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Synthesis of polyphosphoinositides in nuclei of Friend cells

Evidence for polyphosphoinositide metabolism inside the nucleus which changes with cell differentiation

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Previous work demonstrated the existence of phosphatidylinositol kinase and phosphatidylinositol phosphate kinase in rat liver nuclei, with the suggestion that these activities are in the nuclear membrane [Smith & Wells (1983) J. Biol. Chem. **258**, 9368–9373]. Here we show that highly purified nuclei from Friend cells, washed free of nuclear membrane by Triton, can incorporate radiolabel from [γ-^{32}P]ATP into phosphatidic acid, phosphatidylinositol phosphate and phosphatidylinositol 4,5-bisphosphate. The degree of radiolabelling of phosphatidylinositol bisphosphate is highly dependent on the state of differentiation of the cells, being barely detectable in growing cells and much greater after dimethyl sulphoxide-induced differentiation; this difference is mostly due to different amounts of phosphatidylinositol phosphate in the isolated nuclei. We suggest that polyphosphoinositides are made inside the nucleus and that they have a role in chromatin function; either the phospholipids themselves play a role, or there is a possibility of intranuclear signalling by inositide-derived molecules.

INTRODUCTION

Phospholipids exist as minor components of chromatin in a number of cell types [1–6]. They are more abundant in active than in repressed chromatin and are altered significantly in their qualitative and quantitative composition in cancer cells as compared with normal cells (e.g. [1,6]). The effect of anionic phospholipids such as phosphatidylserine, phosphatidylinositol and cardiolipin on chromatin structure and function has been studied in cell-free systems by exposure of isolated nuclei to phospholipid vesicles. The results show that phospholipids, and in particular phosphatidylserine, can decondense chromatin, affect nucleosome structure and stimulate RNA synthesis, both total and that of specific mRNA species [5]. In addition, phospholipids have been identified as components of the nuclear matrix [2], and it has been suggested that they are involved in the binding of nucleic acids to the fibrils of this subnuclear structure [4]. Finally, Sylvia *et al.* [7,8] have reported stimulatory effects of phosphoinositides on a DNA polymerase.

Taken together, these data suggest the involvement of a lipid component in key aspects of nuclear function. Phosphatidylinositol (PtdIns) kinase and phosphatidylinositol phosphate kinase have been shown to be present in rat liver nuclei, with the suggestion that they are in the nuclear membrane [9]. Smith & Wells [9] speculated on a possible role in the regulation of nuclear membrane function as an adjunct to the importance of PtdInsP_2 as an intermediate in the generation of second-messenger signals from the plasma membrane [10,11]. In view of the possible role of phospholipids in chromatin function, we decided to examine isolated nuclei, completely stripped of the nuclear membrane, for the capacity to phosphorylate inositol lipids.

Here we present evidence that isolated membrane-free nuclei from Friend erythroleukaemia cells are able to produce both PtdIns$P$ and PtdInsP_2, and that the amounts of the former are markedly influenced by the DMSO-induced terminal differentiation of these cells.

MATERIALS AND METHODS

Cell culture

Murine erythroleukaemia cells (Friend cells, clone 707) were cultured as previously described [12], and haemoglobin synthesis was induced by addition of 1.5% (v/v) DMSO to the medium for 4 days. In some experiments 100 nM-PMA was added to the medium for inhibition of DMSO-induced differentiation [13].

Isolation of nuclei

Nuclei were purified in the presence of Triton X-100 essentially as described by Marzluff *et al.* [14]. All the buffers contained 5 mM-$MgCl_2$ instead of Ca^{2+} to prevent phospholipase action, and at the end of the preparation purified nuclei were washed again in the presence of 0.4% Triton X-100.

To verify the purity of nuclear preparations the following procedures were carried out.

