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A product of phosphatidylinositol-3 kinase is elevated in dividing HT29 colonic epithelial cells

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Phosphatidylinositol 3 kinase (PI-3 kinase) activity has been linked to cell proliferation and growth regulation. Therefore, we studied changes in phosphoinositide metabolism during the cell cycle of HT-29 cells, a colonic epithelial cell line HT29 cells were treated with the microtubule disrupter, nocodozole, separated into mitotic and quiescent populations and their phospholipid composition was analyzed. Radiolabelled phospholipids from cells labelled with $^{32}PO_4$ or [^{3}H]myoinositol were analyzed by TLC and/or deacylated and analyzed by HPLC. In all cases, levels of phosphatidylinositol 3 phosphate from mitotic phase cells were double that in resting populations. Therefore, levels of a product of PI-3 kinase are elevated and may play a role in cell division.

PI-3 kinase; Phosphatidylinositol 3 phosphate; Colonic epithelia; Cell cycle

1. INTRODUCTION

Evidence is mounting for a role for phosphoinositides, particularly novel phosphoinositides phosphorylated on the D-3 position of the inositol ring, in growth control. Inositol, a precursor for phosphoinositides, is a nutrient which is essential for cell proliferation. Two enzymes involved in phosphoinositide metabolism, phospholipase C (PLC) and phosphatidylinositol-3 kinase (PI-3 kinase), contain src homology (SH-2) domains which permit association with growth factor receptors possessing intrinsic tyrosine kinase activity. In accordance with this, D-3 phosphorylated phosphoinositides become transiently elevated in cells which have been exposed to these growth factors [1–6]. Furthermore, PI-3 kinase appears to play a role in transformation by the middle T antigen [7].

There is reason to suspect that PI-3 kinase activity may be altered in mitotic phase cells. Tyrosine phosphorylation of PI-3 kinase promotes its association with SH-2 domains of pp60^{src} and growth factor receptors and thereby with the plasma membrane (for review see [7]). PI-3 kinase has been shown to be elevated in cells with elevated src protein tyrosine kinase activity [8]. Recently, increased pp60^{csrc} tyrosine kinase activity was observed during mitotis [9]. Human colon carcinoma cell lines, including HT29 have elevated pp60^{csrc} activity relative to normal colonic mucosal cells [10]. Therefore we were interested in investigating the occurrence of PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 in this cell line and their variance over the cell cycle.

Here, we demonstrate that levels of [^{32}P]PI(3)P are elevated during the G_2/M phase of the cell cycle. However, there were no detectable changes in two other known products of PI-3 kinase, PI(3,4)P_2 or PI(3,4,5)P_3.

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Abbreviations. PLC, phospholipase C; PI(3)P, phosphatidylinositol 3 phosphate; PI(4)P, phosphatidylinositol 4 phosphate; PI-3 kinase, phosphatidylinositol-3 kinase; PIP, phosphatidylinositol phosphate, PI(3,4)P_2, phosphatidylinositol 3,4 bisphosphate; PI(3,4,5)P_3, phosphatidylinositol 3,4,5 trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; GroPI(3)P, glycerophosphorylinositol 3 phosphate; GroPI(4)P, glycerophosphorylinositol 4 phosphate.

2. MATERIALS AND METHODS

2.1. *Materials*

Nocodozole (methyl-(5-(2-thienylcarbonyl)-1*H*-benzimidazol-2-yl)-carbamate) was purchased from Sigma Chemical Company, St. Louis, MO; DME/F-12 (1:1) from JRH Bioscience, Irvine, CA; γ-$^{32}PO_4$ was obtained from New England Nuclear and [^{3}H]myoinositol was obtained from American Radiochemical (ARC). [^{32}P]PI(3)P was generously donated by Dr. Phillip Majerus, Washington Univ., St. Louis.

2.2. *Cell culture*

HT29 cells were obtained from ATCC (Accession no. HTB38 F5451) Passages 16–40 were used during these experiments. They were grown in a 1.1 mixture of Dulbecco-Vogt modified Eagle's (DME) and Ham's F-12 medium, containing 5% newborn calf serum (Hyclone), supplemented with 15 mM HEPES buffer, pH 7.5, l-glutamine, 40 mg/l penicillin, 8 mg/l ampicillin, and 90 mg/l streptomycin. Cells were passaged every 4 days by splitting at a ratio of 1:4 using trypsin/EDTA and grown in 100 mm tissue culture dishes (Costar, Cambridge, MA) in a humidified atmosphere of 5% CO_2, 95% air.

