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The activation of nuclear phosphoinositide 3-kinase C2β in all-*trans*-retinoic acid-differentiated HL-60 cells

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Abstract The activity of nuclear phosphoinositide 3-kinase C2β (PI3K-C2β) was investigated in HL-60 cells induced to differentiate along granulocytic or monocytic lineages. A significant increase in the activity of immunoprecipitated PI3K-C2β was observed in the nuclei and nuclear envelopes isolated from all-*trans*-retinoic acid (ATRA)-differentiated cells which was inhibited by the presence of PI3K inhibitor LY 294002. High-performance liquid chromatography analysis of inositol lipids showed an increased incorporation of radiolabelled phosphate in both PtdIns(3)P and PtdIns(3,4,5)P_3 with no changes in the levels of PtdIns(4)P, PtdIns(3,4)P_2 and PtdIns(4,5)P_2. Western blot analysis of the PI3K-C2β immunoprecipitates with anti-P-Tyr antibody revealed a significant increase in the level of the immunoreactive band corresponding to PI3K-C2β in the nuclei and nuclear envelopes isolated from ATRA-differentiated cells.

Key words: Phosphoinositide 3-kinase C2β; Nucleus; HL-60 cell; All-*trans*-retinoic acid; Tyrosine phosphorylation

1. Introduction

Recent studies documented the nuclear production of the 3-phosphorylated phosphoinositides by the phosphoinositide 3-kinase (PI3K) in the nuclei of different cell types. PI3Ks form a large family of enzymes that phosphorylate inositol lipids at the D-3 position of the inositol ring and are divided into three classes on the basis of their primary structure, regulation and in vitro lipid substrate specificity [1]. The most studied are class I PI3Ks which produce mostly phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) in vivo. The class IA enzymes consist of a 110 kDa catalytic subunit and a 85 kDa adapter protein which links the enzyme to tyrosine kinases in cell membranes; the class IB enzyme is composed of a p110γ catalytic subunit and a p101 subunit regulated by G-proteins. PI3Ks of class II generate phosphatidylinositol 3-phosphate (PtdIns(3)P) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) and are large (170–210 kDa) proteins which are distinguished by the presence of a PX domain and C2 domains at their C-termini [1–3]. The human class III enzyme is a homolog of yeast Vps34 gene product which produces only PtdIns(3)P and regulates vesicle trafficking [4]. Immunocytochemical and biochemical analysis demonstrated the presence of the p85α regulatory subunit in the nuclei of rat and human cells [5–7], and the growth factor-dependent nuclear translocation of the p110β catalytic subunit in osteoblast-like cells [8]. A study based on the immunolocalization of epitope-tagged p110γ in HepG2 cells reported that PI3Kγ translocates to the nucleus after serum stimulation [9], and the presence of pertussis-toxin sensitive PI3K immunologically related to PI3Kγ was determined in the nuclei of vascular smooth muscle cells [10]. Recent studies demonstrated the activation of phosphoinositide 3-kinase C2β (PI3K-C2β) and an increase in the level of PtdIns(3)P in the membrane-depleted nuclei at the peak of rat liver regeneration after partial hepatectomy [11], while PI3K-C2α was reported to contain a nuclear localization sequence and to be associated with nuclear speckles [12].

Human promyelocytic leukemia HL-60 cells have been extensively studied as an experimental model of the molecular mechanisms of leukemic and myelocytic differentiation. HL-60 cells differentiate into monocyte-like cells following exposure to interferon-γ (IFN-γ), and vitamin D_3. All-*trans*-retinoic acid (ATRA) and dimethyl sulfoxide (DMSO) induce maturation along neutrophilic pathways and phorbol 12-myristate 13-acetate (PMA) causes the cells to differentiate into a macrophage-like phenotype [13]. Although ATRA is generally considered to act through the RAR/RXR nuclear receptors, recent studies demonstrated that the ATRA-induced granulocytic differentiation of the HL-60 cell line is accompanied by a specific nuclear pattern of expression and activity of enzymes related to the phosphoinositide signaling pathway [14,15]. The presence of p85 protein and PI3K activity was found to be tightly bound to the nuclear matrices and to increase during the ATRA-induced granulocytic differentiation [16], and the inhibition of PI3K activity by wortmannin and its expression by an antisense fragment of p85a prevented the differentiation