(i) Purified nuclear samples were immediately fixed in 2.5% glutaraldehyde/0.1 M-phosphate buffer, pH 7.2, post-fixed in 1% OsO_4, embedded in Araldite, and

Abbreviations used: DMSO, dimethyl sulphoxide; PtdOH, phosphatidic acid; PtdIns, phosphatidylinositol; PtdIns$P$, phosphatidylinositol 4-phosphate; PtdInsP_2, phosphatidylinositol 4,5-phosphate; PMA, phorbol 12-myristate 13-acetate; Gro$P$Ins(P), glycerophosphoinositol and phosphorylated forms; InsP_3, inositol trisphosphate.

sections were stained with uranyl acetate and lead citrate and then observed in a Zeiss E109 electron microscope to check the purity of the preparation and the absence of nuclear membrane.

(ii) A parallel nuclear preparation was carried out in the presence of radiolabelled cytosolic lipids, and the purified nuclei were analysed as described previously [5]. The absence of labelled lipids rules out the possibility that nuclear phospholipids arise as an artefact of the Triton X-100 treatment.

(iii) Nuclear preparations were tested for glucose-6-phosphatase activity as described by Garland and Cori [15]. Routinely this was less than 1% of the activity present in the total cell homogenate.

For some experiments, control rat liver nuclei were prepared with or without outer membranes as described elsewhere [16].

Preparation of total cell homogenate

Cells were homogenized by 20 strokes of a Dounce glass homogenizer in 0.32 M-sucrose/5 mM-$MgCl_2$/1 mM-β-mercaptoethanol/10 mM-Tris/HCl, pH 8.0, at a concentration of 4 mg of protein/ml.

Phosphorylation of nuclear polyphosphoinositides

The standard phosphorylation mixture, in a final volume of 100 μl, contained 15×10^6 nuclei (i.e. about 200 μg of nuclear proteins), or total cell homogenate also containing 200 μg of protein, with sucrose 0.32 M, β-mercaptoethanol 5 mM, $MgCl_2$ 5 mM, Tris/HCl (pH 8.0) 10 mM, ATP 1 mM and 1 μCi of [γ-^{32}P]ATP (5000 Ci/mmol). This mixture was incubated at 30 °C for 2 min. The reaction was terminated by addition of 10% (v/v) trichloroacetic acid (final concn.). To test the possible non-specific effect of DMSO on isolated nuclei, in some samples DMSO (1.5%, w/v) was added to the mixture.

Analysis and identification of ^{32}P-labelled lipids

Portions of lipid extracts of ^{32}P-labelled nuclei (obtained as above) were dried down and deacylated by the method of Clarke & Dawson [17]. The water-soluble glycerophosphate backbones of the lipids were then analysed by high-voltage ionophoresis either in pyridine/acetic acid at pH 3.7 [18] or in sodium oxalate at pH 1.8 [19]. Internal standards were included and, after autoradiography, they were located by spraying for phosphorus [17]. In one experiment the deacylated extract was further degraded by removal of the glycerol moiety (ref. [20], adapted by Irvine *et al.* [21]) and analysed by h.p.l.c. [21] either before or after treatment for 60 min with human red-cell membranes exactly as described by Irvine *et al.* [22].

T.l.c. of lipid extracts

^{32}P-labelled lipids were analysed by t.l.c., usually on 1%-oxalate-sprayed plates developed with chloroform/methanol/water/satd. ammonia (45:35:8:2, by vol.) [23], but in some experiments an alternative system of pre-spraying plates with 1 mM-NaEGTA, pH 5.5, and developing in chloroform/methanol/acetic acid/water (65:50:2:5, by vol.) was used [24], as this separates phosphatidate from other phospholipids [23]. T.l.c. plates were autoradiographed before exposure to iodine, to detect internal lipid standards.

RESULTS

Purity of nuclear preparations

As shown by electron microscopy (Fig. 1), the nuclear preparations used in phosphorylation experiments are highly pure and lack the nuclear envelope, and there is no evidence of extra-nuclear debris.

