Inositol lipids are regulated during cell cycle progression in the nuclei of murine erythroleukaemia cells

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Previous data suggest the existence of discrete pools of inositol lipids, which are components of a nuclear phosphoinositide (PI) cycle. However, it is not known whether the contents of these pools are regulated during cell proliferation. In the present study we demonstrate that the mass levels of three important constituents of the nuclear PI cycle are regulated during the cell cycle. Radioactive label incorporation into PtdIns(4,5)P_2 was seen to increase dramatically as synchronized cells entered S-phase. This did not coincide with any significant changes in the nuclear mass levels of this lipid, suggesting that the rate of turnover of this molecule was increased. Levels of PtdIns4*P*, the major substrate for PtdIns(4,5)P_2 production by Type I PtdIns*P* kinases (PIPkins), were regulated during the cell cycle and indicated a complex relationship between these two lipids. An alternative substrate for PtdIns(4,5)P_2, PtdIns5*P*, phosphorylated by Type II PIPkins, was present in nuclei at much smaller amounts than the PtdIns4*P*, and thus is unlikely to contribute significantly to PtdIns(4,5)P_2 turnover. However, a large increase in nuclear PtdIns5*P* mass was observed when murine erythroleukaemia cells are in G_1, and this could represent a potential pool of nuclear inositol lipid that has a specific signalling role. Analysis of extracted lipid fractions indicated the absence of any PtdIns3*P* in these nuclei.

Key words: nuclear phosphoinositide cycle, phosphatidylinositides, PtdIns*P* kinase.

INTRODUCTION

Phosphoinositide (PI) turnover in cells, and the production of lipid and water-soluble second messengers in response to external stimuli, has been extensively characterized [1,2]. The separate existence in the cell nucleus of the molecular machinery required to phosphorylate various lipid intermediates has also been documented [3–5]. This PI cycle is capable of producing a range of signalling molecules, and has been shown to be under the control of receptor stimulation at the plasma membrane [6]. However, the downstream function of inositol lipid processing in the nucleus has proved to be a complex issue, and the cycling of these substrates and second messenger molecules may impinge on a number of cellular processes, which have not yet been fully elucidated [7].

The production of the second messengers InsP_3 and diacylglycerol (DAG) is achieved by the phospholipase cleavage of PtdInsP_2. Whereas the inositol phosphate may be involved in calcium homoeostasis through release of luminal stores in the nuclear envelope [8–10], nuclear DAG may have a role in regulating cell growth and differentiation [6,7,11]. Evidence has suggested that this DAG activates a nuclear PKC and is regulated by the translocation of a DAG kinase [12]. Furthermore, the discovery of the existence of differentially regulated pools of DAG in the nucleus suggests that downstream effects can be localized to regions within the nuclear matrix [13]. Cell cycle analysis has shown a substantial increase in levels of phosphatidic acid in murine erythroleukaemia (MEL) cells during G_1 and that up to 70% of this change is from PI-derived DAG [13]. It is, therefore, possible that the channelling of DAG produced from cleavage of inositol lipids, rather than that derived from phosphatidylcholine [14], through a discrete nuclear pool associated with a DAG kinase, may have an important role in cell cycle progression [13].

Production of this pool of DAG may require the presence of a PI-specific phospholipase (PIC). Evidence has shown that the predominantly nuclear PICβ1b subtype [15,16] plays a direct role in G_1 progression during proliferation of MEL cells [17]. Different non-detergent-extractable pools of inositol lipids have been reported in the nucleus [18], and association of PIC activity with a PtdInsP_2 pool may provide a means for functional localization of second messengers produced by components of the nuclear inositol cycle.

We have studied the turnover of nuclear inositol lipids during the cell cycle of MEL cells. Our data suggest that the observed increase in PtdIns(4,5)P_2 labelling in isolated nuclei supplied with exogenous [γ-^{32}P]ATP is cell cycle dependent. We have also analysed the mass levels of PtdIns(4,5)P_2, PtdIns4*P* and PtdIns5*P* during cell cycle progression. Changes in PtdIns5*P* levels in response to thrombin have been recently reported in platelets [19], and we suggest here that changes in the nuclear mass of this lipid may have a role in cell proliferation.

MATERIALS AND METHODS

Cell culture reagents were obtained from Gibco BRL and radioisotopes were from Amersham International. All other reagents used were of the highest grade available.

Abbreviations used: DAG, diacylglycerol; PI, phosphoinositide; PIPkin, PtdIns*P* kinase; MEL, murine erythroleukaemia; PIC, PI-specific phospholipase.