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Abbreviations: PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdSer, phosphatidylserine; ATRA, all-*trans*-retinoic acid; IFN-γ, interferon-γ; DMSO, dimethyl sulfoxide; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; db cAMP, dibutyryl-adenosine 3′, 5′-cyclic monophosphate; PMA, phorbol 12-myristate 13-acetate; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor

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process [17]. The activation of nuclear PI3K of class IA is not unique to the ATRA-mediated granulocytic differentiation as the activation of nuclear PI3K was demonstrated in vitamin D_3-treated HL-60 cells which were induced to differentiate along the monocytic lineage [18]. The mechanism of the PI3K activation in the nucleus is incompletely understood, but a possible role of Vav adapter was suggested in the activation of a nuclear class IA PI3K as an association of Vav adapter molecule to PI3K was described in the nuclei of ATRA-differentiated HL-60 cells [19,20].

In the present study we investigated further the metabolism of 3-phosphorylated phosphoinositides in the nuclei of HL-60 cells induced to differentiate along granulocytic or monocytic lineages. Our results show that the activity of immunoprecipitated PI3K-C2β and the level of PtdIns(3)P increase in the nuclei and nuclear envelopes of ATRA-differentiated HL-60 cells.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: IFN-γ, PMA, DMSO, EGTA, EDTA, HEPES, Tris, dibutyryl-adenosine 3′, 5′-cyclic monophosphate (db cAMP), leupeptin, phenylmethylsulfonyl fluoride, phosphatidylserine (PtdSer), PtdIns, DNase I, RNase A, Triton X-100, Na^+ deoxycholate, protein A-Sepharose, sodium dodecyl sulfate (SDS), fetal bovine serum (FBS), and aprotinin from Sigma, St. Louis, MO, USA; ATRA, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), LY 294002 from Calbiochem, Nottingham, UK; [γ-^{32}P]ATP, and enhanced chemiluminescence kit from Amersham Pharmacia Biotech, Amersham, UK; anti-P-Tyr antibody from Upstate Biotechnology, Lake Placid, NY, USA; anti-epidermal growth factor receptor (anti-EGFR) and anti-platelet-derived growth factor receptor (anti-PDGFR) antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA, USA; FITC-conjugated monoclonal antibodies (MoAbs) CD11b, murine IgG2a, murine IgG1 were purchased from Becton Dickinson, Immunochemistry Systems, San Jose, CA, USA; MoAb versus CD64, control murine IgG1 and rat $F(ab)_2$-FITC against murine IgG from Serotec, Oxford, UK. All other chemicals were of analytical grade.

2.2. Cell culture, cell differentiation, and flow cytometric analysis

HL-60 cells (ECCACC no. 88112501) were obtained from the European Collection of Animal Cell Cultures, PHLS, Porton, Salisbury, UK. The cells were maintained in exponential growth in RPMI 1640 medium with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 humidified atmosphere at 37°C. For cell differentiation, cells were treated with one of the following agents: 1 μM ATRA (4 days), 500 nM $1,25(OH)_2D_3$ (4 days), 2000 units/ml IFN-γ (5 days), 1.25% DMSO (5 days), 500 μM db cAMP (5 days), or 500 nM PMA (3 days) at a cell density of 0.5×10^6/ml. At the indicated times, cells were sampled and prepared for the flow cytometric analysis. The surface expression of CD11b, CD64/FcγRI or binding of FITC-conjugated mouse IgG2a was determined by flow cytometry as previously described [21]. For each marker, a total of 10 000 events was collected from the gated population and the fluorescence displayed on a single histogram with the cursor of the control samples set to include up to 1.0% fluorescence in the positive region. The results were collected as a percentage of events in the positive region of the histogram.

2.3. Isolation of nuclei, postnuclear membranes and nuclear envelopes and assessment of their purity

At the indicated time points, cells were sampled, washed three times with cold phosphate-buffered saline and resuspended in 50 mM Tris–HCl, pH 7.4, 250 mM sucrose, 5 mM $MgSO_4$ (STM buffer) containing 0.1 mM sodium metavanadate ($NaVO_3$), 20 mM sodium fluoride, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1% (v/v) 2-mercaptoethanol. After 5 min, the cells were lysed with 20 strokes of a power-driven teflon pestle. The lysate was layered over a cushion of 2.1 M sucrose, 50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$ containing inhibitors in the same concentration as the STM buffer. The samples were then spun at $70\,000\times g$ for 60 min in an SW 28.1 rotor. The pellet at the bottom of the cushion was considered to be the nuclear fraction [22]. Preparation of postnuclear membranes was achieved by centrifugation of supernatant, which remained above the cushion after the nuclear fraction had been obtained. The supernatant was diluted in STM buffer to give a final concentration of 162 mM sucrose and spun at $106\,000\times g$ for 90 min at 4°C in a Beckman SW 28.1 rotor. The resultant pellet was considered to contain postnuclear membranes [11].