2.3 *Segregation of G_0/G_1 and G_2/M phase cells*

Cells were detached by trypsinization with 0.1% trypsin and 0.9 mM

EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and were seeded at a density of 10^6 cells/5 ml in 3-cm dishes for 2–3 days. Cells were then treated with 5 μM nocodozole [11] for 14 to 16 h. Mitotic phase cells were gently detached by agitation on a shaker tray for 5 min in Ringers solution or 0.5 mM EDTA (Group M). G-phase cells were more adherent and remained attached following agitation (Group G) In some experiments these cells were detached by incubation for 15 min with 0.5 mM EDTA in phosphate buffered saline (pH 7.4) before lipid extraction Nocodozole treated cells were compared with cells which were not treated with nocodozole and were freely cycling (Group C). HT29 cells were fixed in 70% ethanol and stained with 4,6-diamidino-2-phenylindole (DAPI) and DNA content per cell was evaluated using an Ortho cytofluorograph. The cell cycle composition of each group is presented in Table I. Viability, tested by Trypan blue exclusion, was similar for all groups (M: 96% ± 0.8; G: 94.4% ± 0.5; 96.2 ± 1.7, means ± S.E M., $n = 4$).

Radiolabelling of HT29 cells with $^{32}PO_4$ was carried out in phosphate-free HEPES buffer containing 30 mM HEPES, 110 mM NaCl, 10 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 1.53 mM $CaCl_2$, and 10 mM glucose, $^{32}PO_4$ (New England Nuclear, 50 μCi/ml) with or without 5 μM nocodozole for 3 h. Coincubated controls without $^{32}PO_4$ were included with each experiment to monitor the cell cycle composition. Following labelling, cells in Group M were harvested in EDTA buffer as described above. Unless otherwise indicated, ice-cold methanol was added to the cells remaining on the dish (Group G), the dishes were sonicated on ice, the cell suspension in methanol was removed to test tubes where the lipid extraction proceeded as described below.

Cells were labelled with [^{3}H]myoinositol by replacing their growth media with 1 ml of inositol-free DMEM/F12 medium (1:1, v/v) supplemented with dialyzed newborn calf serum (5%) and myo-[2-^{3}H]inositol (12.8 Ci/mmol, 50 μCi/ml) Cells were then incubated for 24 h after which time another 1 ml of medium containing dialyzed serum was added and followed by an additional 48 h incubation (72 h total).

2.4. *Phospholipid determination*

Lipid extraction was performed as described elsewhere [12] Two TLC protocols were used. TLC System I was used to separate general classes of lipids and used silica gel 60 (EM Science) TLC plates impregnated with potassium oxalate. The developing solvents were chloroform/acetone/methanol/acetic acid/water, 80:30:26.24.14 (v/v). For resolution of PI(3)P and PI(4)P we used a second TLC technique (II) described by [13] where the TLC plates (silica gel 60, EM Science) were preimpregnated with borate. We found that allowing the plate to develop in the impregnation solution overnight rather than dipping the plate in the solution leads to better resolution of PI(3)P and PI(4)P. Lipids were quantitated with an AMBIS beta scanner or by scraping the appropriate bands of silica gel which were subjected to scintillation counting.

