Nuclei contain two differentially regulated pools of diacylglycerol

Clive S. D'Santos*, Jonathan H. Clarke*, Robin F. Irvine† and Nullin Divecha*

A number of recent studies have highlighted the presence of a nuclear pool of inositol lipids [1,2] that is regulated during progression through the cell cycle [1,3], differentiation [1,2] and after DNA damage [2], suggesting that a number of different regulatory pathways impinge upon this pool of lipids. It has been suggested that the downstream consequence of the activation of one of these nuclear phosphoinositide (PI) regulatory pathways is the generation of nuclear diacylglycerol (DAG) [1,3,4], which is important in the activation of nuclear protein kinase C (PKC) [5–7]. Activation of PKC in turn appears to regulate the progression of cells through G1 and into S phase [4] and through G2 to mitosis [3,8–11]. Although the evidence is enticing, there is as yet no direct demonstration that nuclear PIs can be hydrolysed to generate nuclear DAG. Previous data in murine erythroleukemia (MEL) cells have suggested that nuclear phosphoinositidase Cβ1 (PIC-β1) activity is important in the generation of nuclear DAG. Here, we demonstrate that the molecular species of nuclear DAG bears little resemblance to the PI pool and is unlikely to be generated directly by hydrolysis of these inositol lipids. Further, we show that there are in fact two distinct subnuclear pools of DAG; one that is highly disaturated and mono-unsaturated (representing more than 90% of the total nuclear DAG) and one that is highly polyunsaturated and is likely to be derived from the hydrolysis of PI. Analysis of these pools, either after differentiation or during cell-cycle progression, suggests that the pools are independently regulated, possibly by the regulation of two different nuclear phospholipase Cs (PLCs).

Addresses: *Department of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. †Department of Pharmacology, Cambridge University, Tennis Court Road, Cambridge CB3 1QJ, UK.

Correspondence: Nullin Divecha

Results and discussion

We have identified the molecular species of DAG, PI, phosphatidylcholine (PC) and phosphatidic acid (PA) present in the nuclei of MEL cells. Nuclear PI or PC was hydrolysed with specific phospholipases and the DAG generated was phosphorylated to form PA, using a non-specific DAG kinase, in the presence of ^{32}P-labelled ATP. This PA was then dimethylated and separated using argentation chromatography, which distinguishes different molecular species primarily on the basis of the number of double bonds that they contain. The DAG derived from nuclear PI was predominantly unsaturated, with over 70% of the total species containing two, four and five double bonds, whereas that derived from nuclear PC was predominantly mono-unsaturated and disaturated (Figure 1a). This pattern of saturation acts as a 'fingerprint', indicating from which precursor lipid the nuclear DAG is derived. Subsequent analysis of nuclear DAG showed that over 90% of the total species were disaturated or mono-unsaturated, showing that it cannot be derived solely from the hydrolysis of PI lipids, but is likely to be derived from a balance of *de novo* synthesis, hydrolysis of precursor lipids such as PC or PI, and the removal of DAG. We have also found this pattern of DAG in the nuclei of Swiss 3T3 cells and porcine endothelial cells (data not shown).

As previous data suggested that incubation of intact nuclei at 30°C generates DAG derived from the hydrolysis of PI [3], we analysed DAG generation using argentation chromatography. A nuclear activity was able to generate DAG, but subsequent analysis revealed that this DAG species was highly mono-unsaturated. There was a decrease in the disaturated DAG that might have been due to a DAG lipase activity or to a desaturase activity; a desaturase activity could not, however, account for the increase in the other unsaturated lipids alone (a decrease of 50 with a comparable increase in the total DAGs of 1150 arbitrary values; Figure 1b, D609). This reaction was not inhibited by D609, (an inhibitor of PC-specific PLC [12]), alkyllysophospholipids (ALP), U73122 (a PIC-specific inhibitor [13]) or butanol (Figure 1b). Indeed, in intact cells, butanol treatment did not lead to either the accumulation of nuclear phosphatidylbutanol (PB) or a decrease in nuclear DAG, suggesting that if a phospholipase D (PLD) activity was present, butanol was unable to act as a substitute for water in the transphosphatidylation reaction. No such activity was present in non-nuclear membranes. These data are consistent with the presence of a pool of nuclear DAG that is not derived from PI but may be derived from the hydrolysis of PC.