Nuclear envelopes were isolated from purified nuclei as described previously [22]. Briefly, purified nuclei were resuspended in STM buffer containing 1% (v/v) 2-mercaptoethanol at 1×10^8 nuclei/ml and incubated for 1 h at 4°C with 100 μg/ml DNase I and 100 μg/ml RNase A. After the incubation, the nuclei were sedimented at $800\times g$ for 10 min and resuspended in 50 mM Tris–HCl, pH 7.4, at 5×10^8 nuclei/ml; and four volumes of 50 mM Tris–HCl, pH 7.4, containing 2 M NaCl and 1% (v/v) 2-mercaptoethanol were added dropwise. After a 30 min incubation, nuclear envelopes were recovered by sedimentation at $5000\times g$ for 30 min.

The activities of cytochrome oxidase and glucose-6-phosphatase were determined according to Wakabayashi et al. [23] and Nordlie and Arion [24], respectively, while the activity of 5′-nucleotidase was measured as described by Burnside and Schneider [25].

2.4. Labeling of inositol lipids with [γ-^{32}P]ATP

For the in vitro labeling of inositol lipids with [γ-^{32}P]ATP nuclei or nuclear envelopes (total protein 100 μg) were resuspended in 90 μl of buffer containing 10 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1.5 mM KCl, 1 mM EGTA, and 0.25 M sucrose. The samples were preincubated for 2 min at 30 °C to hydrolyze any remaining endogenous ATP. Then 10 μl of phosphorylation mixture (40 μCi of [γ-^{32}P]ATP, 2 μl of 5 mM non-radiolabeled ATP, made up to 10 μl with the above-mentioned buffer) was added. Incubation was carried out for 5 min at 30 °C and terminated by the addition of 1 ml of chloroform/methanol (1:1). Lipids were extracted and deacylated, and the separation of all the glycerophosphoinositides was achieved using a high-performance liquid chromatography (HPLC) high resolution 5 μM Partisphere SAX column (Whatman) with a discontinuous gradient up to 1 M $(NH_4)_2HPO_4$-H_2PO_4 (pH 3.8) exactly as described in the previous study [11].

2.5. Immunoprecipitation of PI3K-C2β

PI3K-C2β isoform-discriminant polyclonal antisera against the first 331-amino acid portion of PI3K-C2β [2], expressed in *Escherichia coli* as an amino-terminally fused glutathione *S*-transferase protein, were raised in rabbits as described previously [11,26]. These antisera were used for all immunoprecipitations and Western blots directed at PI3K-C2β. Purified nuclei, postnuclear membranes, and nuclear envelopes were resuspended in 0.5 ml of buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100 (w/v), 0.5% Na^+deoxycholate (w/v), 0.1% SDS (w/v), 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin and spun at $100\,000\times g$ for 90 min at 4 °C. PI3K-C2β was immunoprecipitated overnight from 450 μl of supernatants with antibody and protein A-Sepharose. Immunoprecipitates were washed once with the above-mentioned buffer, then three times with 5 mM HEPES/2 mM EDTA (pH 7.5) and then the phosphorylation assay was carried out as described above, except that immunoprecipitates were resuspended in 40 μl of buffer containing 10 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1.5 mM KCl, 1 mM EGTA, and 0.25 M sucrose and made up to 90 μl with lipid vesicles which consisted of 50 mM PtdIns and 100 mM PtdSer formed by sonication. Incubation was terminated, lipids were extracted, deacylated and separation of glycerophosphoinositide was achieved as described above [11].

