Changes in the components of a nuclear inositide cycle during differentiation in murine erythroleukaemia cells

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Differentiation of murine erythroleukaemia cells with the chemical agent DMSO leads to a cessation of proliferation and the production of a number of erythrocyte markers such as haemoglobin. We have previously demonstrated that activation of proliferation leads to an increase in the production of nuclear diacylglycerol (DAG). Here we demonstrate that differentiation leads to a decrease in the levels of nuclear DAG and the activity of the nuclear-associated phosphoinositidase C (PIC). The change in activity appears to be due to a decrease in the mass levels of the β1 isoform, as demonstrated by the use of isoform-specific antibodies. Moreover, the changes correlate with the cessation of proliferation and an increase in the number of cells in G_1 phase of the cell cycle, rather than with the number of cells which have differentiated. Indeed, although treatment of the cells with phorbol 12-myristate 13-acetate (PMA) inhibits the differentiation programme as assessed by haemoglobin staining, it does not inhibit the number of cells blocking in G_1 of the cell cycle or the changes in nuclear DAG or PIC activity. The possible involvement of this nuclear inositide cycle during progression through the cell cycle is discussed.

INTRODUCTION

The role of the breakdown of inositides during receptor activation is well documented (see [1]) and it leads to the production of three second messengers, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and diacylglycerol (DAG). This role of inositides has been broadened by the observation that the lipids and the enzymes of this cycle are also present in the nucleus [2–10]. Previous work has demonstrated that, in Swiss 3T3 cells stimulated with insulin-like growth factor 1 (IGF1), there was a rapid and transient decrease in both of the polyphosphoinositides present in the nucleus. This was accompanied by an increase in the levels of DAG associated with this subcellular fraction [7]. In another study we also demonstrated that, during rat liver regeneration after partial (two-thirds) hepatectomy, there was an increase in nuclear DAG after 20 h [9]. The majority of cells present in rat liver are quiescent, and regeneration leads to a synchronous re-entry into the cell cycle. The 20 h time point has previously been shown to correlate with an increase in the number of cells moving into S phase of the cell cycle [11].

All of the above systems suggest that during stimulation of proliferation there is an activation of the nuclear inositide cycle. In contrast with this, a study carried out on murine erythroleukaemia (MEL) cells, which undergo differentiation in response to DMSO, followed by a cessation of proliferation [12], showed that there were changes in the labelling profile of nuclear inositides, consistent with a decrease in the activity of the cycle [2]. Further work carried out by this group also demonstrated that during the differentiation induced by DMSO there was a decrease in the phosphoinositidase C (PIC) β1 associated with the nucleus [13]. However, no measurements of changes in second messengers were carried out. This is particularly important, as in transfection studies it has been demonstrated that the overexpression of PIC isoenzymes does not lead to a change in the basal levels of the second messengers, DAG and Ins(1,4,5)P_3, but was increased during growth-factor stimulation [14].

In this study using MEL cells, we confirm that differentiation leads to a decrease in the amount of PIC β1 associated with the nuclear fraction. Furthermore, we also demonstrate that there is a decrease in the level of nuclear DAG, but without a change in the levels of PtdIns(4,5)P_2. Phorbol 12-myristate 13-acetate (PMA), which has previously been shown to be an inhibitor of differentiation, as determined by the increase in haemoglobin synthesis, did not inhibit the decrease in the nuclear DAG levels, nor did it inhibit the accumulation of cells in G_1 phase of the cell cycle. The data suggest that the change in the nuclear DAGs occurs before the production of the differentiation markers such as haemoglobin, and that the changes may be more related to the cessation of proliferation and block in G_1 of the cell cycle rather than to the differentiation process.

MATERIALS AND METHODS

Horseradish-peroxidase-conjugated antibodies, prestained protein markers, ECL reagents and [γ-^{32}P]ATP were from Amersham International, Amersham, Bucks., U.K. ^{32}P-labelled PtdIns(4,5)P_2 was made by the phosphorylation of PtdIns4P using a purified PtdIns4P 5-kinase. All other reagents were of analytical grade.