In addition, as reported in the Materials and methods section, the purification of nuclei in the presence of pre-radiolabelled membrane phospholipids does not give rise to contamination, in agreement with previous data [5]. Glucose-6-phosphatase activity, recognized as a cytoplasmic marker [15], is virtually absent from these preparations, and other criteria of purity are described further, below.

Synthesis of ^{32}P-labelled lipids in nuclei

Incubation of purified nuclei from Friend cells differentiated with DMSO resulted in the appearance of radiolabelled lipids identified as PtdOH, PtdIns*P* and PtdIns(4,5)P_2, plus another unidentified compound (see below). However, in Friend cells growing before DMSO differentiation, formation of PtdInsP_2 was barely detectable even when the same number of nuclei were present, and PtdIns*P* and PtdOH formation was similar to that in differentiated nuclei (Fig. 2*a*). That this difference was not a trivial artefact of DMSO, but was due to some change when the cells cease dividing and differentiate, was demonstrated by preventing the DMSO differentiation with PMA [Fig. 2*a*(c)]. In addition, the addition of DMSO directly to nuclei had no effect on PtdInsP_2 labelling (Fig. 2*b*).

The difference between differentiated and growing cells not only suggests a possible functional role for polyphosphoinositides in some aspect of nuclear function (see below), but also gave us the opportunity to eliminate the possibility that the lipid kinase activities are due to contamination with cytosolic membranes. This is shown in Fig. 2(*c*), where whole-cell homogenates showed no difference in PtdInsP_2 formation caused by differentiation, even though in the isolated nuclei the same pattern as in Figs. 2(*a*) and 2(*b*) was observed (results not shown). We believe this constitutes very strong evidence that lipid phosphorylation is indeed occurring in the nuclei.

Identification of ^{32}P-labelled lipids

The exact coincidence of the principal radioactive lipids with internal PtdOH, PtdIns*P* and PtdInsP_2 markers in the two t.l.c. separation methods made their identification reasonably certain. However, to prove their identity, we deacylated three of these lipid extracts and analysed the glycerophosphate backbones by ionophoresis (see the Materials and methods section). In both ionophoretic systems, the radioactivity ran as three major spots, coinciding exactly with glycerophosphate, Gro*P*Ins*P* and Gro*P*InsP_2 internal standards. This confirms unambiguously the identity of the three major radiolabelled lipids; it should be noted, however, that there is another clear spot on the t.l.c. autoradiograms (above PtdIns*P*). We have not examined this in isolation, but we suspect it is another distinct lipid (rather than a lysophospholipid), as in the deacylated samples there was always at least one (sometimes two) fainter spots migrating slower than glycerophosphate in both ionophoretic systems. The identity of this (these) com-