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Cell culture conditions and synchronization

MEL cells were routinely maintained at logarithmic phase in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells grown to a density of 0.3×10^6 cells/ml, were blocked at G_2/M by the addition of nocodazole (100 ng/ml) to the growth medium for 8 h. A synchronously growing population was then released by resuspending the cells in fresh pre-warmed CO_2-equilibrated medium. At each subsequent sample point, 10^6 cells were fixed in cold 70% ethanol, treated with RNase (50 μg/ml), and DNA stained with propidium iodide (1 μg/ml) at 37 °C for 30 min. Cell cycle analysis was performed using a FACScan flow cytometer and ModFit Cell Cycle Analysis software (Verity Software House, Inc., Topsham, ME, U.S.A.).

Purification of MEL cell nuclei

The procedure used was an adaptation of a previous method [6]. MEL cells were harvested by centrifugation at 190 ***g*** for 5 min and washed once with ice-cold PBS, pH 7.4, and all subsequent steps were carried out at 4 °C. Cells were swelled in hypotonic buffer (5 mM Tris/HCl, pH 7.4, 1.5 mM KCl and 2.5 mM $MgCl_2$) for 5 min. The solution was adjusted to 13 mM EGTA and the cells were mechanically sheared using a hypodermic needle. The final sucrose concentration of the cell lysate was adjusted to 320 mM, and $MgCl_2$, EGTA and PMSF concentrations were adjusted to 7.7 mM, 2.1 mM and 100 μM respectively. Nuclei were separated from cell debris by centrifugation (320 ***g*** for 5 min at 4 °C) through a sucrose cushion (10 mM Tris/HCl, pH 7.4, 1 mM EGTA, 1.5 mM KCl, 5 mM $MgCl_2$ and 500 mM sucrose). The sedimented nuclei were washed thoroughly with 4 vol. of cold resuspension buffer (320 mM sucrose, 10 mM Tris/HCl, pH 7.4, 1 mM EGTA, 1.5 mM KCl and 5 mM $MgCl_2$) and resuspended in 200 μl of this solution.

Nuclear lipid extraction and labelling

Total nuclear lipid was extracted from aliquots (equivalent by protein concentration) of nuclei from different cell cycle points. Lipids were extracted by the Bligh–Dyer method [20], and the aqueous phase was back-extracted twice to ensure maximal recovery of the lipid fraction.

Incorporation of [γ-^{32}P]ATP into nuclear phosphatidylinositides was performed as an adaptation of the method originally described by Smith and Wells [3,18]. Briefly, nuclei (total protein 10 μg) were pre-incubated at 30 °C for 2.5 min in a total volume of 90 μl of resuspension buffer and then incubated for a further 2.5 min in the presence of 10 μl of phosphorylation mixture (10 μCi of [γ-^{32}P]ATP and 200 μM non-radiolabelled ATP in resuspension buffer). The assay was stopped by the addition of 0.5 ml of cold chloroform/methanol (1:1, v/v) and the lipids extracted as described above.

Identification of inositol lipid species

Phosphatidylinositol lipids were separated by TLC on silica gel-coated glass plates, which had been activated [1 mM EDTA, 1% oxalate in methanol/water (1:1, v/v)] and dried at 110 °C for 1 h. Radiolabelled lipids were resolved in a chloroform/methanol/water/ammonia (14:20:5:3, by vol.) solvent system and quantified using a PhosphorImager (Molecular Dynamics) and ImageQuant software.

Radiolabelled lipids were extracted from these silica plates and polyphosphatidylinositide species were further identified as follows. Glycerophosphatidylinositides, produced by monomethylamine deacylation of labelled lipids [21], were dried and resuspended in water. Samples were separated by chromatography on poly(ethyleneimine)–cellulose plates in a 450 mM HCl solvent system, or deglycerated using $NaIO_4$ [6]. Radiolabelled inositol phosphate species were separated by HPLC using isocratic elution (55 mM NaH_2PO_4, pH 3.75) from a 250×4.6 mm Partisil 10 SAX column (Whatman). The ^{32}P and ^{3}H content of the fractions containing samples and internal standards were measured by liquid-scintillation counting.

Radiolabelled PtdIns(4,5)P_2 standard was produced by incubation of 5 nmol of PtdIns5*P* (Echelon Research Laboratories, Salt Lake City, UT, U.S.A.) as lipid micelles in 10 mM Tris/HCl, pH 7.4, with excess Type IIα PtdIns*P* kinase (PIPkin) for 2 h at 30 °C, and then extracted as above. PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 and PtdIns3*P* standards were similarly produced by incubation of PtdIns(4,5)P_2, PtdIns4*P* or PtdIns (Echelon Research Laboratories) with a phosphoinositide 3-kinase activity, immunoprecipitated using α-phosphotyrosine antibodies, from lysates of platelet-derived growth factor-stimulated Rat1 cells. Labelled PtdIns3*P* was further phosphorylated to PtdIns(3,5)P_2 by incubation with Type I PIPkin. Labelled glycerophosphatidylinositide standards were prepared from these samples as described above. For HPLC analysis, ^{3}H-labelled Ins(1,3,5)P_3 was prepared by deglyceration of [^{3}H]*Gro*PtdIns(3,5)P_2 (generously given by Dr S. Dove, University of Birmingham), and [^{3}H]Ins(1,4,5)P_3 was obtained from NEN Life Science Products.

