Complex changes in cellular inositol phosphate complement accompany transit through the cell cycle

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Inositol polyphosphates other than $\text{Ins}(1,4,5)\text{P}_3$ are involved in several aspects of cell regulation. For example, recent evidence has implicated InsP_6 , $\text{Ins}(1,3,4,5,6)\text{P}_5$ and their close metabolic relatives, which are amongst the more abundant intracellular inositol polyphosphates, in chromatin organization, DNA maintenance, gene transcription, nuclear mRNA transport, membrane trafficking and control of cell proliferation. However, little is known of how the intracellular concentrations of inositol polyphosphates change through the cell cycle. Here we show that the concentrations of several inositol polyphosphates fluctuate in synchrony with the cell cycle in proliferating WRK-1 cells. InsP_6 , $\text{Ins}(1,3,4,5,6)\text{P}_5$ and their metabolic relatives behave similarly:

concentrations are high during G_1 -phase, fall to much lower levels during S-phase and rise again late in the cycle. The $\text{Ins}(1,2,3)\text{P}_3$ concentration shows especially large fluctuations, and PP-InsP_5 fluctuations are also very marked. Remarkably, $\text{Ins}(1,2,3)\text{P}_3$ turns over fastest during S-phase, when its concentration is lowest. These results establish that several fairly abundant intracellular inositol polyphosphates, for which important biological roles are emerging, display dynamic behaviour that is synchronized with cell-cycle progression.

Key words: cell cycle, inositol polyphosphate, inositol 1,2,3-trisphosphate, iron, WRK-1 cells.

INTRODUCTION

Phosphoinositide-based regulatory systems are ubiquitous in eukaryotes. They contribute to many processes, including signalling from cell-surface receptors, vesicle trafficking and assembly/disassembly of the actin cytoskeleton. Despite this, there is little information on how inositol phosphates behave during the cell cycle. This is particularly true of several inositol polyphosphates that are quite abundant in cells but are not closely related to lipid-derived second messengers metabolically. Processes that may be regulated by one or more of these include serine/threonine-directed protein phosphatases [1], vesicle trafficking [2–4], chemotaxis [5], DNA-dependent protein kinase [6–8], interferon β -mediated apoptosis [9], nuclear mRNA export [10–12], gene transcription [11,13], chromatin remodelling [14,15] and cell proliferation [16]. The metabolic pathways that handle inositol polyphosphates in eukaryote (especially mammalian) cells are complex and incompletely defined. Scheme 1 summarizes much of what is known [17–22].

The available information on what happens to these inositol polyphosphates during the cell cycle does not provide a detailed picture, either because the various isomeric inositol phosphates were not separated [23] or because less than a complete cell cycle was examined [24]. However, it is known for example, that $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 levels fluctuate in growing rat thymocytes [25] and that the $\text{Ins}(1,4,5)\text{P}_3$ concentration changes during sea-urchin egg mitosis [26].

Some time ago, we defined the complex inositol phosphate complement of the rat mammary tumour cell line WRK-1, and described how activating G_q -coupled V_1 -vasopressin receptors influences the formation and metabolism of inositol phosphates in these cells [27–34]. Many of the inositol polyphosphates res-

ponded in ways consonant with their known metabolic relationships with $\text{Ins}(1,4,5)\text{P}_3$, the primary second messenger product of receptor-stimulated $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis by PIC (phosphoinositidase C). However others, including $\text{Ins}(1,3,4,5,6)\text{P}_5$, $\text{Ins}(3,4,5,6)\text{P}_4$ and ‘GroPIns’ (glycerophosphoinositol), showed surprisingly variable responses. In part, this was because their ‘basal’ levels seemed to vary in an unpredictable manner [32]. It seemed possible that some of this variability might be caused by changes in the concentrations of these compounds during the cell cycle. In this study we confirm that striking changes in the concentrations of several inositol polyphosphates do occur during the WRK-1 cell cycle.

MATERIALS AND METHODS

Cell culture and labelling

WRK-1 cells were cultured in a modified Eagle’s minimal essential medium (EMEM) with 5% foetal calf serum and 2% rat serum, as described previously [31,32,34]. Inositol-free EMEM was mixed with dialysed (1000 M_r cut-off) bovine and rat sera and 10 μM [^3H]inositol (5 $\mu\text{Ci/ml}$; American Radiolabeled Chemicals Ltd., St. Louis, MO, U.S.A.). This medium supports normal rates of proliferation (results not shown). Cells were plated at $\leq 10\%$ of confluent density and cultured for up to 144 h (see the Results section). The confluent cells were re-plated in identical radiolabelled medium, either at 10% of confluent density (for experiments in Figure 1) or at 20% of confluent density. In experiments to examine turnover rates, cells were initially grown in unlabelled medium (see Figure 4 legend).