2.6. Western blot analysis

Proteins for electrophoresis were prepared so that the concentration of each sample was 50 μg/25 μl of sample loading buffer [27], and electrophoresis was carried out using a Bio-Rad Minigel apparatus at an acrylamide concentration of 5% (v/v) or separation of proteins was achieved on gradient gel 3–7% (v/v) when PI3K-C2β immunoprecipitates were analyzed. After electrophoresis, the proteins were transferred to nitrocellulose using a Bio-Rad wet-blotting system. The blot was blocked with a buffer containing 4% (w/v) dried milk,

Table 1
The expression of surface markers and the activity of immunoprecipitated PI3K-C2β in nuclei and postnuclear membranes isolated from HL-60 cells induced to differentiate with ATRA, $1,25(OH)_2D_3$, IFN-γ, PMA, DMSO, and db cAMP

	Surface markers (% positive cells)		PI3K-C2β activity (dpm of PtdIns(3)P/100 μg of protein)	
	CD11b	CD 64	Nuclei	Postnuclear membranes
Control	6.7 ± 3.5	1.4 ± 0.5	1679 ± 78	1758 ± 97
ATRA	58.6 ± 11.6*	n.d.	4128 ± 172*	1802 ± 103
$1,25(OH)_2D_3$	62.3 ± 6.7*	n.d.	1712 ± 105	1659 ± 115
IFN-γ	n.d.	26.9 ± 1.4*	1755 ± 127	1664 ± 181
PMA	60.4 ± 5.2*	n.d.	1662 ± 146	1759 ± 134
DMSO	52.2 ± 12.3*	n.d.	1805 ± 128	1683 ± 142
db cAMP	47.5 ± 8.3*	n.d.	1752 ± 161	1699 ± 155

HL-60 cells were incubated in the presence of one of the following agents: 1 μM ATRA (4 days), 500 nM $1,25(OH)_2D_3$ (4 days), 2000 units/ml IFN-γ (5 days), 500 nM PMA (3 days), 1,25% vol/vol DMSO (5 days), or 500 μM db cAMP (5 days). After the incubation, an aliquot of cells was analyzed by flow cytometry for the expression of surface markers. The rest of the sample was used to isolate nuclei and postnuclear membranes and the immunoprecipitation of PI3K-C2β and the kinase assay using PtdIns as a substrate were performed as described in Section 2. The results are means ± S.E.M. for at least three different experiments, each performed in duplicate. *$P < 0.05$ (Student's *t*-test) with respect to the control. n.d., not determined.

20 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween 20. It was then probed for 2 h with primary antibody (1:1000), washed with a blocking buffer and incubated with the secondary antibody conjugated to horseradish peroxidase. When PI3K-C2β immunoprecipitates were analyzed nitrocellulose was first probed with anti-PI3K-C2β antibody, then stripped off antibodies using buffer containing 62.5 mM Tris (pH 6.8), 2% SDS (w/v) and 100 μM 2-mercaptoethanol for 30 min at 50 °C and reprobed with anti-P-Tyr antibody. Visualization was carried out using the ECL kit.

2.7. Statistical analysis

Results are shown as means ± S.E.M. For statistical analyses, Student's *t*-test for unpaired samples at a level of significance of 0.05 was used.

3. Results

3.1. Assessment of purity of nuclei and postnuclear membranes

The purity of nuclei and postnuclear membranes was assessed by measurement of activity of marker enzymes for microsomes (glucose-6-phosphatase), plasma membrane (5′-nucleotidase) and mitochondria (cytochrome oxidase). In isolated nuclei no specific activity of 5′-nucleotidase and cytochrome oxidase could be measured, while the specific activity of glucose-6-phosphatase dropped from 0.324 ± 0.041 (μmol P_i/mg protein per h) which was measured in original homogenate to 0.035 ± 0.007 (μmol P_i/mg protein per h) in isolated nuclei, showing only residual contamination of nuclei with microsomal marker enzyme similar to the level observed in our previous studies [11,28], when liver nuclei were purified even in the presence of strong detergents, suggesting that microsomal marker enzymes are present in the nuclear membrane. On the other hand, when the specific activity of the above enzymes was measured in postnuclear membranes and compared with specific activity in original homogenate the enrichment factors were 2.69 for glucose-6-phosphatase, 2.17 for 5′-nucleotidase and 2.49 for cytochrome oxidase, showing that postnuclear membranes as well as nuclei were purified to a satisfactory degree and could be used for further biochemical studies.