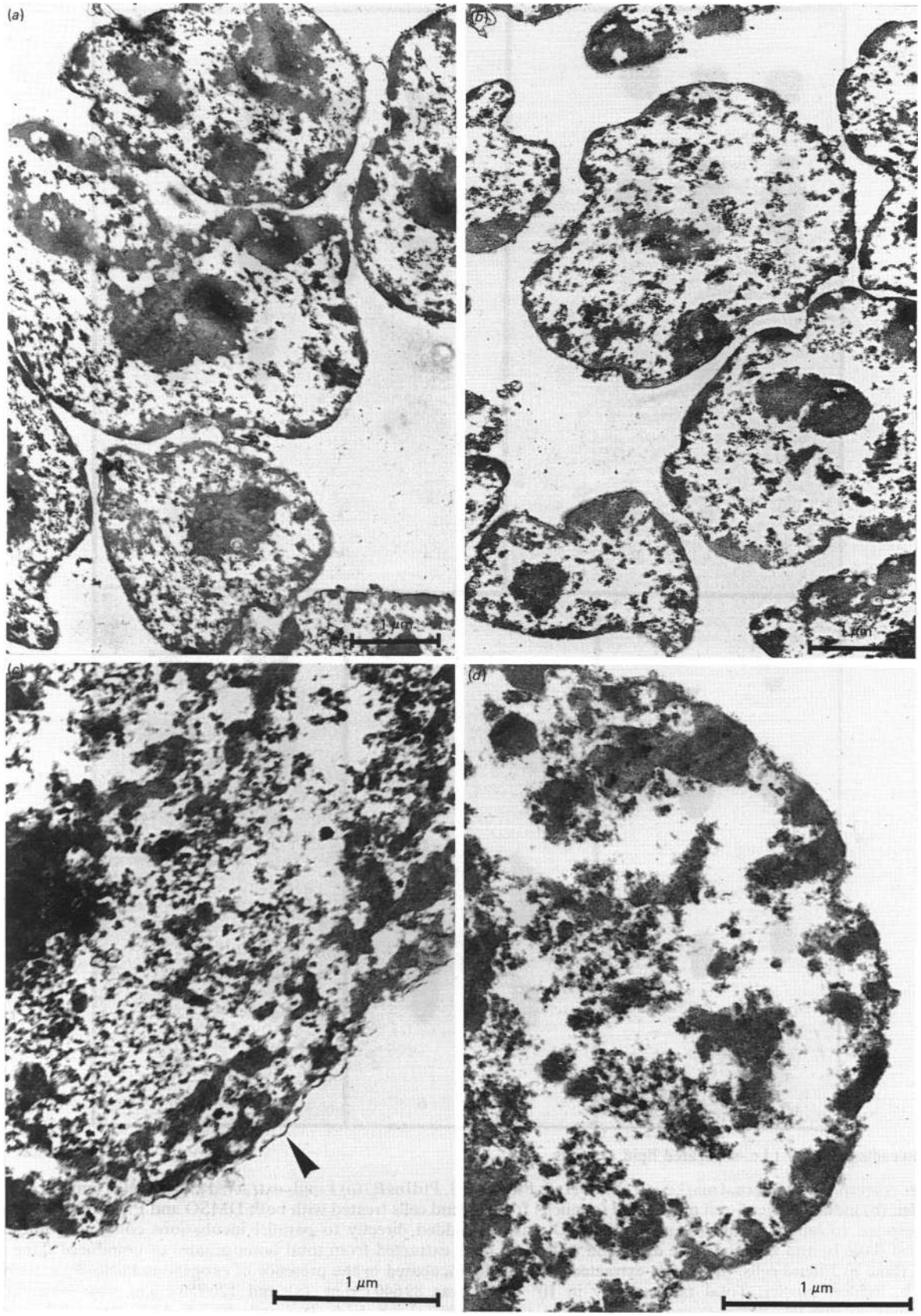

Fig. 1. Electron microscopy of nuclear preparations

(*a*) Isolated nuclei from uninduced Friend cells. Both dispersed and condensed chromatin domains, as well as interchromatin areas, are visible. (*b*) Isolated nuclei from induced Friend cells. Note the presence of condensed chromatin and the loss of interchromatin material. (*c*) Rat liver nuclei isolated without detergent. The arrow indicates the nuclear membrane. (*d*) Rat liver nuclei isolated in the presence of Triton X-100. Note the absence of the nuclear membrane.