Two cell synchronization protocols were used. In protocol A, labelled confluent cells were trypsinized and re-plated in fresh

Abbreviations used: EMEM, Eagle’s minimal essential medium; GroPIns, glycerophosphoinositol; PIC, phosphoinositidase C; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

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Scheme 1 Known pathways of inositol polyphosphate metabolism in mammalian cells, indicating principal routes of synthesis and degradation

The scheme shows both direct (unbroken lines) and indirect (dashed lines) routes for the synthesis of InsP_6 and its close metabolic relatives. Dephosphorylation routes, particularly from InsP_6 , are less well defined. A likely route to $\text{Ins}(1,2,3)\text{P}_3$ and $\text{Ins}(1,2,3)\text{P}_2$ is depicted (where 'X' is a phosphate at an unknown position). The unexpected relationship between the metabolism of $\text{Ins}(1,3,4,5,6)\text{P}_5$ and of $\text{PtdIns}(3,4,5)\text{P}_3$, due to the multiple specificity of the 3-phosphatase PTEN, is shown on the right-hand side. MIPP, multiple inositol polyphosphate phosphatase; MK, multi-kinase.

Figure 1 Cell numbers and relative concentrations of inositol phosphate classes following release of contact-inhibited cells into cycle

(A) InsP_3s (●). (B) GroPIns (■) and $\text{InsP}_5/\text{InsP}_6$ (○). Cells were labelled for 144 h with $[2\text{-}^3\text{H}]\text{myo-inositol}$ and re-plated (protocol A, see the Materials and Methods section), and inositol phosphates were extracted and separated on Dowex-1 columns at the indicated times. The plotted data are from one of two experiments (in triplicate; mean $\pm$ S.E.M.) that gave similar results. Values through each time-course were compared using an unpaired, two-tailed t test (Instat, Graphpad), and the bars at the top of each panel indicate when values were significantly different from the adjacent values in the same curve (* $P < 0.05$, ** $P < 0.02$ and *** $P < 0.002$). In both panels the cell numbers are represented by dashed lines and are an average of duplicate determinations, again representative of two experiments.

medium. For protocol B, re-plated cells (as in protocol A) that had completed approximately three-quarters of a cycle were treated for 5.5 h with 50 ng/ml nocodazole, after which the nocodazole was washed out with PBS (pH 7.4) and culture was continued.

Cell cycle measurements

Cell volume

Labelled cells were trypsinized from plates, washed and suspended. Diameters of the now-spherical cells were measured microscopically using a haemocytometer (≥ 100 cells per sample).

Thymidine incorporation

Growing cells were pulsed for 2 h with $[^3\text{H}]\text{thymidine}$ (0.5 $\mu\text{Ci}/\text{ml}$, 1 μM) and washed three times with PBS. Cells were killed and fixed with methanol/glacial acetic acid (1:1, v/v), solubilised in 0.5 ml of 1% (w/v) SDS, and radioactivity was determined.

FACS analysis

Cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, trypsinised, gently sedimented, fixed (70% ethanol) and stored at -20°C . After washing twice in PBS, they were incubated with RNase (1 $\mu\text{g}/\text{ml}$; Boehringer) for 30 min at 37°C , treated with 10 $\mu\text{g}/\text{ml}$ propidium iodide and analysed by flow cytometry (Becton-Dickinson FACS 440).

Inositol phosphate extraction and analysis

Inositol phosphates were extracted as described previously [33]. Briefly, cells were killed in 5% (w/v) trichloroacetic acid containing 125 $\mu\text{g}/\text{ml}$ unlabelled InsP_6 (Sigma) and sedimented.

Supernatants were quickly neutralized by washing with diethyl ether, adjusted to pH 6–7 (50 μ l 0.1 M EDTA, pH 7) and stored at -70°C . Some samples were analysed on small columns of AG-1X8 resin (200–400 mesh, formate form; BioRad) to separate inositol phosphate classes [31]. Most were subjected to anion-exchange HPLC on 25 cm Whatman Partisphere-SAX columns (Whatman Biosystems Ltd., Maidstone, Kent, U.K.), with elution by a complex $[\text{NH}_4]_2\text{HPO}_3$ gradient [33].

RESULTS

Equilibrium labelling of inositol phosphates in growing cells

When changes in the concentrations of cell constituents are to be followed by isotopic methods, isotopic equilibrium must first be established. We used WRK1 cells plated in $[\text{H}]$ inositol medium at approximately one-tenth of their confluent density to determine when each HPLC-separated inositol phosphate species approached isotopic equilibrium. $\text{Ins}(1,3)\text{P}_3$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(3,4)\text{P}_2$, $\text{Ins}(1,4,5)\text{P}_3$, the InsP_5 s and GroPIns equilibrated in ≤ 2 days; $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,3,4)\text{P}_3$ and PP-InsP_5 took ≤ 3 days; InsP_6 and several metabolically related compounds [including $\text{Ins}(1,3,4,6)\text{P}_4$, $\text{Ins}(1,3,4,5,6)\text{P}_5$, $\text{Ins}(1,2,3,4,5)\text{P}_5$, $\text{Ins}(3,4,5)\text{P}_3$, $\text{Ins}(1,2,3)\text{P}_3$, $\text{Ins}(1,2)\text{P}_2$ and $\text{Ins}(1,4,5,6)\text{P}_4/\text{Ins}(3,4,5,6)\text{P}_4$] equilibrated in 4–5 days, and InsP_5 s [$\text{Ins}(1,2,4,5,6)\text{P}_5$ and $\text{Ins}(1,2,3,4,6)\text{P}_5$] after 5–6 days (results not shown).