3.2. The activity of PI3K-C2β increases in nuclei and nuclear envelopes of ATRA-differentiated HL-60 cells

To allow differentiation along different pathways, HL-60 cells were incubated in the presence of one of the following agents: 1 μM ATRA (4 days), 500 nM $1,25(OH)_2D_3$ (4 days), 2000 units/ml IFN-γ (5 days), 1,25% vol/vol DMSO (5 days), 500 μM db cAMP (5 days), or 500 nM PMA (3 days). After the incubation, an aliqout of cells was analyzed by flow cytometry to confirm the expression of surface markers, and the rest of the sample was used to isolate nuclei and assayed for the activity of immunoprecipitated PI3K-C2β. As shown in Table 1, the activity of immunoprecipitated PI3K-C2β in nuclei isolated from ATRA-differentiated HL-60 cells was significantly increased in comparison to the activity of the enzyme in nuclei isolated from cells treated with vehicle alone. No increase in the level of immunoprecipitated PI3K-C2β activity was observed in postnuclear membranes isolated from ATRA-treated HL-60 cells. Although the presence of DMSO, db cAMP, $1,25(OH)_2D_3$, IFN-γ, or PMA induced the expression of surface markers corresponding to differentiation, none of the agents tested induced any changes in the level of PI3K-C2β activity in nuclei or postnuclear membranes (Table 1). Highly purified nuclear envelopes were prepared

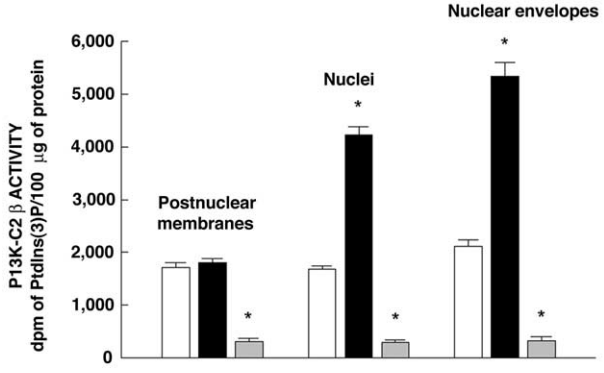

Fig. 1. Activity of immunoprecipitated PI3K-C2β in postnuclear membranes, nuclei and nuclear envelopes of either the control or ATRA-treated HL-60 cells. Postnuclear membranes, nuclei and nuclear envelopes were isolated from HL-60 cells treated for 96 h with either 1 μM ATRA (black bars), or vehicle alone (open bars). The immunoprecipitation of PI3K-C2β and the kinase assay using PtdIns as a substrate were performed as described in Section 2. The effect of the presence of LY 294002 (100 μM) during the phosphorylation assay on the postnuclear membranes, nuclei and nuclear envelopes isolated from ATRA-treated cells is shown (gray bars). Results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's *t*-test) with respect to the control.

Table 2
Effect of LY 294002 on immunoprecipitable PI3K-C2β activity in the nuclear envelopes isolated from either control or ATRA-treated HL-60 cells

Concentration of LY 294002 (μM)	Control	ATRA
0	2109 ± 145	5337 ± 272
1	2004 ± 132	5128 ± 225
5	1796 ± 121	3722 ± 207*
10	1002 ± 88*	2522 ± 181*
50	593 ± 71*	1722 ± 103*
100	150 ± 37*	322 ± 72*

Nuclear envelopes were isolated from HL-60 cells treated for 96 h with either 1 μM ATRA or vehicle alone, and the immunoprecipitation of PI3K-C2β and kinase assay was carried out using PtdIns as substrates as described in Section 2. The results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's t-test) with respect to the controls.

from ATRA-treated cells by sequential treatment of nuclei with nucleases using the procedure which was previously shown to remove nucleic acid, histones and some non-histone proteins [22]. As shown in Fig. 1, a significant increase in the level of immunoprecipitated PI3K-C2β activity was observed in both nuclei and nuclear envelopes isolated from ATRA-differentiated HL-60 cells and when the phosphorylation assay was performed in the presence of PI-3 kinase inhibitor LY 294002 (100 μM) the activity of immunoprecipitated PI3K-C2β was almost completely inhibited. More detailed analysis of the sensitivity of the immunoprecipitable lipid kinase activity in the nuclear envelopes isolated from either control or ATRA-treated HL-60 cells to the LY 294002 (Table 2) revealed that IC_{50} in both control and ATRA-treated cells is similar (9.6 μM versus 9.3 μM) and is comparable with that which was observed when inhibitor was tested on purified recombinant PI3K-C2β activity [3].