The above data did not show whether DAG could be derived from PI hydrolysis. Previously, it was demonstrated that PA can be generated in intact isolated nuclei in the presence of ATP, through the phosphorylation of the endogenous DAG by an endogenous nuclear DAG kinase

Figure 1
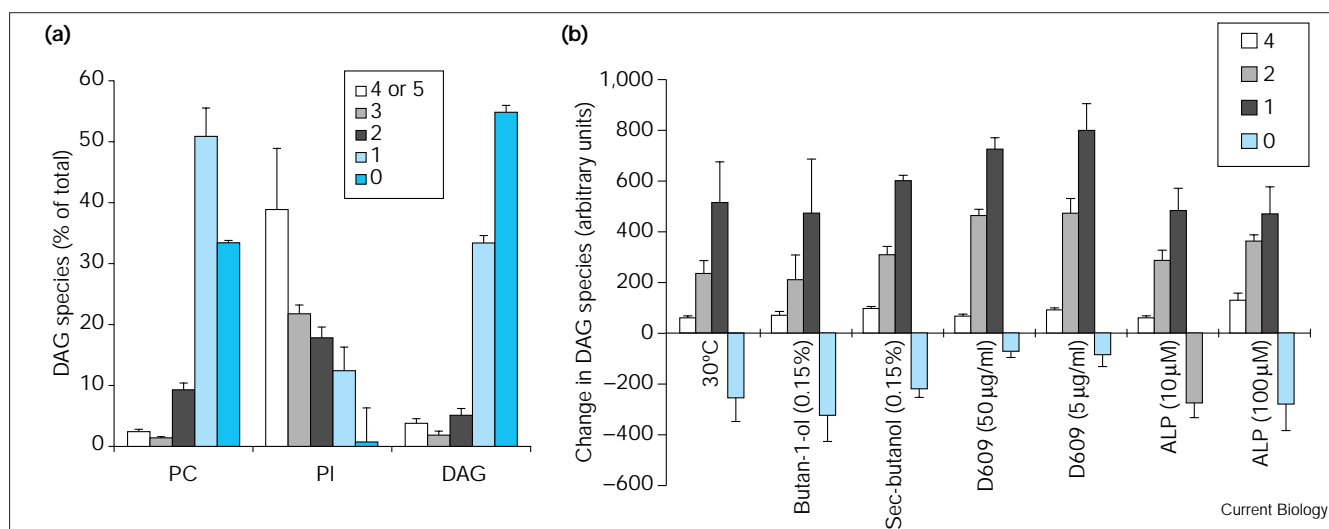
(a) Analysis of nuclear DAG species. Nuclei were purified from MEL cells as described and a total lipid extract was isolated. The DAG was removed by chromatography on a neomycin column and the bound PC and PI were re-extracted from the column. The PC and PI were then hydrolysed using specific phospholipases and the DAG generated was phosphorylated to PA using a non-specific DAG kinase. The PA was analysed using argentation chromatography to reveal the saturation pattern of the DAG species. The data are presented as the percentage of the total DAG ± standard error of the mean (SEM) ($n = 3$). (b) Intact isolated nuclei are able to generate mono-unsaturated DAG. Nuclei were incubated either on ice or at 30ºC in the presence or absence of the various inhibitors. Nuclear DAG was extracted and phosphorylated to PA and analysed by argentation chromatography. The data are presented as the change in DAG species between nuclei that were incubated on ice and at 30°C. Data are shown ± SEM ($n = 3$). In (a,b) the numbers in the inset indicate the number of double bonds.

[1,2]. Argentation analysis of this PA showed that it was highly polyunsaturated (Figure 2a, lane 1). This could occur if the nuclear DAG kinase has a specificity for polyunsaturated DAG or if this specific DAG is co-compartmentalised with the nuclear DAG kinase. Addition of exogenous DAG showed that the enzyme had no specificity even when the various species were added together and the products separated after the reaction (Figure 2a, lane 2). Moreover, disruption of the nuclei using deoxycholate and subsequent labelling of endogenous PA produced a species profile that was similar to the nuclear DAG profile previously described (Figure 2a, lane 3).

Neither inhibitors of PLD nor inhibitors of PC–PLC had any effect on the *in vitro* labeling of PA (Figure 2b). Two structurally different PIC inhibitors, U73122 and ALP, however, completely inhibited the production of nuclear PA (Figure 2b), whereas the inactive analogue, U73343, had no effect (data not shown). These data demonstrate that there are two distinct pools of DAG in the nucleus, one of which is highly mono-unsaturated and disaturated and is not accessible to the nuclear DAG kinase. The second pool is highly polyunsaturated and the data are consistent with this being produced by a nuclear PIC, which, importantly, is able to utilise endogenous nuclear PIs. This second pool of DAG is then utilised directly by a co-compartmentalised DAG kinase to generate PA. These data also demonstrate that the production of labelled polyunsaturated PA in intact nuclei is a direct measure of the nuclear PIC activity using endogenous substrates.