We conclude that changes in the radioactivity in these compounds will accurately reflect relative changes in their concentrations in cells that have been continuously labelled for 6 days or more.

Inositol phosphate classes show repetitive changes during proliferation

We first looked for fluctuations in the concentrations of mixed inositol phosphate classes (InsP_2 s, InsP_3 s, etc., separated by simple anion-exchange chromatography), through several cell cycles, in cells that had been re-plated after growing to confluence. During the first 24 h, whilst cells were re-entering cycle from G_0 , concentrations of GroPIns (Figure 1B), InsP_5 s (results not shown), InsP_2 s (results not shown) and InsP_3 s (Figure 1A) were higher than during subsequent proliferation.

Once proliferation was underway (from approx. 20–100 h in Figure 1), each inositol phosphate class displayed one of three temporal patterns. GroPIns (Figure 1B) and inositol monophosphates (results not shown) remained low and quite constant whilst cells were proliferating, and accumulated when they stopped multiplying. Other inositol phosphate classes showed sustained fluctuations, with a period of approx. 25–30 h, that were statistically vindicated over at least 3 days (Figure 1). The repetitive fluctuations in the InsP_3 and $\text{InsP}_5/\text{InsP}_6$ fractions were substantial and well sustained (Figures 1A and 1B), and the InsP_2 and InsP_4 fluctuations were smaller (results not shown). When InsP_6 and a mixed InsP_5 s fraction were separated on Sep-Pak columns (Waters U.K. Ltd., Watford, Herts., U.K.), both behaved similarly (results not shown).

Parallel cultures that were pulsed with $[\text{H}]$ thymidine showed repetitive changes in DNA synthesis with a period similar to the inositol phosphate fluctuations (results not shown). This indicated that re-plated WRK1 cells proliferate in a largely synchronized manner for at least 2–3 cell cycles, with inositol phosphate concentrations fluctuating in time with the cell cycle. The fluctuations were clearest during the first and most synchronized cycle, so we focused on this period.

Figure 2 The cell cycle behaviour of growth-arrested cells re-plated in serum (protocol A) and of cells released from a nocodazole block (protocol B)

The left-hand column (panels A, B and D) represents data from protocol A, the right-hand column (panels C and E) from protocol B. (A) Cells were pulsed with $[\text{H}]$ thymidine for 2 h, ending at the times plotted. (B) and (C) Single-cell volume changes through the cell cycle (means $\pm$ S.E.M. for 60 measurements per point). (D) and (E) Cell cycle distributions, analysed by propidium iodide staining and FACS analysis (means of duplicate determinations). The bars under the panels denote the cell cycle assignments that also appear in Figure 4. The results are from one of three experiments that yielded similar results. The inositol phosphate data in Table 1 and Figure 3 are from the same experiments.

Definition of cell-cycle-related parameters

We analysed several parameters during the first 24–35 h after arrested cells were released into cycle, including: cell volume, the cell cycle (analysed by FACS), pulsed incorporation of $[\text{H}]$ thymidine and changes in the concentrations of individual inositol derivatives. To discriminate between genuine cell-cycle-related events and cycle-independent effects that were consequences of particular cell-synchronization procedures, we undertook parallel measurements on cells synchronized in two ways.

In protocol A, cells were grown to confluence (to a G_0 -like state) and released into cycle by re-plating in the presence of serum (Figures 2A, 2B and 2D). The first S-phase occurred after approx. 20–30 h (Figures 2A and 2D) and G_2/M after approx. 30 h. Average cell volume increased by approx. 75% before mitosis (Figure 1B), and cell number increased by 60–70% over approx. 4 h at the end of the cycle (results not shown). Therefore it appeared that approximately two-thirds of the cells cycled in a well-synchronized manner, and this was confirmed by the FACS analyses (Figure 2D). The remaining approximately one-third of the cells failed to leave G_0/G_1 .

Table 1 Cellular concentrations of inositol derivatives through the cell cycle, from two synchronization protocols

Cells were labelled and their inositol phosphates extracted and analysed as described in the Materials and methods section. Inositol phosphate concentrations (μM), averaged throughout the volume of the cells, were calculated from the specific radioactivity of the medium inositol, the ^3H content of each compound and the average cell volume at the time of cell killing. Three protocol A and two protocol B experiments were documented, with triplicate cultures for each point. Data representative of each protocol are presented (Figure 4 presents more detailed temporal analyses for some compounds). Values are presented for the G_0/G_1 period of re-entry into cycle, from protocol A only, and for the G_1 , S and G_2/M phases from both protocol A and protocol B. When an obvious change occurred between the start and end of a cell cycle phase, the concentration range is presented as the temporal trend of that change (i.e. low $\rightarrow$ high or high $\rightarrow$ low). n.d., not detected.

