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\(a\) 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 \(\alpha\) 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.

Materials and methods

Abbreviations: PI3K, phosphoinositide 3-kinase; RIE, rat intestinal epithelial; PCNA, proliferating cell nuclear antigen; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF-\(\alpha\), transforming growth factor \(\alpha\); PDK-1, phosphoinositide dependent kinase-1; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; FBS, fetal bovine serum; IPTG, isopropyl-\(-\)\(\beta\)-\(D\)-galactopyranoside; MEK, MAPK/ERK kinase; CdK, cyclin dependent kinase
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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 purchase 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/C 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). 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 (10^5 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.

[^H]-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 Heps, pH 7.4, 1% Triton X-100, 70 mM NaCl, 1 mM Na_2VO₄, 25 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM 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).

Ectopic expression of PI3K
To express p110α or Akt1 in RIE cells, an inducible expression system, LacSwitch (Stratagene, La Jolla, California, USA) was employed.21 22 Myristoylated p110α (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, pO普RSV/ MCS. These vectors were transfected into RIE cells that were previously transfected with a eukaryotic Lac repressor expressing vector, pCMVlact. Double transfected RIE cells were selected with hygromycin (150 μg/ml) and geneticin (600 μg/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, Ap85α (a gift from Dr B Vanhaesebroeck, Ludwig Institute for Cancer Research) which lacks the binding site for the catalytic subunit p110α23 was inserted into the LZR5 retroviral system (a gift from Dr Gary Nolan, Stanford University). The construct, designated LZR5-Ap85α, was transfected into Phoenix packaging cells (ATCC). RIE cells were infected with fresh viral supernatants that contained either empty vector or LZR5-Ap85α, selected with 4 mg/ml puromycin for 10 days, and referred to as RIE/LZR5 and RIE/ Ap85α 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.14 Treatment with a selective PI3K inhibitor, LY-294002 (20 μM), 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

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10 μM 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 PI3Kz and Akt1 are involved in EGF induced mitogenic signalling,1 11 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, Δp85α, which lacks the binding site for the catalytic subunit, p110α, and inhibits activation of PI3K.12 Ecotropic expression of Δp85α significantly reduced levels of pAkt and cyclin D1 (fig 2A). DNA synthesis was decreased by 50% in Δp85α 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 p110α (myr-p110α) can be induced by IPTG.13 Levels of phosphorylated Akt (pAkt) which reflect PI3K activity14 increased following induction of myr-p110α; 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 PI3Kz isoform plays a...
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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. 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 PI3K and can be used to block PI3K activity in vivo. To determine whether induction of PI3K activity is essential for intestinal mucosal proliferation, a relatively low dose of LY-294002 (10 mg/kg body weight) was administered intraperitoneally 1.5 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). Wortmannin treatment. Balb/c mice were subjected to a normal diet (C), 72 hours of a liquid diet (L), or two hours of 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). Wortmannin treatment. Balb/c mice were subjected to a normal diet (C), 72 hours of a liquid diet (L), or two hours of 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).

**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 colon cancer cell lines that continuously proliferate and undergo differentiation under certain circumstances have provided important experimental models for human intestinal stem cells. In the present study, we employed two well differentiated colorectal cancer cell lines, HCA-7 and LS-174. 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). 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/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/Akt pathway in cell cycle progression.
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. 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 of cyclin D1 and pAkt, which remained at high levels for at least 24 hours (fig 6A).

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 (x200). (B) HCA-7 and LS-174 cell growth. HCA-7 or LS-174 cells (10⁴) were seeded in 12 well plates and treated with 20 μM LY-294002 (LY) or 50 μM 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) [³H]-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 μM) or PD-98059 (50 μM) for 24 hours. [³H]-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 μM), PD-98059 (50 μM), 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 separate experiments.

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 μM) (upper panel), transforming growth factor α (TGF-α 100 ng/ml), or TGF-α plus LY-294002 (20 μM) [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,46 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.37 PI3K catalytic subunit p110alpha, which encodes the class IA PI3K catalytic subunit p110alpha, is essential for embryonic development.38 Homozygous knockout of PI3K catalytic subunit p110alpha, which encodes the class IA PI3K catalytic subunit p110alpha, is essential for embryonic development. Our studies have examined the role of the PI3K/Akt pathway on cell proliferation in intestinal epithelial cells 1477

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