Phospholipids were also analyzed by deacylation followed by HPLC as described [12]. The aqueous products of deacylation were resuspended in water and injected on HPLC (see below). Glycerophosphorylinositol phosphates and inositol phosphates were resolved by anion-exchange HPLC as described [4] on a 4.6 × 250 mm Partisil 10 SAX column (Whatman, Clifton, NJ) The radioactivity in the effluent was continuously measured by a Beckman 171 dual channel radioisotope detector (Beckman, San Ramon, CA), which performed automatic peak integration using Beckman System Gold program software Identification of the inositol phosphates was based on comparison of their elution times with those of authentic radiolabelled standards (New England Nuclear, Boston, MA), myo-[2-^{3}H]inositol, D-[inositol-2-^{3}H(N)]inositol 1-phoshate, D-[inositol-2-^{3}H(N)]inositol 1,4-bisphosphate, D-[inositol-2-^{3}H(N)]inositol 1,3,4-trisphosphate, and D-[inositol-2-^{3}H(N)]inositol tetrakisphosphate. ^{32}P-labelled glycerophosphoinositol standards were prepared from neutrophils as described previously [12] These included [^{32}P]GroPIP$_3$, [^{32}P]GroPI(3,4)P$_2$, [^{32}P]GroPI(4,5)P$_2$, and [^{32}P]GroPI(4)P. [^{3}H]GroPI(3)P was identified by its elution time relative to [^{32}P]GroPI(3)P prepared from authentic [^{32}P]PI(3)P provided by Dr. Phillip Majerus, St. Louis, MO.

3. RESULTS

3.1. *Identification of PI(3)P, PI(3,4)P$_2$ and PI(3,4,5)P$_3$ in HT29 cells*

HT29 cell phospholipids were labelled with [^{3}H]myoinositol, and extracted as described in section 2. TLC bands containing phosphoinositides were scraped and deacylated. The radiolabelled products were then detected using a HPLC equipped with a radioisotope detector. As can be seen in Fig. 1, putative [^{3}H]glycerophosphorylinositol-3 phosphate (GroPI(3)P) prepared from [^{3}H]inositol-labelled HT29 cells comigrated with [^{32}P]GroPI(3)P prepared from authentic [^{32}P]PI(3)P provided by Dr. Phillip Majerus. This was true of [^{3}H]PIP prepared from cells of groups M, C and G.

GroPI(3,4)P$_2$ and GroPI(3,4,5)P$_3$ were also observed and identified by comparison to authentic [^{32}P]GroPI(3,4)P$_2$ and [^{32}P]GroPI(3,4,5)P$_3$ prepared from human neutrophils as described [12] (data not shown). However, the levels of the deacylated products of [^{32}P]PI(3,4)P$_2$ and [^{32}P]PIP$_3$ were less than 10% of that of [^{32}P]PI(3)P and were therefore not always detectable. However, when detected there was no difference in the levels of PI(3,4)P$_2$ (M: [^{32}P]PI(3,4)P$_2$ = 1.67% of total [^{32}P]PIP$_2$; G: [^{32}P]PI(3,4)P$_2$ = 1.68% total [^{32}P]PIP$_2$, means of $n = 2$).

3.2. *PI(3)P is elevated in cell populations enriched in dividing cells*

HT29 cells were radiolabelled with ^{32}P for three hours and then segregated into G_2/M phase cells (M) and G_0/G_1 phase cells (G or C) as described in section 2. The

Table 1

PI(3)P is elevated in populations of HT29 cells containing a high proportion of mitotic phase cells

	Cell cycle compartment		[^{32}P]PI(3)P (% total [^{32}P]PIP)
	$G_1 + G_0$ (% total cells)	$G_2 + M$ (% total cells)	
C	80.8 ± 1.97	12.8 ± 1.39****	7.95 ± 2.65** ***
G	40.95 ± 5.91	30.76 ± 4.81****	8.9 ± 1.08***
M	2.35 ± 0.46	80.1 ± 7.51*	21.2 ± 2.45**
	($n = 7$)	($n = 7$)	($n = 5$)

Cells were labelled with $^{32}PO_4$ and segregated into populations enriched in the various cell cycle compartments as described in section 2. Coincubated cells were analyzed for cell cycle composition by flow cytometry as described in section 2. Data is presented as percent of total cells in group C, G, or M in the indicated compartment. ^{32}P-labelled phospholipids were extracted and subjected to TLC to separate the phosphoinositide classes. Bands of silica gel containing phosphoinositides were scraped, the phospholipids were deacylated and the deacylated products were resolved by HPLC Data is presented as percent total cpm in PIP. All data is presented as means ± S.E M Statistical significance was estimated using the unpaired Student's t-test. *$P < 0.0001$ M vs. C or G; **$P < 0.0005$ C vs. M, ***$P < 0.001$ G vs. M; ****$P < 0.005$ C vs. G.