Inositol derivative	Cell cycle phase . . .	Inositol phosphate concentration (μM)						
		G_0/G_1		G_1		S		G_2/M
		Protocol A	Protocol A	Protocol B	Protocol A	Protocol B	Protocol A	Protocol B
GroPIns		58	42 $\rightarrow$ 24	53	17	33	15	42
*Ins1/3P		60	23 $\rightarrow$ 35	28	16	17	14	23
Ins2P		3.8	12	18	7.0	7.8	6.4	11
Ins4P		1.3	1.7 $\rightarrow$ 3.0	n.d.	1.1	n.d.	1.8	n.d.
Ins(1,3)P ₂		0.93	0.64 $\rightarrow$ 0.36	0.60	0.21	0.32	0.18 $\rightarrow$ 0.59	0.66
Ins(1,4)P ₂ + Ins(3,4)P ₂		4.2	5.9 $\rightarrow$ 3.4	5.6	2.1	4.7	1.9	5.1
*Ins(1,2/2,3)P ₂		2.7	2.1 $\rightarrow$ 2.6	1.4	0.85	1.1	0.98	1.1
Ins(4,5)P ₂		2.0	1.4	1.6	0.63	0.87	0.85	1.2
Ins(1,2,3)P ₃		0.54	0.54 $\rightarrow$ 0.97	0.67 $\rightarrow$ 0.90	0.35	0.25	0.38 $\rightarrow$ 0.43	0.31
Ins(1,3,4)P ₃		1.4	1.2 $\rightarrow$ 0.7	0.73	0.55	0.44	0.38 $\rightarrow$ 0.87	0.64
*Ins(1,5,6/3,4,5)P ₃		0.69	0.68	0.57	0.18	0.27		0.37
Ins(1,4,5)P ₃		2.2	1.4 $\rightarrow$ 1.1	1.4	1.0	2.7	0.8 $\rightarrow$ 0.92	2.5
Ins(2,4,5)P ₃		n.d.		0.51	n.d.	0.27	n.d.	0.35
Ins(1,3,4,5)P ₄		3.5	3.2 $\rightarrow$ 0.50	0.54	0.22	0.1	0.08 $\rightarrow$ 0.35	0.38
Ins(1,3,4,6)P ₄		1.1	0.25 $\rightarrow$ 0.68	1.1	0.44	0.72	0.76	0.77
*Ins(1,4,5,6/3,4,5,6)P ₄		8.8	6.7 $\rightarrow$ 10.5	6.7	4.3	3.6	3.7 $\rightarrow$ 4.5	4.3
Ins(1,3,4,5,6)P ₅		91	80	66	47	47	41 $\rightarrow$ 65	62
*Ins(1,2,3,4,5/1,2,3,5,6)P ₅		0.80	0.37 $\rightarrow$ 0.56	0.64	0.26	0.34	0.22 $\rightarrow$ 0.49	0.44
*Ins(1,2,4,5,6/2,3,4,5,6)P ₅ + Ins(1,2,3,4,6)P ₅		1.6	2.9	1.8	1.5	1.2	1.3 $\rightarrow$ 2.0	1.5
InsP ₆		35	32	43	21	31	18 $\rightarrow$ 34	47
PP-InsP ₅		0.6	0.6 $\rightarrow$ 1.4	1.4	0.65	0.56	0.61 $\rightarrow$ 0.8	0.85

* Denotes enantiomer pair.

Events that occur whilst cells are re-entering G_1 from G_0 can influence the information obtained from confluent cells that are trypsinized and re-plated. In protocol B, therefore, cells that had been released into cycle were accumulated at the G_2/M -phase boundary with nocodazole (Figures 2C and 2E) and then released by its removal. FACS analyses and measurements of cell size showed that somewhat more than half of these cells re-entered cycle approximately in synchrony (Figure 2E) and progressed through mitosis and at least one more cell cycle. This G_1 - and S-phase occupied approximately hours 4 to 16 (Figures 2C and 2E), suggesting that the first cell cycle in protocol B was somewhat shorter than in protocol A.

Changes in individual inositol phosphate species through the cell cycle

WRK1 cells make little or no inositol *de novo* from glucose 6-phosphate [30]. In Table 1 and Figure 3, therefore, we have calculated intracellular concentrations for each inositol derivative from: (a) the specific radioactivity of the [^3H]inositol used to label the cells, (b) the radioactivity found in a particular cell constituent, and (c) the average cell volume at the relevant stage in the cell cycle. The calculated average concentrations are based on the entire cell volume, and any intracellular compartmentation will make local concentrations within particular cell compartments higher or lower. Cycling cells double their size during the progress from early G_1 to mitosis, so the quantity of any compound in a cell must double during that period if it is to maintain a constant intracellular concentration.

We defined the inositol polyphosphate changes that occurred through the first approx. 25–30 h after cells re-entered the cycle

in each protocol. We focus mainly on those changes that showed a similar pattern through the various stage(s) of the cycle under both protocol A and protocol B, and so are likely to be linked to cycle transit.

Table 1 summarizes the concentrations of twenty resolved peaks, through three phases of the cycle, from each protocol. Protocol A also yielded data for the G_0/G_1 period during which cells were entering cycle from quiescence (cf. Figure 1). Figure 3 depicts the most striking of these changes with greater temporal resolution (panels A and B presents results obtained with protocol A, and panels C and D shows those from protocol B).