Mass determination of nuclear phosphoinositides

PtdIns*P*

The assay method used to determine the mass levels of PtdIns*P* was essentially as described in [19]. Lipids from nuclei (20 μg of total protein for PtdIns4*P* or 200 μg of total protein for PtdIns5*P* mass determination) were extracted as described above. Dried lipids were resuspended in 500 μl of chloroform/methanol/50 mM ammonium formate (5:10:2, by vol.) and transferred to Helmenex II treated glass tubes. A 50 μl volume [50% suspension in chloroform/methanol/425 mM ammonium formate (5:10:2, by vol.)] of neomycin-coated glass beads [22] was added to the nuclear lipid extracts and incubated at room temperature for 20 min. The beads were centrifuged at 5000 ***g*** for 30 s and washed twice with chloroform/methanol/425 mM ammonium formate (5:10:2, by vol.). Phosphoinositides were eluted from the beads by incubation for 1 h at room temperature with 250 μl of 2 M triethylamine, pH 4.5 [19]. Phosphatidylserine was added as a lipid carrier and phosphatidic acid as an enzyme activator in the PtdIns4*P* assay (5 nmol each). Lipid extracts were resuspended in 50 μl of diethyl ether and sonicated with the addition of 50 μl of 10 mM Tris/HCl, pH 7.4. The conversion of PtdIns4*P* or PtdIns5*P* into PtdIns(4,5)P_2 was achieved by incubation of the purified lipids with either Type Iα or Type IIα PIPkin [23]. The assay was carried out in a total of 100 μl of PIPkin buffer (50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 80 mM KCl and 2 mM EGTA), containing 2 μCi of [γ-^{32}P]ATP, 5 μM non-radiolabelled ATP and 1 μl of enzyme, and incubated at 30 °C for 2 h. The reaction was terminated by the addition of 500 μl of cold chloroform/methanol (1:1, v/v). Lipids were extracted as above and dried by vacuum centrifugation. Mass levels of nuclear PtdIns*P*s were determined by comparison with assays performed with lipid standards.

PtdInsP_2

Lipids from nuclei (50 μg of total protein) were extracted and converted into inositol phosphates as above. The mass of the

Ins(1,4,5)P_3 produced was determined by a receptor/ligand-binding assay [24]. Competition for binding to Ins(1,4,5)P_3 receptors was monitored using [γ-^{32}P]ATP-labelled Ins(1,4,5)P_3. The mass of PtdInsP_2 in the experimental samples was directly proportional with the Ins(1,4,5)P_3 displaced in the assay. Bound [γ-^{32}P]ATP-labelled Ins(1,4,5)P_3 was detected by liquid-scintillation counting of Cerenkov radiation.

RESULTS

Lipid labelling during the cell cycle

MEL cells were grown in suspension culture with an average cell doubling time of 11 h. Synchronization of the cell population was achieved using nocodazole, an inhibitor of microtubule formation, which resulted in over 90 % of the cells arresting in G_2/M (Figure 1b and Table 1). Removal of the inhibitor resulted in a continued progression of the cells through the cell cycle as a synchronized population (Figure 1c–e).

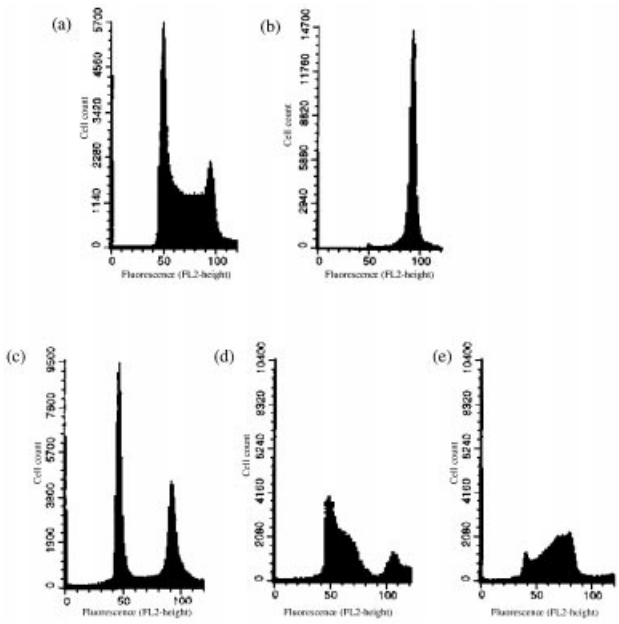

Figure 1 Analysis of the progression through the cell cycle of a population of MEL cells, synchronized by block and release from G_2/M

By comparison to an exponentially growing cell population (**a**), cells treated with nocodazole were blocked at G_2/M (**b**). Samples were taken at 2 h (**c**), 4.5 h (**d**) and 8 h (**e**) after release from the cell cycle block and subjected to propidium iodide DNA staining and FACS analysis. As arbitrary units, cells expressing fluorescence at 50 were in G_1 and at 100 were in G_2/M. For each sample 1×10^5 positive counts were monitored. FL2, fluorescence channel 2.