The nuclear DAG levels in MEL cells during progression through the cell cycle or after differentiation were studied to establish whether these pools of DAG are independently regulated. Differentiation of these cells along the erythroid pathway leads to a decrease in the levels of nuclear DAG, suggested to occur through the downregulation of nuclear PIC-β1 activity [1,2]. The data in Figure 3a, however, show that the decrease in nuclear DAG occurs predominantly through a decrease in the disaturated and the mono-unsaturated DAG (as these are the predominant species in control nuclei), showing that an inhibition of nuclear PIC activity cannot be the direct reason for this decrease (Figure 3a). Although we and others [1,2] have shown that nuclear PIC-β1 is downregulated after differentiation, and that when assayed with exogenous substrate the total nuclear PIC activity decreases, we found that, when assayed using intact nuclei and endogenous PI, the nuclear PIC activity remained unchanged (Figure 3b). Analysis of the generation of nuclear DAG in intact nuclei showed that this activity was decreased by 50% after differentiation (Figure 3c).

We then characterised changes in the nuclear DAG as a consequence of progression through the cell cycle. Nuclei were isolated at various time points after release from a

Figure 2

(a) Nuclear PA, generated by labelling of the endogenous DAG by an endogenous DAG kinase, is highly polyunsaturated and likely to be derived from PIC-mediated hydrolysis of PI. Arrows indicate the positions of DAG species with the indicated numbers of double bonds. Lane 1 shows the argentation separation of labelled PA after incubation of intact nuclei with ^{32}P-labelled ATP. Lane 2 shows the argentation separation of labelled PA after incubation of nuclei with exogenously added DAG (an equimolar mixture of all four types). Lane 3 shows the argentation separation of nuclear PA labelled *in vitro* in the presence of deoxycholate (2 mM). (b) Intact nuclei were incubated for 2.5 min with ^{32}P-labelled ATP either on ice in the presence of ALP or at 30°C in the presence or absence of various phospholipase inhibitors. The labelled PA was isolated and separated by argentation chromatography. The numbers in the inset refer to the number of double bonds in the DAG species. The data are the means of duplicates and the range of the values is shown. Each experiment was carried out at least three times.
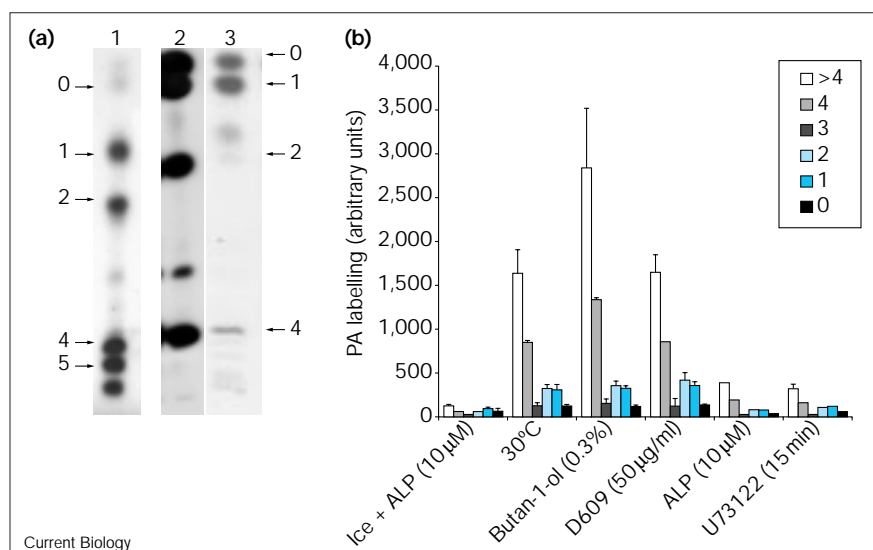

nocodazole block and either the DAG or the PIC activity in intact nuclei was assayed. No changes in either the levels of nuclear DAG or in the nuclear activity that generates mono-unsaturated and disaturated DAG were seen as cells progressed through the cell cycle (data not shown). In contrast to this, there was a threefold increase in the nuclear PA labelling 2 hours after release from the nocodazole block (Figure 4a), which was completely