Ins(1,4,5)P₃ and its immediate metabolites

In protocol A, the concentrations of Ins(1,3,4,5)P₄ and Ins(1,4,5)P₃ were high soon after re-plating. Ins(1,4,5)P₃ concentration declined for 8–12 h and stabilized, but Ins(1,3,4)P₃ and Ins(1,3,4,5)P₄ levels continued to decline (Table 1). The Ins(1,4)P₂/Ins(3,4)P₄ peak (which contained the two isomers in an approx. 4:1 ratio respectively [31]), Ins(4,5)P₂ and Ins(1/3)P behaved much like Ins(1,4,5)P₃ (Table 1).

Levels of Ins(1,4,5)P₃ and its immediate metabolites during G_1 -phase were similar in both protocols (Table 1). Ins(1,4,5)P₃ and Ins(1,4)P₂ concentrations increased substantially when the cells entered S-phase under protocol B, but Ins(1,3,4,5)P₄, Ins(1,3,4)P₃ and Ins(1,3,4,6)P₄ concentrations stabilized (Table 1).

InsP₆, Ins(1,3,4,5,6)P₅, Ins(1,2,3)P₃ and metabolically related inositol phosphates

The main cell-cycle-related fluctuations in InsP₆, Ins(1,3,4,5,6)P₅ and Ins(1,4,5,6/3,4,5,6)P₄ concentrations paralleled one another

Figure 3 Cell-cycle-related changes in $\text{Ins}(1,2,3)\text{P}_3$, $\text{D/L-Ins}(3,4,5,6)\text{P}_4$, $\text{Ins}(1,3,4,5,6)\text{P}_5$, InsP_6 and PP-InsP_5

WRK-1 cells were labelled for 144 h, re-plated in similar medium, and synchronization protocol A (panels A and B) or protocol B (panels C and D) was applied. At intervals, inositol phosphates were extracted and analysed by HPLC. The results are from single experiments carried out in triplicate and are representative of three (protocol A) or two (protocol B) experiments. Cell cycle progression is summarized above each panel.

and were similar in the two protocols. They were less marked in protocol B, probably because fewer cells cycled (see above). Concentrations were highest in G_1 , decreased during S-phase and tended to rise again during G_2/M -phase (Table 1; Figures 3B and 3D). The two less-abundant InsP_5 peaks, which are likely to be InsP_6 metabolites, behaved similarly (Table 1).

Two pyrophosphate derivatives of InsP_6 , PP-InsP_5 , sometimes termed ' InsP_7 ', and a trace of $(\text{PP})_2\text{-InsP}_4$ (or ' InsP_8 '), eluted after InsP_6 and exhibited notable fluctuations. The $[\text{PP-InsP}_5]$ changes are illustrated (Table 1, Figures 3A and 3C). It was most abundant in G_1 -phase, fell during S-phase and may have started to rise again during G_2/M -phase.

$\text{Ins}(1,2,3)\text{P}_3$ is probably a product of InsP_6 dephosphorylation [34], but its concentration changed more strikingly than other inositol polyphosphates during the cell cycle (Figures 3A and 3C). It was most abundant around mid G_1 -phase, decreased by approximately two-thirds during S-phase, and tended to accumulate again towards the end of the cycle.

$\text{Ins}2\text{P}$, probably the penultimate product of InsP_6 dephosphorylation, may be reached via $\text{Ins}(1,2,3)\text{P}_3$. Its concentration rose rapidly as cells re-entered the cycle (protocol A), was highest at 8–12 h when concentrations of its more highly phosphorylated precursors were starting to decline (results not shown), and then showed cycle-related changes that were intermediate in magnitude between those of $\text{InsP}_6/\text{Ins}(1,3,4,5,6)\text{P}_5$ and of $\text{Ins}(1,2,3)\text{P}_3$ (Table 1).

Overall, therefore, the concentrations of most of the molecules that are metabolically most closely related to InsP_6 were highest in G_1 -phase, lower in S-phase and returned towards G_1 levels during G_2/M -phase. Within this group, the fluctuations in $\text{Ins}(1,2,3)\text{P}_3$ and PP-InsP_5 were most marked, and this may indicate that these compounds have distinctive functions (see the Discussion).

Turnover rates vary during the cell cycle. Average intracellular concentrations of most of the more abundant inositol polyphosphates were higher during G_1 -phase than during S-phase by a factor of two or more (see above, and the Discussion).

Paradoxically, however, some reports have suggested that inositol lipids and phosphates incorporate radioactive precursors more readily during S-phase [24,35], which might indicate that they turn over faster during that part of the cell cycle. To address this, we pulse-labelled unlabelled protocol A cells with $[\text{H}]\text{inositol}$ for 4 h periods at three stages of the cycle: from 4–8 h (during G_1 -phase); from 20–24 h (during S-phase); and from 25–29 h (during G_2/M -phase). Figure 4 compares HPLC analyses of the inositol phosphates that were labelled during the 4–8 h and 20–24 h windows. GroPIns , $\text{Ins}1/3\text{P}$, $\text{Ins}2\text{P}$ and $\text{Ins}(1,2,3)\text{P}_3$ labelled most readily, and smaller amounts of $[\text{H}]\text{inositol}$ were incorporated into $\text{Ins}(1,4)\text{P}_2/\text{Ins}(3,4)\text{P}_2$, $\text{Ins}(1,4,5)\text{P}_3$, three InsP_4 isomers and $\text{Ins}(1,3,4,5,6)\text{P}_5$. Very little label was incorporated into InsP_6 during these 4 h pulses (results not shown). Quiescent cells do not show this rapid $\text{Ins}(1,2,3)\text{P}_3$ labelling [34]. $\text{Ins}(1,2,3)\text{P}_3$ labelled fastest during S-phase, when its concentration is lowest (see above).