Table 1 Comparison of MEL cell populations in various stages of the cell cycle at different times after release from cell block

Data represent percentages of the total counted population in each cell cycle phase. The control population (exponentially growing cells) are used for comparison of normal cell phase ratios for this cell type.

Cell sample	G_1	S phase	G_2/M
Control	26	62	13
Blocked	2	7	91
2 h after release	45	7	48
4.5 h after release	46	40	15
8 h after release	10	86	4

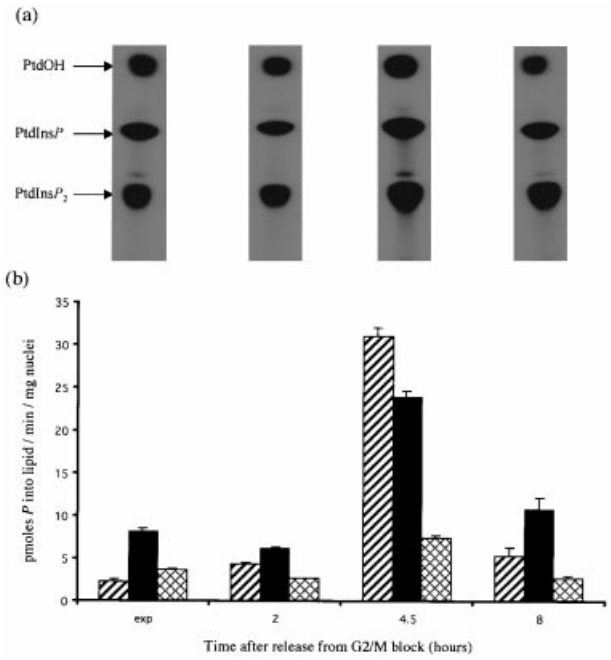

Figure 2 Autoradiograph of a TLC plate of a typical experiment showing the incorporation of radiolabel from [γ-^{32}P]ATP into phosphatidylinositides in synchronized MEL cell nuclei at different points in the cell cycle.

(**a**) Sample spots indicate separation of the inositol lipids as labelled. (**b**) Pooled data of duplicate samples from three biological replicates, as exemplified by (**a**), are quantified ($\pm$S.E.M, $n = 6$); PtdInsP_2 (▨); PtdIns$P$ (■); phosphatidic acid (PtdOH; ⊠). The time points indicated correspond to both (**a**) and (**b**). Control cells (exp) are labelled nuclei from an exponentially growing MEL cell population.

Nuclei isolated from cells at different points of the cell cycle were used for subsequent experiments. Approx. 3 mg of nuclei (by total protein) were prepared from three biological replicates at each of the chosen time points. Nuclei were kept at 4 °C during extraction and used immediately for ^{32}P-incorporation assays. The remaining nuclei were frozen in liquid N_2 and stored at −80 °C for further analysis in order that all mass assays could be carried out on the same batches of nuclei. Control nuclei were prepared from an exponentially growing cell culture, which provided a standard ratio of cells in each cell cycle phase (Figure 1a). Cell number ratios indicated that nuclei at 2 h after release from the cell block were predominantly in G_1, nuclei at 4.5 h were at the transition between G_1 and S-phase, and the majority of nuclei at 8 h were in S-phase (Table 1).

Phosphorylation of nuclear DAG, PtdIns and PtdIns$P$ by isolated nuclei [3,5] in the presence of exogenous [γ-^{32}P]ATP allows the monitoring of these incorporation events. Phosphorylation assays, using nuclei from the experimental time points, indicated differential rates of radiolabel incorporation into PtdIns$P$ and PtdInsP_2, without significant increases in the incorporation rate into phosphatidic acid (Figure 2). Similar assays carried out using preparations of light membranes indicated that this incorporation profile specifically occurred in nuclei, suggesting that the preparations were free from contamination with other cellular membranes (results not shown; compare with [4]). Analysis of cells synchronized with aphidicolin, and labelling assays of nuclei from cells monitored