Figure 3

(a) DAG was extracted from nuclei isolated from either control cells (control) or from DMSO-differentiated cells (DMSO), converted to PA and separated by argentation chromatography. The figure shows a thin-layer chromatography (TLC) plate representative of at least three experiments. To determine the statistical relevance, the decrease in the particular DAG species after differentiation was expressed as a percentage and the SEM for these values is as follows: no double bonds, 44.75 ± 3.4 ($n = 4$); 1 double bond, 53 ± 2.4 ($n = 4$); 2 double bonds, 36.6 ± 13.8 ($n = 3$) and 4 double bonds, 36.6 ± 8.5 ($n = 3$). All of these values are significantly different ($p < 0.01$) from the control value. (b) Nuclei from control cells or from differentiated cells were incubated for 2.5 min with ^{32}P-labelled ATP, after which time the PA was extracted and analysed by argentation chromatography. The data shown are the means of triplicate measurements from one of three independent experiments. (c) Isolated nuclei from either control or differentiated cells were incubated for 15 min either on ice or at 30°C. The DAG was extracted, phosphorylated to form PA and subsequently analysed by argentation chromatography. The data are represented as the difference in nuclear DAG production
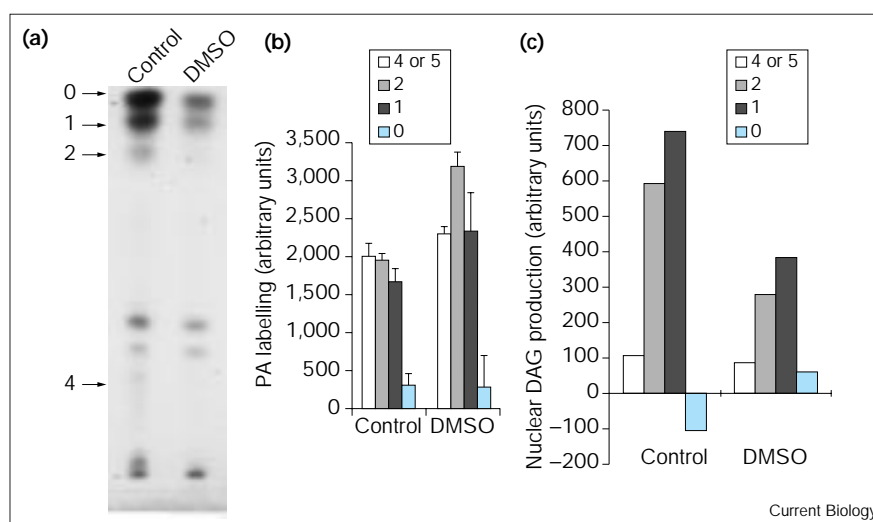
between incubation on ice or at 30°C and represent the PC-specific PLC activity. The data shown are the means of duplicate samples for a single experiment. For a statistical analysis, the percentage decrease in the different DAG species was determined for three separate experiments and expressed as a percentage $\pm$ SEM as follows: 1 double bond, 42 ± 4; 2 double bonds, 45.3 ± 0.71; and 4 double bonds, 56 ± 10. All of these numbers are significantly different ($p < 0.01$) from the control values. In (b,c), the numbers in the inset refer to the number of double bonds in the DAG species.

Figure 4
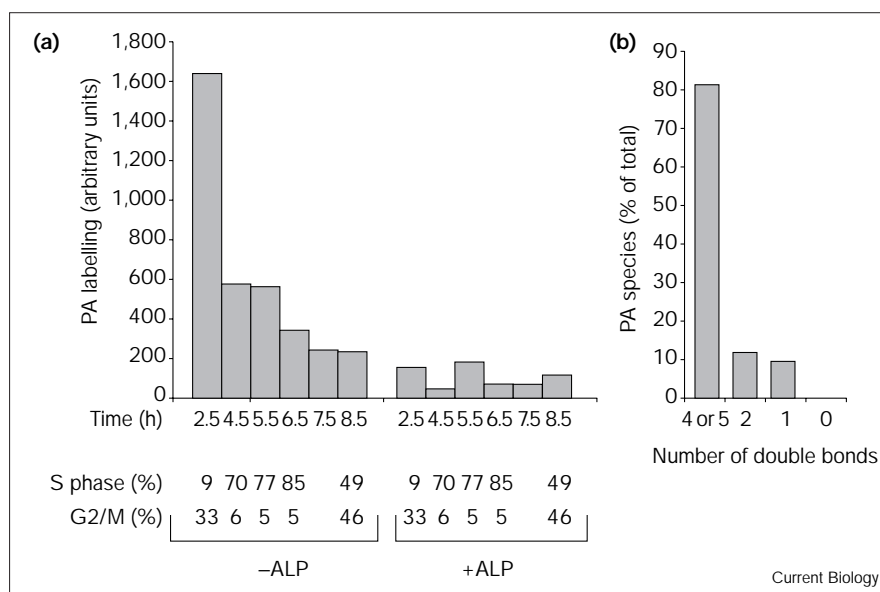
(a) Nuclei were isolated from cells progressing through the cell cycle at the indicated times after release from nocodazole and were incubated with ^{32}P-labelled ATP for 2.5 min in the presence or absence of ALP (10 μM). The PA was extracted and analysed by TLC to determine the total incorporation of ^{32}P. The percentage of cells in the various phases was determined by fluorescence-activated cell sorting (FACS) analysis. (b) The PA extracted in (a) was analysed by argentation chromatography to determine the molecular species and the data shown are the percentage difference in PA species between the 2.5 h and the 4.5 h time point.