Cell culture

Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal-calf serum (FCS) at a density of 0.1×10^6/ml. For differentiation studies cells were set up at a starting density as above, with or without DMSO (1.5%). Differentiation status was studied by staining cells with 3,3-diaminofluorene exactly as described by Kaiho and Mizuno [15].

Abbreviations used: DAG, diacylglycerol; IGF1, insulin-like growth factor 1; MEL cells, murine erythroleukaemia cells; PIC, phosphoinositidase C; PMA, phorbol 12-myristate 13-acetate.

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Cell-cycle analysis

Cells were washed twice with PBS, and 10^6 cells were resuspended in 100 μl of PBS. To this was added 900 μl of ice-cold 70% ethanol. The samples were either stored at 4 °C or analysed by fluorescence-activated cell sorting (FACS). For this, the cells were washed in PBS (3 × 1 ml) and resuspended in PBS (1 ml) containing RNase (1 μg/ml) and propidium iodide (10 μg/ml) and incubated at 37 °C for 30 min. DNA fluorescence analysis was carried out with a FacScan flow-cytometer (Becton Dickinson) with a 488 nm argon laser and detection by either a long-pass 650 nm filter or a 575 nm band-pass filter [15a].

Nuclei isolation

Cells (5×10^7) were spun down (5 min at 500 *g*), washed twice with PBS and resuspended in 8 ml of buffer 1 (5 mM Tris/HCl, pH 7.4, 1.5 mM KCl, 2.5 mM $MgCl_2$), and the cells were allowed to swell on ice for 5 min. The cells were disrupted by addition of 0.3 ml of the above buffer containing 10% Triton X-100 and 33 mM EGTA. PMSF and leupeptin were added, and the cells were syringed through a 22-gauge needle. Sucrose was added to a final concentration of 0.3 M, and $MgCl_2$ (1 M) to give a final concentration of 5 mM. The nuclei were sedimented by centrifugation (5 min at 600 *g*) and resuspended in 1 ml of buffer 2 (10 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 1.5 mM KCl, 1 mM EGTA) containing 0.3 M sucrose and 0.3% Triton. Then 4 ml of buffer 2 containing 0.5 M sucrose was carefully layered underneath, and the nuclei were pelleted by centrifugation as above. The nuclei were then washed in buffer 2 and finally resuspended in 0.5 ml of this buffer. All steps were carried out on ice, and all centrifugation steps were conducted at 4 °C.

The DAG assay was carried out with 250 μg of nuclear proteins or with 10^6 cells as described in Divecha et al. [7], except that in place of the silica acid columns neomycin beads were utilized. All glassware used during the assay was treated with chromic acid, and any plastic was autoclaved.

For measurement of PtdIns(4,5)P_2, the phosphoinositides were extracted by the method of Bligh and Dyer [16] and deacylated and deglycerated by the procedure of Clarke and Dawson [17] exactly as described in Divecha et al. [7]. The mass of Ins(1,4,5)P_3 was measured by the mass assay described by Palmer and Wakelam [18].

PIC assay

A 30 μg sample of nuclear protein was assayed in a final volume of 200 μl of PIC buffer (50 mM Hepes, pH 7.5, 140 mM NaCl, 1 mM EGTA, 0.14% deoxycholate) containing ^{32}P-labelled PtdIns(4,5)P_2 (60000 c.p.m.) together with 3 nmol of unlabelled PtdIns(4,5)P_2 and a free Ca^{2+} concentration of 500 μM. Assays were quenched by addition of 30 μl of 16% trichloroacetic acid and 50 μl of 5% BSA. The reactions were placed on ice for 10 min, and the particulate matter was sedimented by centrifugation (13000 *g* for 5 min). A 450 μl sample of supernatant was removed, and radioactivity was measured by scintillation counting. Assays were carried out in the presence and absence of Ca^{2+} to exclude the possibility of non-specific phosphatase action. Also, the products of the reaction were analysed by HPLC and determined to contain only [^{32}P]Ins(1,4,5)P_3. The assays were carried out for 5 min such that less than 10% of the substrate was utilized.