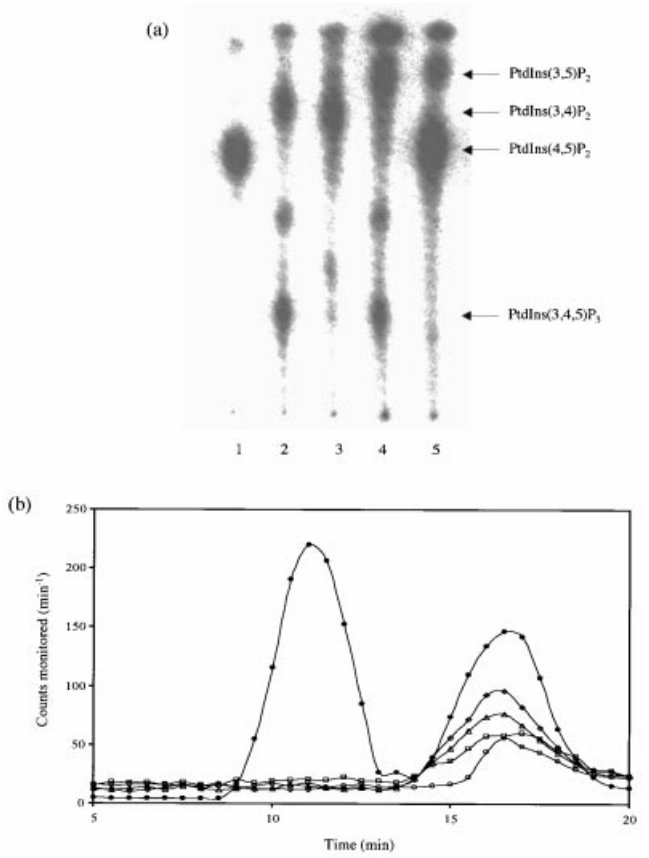

Figure 3 Analysis of PtdInsP_2 species obtained from the labelling of MEL cell nuclear phosphatidylinositides with [γ-^{32}P] from ATP

(**a**) ^{32}P-labelled PtdInsP_2 from the 4.5 h time point was converted into GroPtdInsP_2 and subjected to poly(ethyleneimine)–cellulose chromatography against lipid standards. Lane 1, nuclear PtdInsP_2; lane 2, PtdIns(3,4,5)P_3; lane 3, PtdIns(3,4)P_2; lane 4, PtdIns(3,5)P_2; lane 5, PtdIns(4,5)P_2. The standards have traces of other labelled lipids due to the specificity of the enzymes used in their manufacture. The experimental sample is devoid of these other lipids. (**b**) ^{32}P-labelled PtdInsP_2 samples from each of the experimental time points were also processed (see the Materials and methods section) and analysed by HPLC. Exponentially growing control nuclei (○); 2 h time-point nuclei (◇); 4.5 h time-point nuclei (△); 8 h time-point nuclei (□). ^{3}H-labelled Ins(1,3,4)P_3, Ins(1,3,5)P_3 and Ins(1,4,5)P_3 standards (●) eluted at 10.5, 11 and 16.5 min respectively.

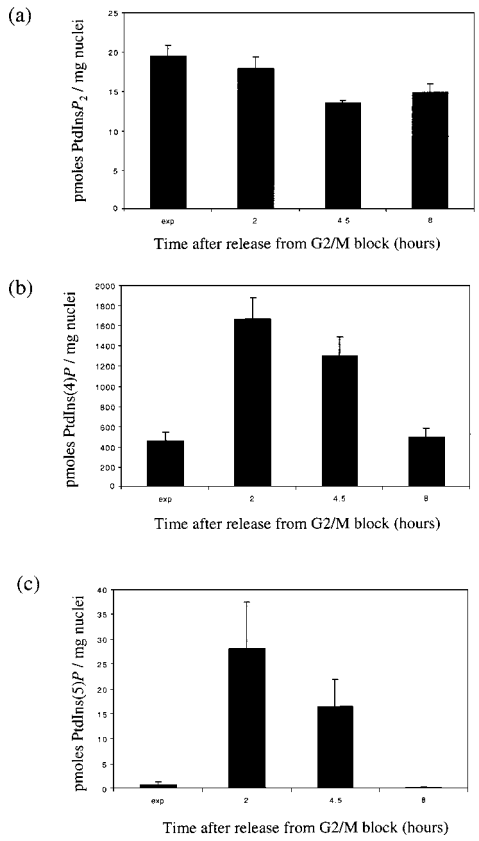

Figure 4 Quantification of mass assay data obtained from incorporation of radiolabel into extracted PtdInsP_2, PtdIns4P and PtdIns5P from MEL cell nuclei at different cell cycle stages

Mass assays were completed as described in the Materials and methods section. Data were calculated as pmol of lipid produced per mg of nuclei (total protein) from phosphoimage analysis. (**a**) PtdInsP_2 mass. Bars represent means of three samples from three biological replicates ($n = 9 \pm$ S.E.M.). (**b**) PtdIns4P mass. Bars represent means of four samples from three biological replicates ($n = 12 \pm$ S.E.M.). (**c**) PtdIns5P mass. Bars represent means of three samples from three biological replicates ($n = 9 \pm$ S.E.M.). Control samples (exp) represent samples extracted from equivalent amounts of nuclei from exponentially growing cell cultures.

through successive cell cycles, indicated that the observed increases were not a direct effect of treatment of cells with nocodazole (results not shown).