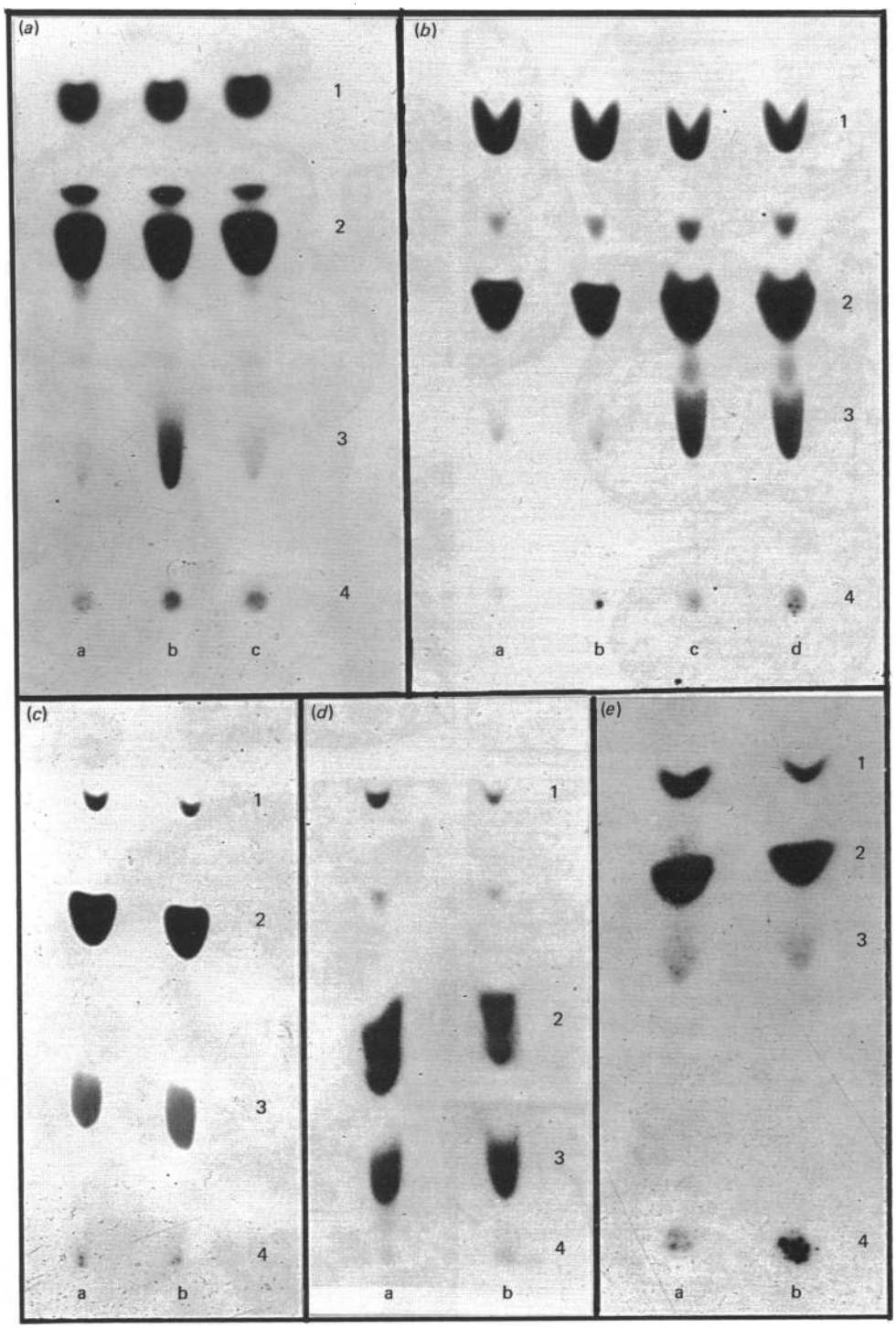

Fig. 2. Autoradiograms of t.l.c.-separated lipid extracts

Numbers correspond to internal markers: 1, PtdOH; 2, PtdIns*P*; 3, PtdInsP_2. (*a*) Lipids extracted [34] from (a) uninduced Friend-cell nuclei, (b) induced Friend-cell nuclei, and (c) nuclei from Friend cells treated with both DMSO and PMA. (*b*) Lanes (a) and (c) correspond to lanes (a) and (b) above. DMSO was also added directly to parallel incubations containing nuclei from uninduced (lane b) and induced (lane d) Friend cells. (*c*) Lipids extracted from total homogenates of uninduced (lane a) and induced (lane b) Friend cells. (*d*) Lipids extracted from nuclei incubated in the presence of exogenous PtdIns*P*: a, uninduced nuclei; b, induced nuclei. Total radioactivity in 10^8 nuclei was 89640 c.p.m. (a) and 126750 c.p.m. (b). Percentages of radioactivity in PtdOH, PtdIns*P* and PtdInsP_2 were respectively (a) 8.8, 62.6, 28.7 and (b) 7.6, 47.3, 45.1. (*e*) Lipids from rat liver nuclei incubated with intact nuclear membrane (lane a) or after removal of membranes with Triton X-100 (lane b). Total radioactivity in 10^8 nuclei was 65040 c.p.m. (a) and 69384 c.p.m. (b). Percentages of radioactivity in PtdOH, PtdIns*P* and PtdInsP_2 were respectively (a) 8.3, 83.0, 8.7 and (b) 6.1, 86.8, 7.1.