SDS/PAGE and Western blotting

This was carried out essentially as described by Laemmli [19], and transfer to nitrocellulose was carried out by using 25 mM Tris/190 mM glycine/20% methanol in a wet-blotting apparatus. Transfer was carried out for 2 h at 0.5 A in the cold-room with cooling. The filters were blocked overnight at 4 °C with Tris-buffered saline containing 5% dried milk and 0.05% Tween 20. Primary incubations were carried out with antibodies as detailed in the Figure legend, for 2 h. The blots were washed with the above buffer and incubated with the appropriate second antibody conjugated to horseradish peroxidase. Blots were revealed with the ECL system exactly as described by the manufacturers.

RESULTS

Previous work by us [7] has demonstrated that stimulation of Swiss 3T3 cells with IGF1 leads to an increase in the mass levels of DAG associated with the nucleus. In these cells IGF1 together with other agonists such as bombesin or epidermal growth factor leads to a synergistic activation of proliferation [20, 21]. We were therefore interested to measure levels of these lipids within the nucleus when cells were inhibited from proliferation. Work carried out previously [2] demonstrated that during differentiation there was a change in nuclear inositide metabolism. A direct repeat of this experiment is shown in Table 1. Cells were differentiated for 4 days, and their nuclei were then isolated. The incorporation of ^{32}P into endogenous nuclear lipids from radiolabelled ATP was then measured *in vitro* as described in Cocco et al. [2]. Differentiation led to an increase in the radioactivity incorporated into nuclear PtdIns(4,5)P_2 and into PtdIns4*P*. It should be noted here that the method utilized for the preparation of nuclei yields membrane-depleted nuclei, as assessed by electron microscopy [2]. The problems of the interpretation of this type of data have been discussed previously [7]. The ability to demonstrate changes in the mass levels of both PtdIns(4,5)P_2 and DAG, when no such changes were demonstrated in the *in vitro* labelling assay [7], led us to study the mass levels of the lipids involved in the cycle.

Nuclear DAG levels decrease during differentiation

The mass levels of DAG and PtdIns(4,5)P_2 were measured both in whole cells and in nuclear fractions (Table 2). These data show that after 4 days of treatment with DMSO there was a 57% decrease in the nuclear DAG levels. However, there was no change in mass levels of the PtdIns(4,5)P_2. At the whole-cell level the mass of DAG decreased dramatically, from 43.7 to 15.8 pmol/10^6 cells. These data agree with those of Faletto et al. [22], and suggest that the decrease in whole-cell DAG is important during the differentiation process. Again, at the whole-cell level, the mass of PtdIns(4,5)P_2 did not change significantly, suggesting that the concentration of this lipid is tightly controlled.

Table 1 Changes in nuclear lipid phosphorylation during differentiation with DMSO

Nuclei from either control or DMSO-stimulated cells were incubated with [γ-^{32}P]ATP for 5 min. The phosphoinositides were then extracted and separated by TLC. The radioactive spots corresponding to the above lipids were scraped and quantified by scintillation counting. The data are expressed as c.p.m. incorporated into the lipid/mg of nuclear protein and are from one experiment typical of three.

	Control cells	DMSO-treated cells
Phosphatidate	324	351
PtdIns4*P*	2581	3820
PtdIns(4,5)P_2	667	3681

Table 2 Changes in the mass of PtdIns(4,5)P_2, DAG and PIC activity during differentiation with DMSO

Whole cells or nuclei were isolated before or after treatment with DMSO, and the masses of the lipids defined in the Table were measured by the mass assays as described in the Materials and methods section. Each set of data are from a minimum of $n = 3$ (or as stated in the text) and are defined as means ± S.E.M.: * denotes that the control and the DMSO samples are statistically different at $P < 0.02$, by a standard Student t test.