Changes in mass levels of PtdIns(4,5)P_2

The previous experiment suggested that levels of PtdInsP_2 in nuclei might be regulated during the cell cycle. The total radiolabelled PtdInsP_2 fraction from exponentially growing MEL cell nuclei subjected to a ^{32}P-incorporation assay was processed (see the Materials and methods section) to allow identification of lipid species using poly(ethyleneimine)–cellulose chromatography. This indicated that the pool was predominantly PtdIns(4,5)P_2 (Figure 3a). Further HPLC analysis of inositol phosphates derived from labelled PtdInsP_2, obtained from nuclei at each of the experimental cell cycle time points, confirmed that this pool did not contain any PtdIns(3,5)P_2 or PtdIns(3,4)P_2, and that ^{32}P counts were only detected at an elution time that coincided with the PtdIns(4,5)P_2 control (Figure 3b). While [^{3}H]Ins(1,3,5)P_3 and [^{3}H]Ins(1,3,4)P_3 controls could be individually separated from [^{3}H]Ins(1,4,5)P_3 (results not shown), the elution peaks of the 3-phosphorylated products were too close to be resolved by this method and ran as a single peak (Figure 3b).

Data from mass assays indicated that the PtdIns(4,5)P_2 mass does not significantly change during the cell cycle (Figure 4a). Thus the specific transient increase in ^{32}P-incorporation as cells enter S-phase (Figure 2) would seem to represent an increase in turnover of PtdIns(4,5)P_2, observed as a percentage increase of radiolabelled molecules in a constant pool of this polyphosphoinositide, rather than an increase in the actual levels of PtdIns(4,5)P_2 within the nucleus.

Changes in mass levels of PtdIns$P$ species

Using the mass assay described above it was possible to quantitatively assay the mass of PtdIns4P in MEL cell nuclei at the different cell cycle stages examined (Figure 4b). A significant peak was observed at the 2 h time point, which had reduced by 4-fold as the cells approached mitosis. A similar assay was carried out using a Type II PIPkin (PtdIns5P 4-kinase) to

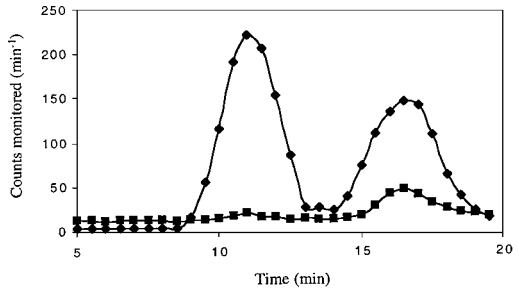

Figure 5 Analysis of PtdInsP_2 species produced from phosphorylation of PtdIns5P extracted from MEL cell nuclei, using HPLC comparison of inositol phosphate head-groups derived from the lipid with Ins(1,3,4)P_3, Ins(1,3,5)P_3 and Ins(1,4,5)P_3 standards

PtdIns5P was extracted from 2 mg (total protein) of nuclei from the 2 h time point (as described in the Materials and methods section) and subjected to processing to phosphorylate the lipid and then derive the inositol phosphate head-group. The sample was analysed by HPLC and the chromatographic separation monitored by reading the ^{32}P-labelled sample (■) against the ^{3}H-labelled standards (◆). Ins(1,3,4)P_3, Ins(1,3,5)P_3 and Ins(1,4,5)P_3 eluted at 10.5 min, 11 min and 16.5 min respectively.

accurately measure the mass of PtdIns5P in cell nuclei during the cell cycle (Figure 4c). Results indicated that although the total mass of this lipid is at least ten times smaller than the PtdIns4P in MEL cell nuclei, a large relative reduction in PtdIns5P was observed as the cells progressed through the cell cycle. HPLC analysis of the product of PtdIns5P phosphorylation was carried out as this enzyme has been reported to utilize PtdIns3P *in vitro* to produce PtdIns(3,4)P_2 [23]. Generation of ^{32}P-labelled PtdInsP_2 from extracted PtdIns5P (2 h sample) was carried out as described above, and the lipid was processed to produce the inositol phosphate head-group (Figure 5). Most of the ^{32}P counts were observed at an elution time that coincided with the ^{3}H-labelled Ins(1,4,5)P_3 standard, and not with the Ins(1,3,4)P_3 or Ins(1,3,5)P_3 standard mix, suggesting that the assay is indeed measuring PtdIns5P (compare with [19]).

DISCUSSION

Previous studies [7,12,13,18,25] have established that the function and compartmentalization of PI cycle components in the nucleus is potentially more complex than originally thought. Although some downstream targets of the second messenger molecules produced from this cycle have been suggested [7], it is still not clear which part or parts of different cellular responses these signalling molecules regulate.

Our data suggest that the metabolism of PtdIns(4,5)P_2 is regulated during the cell cycle, and hence may have a direct effect on the regulation of nuclear processes. The incorporation of radioactive phosphate into this molecule increases dramatically (6-fold) as the cell approaches the transition from G_1 to S-phase. The observation that there is no concomitant increase in mass levels of PtdIns(4,5)P_2 suggests that the turnover of this molecule is rapidly increased at this point of the cell cycle, rather than a direct utilization of the PtdIns(4,5)P_2 as a second messenger.