3.3. *Phosphorylation of phosphoinositides in the nuclei and nuclear envelopes isolated from ATRA-differentiated HL-60 cells*

Nuclei and nuclear envelopes were isolated from either control or HL-60 cells treated with ATRA for 96 h and labeled with [γ-^{32}P]ATP for 5 min. As shown in Fig. 2, HPLC analysis of deacylated phospholipids showed no difference in the level of incorporation of ^{32}P into either phosphatidylinositol 4-phosphate (PtdIns(4)P) or phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) in ATRA-differentiated cells in comparison to the control. No incorporation of ^{32}P into PtdIns(3,4)P_2 was detected. However, a significant increase was observed in the incorporation of radioactivity in both PtdIns(3)P and PtdIns(3,4,5)P_3 in the nuclei and nuclear envelopes of ATRA-differentiated cells in comparison to the cells treated with vehicle alone. Furthermore, this increased incorporation was completely inhibited by the presence of 50 μM LY 294002. The increase in the production of PtdIns(3,4,5)P_3 is in agreement with previous studies, showing the activation of class I PI3K in nuclei of ATRA-differentiated cells [17]. However, the observed increase in radiolabeling of PtdIns(3)P in the nuclei and nuclear envelopes of ATRA-differentiated cells is unlikely to be due to the activation of class I PI3K or subsequent dephosphorylation of PtdIns(3,4,5)P_3 as no incorporation of ^{32}P into PtdIns(3,4)P_2 could be detected. To further distinguish between the activation of class I and class II enzymes, the phosphorylation assay was carried out in the presence of Ca^{2+} instead of Mg^{2+}, which would improve the specificity of the assays towards that of the class II PI3K enzymes as described by Arcaro et al. [3]. As shown in Table 3, no difference in the incorporation rate of ^{32}P into PtdIns(3)P could be observed, while the incorporation rate of ^{32}P into PtdIns(3,4,5)P_3 was reduced to about 20% of the level observed when phosphorylation assay was carried out in the presence of Mg^{2+}, further ruling out a possible involvement of class I PI3K in the increase of nuclear PtdIns(3)P.

Since it is known that PI3K-C2β is able to phosphorylate PtdIns, but not PtdIns(4)P in the presence of Ca^{2+}, while some phosphorylation of PtdIns(4)P could be observed in the presence of Mg^{2+} [3,29], in vitro substrate specificity for immunoprecipitable PI3K-C2β was tested using Mg^{2+} for phosphate transfer. As shown in Table 4, the basal level of PtdIns phosphorylation is about four times higher than the phosphorylation of PtdIns(4)P and this proportion increases to about six times in the nuclei and nuclear envelopes harvested after stimulation of HL-60 cells with ATRA, showing that there is a strong preference for PtdIns over PtdIns(4)P as a substrate in both basal and stimulated condition, which could be completely inhibited by 100 μM LY 294002 as has been demonstrated for purified recombinant enzyme [3]. Furthermore, no phosphorylation of PtdIns(4,5)P_2 could be observed, showing that we were able to immunoprecipitate only class II PI3K, since the enzyme activity in immunoprecipitates failed to phosphorylate this substrate which should get phosphorylated if any class I PI3K co-immunoprecipitated with class II PI3K. Moreover, as the PI3K activity in immunoprecipitates was unable to phosphorylate PtdIns(4,5)P_2 but could phosphorylate PtdIns(4)P into PtdIns(3,4)P_2, the possible role

Table 3
Incorporation of ^{32}P into PtdIns(3)P and PtdIns(3,4,5)P_3 in the nuclei and nuclear envelopes isolated from either the control or ATRA-treated HL-60 cells

	Incorporation of ^{32}P into PtdIns(3)P (dpm/100 μg of protein)		Incorporation of ^{32}P into PtdIns(3,4,5)P_3 (dpm/100 μg of protein)	
	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
Control nuclei	311 ± 56	298 ± 61	476 ± 78	86 ± 18
ATRA nuclei	1078 ± 105*	1111 ± 102*	1292 ± 105*	196 ± 37*
Control nuclear envelopes	372 ± 69	356 ± 70	455 ± 55*	91 ± 16
ATRA nuclear envelopes	1242 ± 172*	1311 ± 128*	1463 ± 147*	227 ± 29*