	Whole cells			Nuclei	
Cells	DAG (pmol/10^6 cells)	PtdIns(4,5)P_2 (pmol/10^6 cells)	PIC (nmol/min per mg)	DAG (pmol/mg)	PtdIns(4,5)P_2 (pmol/mg)
Control	43.7 ± 4.3	100.8 ± 15	0.200	61.1 ± 8.4	58.1 ± 11
DMSO-treated	15.8 ± 5.2	109.8 ± 17 ($n = 8$)	*0.150 ($n = 5$)	26.1 ± 6.1 ($n = 8$)	48.7 ± 7.3

Table 3 Time course of the changes in the nuclear DAG and PIC activities during differentiation with DMSO

Nuclei were isolated either from control cells or from cells treated with DMSO for the number of days shown. The lipids were then either used for the determination of the mass levels of DAG or used to determine the activity of the nuclear PIC. Cell-cycle data were obtained by using propidium iodide staining of the DNA and quantification using a fluorescence-activated cell sorter. The data are from one experiment, but were typical of three, with a variation of less than 10%.

DMSO induction	DAG (% of control)	PIC (% of control)	Cells in G_1 (%)
Day 1	100	138	32
Day 2	65	50	46
Day 3	54	59	68
Day 4	51	57	67

Analysis of the time course showed that the decrease in the nuclear DAG was evident after about 2 days of differentiation (Table 3). It is interesting that the decrease in the DAG does not reflect the number of cells that have differentiated, as assessed by staining for haemoglobin. In fact, at this time point only 5–10% of the cells appear to be haemoglobin-positive (results not shown). Analysis of the cell cycle at the various stages during differentiation, however, demonstrates that by 3 days there is a doubling of the number of cells which become blocked in G_1 (Table 3), suggesting a possible relationship between this parameter and nuclear DAG levels.

Differentiation of MEL cells leads to a decrease in the levels of the β1 PIC isoenzyme

The decrease in the levels of nuclear DAG may be a consequence of a decrease in the levels of the nuclear PIC activity. Measurement of this activity showed that differentiation led to a 50% decrease in the activity of PIC associated with the nucleus (Table 4), in agreement with Martelli et al. [13]. Although measurements of the activity in whole cells were carried out (Table 2), it was difficult to draw conclusions from this. We found that separation of the various isoforms of this activity using heparin–agarose led to the appearance of 5 times more total activity than when measured in the whole cell lysate (results not shown). This suggests that other activities which utilize PtdIns(4,5)P_2 were present and were removed by chromatography, or that specific isoforms of the enzyme are not measured in the whole cell lysate due to the presence of an inhibitor. Analysis of the time course of the changes in the nuclear PIC activity demonstrated a correlation with the changes in the levels of the nuclear DAG (Table 3).

Analysis using isoform-specific antibodies showed that only the β1 isoform and a small amount of γ1 was present (Figure 1). Neither β3 nor δ1 isoform was present. Interestingly, differentiation led to a decrease in the level of the β1 isoform present at the nucleus.

PMA, which does inhibit the differentiation programme as assessed by haemoglobin staining, does not inhibit the decrease in nuclear DAG

PMA has been shown to inhibit the differentiation programme induced by DMSO in MEL cells [22]. Treatment with PMA for

Table 4 Differentiation of MEL cells in the presence of PMA leads to an inhibition of the number of cells synthesizing haemoglobin, but has no effect on the decrease in nuclear DAG, PIC or the number of cells becoming blocked in G_1 of the cell cycle

Cells were grown for 4 days in the absence (CONT) or in the presence of DMSO, PMA or both agents. The DAG, PIC, differentiation and the cell-cycle analysis were all performed on the same batch of cells. The experiments were carried out in biological triplicates, with each assay being analysed in triplicate. The data are presented as means ± S.D. and are typical of three experiments: * denotes that the sample is significantly different ($P < 0.02$) from the control value.