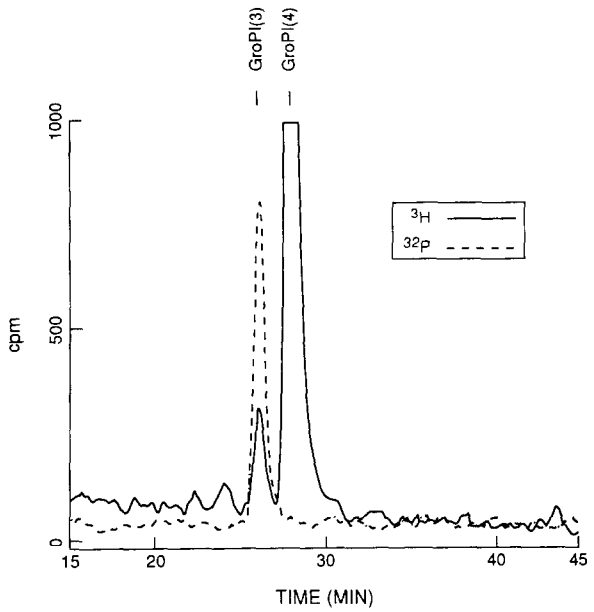

Fig. 1. Putative [^{3}H]GroPI(3)P from [^{3}H]myoinositol-labelled HT29 cells comigrates with authentic [^{32}P]GroPI(3)P. Phospholipids from HT29 cells labelled with [^{3}H]myoinositol as described above were extracted and deacylated Authentic [^{32}P]PI(3)P generously donated by Dr. Phillip Majerus was also deacylated and coinjected with the aqueous products of deacylation of ^{3}H-labelled phospholipids from both nocodozole treated and untreated cells. Radioactivity was detected by a Beckman 171 flow-through detector equipped with a 1 ml liquid flow cell. This experiment was repeated 3 times with similar results. The standard comigrated with putative GroPI(3)P from each group of cells, M, G and C.

cell cycle composition of the three groups is presented in Table I. Phosphoinositide levels in phospholipid extracts were measured using two techniques: (1) TLC (II) using a technique which resolved PI(3)P and PI(4)P but poorly separated other phospholipids (Fig. 2), or (2) TLC (I) which resolved PI, PIP, PIP_2 and PIP_3 but did not resolve PI(3)P and PI(4)P which were subsequently resolved by deacylation and HPLC. As can be seen in Table I and Fig. 2, levels of PI(3)P relative to PI(4)P doubled in populations of HT29 cells enriched in G_2/M phase cells (Group M) compared to populations enriched in G_0/G_1 phase cells (Group G), when the phospholipids were separated by TLC (II) or TLC (I) followed by HPLC. The composition of PIP from cells not treated with nocodozole (Group C), where the proportions of cells in the M phase was relatively low, was comparable to the nocodozole treated, G_0/G_1 enriched population.

3.3. *[^{3}H]PI(3)P is elevated relative to [^{3}H]PI(4)P in mitotic phase cells*

The 3, 4 and 5 phosphates on polyphosphoinositides turn over more rapidly than the 1 phosphate or the phosphate in other phospholipids. As a result polyphosphoinositides are the first phospholipids to be labelled in cells loaded with $^{32}PO_4$ and reach equilibrium within a few hours. In contrast, [^{3}H]myoinositol becomes slowly incorporated into phosphoinositides and takes days to reach equilibrium. Hence, [^{3}H]myoinositol may label a somewhat different pool of phosphoinositides than ^{32}P. Therefore, we studied phosphoinositide levels in HT29 cells labelled with [^{3}H]myoinositol. HT29 cells were labelled with [^{3}H]myoinositol for 3 days (50 μCi/ml) in inositol-free medium after which the cells were incubated with regular medium containing myoinositol and nocodozole (5 μM) for 14 h as described in section 2. Coincubated controls were separated as before and checked for cell cycle composition as described above. The inositol labelling procedure did not interfere with the subsequent generation of populations of cells in which G_2 + M phase cells were enriched (79%). When the phospholipids were extracted from these cells, separated by TLC (I), scraped from the TLC plates, deacylated and subjected to HPLC, the results reflected the findings with the ^{32}P-labelled cells. PI(3)P accounted for over 20% of the radioactivity in PIP in group M whereas it accounted for approximately 9% of the radioactivity in PIP in group G.

