates signals from EGFR to cell cycle progression

To address mechanisms by which PI3K mediates intestinal epithelial proliferation, we determined the role of the PI3K/

Figure 5 Phosphoinositide 3-kinase (PI3K) activity in human intestinal epithelial cell proliferation. (A) Differentiation of HCA-7 and LS-174 cells. Cells were seeded in Matrigel for 14 days. Colonies were fixed, paraffin embedded, sectioned, and alcian blue stained ($\times 200$). (B) HCA-7 and LS-174 cell growth. HCA-7 or LS-174 cells (10^4) were seeded in 12 well plates and treated with 20 μ M LY-294002 (LY) or 50 μ M PD-98059 (PD) for four days. Cell numbers were counted and values are means (SD) from triplicate wells. * $p < 0.05$. Results are representative of three separate experiments. (C) [3 H]-Thymidine incorporation in human intestinal epithelial cells. HCA-7 or LS-174 cells were seeded in 24 well plates and treated with LY-294002 (20 μ M) or PD-98059 (50 μ M) for 24 hours. [3 H]-Thymidine incorporation was measured after a three hour pulse. Results are expressed as cpm/50 000 cells from quadruplicate wells. * $p < 0.05$. Results are representative of three separate experiments. (D) Cell cycle profile. HCA-7 and LS-174 cells were treated with dimethyl sulphoxide (DMSO), LY-294002 (20 μ M), PD-98059 (50 μ M), or PD-98059 plus LY-294002 for 48 hours. DNA was analysed by a flow cytometer and the cell cycle profile is presented as percentage of cells in each stage of the cell cycle. Values are means (SD) from three experiments.

Akt pathway in growth factor stimulated cyclin D1 expression. Progression through the mid to late G1 phase of the mammalian cell cycle is dependent on cyclin D1 mediated activation of cyclin dependent kinase 4 (Cdk4) or the related Cdk6.³¹ Both cyclin D1 and pAkt proteins were detected in growing RIE cells. Deprivation of serum for 72 hours reduced levels of both cyclin D1 and pAkt. Stimulation with EGF or TGF- α rapidly increased the levels of cyclin D1 and pAkt, which remained at high levels for at least 24 hours (fig 6A).

The presence of LY-294002 attenuated EGF or TGF- α induced pAkt and cyclin D1 expression.

Similar experiments were performed in human LS-174 cells. Stimulation with EGF induced expression of both cyclin D1 and pAkt (fig 6B). Treatment with LY-294002 completely attenuated EGF induced expression of cyclin D1 and pAkt.

Cyclin D1 protein was detected in normal mouse intestinal mucosa. Disuse of the gut decreased levels of cyclin D1 in the ileal mucosa. Resumption of solid food stimulated expression

Figure 6 Regulation of cyclin D1. (A) Cyclin D1 (CcnD1) in rat intestinal epithelial (RIE) cells. RIE cells were grown in serum deprived medium for 72 hours (–S). Cells were restimulated with epidermal growth factor (EGF 100 ng/ml), EGF plus LY-294002 (20 μ M) (upper panel), transforming growth factor α (TGF- α 100 ng/ml), or TGF- α plus LY-294002 (20 μ M) (lower panel) for the indicated times. Expression of cyclin D1, pAkt, and cyclin dependent kinase 4 (Cdk4) was determined by western analysis. S = cells grown in serum containing medium. All western analyses were repeated three times. (B) Cyclin D1 regulation in LS-174 cells. Cells were grown in serum deprived medium for 72 hours (–S) and then restimulated with EGF (100 ng/ml) or EGF plus LY-294002 for the indicated times. Expression of cyclin D1, pAkt, and Cdk4 was determined by western analysis. (C) Cyclin D1 regulation in mouse intestinal mucosa. Balb/c mice were subjected to a normal diet (C), 72 hours of a liquid diet (L), two hours resumption of solid food (S), or resumption of solid food plus wortmannin (W) or LY-294002 (LY). Levels of cyclin D1 and pAkt in jejunal mucosa (upper panel) and ileal mucosa (lower panel) were determined by western analysis.

of cyclin D1 in both the jejunum (fig 6C upper panel) and ileum (fig 6C bottom panel) and coincided with induction of pAkt. Administration of wortmannin or LY-294002 attenuated solid food induced expression of cyclin D1 and pAkt.