DISCUSSION

All eukaryote cells contain a complex mixture of inositol phosphates. The inositol polyphosphate complement of WRK1 cells is uniquely well defined [28,29,31,33]. Our results establish that the concentrations of several inositol polyphosphates fluctuate substantially during the WRK1 cell cycle. The key observations include the following: the concentrations of InsP_6 and of its close metabolic relatives decline as cells move from G_1 - into S-phase; $\text{Ins}(1,4,5,6/3,4,5,6)\text{P}_4$, PP-InsP_5 and $\text{Ins}(1,2,3)\text{P}_3$ concentrations accumulate transiently during G_1 -phase; and the $\text{Ins}(1,2,3)\text{P}_3$ concentration falls sharply as cells move from G_1 - through S-phase, but it turns over faster during S-phase. No previous study has approached this level of detail, and we suspect that equivalent analyses of other mammalian cells would yield at least partially similar information.

What are the true magnitudes of the inositol polyphosphate fluctuations during the cell cycle?

The experimental observations significantly understate the magnitudes of the cell-cycle-related fluctuations for several reasons. First, and most important, the cells that did not cycle contributed a substantial proportion of the analysed inositol phosphate mixtures, on average of the order of 30% in protocol A and 50% in protocol B. The inositol phosphate concentrations in these cells would have been quite stable, and will have 'diluted' the fluctuations that occurred in the cells that cycled. Secondly, less-than-perfect cell synchronization will have broadened and suppressed the peaks and troughs. Thirdly, inositol polyphosphates may be compartmentalized within cells, but the recorded concentrations are averaged throughout the cell.

By correcting the experimental values to take account of the first of these concerns, we can get a truer impression of the magnitudes of the underlying cycle-related changes. For this, we assumed that the inositol phosphate concentrations in non-cycling cells remain fairly stable throughout, and at concentrations similar to the starting values. The proportion of non-cycling cells was approx. 30% in protocol A and approx. 50% in protocol B: for the calculations, we took a mean value of 40%.

As a first example, take the changes in concentration of InsP_6 , one of the most abundant inositol polyphosphates. The average intracellular InsP_6 concentration was quite similar in both

protocols at the start (approx. 30–40 μM) and the end (approx. 40–45 μM) of cycle, and it went through a minimum of approx. 20–30 μM during mid and late S-phase. For the calculations, we took approximate central InsP_6 concentrations of approx. 40 μM (for non-cycling cells and for cells in G_1 - and M-phase) and approx. 25 μM (during mid/late S-phase).

Throughout the experiments the non-cycling cells contributed $0.4 \times 40 \mu\text{M}$, or approx. 16 μM , to the InsP_6 concentration of the entire population. During mid S-phase, the overall InsP_6 concentration fell to 25 μM , to which the cycling cells must have contributed only approx. 9 μM (25 μM minus 16 μM). These cycling cells (approx. 60%) must, therefore, have averaged approx. 15 μM InsP_6 ($9/0.6 = 15 \mu\text{M}$). Synchrony of the cycling cells was imperfect, so the real nadir in the InsP_6 concentration during late S-phase was even lower (less than 15 μM , but probably more than 10 μM). It seems, therefore, that the InsP_6 concentration fluctuates through an approx. 3-fold range during the cell cycle.

The observed changes in Ins2P , which is probably the penultimate metabolite of the above two compounds, correct to a roughly 4-fold decline from early G_1 - to late S-phase.

When the $\text{Ins}(1,2,3)\text{P}_3$ concentration values are corrected, it becomes apparent that cellular $\text{Ins}(1,2,3)\text{P}_3$ concentrations fluctuated over an even wider range. The population average concentration varied from approx. 0.6 μM $\text{Ins}(1,2,3)\text{P}_3$ (before entry into cycle), through a transient peak of approx. 0.8 μM (during G_1 -phase) to approx. 0.35 μM (during S-phase). If the non-cycling cells remained at approx. 0.6 μM throughout, they will have contributed a constant 0.24 μM ($0.4 \times 0.6 \mu\text{M}$) to the whole population. For this population to have achieved an average concentration of 0.8 μM during G_1 -phase, the $\text{Ins}(1,2,3)\text{P}_3$ concentration in the cycling cells must have passed through a peak of approx. 1 μM [$0.56 \mu\text{M} (\equiv 0.8 \mu\text{M} - 0.24 \mu\text{M})/0.6 = 0.92 \mu\text{M}$] during G_1 -phase. And when these cells cycled through late S-phase, they will have contributed only approx. 0.11 μM ($0.35 - 0.24 \mu\text{M}$) of the detected approx. 0.35 μM $\text{Ins}(1,2,3)\text{P}_3$. Given the imperfect synchrony of the populations, it therefore seems that $\text{Ins}(1,2,3)\text{P}_3$ concentrations fluctuated between approx. 1 μM in G_1 -phase and approx. 0.1 μM during S-phase.