	Whole cells	Nuclear			
	DAG (pmol/10^6 cells)	DAG (pmol/mg)	PIC (pmol/min per mg)	Differentiation (%)	G_1 cells (%)
CONT	31.6 ± 7.9	54.9 ± 12	318 ± 11.6	0	33
DMSO	9.3 ± 2.3*	18.2 ± 5*	154 ± 15.6*	89	66
PMA	13.6 ± 6.3*	78.2 ± 3.8	301 ± 8.5	0	41
DMSO + PMA	8.3 ± 1.32*	18.7 ± 2*	160 ± 11*	25	76

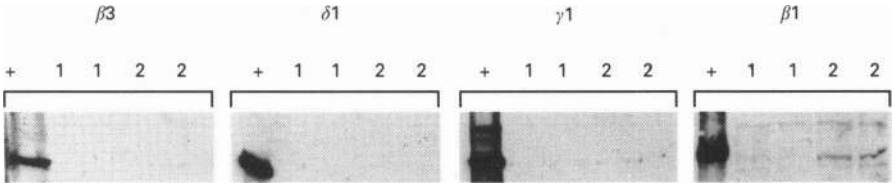

Figure 1 Levels of β1 PIC associated with the nucleus decrease after differentiation

Nuclei were isolated from either control cells (lanes 1) or DMSO-treated cells (lanes 2), and 50 μg samples were subjected to SDS/PAGE, transferred to nitrocellulose and probed with the anti-PIC-isoform-specific antibodies shown above the blots. + designates purified enzyme used as a positive control. Neither the β3 nor the δ1 isoform was found in the nucleus. In contrast, a small amount of γ1 PIC was found: however, no change in levels was detected after differentiation with DMSO. β1 PIC was also found to be present, and during differentiation the levels of this enzyme associated with the nucleus decreased.

4 days inhibited the number of cells which appeared haemoglobin-positive from 89% down to 25% (Table 4). Measurement of the changes in whole-cell DAG demonstrated that treatment with PMA, or PMA + DMSO, or DMSO on its own, led to a decrease in the levels of whole cell DAG. Again, these data are in agreement with those previously obtained by Faletto et al. [22]. Measurement of the nuclear DAG, however, showed that PMA pretreatment was unable to inhibit the decrease in the nuclear DAG levels. Measurement of the nuclear PIC levels in the same samples also showed that these still decreased, even though the differentiation was inhibited. Analysis of the cell cycle showed that, although fewer cells appeared to be haemoglobin-positive after treatment with PMA + DMSO, these cells still committed themselves to the differentiation programme as determined by their entry into a G_1 block (Table 4). As shown in Table 3, differentiation with DMSO leads to a progressive increase in the number of cells that appear in G_1, such that after 3 days approx. 65% of the cells are in this phase of the cell cycle, whereas only 15% are present in S phase. In contrast with this, 55% of the control cells are in S phase, with only 30% in G_1. Treatment of the cells with PMA in either the presence or the absence of DMSO had no affect on the number of cells in the different parts of the cell cycle.