pound(s), and therefore of the extra lipid, is not known. However, we can be certain that radiolabelled PtdOH, PtdIns*P* and PtdInsP_2 are formed and are the major radiolabelled lipids.

In one experiment we sought indirect evidence that the PtdInsP_2 is PtdIns(4,5)P_2, something that in these novel observations we cannot take for granted. After deacylation of the lipids we removed the glycerol moiety (see the Materials and methods section), isolated the InsP_3 fraction by ion exchange on Sep-Paks [25], and desalted it by repeated freeze-drying. The Ins[^{32}P]P_3 was then spiked with [^{3}H]Ins(1,4,5)P_3 (Amersham International, Amersham, Bucks., U.K.) and analysed by h.p.l.c. [21], and the two compounds coincided exactly. We also treated the InsP_3 mixture with human red-cell membranes (see the Materials and methods section), whose non-specific phosphatase activity is very low, and is insignificant compared with the specific Ins(1,4,5)P_3 5-phosphatase [22,26]; this was confirmed here by the quantitative conversion of [^{3}H]Ins(1,4,5)P_3 into inositol 1,4-bisphosphate, with negligible formation of inositol monophosphate or inositol (results not shown). The Ins[^{32}P]P_3 formed from the nuclear PtdInsP_2 was entirely hydrolysed under these conditions, and, although we did not have sufficient of this InsP_3 to do semi-kinetic experiments of the sort described previously [22], these data overall lead us to the more or less unequivocal conclusion that the lipid formed in isolated Friend-cell nuclei is PtdIns(4,5)P_2.

Inclusion of PtdIns*P* in assay

The experiment described above, in which we degraded the InsP_3 derived from nuclear PtdInsP_2 with human red-cell membranes, gave us the opportunity to examine the relative distribution of radioactivity between the 4- and 5-phosphate groups of the PtdInsP_2 (see [27]). Although this was a single experiment, the result was very clear in showing that $> 90\%$ of the radioactivity was recovered in P_i rather than inositol bisphosphate, which shows that most of the radioactivity was in the 5-phosphate, and this in turn indicates that most of the PtdIns*P* substrate was not radiolabelled, i.e. it was already there before addition of the [γ-^{32}P]ATP. This in turn suggested the possibility that the difference between differentiated and growing cells (Fig. 2*a*) may be the presence or absence not of PtdIns*P* kinase, but of its substrate. To examine this further, we included in the incubations some exogenous PtdIns*P*.

If exogenous PtdIns*P* is included in the assays, then a considerable amount of the difference seen in Fig. 2(*a*), lanes a and b, between differentiated and undifferentiated cells disappears (Fig. 2*d*). However, it did not entirely eliminate the difference between differentiated and undifferentiated nuclei, and therefore we cannot from these experiments distinguish between whether there is indeed a different amount of PtdIns*P* kinase (in addition to the different amount of its substrate), or whether the exogenous substrate could not reach the PtdIns*P* kinase to replace missing substrate entirely, and consequently the activities of PtdIns*P* kinase are identical. Nevertheless, it is clear that much of the difference between differentiated and undifferentiated nuclei lies in the presence or absence of PtdIns*P*. We cannot tell whether this is so *in vivo*, or whether losses of PtdIns*P* occur during isolation of the nuclei (in undifferentiated cells). But, as there is no difference in PtdIns kinase activity (because radiolabelling of PtdIns*P* *in vitro* is similar; Fig. 2*a*), and only a little difference (see above) in PtdIns*P* kinase activity, between differentiated and undifferentiated nuclei, we can conclude tentatively that the differences observed here (caused by differentiation) are probably due mostly to different amounts of polyphosphoinositide monoesterases or diesterases. Obviously more detailed kinetic studies of the radiolabelling *in vitro* (we have here employed a routine 2 min incorporation) and examination of amounts of hydrolase (e.g. the PtdIns*P* monoesterase described by Smith & Wells [28]) will help to answer these questions. For the present, the principal observation is the clear qualitative difference caused by differentiation, and the probability that it is caused, at least in part, by different amounts of lipid hydrolases.