These calculations yield all-cell averages, but individual inositol polyphosphates are likely to be even more abundant in whichever intracellular compartment(s) they are concentrated or confined. This compartmentation is little understood. In animal cells, the second messenger $\text{Ins}(1,4,5)\text{P}_3$ (and its immediate metabolites) is likely to be largely cytosolic, and InsP_6 and other inositol polyphosphates seem to be mainly cytosolic in HL60 cells [36]. However, other information, such as that from *Saccharomyces cerevisiae*, seems contradictory. The phenotypes of yeast that lack either PIC (Plc1p) or the enzymes that convert $\text{Ins}(1,4,5)\text{P}_3$ to other inositol polyphosphates, notably $\text{Ins}(1,4,5,6)\text{P}_4$, InsP_6 and $PP\text{-InsP}_5$, suggest that these $\text{Ins}(1,4,5)\text{P}_3$ metabolites influence both cytosolic processes [37,38] and nuclear events [10,11,13–15,39]. The $\text{Ins}(1,4,5)\text{P}_3$ that is formed when PIC is hypo-osmotically activated in *S. cerevisiae* is converted into InsP_6 rapidly [40], which might mean that part or all of this process occurs in the cytosol.

There is scant information from other animal cells with which to compare the estimates reported here. Cell-cycle-related changes in a rat thymocyte cell line are at least partially similar: $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 concentrations changed little during G_2/M -phase and G_1 -phase, but halved during S-phase [25]. Another study, in apparent contrast, reported increased labelling of InsP_5 and InsP_6 as Rat-1 fibroblasts entered S-phase [24]. In this study, however, the inositol phosphates were far from isotopic equilibration, so these results give little insight into InsP_5 and

Figure 4 Labelling of inositol phosphates during a brief [^3H]inositol pulse

Unlabelled cells were synchronised by protocol A and 100 $\mu\text{Ci}/\text{ml}$ [^3H]myo-inositol was added at 4 h (A) and 20 h (B). Cells were killed 4 h later and inositol phosphates were extracted and analysed. The break in the traces between 60 and 80 minutes is to eliminate a peak that represented a contaminant in the [^3H]inositol: the cells contained negligible quantities of GroPIns4P , the only inositol phosphate derivative that elutes in this region of the chromatogram. The positions of ^{14}C -labelled inositol phosphate standards are shown with a broken line.

InsP_6 concentrations. Myeloid differentiation is accompanied by major changes in inositol polyphosphate complement, the most striking of which is a rise in $\text{Ins}(1,3,4,5,6)\text{P}_5$ concentration after 2–4 days [41–44], at approximately the time that the differentiating cells leave the cell cycle [45,46].

What metabolic pathways underlie the cell-cycle-related inositol polyphosphate changes?

$\text{Ins}(1,4,5)\text{P}_3$ and its immediate metabolites

In this study, the inositol phosphates that are formed rapidly as a result of PIC activity, such as $\text{Ins}(1,4,5)\text{P}_3$ and its immediate metabolites, showed different behaviour under each protocol, so we learned nothing new about the relationship between their dynamics and the cell cycle.

$\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6

Routes to and from $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 remain poorly understood in mammalian cells. The route that was described first, from $\text{Ins}(1,4,5)\text{P}_3$, via $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,3,4)\text{P}_3$, and $\text{Ins}(1,3,4,6)\text{P}_4$, is circuitous ([17], Scheme 1). Recent studies have identified yeast and mammalian (multi)kinases that can make $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 more directly from $\text{Ins}(1,4,5)\text{P}_3$, via

Ins(1,4,5,6) P_4 ([15,22,47–51], Figure 4): an early hint of this came from over-expression of Ins(1,4,5) P_3 '3-kinase' in fibroblasts [24].

Despite the possible influence of the latter pathways, we saw no correlation between the behaviour of metabolically dynamic Ins(1,4,5) P_3 (and its immediate metabolites) and of the metabolically more sluggish Ins(1,3,4,5,6) P_5 and Ins P_6 (and their close metabolic relatives). This is not surprising for two reasons. First, these two metabolite families display very different dynamics. Secondly, even when the more direct pathways to Ins(1,3,4,5,6) P_5 and Ins P_6 are active, little of the inositol that goes through the Ins(1,4,5) P_3 pool is likely to find its way to Ins P_6 and its metabolic relatives.

Routes of Ins(1,3,4,5,6) P_5 /Ins P_6 /PP-Ins P_5 dephosphorylation in mammalian cells remain poorly understood. Multiple inositol polyphosphate phosphatase (MINPP1), the primary candidate as a mammalian inositol polyphosphate phosphatase, is restricted to the endoplasmic reticulum lumen [52–54] but it somehow gains access to cytosolic inositol polyphosphates [55], and the cytoplasmic surface of red-cell plasma membranes displays a closely related enzyme that 3-dephosphorylates Ins(1,3,4,5) P_4 [55,56]. At least one similar enzyme can partially compensate if MINPP1 is ablated [55]. The PtdIns(3,4,5) P_3 phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) can 3-dephosphorylate Ins(1,3,4,5,6) P_5 , but not Ins P_6 , and PTEN over-expression slightly reduces cellular Ins(1,3,4,5,6) P_5 levels [57].