Nuclei and nuclear envelopes were isolated from HL-60 cells treated for 96 h with either 1 μM ATRA or vehicle alone. Lipids in the nuclei and nuclear envelopes were radiolabeled using [γ-^{32}P]ATP, and the separation of glycerophosphoinositides was achieved as described in Section 2. When phosphorylation assay was carried out in the presence of Ca^{2+}, 5 mM $CaCl_2$ was added to the buffer instead of 5 mM $MgCl_2$ and 1 mM EGTA was replaced by 1 mM EDTA. The results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's t-test) with respect to the control.

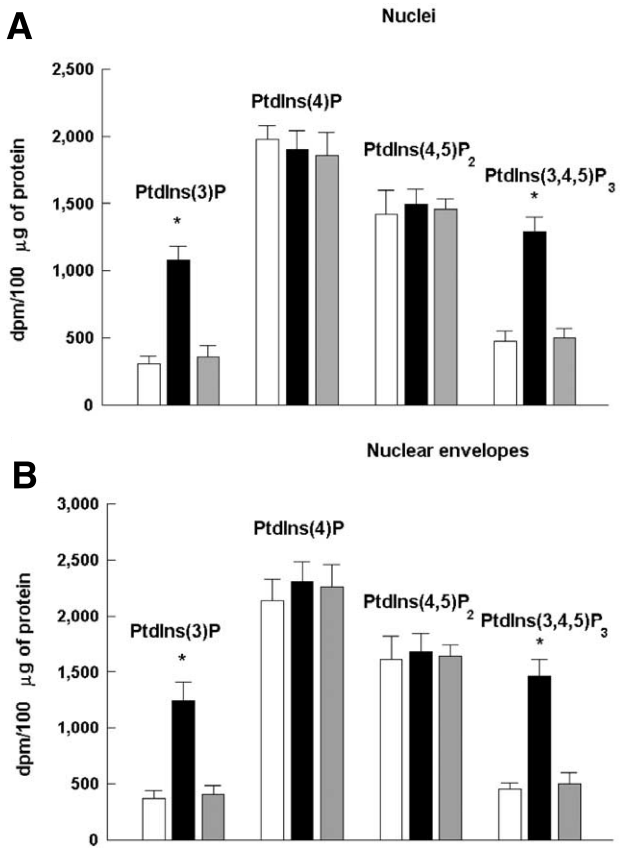

Fig. 2. Incorporation of ^{32}P into PtdIns(3)P, PtdIns(4)P, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 in the nuclei (A) and nuclear envelopes (B) isolated from either the control or ATRA-treated HL-60 cells. Nuclei (A) and nuclear envelopes (B) were isolated from HL-60 cells treated for 96 h with either 1 μM ATRA (black bars), or vehicle alone (open bars). Lipids in the nuclei and nuclear envelopes were radiolabeled using [γ-^{32}P]ATP, and the separation of glycerophosphoinositides was achieved as described in Section 2. The effect of the presence of LY 294002 (50 μM) during the phosphorylation assay on the nuclei and nuclear envelopes isolated from ATRA-treated cells is shown (gray bars). The results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's t-test) with respect to the control.

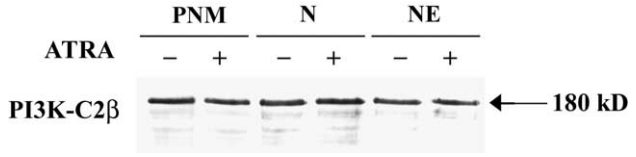

Fig. 3. Western blot analysis of PI3K-C2β in subcellular fractions of either the control or ATRA-treated HL-60 cells. Postnuclear membranes (PNM), nuclei (N) and nuclear envelopes (NE) were isolated from either the control (−) or ATRA-treated (+) HL-60 cells. Protein (50 μg) was subjected to SDS–PAGE, transferred to nitrocellulose, and probed with anti-PI3K-C2β antibody. The position of the molecular mass marker for α_2-macroglobulin (180 kDa) is indicated on the right by the arrow.

of class III activity could be ruled out. Knowing that in the nuclei the concentration of PtdIns is about 15 times higher than PtdIns(4)P [28,30], and that in in vitro conditions with equimolar concentration of substrates, PtdIns(4)P could be phosphorylated only in the presence of Mg^{2+} with substantially less efficiency than PtdIns [3,29], it seems obvious that in vivo PI3K-C2β phosphorylates PtdIns to produce PtdIns(3)P.