DISCUSSION

Friend erythroleukaemia cells are a good model for the investigation into signals involved in differentiation along the erythroid pathway. Friend cells grow indefinitely in suspension culture, but can be induced to differentiate along the erythroid pathway by DMSO [12], hypoxanthine [23] and a variety of other chemically unrelated agents [24,25]. Indeed, this was the first system in which a change in nuclear inositide metabolism was demonstrated [2]. Further work carried out by the same group has recently demonstrated that there is also a decrease in the levels of PIC β1 associated with the nucleus accompanying differentiation [13]. However, no results were presented on whether this led to any changes associated with this signalling system. In this study we also show that differentiation leads to a decrease in the PIC activity associated with the nucleus, and that indeed this corresponds to a down-regulation of the β1 enzyme. Moreover, measurement of the nuclear DAG levels showed a concomitant decrease. While we were preparing this manuscript, the above-mentioned workers have shown that there are indeed changes in the levels of the nuclear DAG during differentiation [26]. In their measurements they also showed an increase in the levels of the nuclear PtdIns(4,5)P_2. This is in contrast with our data, and may reflect a difference in the cell clone and/or the extent to which the different clones progress through the differentiation programme and/or in the methodology used to detect this lipid. We utilized a mass assay, whereas they radiolabelled the lipids with [^{3}H]inositol. It is difficult, however, to ensure that the nuclear pool of inositides is indeed labelled to equilibrium, and thus any changes seen are related to the mass of the lipids. As the mass of the PtdIns4*P* is changing ([26]; results not shown), if the pool of PtdIns(4,5)P_2 is not labelled to equilibrium then this could lead to changes in the amount of radioactivity incorporated into PtdIns(4,5)P_2, regardless of any changes in the mass of this lipid.

In our cell clone it is particularly interesting that no changes in the nuclear PtdIns(4,5)P_2 levels were detected during the differentiation, despite the measured decrease in PIC activity (at least as assayed *in vitro*) and the decrease in nuclear DAG. Martelli et al. [26] have reported an increase in the levels of PtdIns4*P* during differentiation, which we have also demonstrated (results not shown). This may suggest that *in vivo* the substrate for the nuclear PIC may be PtdIns4*P*; however, there is extensive evidence for homoeostatic mechanisms controlling inositide kinases to maintain PtdIns(4,5)P_2 levels (see [27] for review), and nuclear inositide metabolism may be similar in this respect. The decrease in DAG that is shown raises again the possibility that another lipid (e.g. phosphatidylcholine) could be acting as the precursor to nuclear DAG, and we cannot address this here. Leach et al. [28] have shown just such a PtdCho contribution, but, as we have discussed elsewhere [8,29], those experiments measured DAG in nuclei with their outer nuclear membrane intact, and all the data that we and others have suggest that outer-nuclear-membrane lipid metabolism is probably a different process from what happens inside the nucleus.

The inhibition of the induction of haemoglobin synthesis, but not of the changes in the nuclear DAG, by PMA is interesting for two reasons. Firstly, the samples that are treated with PMA alone show a decrease in the levels of the whole-cell DAG, whereas no such decreases are observed in the nucleus, and this argues against the nuclear DAG that we measure being a contaminant (see also [7] and [9]). Secondly, although treatment with PMA appears to inhibit the differentiation as assessed by haemoglobin staining, the cells are still blocked in G_1 of the cell cycle. Thus it is suggestive that the decrease in the nuclear inositide cycle may be related to the cell cycle phase in which the cells are found, such that in S phase the nuclear cycle is activated, whereas in G_1 the cycle is inactivated. Indeed preliminary results in our laboratory have demonstrated that synchronizing cells in S phase leads to higher DAG levels than those found in an asynchronous population. York and Majerus [30] found changes in nuclear phosphoinositide metabolism during S phase of the cell cycle after synchronization of cells with aphidicolin. A number of proteins which are associated with activation during S phase, such as DNA polymerase and topoisomerase, have been

shown to be substrates *in vitro* for protein kinase C (for a longer discussion of this see [29]). Indeed, the phosphorylation *in vitro* of DNA polymerase α by protein kinase C leads to a 5-fold increase in the activity of this enzyme [31]. Moreover, the same sites that are phosphorylated *in vitro* are also phosphorylated *in vivo* in a cell-cycle-dependent fashion [32]. It has also been suggested that this enzyme is regulated by Ins(1,4)P_2, the product of the phosphodiesteratic cleavage of PtdIns4P [33]. It is possible then that the activation of the nuclear inositide cycle leads to the generation of two signals, DAG and Ins(1,4)P_2, possibly involved in the activation of the same enzyme.

Further work will be required to elucidate the exact role of the nuclear inositide cycle during proliferation or differentiation.

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