WRK-1 cell extracts dephosphorylate Ins P_6 to Ins(1,2,3) P_3 [33,34]. In the present study, Ins(1,2,3) P_3 was radiolabelled most quickly in S-phase, when its concentration was very low (see calculation above). This implies that at least two independent controls, on synthesis and on degradation, must contribute to the striking changes in Ins(1,2,3) P_3 concentration and turnover that accompany the cell cycle. Its only known precursor, Ins P_6 , labels slowly during S-phase. The small pool of Ins(1,2,3) P_3 that is rapidly renewed during S-phase must be made either by some novel, and Ins P_6 -independent, route or from a small and metabolically autonomous sub-compartment of a cell's substantial Ins P_6 complement.

Possible functions of cell-cycle-regulated changes in inositol polyphosphate complement

Recent reports have implicated inositol derivatives containing four or more phosphates in a number of important cell events, including regulation of serine/threonine-directed protein phosphatases [1], vesicle trafficking and vacuolar function [12], neurotransmission [58], nuclear mRNA export [11,59], gene transcription [10], DNA end-joining by DNA-dependent protein kinase and its DNA-binding subunit (Ku) complex [6–8], chromatin remodelling [14,15] and cell proliferation [16].

Which of these are the cell-cycle-related fluctuations in inositol polyphosphate complement most likely to influence? At the concentrations encountered during the cell cycle, and particularly during G₁-phase, Ins P_6 [and, to a lesser extent, Ins(1,3,4,5,6) P_5] may partially inhibit protein phosphatases PP1 and PP2A [1]. Most changes in protein dephosphorylation rates during the cell cycle involve specific regulation of individual protein phosphatases, but 'global' effects of cytosolic inositol polyphosphates might amplify or restrain some of these effects. One such effect might be to enhance PP1 inhibition during G₁-phase and so facilitate hyper-phosphorylation of the Rb (retinoblastoma) protein.

There may be other cytosolic effects, for instance on the PIC and phosphoinositide 3-kinase signalling pathways. High cytosolic

Ins(1,3,4,5,6) P_5 concentrations can diminish Ins(1,4,5) P_3 binding to its receptor [60], so Ins(1,3,4,5,6) P_5 variations through the cell cycle might modulate Ca²⁺ signalling. PTEN has a modest affinity for Ins(1,3,4,5,6) P_5 , and Ins(1,3,4,5,6) P_5 variation may temper the phosphatase activity of PTEN against PtdIns(3,4,5) P_3 [57], a reaction that is important to the regulation of cell survival and proliferation. High PTEN activity tends to restrain G₁ progression [61], and the high Ins(1,3,4,5,6) P_5 concentration during G₁-phase might limit PTEN activity, prolong the lifetime of PtdIns(3,4,5) P_3 and facilitate G₁ progression. Recent studies of an artificial PTEN mutant that hydrolyses Ins(1,3,4,5,6) P_5 but not PtdIns(3,4,5) P_3 has provided evidence compatible with the idea that Ins(1,3,4,5,6) P_5 may have a pro-proliferative role [16].

Possible roles for PP-Ins P_5 fluctuations

Recent reports suggest that PP-Ins P_5 is made by at least three Ins P_6 kinases, and that there may be separate nuclear and cytosolic pools of PP-Ins P_5 [62]. PP-Ins P_5 formation by one kinase has been linked to interferon- β -stimulated apoptosis [9], and by another, to neuronal vesicle trafficking and exocytosis [63] (possibly due to the high affinity of PP-Ins P_5 for clathrin adaptor proteins [64–69]). Very recently, it was demonstrated that cAMP co-ordinately causes activation of PP-Ins P_5 synthesis, chemotaxis and aggregation in *Dictyostelium discoideum* [5].

Possible effects of Ins(1,2,3) P_3 and related compounds on cell-cycle-dependent iron metabolism

The striking cell-cycle-related changes in Ins(1,2,3) P_3 concentration, between approx. 1.2 μ M in early G₁-phase and approx. 0.1 μ M in S-phase (see above), and its speedy turnover whilst its concentration is lowest were both unexpected. It seems that the metabolism of this little-studied inositol trisphosphate is under tight control, and it may fulfil some cell-cycle-related function.

All inositol phosphates possessing the axial/equatorial/axial 1,2,3-trisphosphate motif, of which Ins(1,2,3) P_3 is the simplest, share remarkable iron-chelating properties. An up-regulation of transferrin receptor number accompanies S-phase entry, and interrupting the iron supply with antibodies to the transferrin receptor or with cell-permeant iron chelators can halt cells in S-phase. At least in part, the iron requirement of S-phase transit is because of the need to maintain the M2 subunit of the ribonucleotide reductase, needed for DNA synthesis, in an active state. The iron component of RNR-M2 undergoes rapid exchange with iron in its environment, so there is a need for constant iron re-supply [70]. One possible role for Ins(1,2,3) P_3 is as a low-molecular-mass intracellular iron shuttle ([33,71–75], C. J. Barker and D. R. Poyner, unpublished work). Any candidate intracellular iron carrier must be able to respond quickly to changes in cellular iron demand.

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