3.4. *The elevation of PI(3)P is not a function of cell attachment*

We then considered the possibility that the difference in PIP between mitotic phase and nonmitotic phase populations might result from the fact that the mitotic phase cells were not attached to the substratum whereas the nonmitotic phase cells were still attached when the lipids were extracted. If this were the case the difference in levels of PI(3)P would correspond to the degree of cell substratum adhesion and not necessarily the phase of the cell cycle. We therefore examined the composition of PIP from G_0/G_1 enriched cells which had been detached by incubation in an EDTA containing phosphate buffer for 15 min prior to lipid extraction. The ratio of PI(3)P to PI(4)P was similar to that from cells which were attached (8.67 ± 0.23%; n = 3; mean ± S.E.M.).

4. DISCUSSION

Here we demonstrate that levels of PI(3)P are elevated in mitotic phase cells. This was the case whether the phospholipids were labelled with [^{3}H]myoinositol or $^{32}PO_4$. Furthermore, this difference was not due to the differences in cell substratum adhesion between mitotic and nonmitotic cells. Labelling of another product of PI-3 kinase, $PI(3,4)P_2$ was 10% of that in PI(3)P and was not detected in every experiment. However, levels of $PI(3,4)P_2$ did not appear to be elevated in mitotic phase cells. Although we do not know whether this elevation of PI(3)P occurs in other cell types during mitosis, Serunian and co-workers [14] reported an elevation of D-3 phosphorylated phosphoinositides in subconfluent

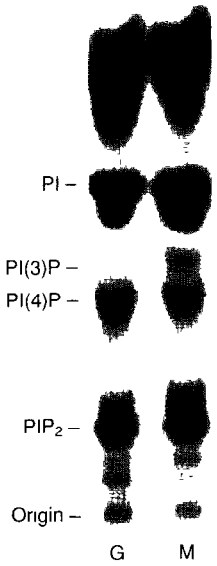

Fig. 2. Autoradiogram of TLC resolution of [^{32}P]PI(3)P and PI(4)P. [^{32}P]Phospholipids from cells consisting of primarily G_2/M(M) or G_0/G_1(G) phase cells were resolved by TLC (II) as described by Walsh et al. [13] Radioactivity in phospholipid bands was quantitated using an AMBIS beta scanner (AMBIS, San Diego). [^{32}P]PI(3)P from mitotic phase cells accounted for 1.075 ± 0.095% of total [^{32}P]phospholipid compared to 0.55 ± 0.025% in G_0/G_1 (Mean of 4 experiments ± S.D., $n = 4$, $P < 0.005$; Student's t-test)

NIH3T3 cells. Because subconfluent cultures have a higher proportion of mitotic phase cells than confluent cultures these results suggest that the phenomenon we report here may be widespread. Differences in the relative levels of PI(3)P and PI(4)P during the cell cycle may have functional significance. Tsai and co-workers have demonstrated that PIP was among the phospholipids which inhibited guanosine triphosphatase activating protein (GAP) activity and bound GAP tightly enough to retain it on an affinity column [15]. In addition, phosphoinositides selectively bind profilin and gelsolin and may thereby regulate cytoskeletal assembly [16–18].

Possible mechanisms which could account for the elevation of PI(3)P include changes in the activities of PI-3 kinase, PI-3 phosphatase, or the transfer protein which transfers PI to the plasma membrane where it may be further phosphorylated. Further studies will be required to differentiate between these possibilities or to ascertain whether the levels of PI-3 kinase or the activity of the enzyme is elevated in dividing cells. It is interesting that only PI(3)P appears to be elevated in mitotic phase cells and not other products of PI-3 kinase. Because levels of PIP_3 and $PI(3,4)P_2$ are often transient, it may be that the experiments described here do not have adequate time resolution to detect elevation of these products. Therefore PIP_3 and $PI(3,4)P_2$ levels may be elevated at a certain point in the G_2 or M phase of the cell cycle but are hydrolyzed to PI(3)P which accumulates and is the only detectable product using our current protocol. On the other hand PI(3)P itself may have a function in dividing cells. In either case, these findings suggest that product(s) of PI-3 kinase are elevated and may play a role during cell division.

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