This interpretation is, however, complicated by the possibility that, as in the cytoplasm, PtdIns(4,5)P_2 may have more than one function, and exist as more than one pool [26]. The behaviour of PtdIns4P also suggests complexity in its metabolism. The mass of PtdIns4P, the major precursor of PtdIns(4,5)P_2 in the nucleus [18], does increase, but even from this limited set of time points it is evident that this mass increase does not coincide temporally with the increase in incorporation of radioactivity into PtdIns(4,5)P_2, implying that there must be a complex relationship between the two phenomena. We have already shown elsewhere that rat liver nuclei probably contain more than one pool of PtdIns4P [18], an idea that is consistent with the different intranuclear localizations of PtdIns kinase and PtdIns4P kinase [5]. If that is so, and given that we cannot analyse these pools separately, we cannot say whether the increase in incorporation of radioactivity into PtdIns(4,5)P_2 is a reflection of an increase in PtdIns4P 5-kinase activity or availability of its substrate, or both.

The increasing complexity of nuclear inositide metabolism is most clearly seen, however, in the behaviour of PtdIns5P. This novel lipid [19,23] is the substrate for the Type II PIPkins, which Rameh et al. [23] have shown are PtdIns5P 4-kinases. The presence of at least one Type II PIPkin, the β isoform, in nuclei [27,28] makes the detection of its principal substrate here not entirely surprising, though it is nevertheless a novel observation, as it adds a new player to the nuclear lipid scene. However, its behaviour during the cell cycle is the most dramatic of all nuclear lipids so far analysed [7]. It is almost absent in proliferating cell nuclei, and it increases more than 20-fold after release from nocodazole block, and by 4.5 h is already decreasing, and almost disappears by 8 h. The simplest interpretation is that there is a transient, but large, increase in PtdIns5P synthesis in G_1. The reason for this is not known, since we do not know presently whether PtdIns5P is made by phosphorylation of PtdIns or 4-dephosphorylation of PtdIns(4,5)P_2. As for the physiological reason for these changes, we do not know whether PtdIns5P serves merely as an alternative precursor to PtdIns(4,5)P_2, or as a messenger in its own right. There is indirect evidence that its synthesis is increased when platelets are activated by thrombin [19], and so the latter possibility is one that merits serious consideration. It is noteworthy that the major precursor of PtdIns(4,5)P_2, PtdIns4P, is present at much higher levels than PtdIns5P (Figure 4). Indeed, the mass levels of PtdIns5P and PtdIns(4,5)P_2 are very similar, though all such direct comparisons are subject to the caveat of the unknown degree to which these lipids are hydrolysed during isolation of the nuclei by the active phosphatases present therein [18]. Nevertheless, within the limits of these constraints we suggest that the remarkable increase in nuclear PtdIns5P around G_1 presents an enticing suggestion that it has a role to play in nuclear function.

In conclusion, our data reveal a complex set of changes that take place in nuclear polyphosphoinositide metabolism through the cell cycle, and we suggest these are reflections of what we increasingly perceive as the multiple roles of inositol lipids and their products in nuclear function.

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REFERENCES

1 Berridge, M. J. and Irvine, R. F. (1989) Inositol phosphates and cell signalling. Nature (London) **341**, 197–205
2 Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science (Washington, D.C.) **258**, 607–614
3 Smith, C. D. and Wells, W. W. (1983) Phosphorylation of rat liver nuclear envelopes. II. Characterization of *in vitro* lipid phosphorylation. J. Biol. Chem. **258**, 9368–9373