DISCUSSION

The occurrence of lipids in chromatin has been well documented previously [1–6], and the results here show that PtdIns*P* and PtdInsP_2 can be added to that list of lipids. More importantly, the results in Fig. 2(*a*), showing marked changes in labelling pattern in nuclei (but not whole-cell homogenates) extend the evidence that the presence of these lipids is not due to cytosolic contamination. We cannot absolutely eliminate the possibility that the lipid phosphorylation that we observe here is in the nuclear membrane (cf. [9]), but the removal of this membrane by Triton is apparently complete (ref. [5], and Fig. 1). To emphasize this point further, we carried out some experiments to show that removal of the membranes from rat liver nuclei (Figs. 1*c* and 1*d*) does not significantly alter their inositide phosphorylation (Fig. 2*e*). The pattern of inositide phosphorylation in liver is different from that in Friend cells, but the important point is that removal of the nuclear membrane has no detectable effect on this pattern. We should note also that Smith & Wells [9] found that only nuclear membranes prepared by a gentle heparin method had inositol-lipid kinase activities, whereas the more vigorous method of sonication followed by sucrose-density-gradient separation removed these. Riedel & Fasold [29] have shown that nuclear envelopes isolated by the mild heparin method retain some components of the nuclear interior (see Fig. 2 of ref. [29]). We can therefore suggest that the activities studied by Smith & Wells [9,28] could have resided in the nuclear interior rather than the nuclear membrane, and this is supported by the data in Fig. 2(*e*).

The interpretation of the difference between differentiated and undifferentiated cells (Figs. 2*a* and 2*b*) is complex, as discussed above. But the crucial point is that there clearly is a difference in the amounts of enzymes or lipids (or both), and this suggests to us that the occurrence of polyphosphoinositides in the nucleus is not trivial, and that they play an important role. The unique physicochemical properties of polyphosphoinositides certainly makes attractive the possibility that they have a structural role in chromatin, as has been suggested for other phospholipids [1–6]. Also, the data of Sylvia *et al.* [7,8] show that PtdIns*P* can alter DNA polymerase activity, and our interpretation of our results, that intranuclear PtdIns*P* contents change with different states of cell division or differentiation, is consistent with PtdIns*P* (or InsP_2) serving a regulatory function on DNA syn-

thesis. Alternatively, the occurrence of protein kinase C in nuclei (e.g. [30]) raises the idea that polyphosphoinositides may generate intranuclear signals of a sort parallel to their well-documented signalling role in the cytoplasm [10,11]. In this context, it is relevant to draw attention to the increasing evidence for intranuclear Ca^{2+} homoeostasis being controlled separately from that of the cytoplasm [31], and the possibility that Ca^{2+} could regulate intranuclear processes [32,33].

Certainly our results point to the likelihood that Nature has put these unique lipids to some important use inside the nucleus, to add to their functions outside it.