DISCUSSION

Our studies have examined the role of the PI3K/Akt signalling pathway in the regulation of intestinal epithelial proliferation. We have found that PI3K/Akt is a major signalling pathway which mediates proliferative signals in intestinal epithelial cells in vitro and in vivo. Blocking PI3K enzyme activity by either pharmacological or molecular methods inhibited proliferation of the RIE cell line, a non-transformed rat intestinal epithelial cell line. In addition, proliferation of well differentiated human colorectal cancer cells, which have been considered as a model of human intestinal stem cells,²⁸ greatly depends on PI3K activity. Moreover, our in vivo experiments are the first to demonstrate the essential role of the PI3K/Akt pathway in normal intestinal mucosal proliferation. We have shown that PI3K activity, cyclin D1 protein, and PCNA were concurrently regulated in rapidly proliferative intestinal epithelial cells. These observations from three independent in vitro and in vivo models suggest the central involvement of PI3K activity in signalling of intestinal epithelial proliferation.

Several lines of investigation indicate that the PI3K/Akt pathway controls cell proliferation in vivo.³² *Pik3ca*, which encodes the class IA PI3K catalytic subunit p110 α , is essential for embryonic development.³² Homozygous knockout of *Pik3ca* results in complete failure of embryonic cells to proliferate and no abnormality in apoptosis is observed. On the other hand, the tumor suppressor gene *PTEN/MMAC1* (phosphatase and tensin homologue deleted on chromosome 10), has been shown to dephosphorylate the 3 position of both PtdIns-3,4,5-P₃ and PtdIns-3,4,-P₂ to reverse the reaction catalysed by PI3K.³³ Homozygous *PTEN* knockout mice die by day 9.5 (E9.5) of development. The proliferative index (BrdU positive nuclei/total cell number) is significantly increased in *PTEN*^{-/-} embryo compared with wild-type embryo.³⁴ These findings suggest that PI3K is critical for the proliferation of a variety of cell types and support our observation that PI3K/Akt is a major signalling pathway for intestinal epithelial cell proliferation in vivo.

Expression of active Akt increases expression of cyclin D1³⁵⁻³⁶ and promotes S phase entry.³⁷ In our experiments, ectopic expression of active p110 α or Akt1 resulted in increased DNA synthesis. PI3K may require additional synergistic signals to stimulate cell proliferation.³⁸ Interestingly, MEK/ERK activity was not correlated with intestinal epithelial proliferation in all three of our experimental models. Levels of pERK were increased in the disused mouse intestine and resumption of solid food did not significantly stimulate MEK/ERK activation. Inhibition of MEK activity did not alter cell cycle progression in RIE, HCA-7, or LS-174 cells. The precise role of MEK/ERK in intestinal epithelial proliferation is not clear. To determine whether a synergistic signalling of basic MEK/ERK and PI3K/Akt is essential for normal intestinal epithelial proliferation requires further experiments.

Cyclin D1 plays an important role in cell cycle progression in intestinal epithelial cells. TGF- β treatment inhibits the growth of rat intestinal epithelial cells through downregulation of levels of cyclin D1.³⁹ Cyclin D1 is thought to play important roles in normal and neoplastic growth of intestinal epithelial cells, and overexpression of cyclin D1 has been implicated in colorectal neoplasia.⁴⁰⁻⁴¹ However, expression of cyclin D1 in normal mouse intestinal mucosa has not been detected.⁴¹ Cytoplasmic, but not nuclear, staining of cyclin D1 was identified in normal human colonic mucosa.⁴⁰ In our

present study, we demonstrated that cyclin D1 protein is expressed in normal mouse intestinal mucosa and resumption of solid food significantly increased expression of cyclin D1, as determined by western analysis. Cyclin D1 is regulated by the PI3K/Akt pathway at both the transcriptional³⁶ and post-transcriptional levels.³⁵ In addition, Akt stabilises cyclin D1 protein through inhibition of GSK-3 β kinase.⁴² Our in vitro and in vivo data show that extrinsic stimuli induced expression of cyclin D1 in intestinal epithelial cells; this expression was attenuated by treatment with PI3K inhibitors. Regulation of the expression and function of D-type cyclins provides the molecular basis through which cells respond to extrinsic mitogenic signals. Our results suggest that the PI3K/Akt pathway may control intestinal epithelial proliferation through regulating the availability of functional cyclin D1 protein.

PI3K activation stimulates growth of transformed intestinal epithelial cells and therefore enhances the transformed phenotype of these cells.^{15-17, 43} In normal gut mucosa, intestinal epithelial proliferation is crucial for maintaining the homeostasis of the intestinal epithelium⁴⁴ and critical for improving the integrity and function of the intestinal tract in patients with intestinal injury and atrophy.^{4, 45} Our present study demonstrates that the PI3K/Akt pathway mediates key signals for intestinal epithelial proliferation and contributes to the understanding of the molecular mechanisms of intestinal epithelial homeostasis. Our results also suggest that the PI3K/Akt pathway may be a potential target for regulation of intestinal mucosal proliferation.

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