inhibited by pre-incubation with ALP (Figure 4a) or with U73122 (data not shown). Analysis of the DNA content showed that the cells were in G1 at this point. Species analysis of the increase in PA over the 4.5 hour time point, showed that more than 70% of the PA was highly polyunsaturated (Figure 4b).

These data are consistent with the idea that nuclei contain two separate pools of DAG that are independently regulated to produce structurally distinct molecular species of DAG. The data strongly suggest that one of these pools is derived from the hydrolysis of PI lipids through the action of a PIC. The other pool of DAG is derived from various pathways, one of which may be through the action of a nuclear PC-specific PLC, as previously suggested [14]. As nuclear DAGs derived from various lipid precursors are different with respect to not only their structure but also their intranuclear localisation, this may represent a mechanism for the distinct spatial and temporal activation of specific isoforms of PKC within the nucleus.

Supplementary material
Additional methodological detail is published with this paper on the internet.

References
1. D'Santos CS, Clarke JH, Divecha N: **Phospholipid signalling in the nucleus: Een DAG uit het leven van de inositide signalering in de nucleus.** *Biochim Biophys Acta* 1998, **1436**:201-232.
2. Cocco L, Capitani S, Maraldi NM, Mazzotti G, Barnabei O, Gilmour RS, Manzoli FA: **Inositol lipid cycle and autonomous nuclear signalling.** *Adv Enzyme Regul* 1996, **36**:101-114.
3. Sun B, Murray NR, Fields AP: **A role for nuclear phosphatidylinositol-specific phospholipase C in the G2/M phase transition.** *J Biol Chem* 1997, **272**:26313-26317.
4. Topham MK, Bunting M, Zimmerman GA, McIntyre TM, Blackshear PJ, Prescott SM: **Protein kinase C regulates the nuclear localisation of diacyglycerol kinase zeta.** *Nature* 1998, **394**:697-700.
5. Leach KL, Ruff VA, Jarpe MB, Adams LD, Fabbro D, Raben DM: **Alpha-thrombin stimulates nuclear diglyceride levels and differential nuclear localization of protein kinase C isozymes in IIC9 cells.** *J Biol Chem* 1992, **267**:21816-21822.
6. Mallia CM, Jeter JR, Jr, Fields AP, Wilson RB, Beckman BS: **Protein kinase C beta from Friend erythroleukemia cells is associated with chromatin and DNA.** *Mol Cell Biochem* 1995, **151**:107-111.
7. Malviya AN, Block C: **Nuclear protein kinase C and signal transduction.** *Receptor* 1993, **3**:257-275.
8. Goss VL, Hocevar BA, Thompson LJ, Stratton CA, Burns DJ, Fields AP: **Identification of nuclear beta II protein kinase C as a mitotic lamin kinase.** *J Biol Chem* 1994, **269**:19074-19080.
9. Thompson LJ, Fields AP: **BetaII protein kinase C is required for the G2/M phase transition of cell cycle.** *J Biol Chem* 1996, **271**:15045-15053.
10. Hocevar BA, Morrow DM, Tykocinski ML, Fields AP: **Protein kinase C isotypes in human erythroleukemia cell proliferation and differentiation.** *J Cell Sci* 1992, **101**:671-679.
11. Fields AP, Tyler G, Kraft AS, May WS: **Role of nuclear protein kinase C in the mitogenic response to platelet-derived growth factor.** *J Cell Sci* 1990, **96**:107-114.
12. Kiss Z, Tomono M: **Compound D609 inhibits phorbol ester-stimulated phospholipase D activity and phospholipase C-mediated phosphatidylethanolamine hydrolysis.** *Biochim Biophys Acta* 1995, **1259**:105-108.
13. Yule DI, Williams JA: **U73122 inhibits Ca^{2+} oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells.** *J Biol Chem* 1992, **267**:13830-13835.
14. Jarpe MB, Leach KL, Raben DM: **Alpha-thrombin-induced nuclear sn-1,2-diacylglycerols are derived from phosphatidylcholine hydrolysis in cultured fibroblasts.** *Biochemistry* 1994, **18**:526-534.