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REFERENCES

1. Rose, H. G. & Frenster, J. R. (1965) Biochim. Biophys. Acta **106**, 577–591
2. Berezney, R. & Coffey, D. S. (1974) Biochem. Biophys. Res. Commun. **60**, 1410–1417
3. Manzoli, F. A., Maraldi, N. M., Cocco, L., Capitani, S. & Facchini, A. (1977) Cancer Res. **37**, 843–849
4. Cocco, L., Maraldi, N. M., Manzoli, F. A., Gilmour, R. S. & Lang, A. (1980) Biochem. Biophys. Res. Commun. **96**, 890–898
5. Cocco, L., Gilmour, R. S., Maraldi, N. M., Martelli, A. M., Papa, S. & Manzoli, F. A. (1985) Biol. Cell **54**, 49–56
6. Maraldi, N. M., Capitani, S., Caramelli, E., Cocco, L., Barnabei, O. & Manzoli, F. M. (1984) Adv. Enzyme Regul. **22**, 447–464
7. Sylvia, V. L., Joe, C. O., Norman, J. O., Curtin, G. M. & Busbee, D. L. (1986) Biochem. Biophys. Res. Commun. **135**, 880–885
8. Sylvia, V. L., Norman, J. O., Curtin, G. M. & Busbee, D. L. (1986) Biochem. Biophys. Res. Commun. **141**, 60–66
9. Smith, C. D. & Wells, W. W. (1983) J. Biol. Chem. **258**, 9368–9373
10. Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
11. Nishizuka, Y. (1984) Nature (London) **308**, 693–698
12. Gilmour, R. S., Harrison, P. R., Windass, J. D., Affara, N. A. & Paul, J. (1974) Cell Differ. **3**, 9–22
13. Faletto, D., Arrow, A. S. & Macara, I. G. (1985) Cell **43**, 315–325
14. Marzluff, W. F., Murphy, E. C. & Huang, R. C. C. (1973) Biochemistry **12**, 3440–3446
15. Garland, R. C. & Cori, C. F. (1972) Biochemistry **11**, 4712–4718
16. Manzoli, F. A., Capitani, S., Mazzotti, G., Barnabei, D. & Maraldi, N. M. (1982) Adv. Enzyme Regul. **20**, 247–262
17. Clarke, N. & Dawson, R. M. C. (1981) Biochem. J. **195**, 301–306
18. Dawson, R. M. C. & Clarke, N. (1972) Biochem. J. **127**, 113–118
19. Seiffert, U. B. & Agranoff, B. W. (1965) Biochim. Biophys. Acta **98**, 574–581
20. Brown, D. M. & Stewart, J. C. (1966) Biochim. Biophys. Acta **125**, 413–421
21. Irvine, R. F., Änggård, E. E., Letcher, A. J. & Downes, C. P. (1985) Biochem. J. **229**, 505–511
22. Irvine, R. F., Letcher, A. J., Lander, D. J. & Downes, C. P. (1984) Biochem. J. **223**, 237–243
23. Irvine, R. F. (1986) in Phosphoinositides and Receptor Mechanisms (Putney, J. W., Jr., ed.), pp. 89–107, Alan Liss, New York
24. Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) Biochem. J. **235**, 869–877
25. Wreggett, K. A. & Irvine, R. F. (1987) Biochem. J. **245**, 655–660
26. Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) Biochem. J. **203**, 169–177
27. Hawkins, P. T., Michell, R. H. & Kirk, C. J. (1984) Biochem. J. **218**, 785–793
28. Smith, C. D. & Wells, W. W. (1984) Arch. Biochem. Biophys. **235**, 529–537
29. Riedel, N. & Fasold, H. (1987) Biochem. J. **241**, 203–212
30. Capitani, S., Girard, P. R., Mazzei, G. J., Kuo, J. F., Berezney, R. & Manzoli, F. A. (1987) Biochem. Biophys. Res. Commun. **142**, 367–375
31. Williams, D. A., Becker, P. L. & Fay, F. S. (1987) Science **235**, 1644–1648
32. White, B. A. (1985) J. Biol. Chem. **260**, 1213–1217
33. Sahyoun, N., Levine, H., Bronson, D. & Cuatrecasas, P. (1984) J. Biol. Chem. **259**, 9341–9344
34. Dawson, R. M. C. & Eichberg, J. (1965) Biochem. J. **96**, 634–643

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