4 Cocco, L., Gilmour, R. S., Ognibene, A., Letcher, A. J., Manzoli, F. A. and Irvine, R. F. (1987) Synthesis of polyphosphoinositides in nuclei of Friend cells. Evidence for polyphosphoinositide metabolism inside the nucleus which changes with cell differentiation. Biochem. J. **248**, 765–770
5 Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A. J. and Van Bergen en Henegouwen, P. M. (1992) A differential location of phosphoinositide kinases, diacylglycerol kinase, and phospholipase C in the nuclear matrix. J. Biol. Chem. **267**, 5078–5084
6 Divecha, N., Banfic, H. and Irvine, R. F. (1991) The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-I) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. EMBO J. **10**, 3207–3214
7 D'Santos, C. S., Clarke, J. H. and Divecha, N. (1998) Phospholipid signalling in the nucleus. Een DAG uit het leven van de inositide signalering in de nucleus. Biochim. Biophys. Acta **1436**, 201–232
8 Sorensen, A. M. and Baran, D. T. (1995) 1α,25-Dihydroxyvitamin D3 rapidly alters phospholipid metabolism in the nuclear envelope of osteoblasts. J. Cell. Biochem. **58**, 15–21
9 Hennager, D. J., Welsh, M. J. and DeLisle, S. (1995) Changes in either cytosolic or nucleoplasmic inositol 1,4,5-trisphosphate levels can control nuclear Ca^{2+} concentration. J. Biol. Chem. **270**, 4959–4962
10 Humbert, J. P., Matter, N., Artault, J. C., Koppler, P. and Malviya, A. N. (1996) Inositol 1,4,5-trisphosphate receptor is located to the inner nuclear membrane vindicating regulation of nuclear calcium signaling by inositol 1,4,5-trisphosphate. Discrete distribution of inositol phosphate receptors to inner and outer nuclear membranes. J. Biol. Chem. **271**, 478–485
11 Banfic, H., Zizak, M., Divecha, N. and Irvine, R. F. (1993) Nuclear diacylglycerol is increased during cell proliferation *in vivo*. Biochem. J. **290**, 633–636
12 Topham, M. K., Bunting, M., Zimmerman, G. A., McIntyre, T. M., Blackshear, P. J. and Prescott, S. M. (1998) Protein kinase C regulates the nuclear localization of diacylglycerol kinase-ζ. Nature (London) **394**, 697–700
13 D'Santos, C. S., Clarke, J. H., Irvine, R. F. and Divecha, N. (1999) Nuclei contain two differentially regulated pools of diacylglycerol. Curr. Biol. **9**, 437–440
14 Jarpe, M. B., Leach, K. L. and Raben, D. M. (1994) Alpha-thrombin-induced nuclear sn-1,2-diacylglycerols are derived from phosphatidylcholine hydrolysis in cultured fibroblasts. Biochemistry **33**, 526–534
15 Bahk, Y. Y., Lee, Y. H., Lee, T. G., Seo, J., Ryu, S. H. and Suh, P. G. (1994) Two forms of phospholipase C-β1 generated by alternative splicing. J. Biol. Chem. **269**, 8240–8245
16 Bahk, Y. Y., Song, H., Baek, S. H., Park, B. Y., Kim, H., Ryu, S. H. and Suh, P. G. (1998) Localization of two forms of phospholipase C-β1, a and b, in C6Bu-1 cells. Biochim. Biophys. Acta **1389**, 76–80
17 Faenza, I., Matteucci, A., Manzoli, L., Billi, A. M., Aluigi, M., Peruzzi, D., Vitale, M., Castorina, S., Suh, P. G. and Cocco, L. (2000) A role for nuclear phospholipase cβ1 in cell cycle control. J. Biol. Chem. **275**, 30520–30524
18 Vann, L. R., Wooding, F. B., Irvine, R. F. and Divecha, N. (1997) Metabolism and possible compartmentalization of inositol lipids in isolated rat-liver nuclei. Biochem. J. **327**, 569–576
19 Morris, J. B., Hinchliffe, K. A., Ciruela, A., Letcher, A. J. and Irvine, R. F. (2000) Thrombin stimulation of platelets causes an increase in phosphatidylinositol 5-phosphate revealed by mass assay. FEBS Lett. **475**, 57–60
20 Bligh, E. G. and Dyer, J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917
21 Clarke, N. G. and Dawson, R. M. C. (1981) Alkaline O leads to N-transacylation. A new method for the quantitative deacylation of phospholipids. Biochem. J. **195**, 301–306
22 Schacht, J. (1978) Purification of polyphosphoinositides by chromatography on immobilized neomycin. J. Lipid Res. **19**, 1063–1067
23 Rameh, L. E., Tolias, K. F., Duckworth, B. C. and Cantley, L. C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature (London) **390**, 192–196
24 Cook, S. J., Palmer, S., Plevin, R. and Wakelam, M. J. (1990) Mass measurement of inositol 1,4,5-trisphosphate and sn-1,2-diacylglycerol in bombesin-stimulated Swiss 3T3 mouse fibroblasts. Biochem. J. **265**, 617–620
25 York, J. D. and Majerus, P. W. (1994) Nuclear phosphatidylinositols decrease during S-phase of the cell cycle in HeLa cells. J. Biol. Chem. **269**, 7847–7850
26 Hinchliffe, K. A., Ciruela, A. and Irvine, R. F. (1998) PIPkins, their substrates and their products: new functions for old enzymes. Biochim. Biophys. Acta **1436**, 87–104
27 Ciruela, A., Hinchliffe, K. A., Divecha, N. and Irvine, R. F. (2000) Nuclear targeting of the β isoform of type II phosphatidylinositol phosphate kinase (phosphatidylinositol 5-phosphate 4-kinase) by its α-helix 7. Biochem. J. **346**, 587–591.
28 Boronenkov, I. V., Loijens, J. C., Umeda, M. and Anderson, R. A. (1998) Phosphoinositide signaling pathways in nuclei are associated with nuclear speckles containing pre-mRNA processing factors. Mol. Biol. Cell. **9**, 3547–3560.

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