3.4. *Tyrosine phosphorylation of PI3K-C2β isolated from nuclei and nuclear envelopes of ATRA-differentiated HL-60 cells*

In postnuclear membranes, nuclei and nuclear envelopes isolated from both control and ATRA-treated cells, Western blot analysis revealed the presence of a PI3K-C2β immunoreactive band of 180 kDa (Fig. 3). No changes in the amount of PI3K-C2β protein were detected in either nuclei or nuclear envelopes isolated from ATRA-treated cells in comparison to the control. In the next experiment (Fig. 4), PI3K-C2β was first immunoprecipitated from postnuclear membranes, nuclei and nuclear envelopes of the control and ATRA-treated cells using anti-PI3K-C2β antibody, then subjected to gradient gel SDS–PAGE and probed first with anti-PI3K-C2β antibody (panel A), which revealed two bands of slightly different molecular weights, showing that gel-shift of immunoreactive band towards higher molecular weight has occurred in nuclei and nuclear envelopes of ATRA-treated cells. Nitrocellulose membrane was then stripped off anti-PI3K-C2β antibody and probed with anti-P-Tyr antibody, which revealed a single immunoreactive band (panel B), which corresponds to a higher molecular weight band found in panel A, with significant increase in the level of immunoreactive band found in the nuclei and nuclear envelopes isolated from ATRA-differentiated cells, suggesting that in the nuclei and nuclear envelopes isolated from ATRA-differentiated cells PI3K-C2β is subjected to tyrosine phosphorylation which causes gel-shift of the enzyme. Moreover, since it is known that upon cell stimulation PI3K-C2β binds to tyrosine kinase receptors such as EGFR and PDGFR [29], in a separate experiment PI3K-C2β was first immunoprecipitated using anti-

Table 4
Substrate specificity of immunoprecipitable PI3K-C2β activity in the nuclei and nuclear envelopes isolated from either control or ATRA-treated HL-60 cells and the effect of LY 294002 (100 μM) during phosphorylation assay on the nuclei and nuclear envelopes isolated from ATRA-treated cells

Treatment	PtdIns(3)P (dpm/100 μg of protein)		PtdIns(3,4)P_2 (dpm/100 μg of protein)		PtdIns(3,4,5)P_3 (dpm/100 μg of protein)	
	Nuclei	Nuclear envelopes	Nuclei	Nuclear envelopes	Nuclei	Nuclear envelopes
None	1678 ± 64	2109 ± 133	388 ± 51	433 ± 33	n.d.	n.d.
ATRA	4222 ± 155*	5337 ± 272*	715 ± 77*	822 ± 59*	n.d.	n.d.
ATRA+LY	301 ± 54*	322 ± 72*	n.d.	n.d.	n.d.	n.d.

Nuclei and nuclear envelopes were isolated from HL-60 cells treated for 96 h with either 1 μM ATRA, or vehicle alone, and the immunoprecipitation of PI3K-C2β and kinase assays were carried out using PtdIns, PtdIns(4)P or PtdIns(4,5)P_2 as substrates as described in Section 2. The results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's t-test) with respect to the controls. n.d., not detected.

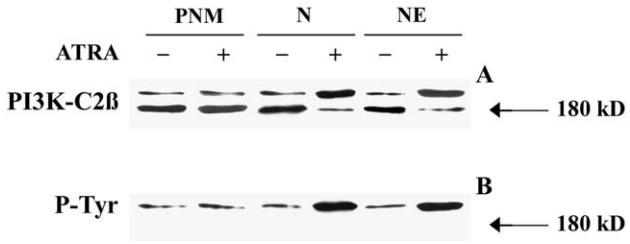

Fig. 4. Western blot analysis of PI3K-C2β immunoprecipitates from postnuclear membranes, nuclei and nuclear envelopes of either the control or ATRA-treated HL-60 cells. Postnuclear membranes (PNM), nuclei (N) and nuclear envelopes (NE) were isolated from either the control (−) or ATRA-treated (+) HL-60 cells, PI3K-C2β was immunoprecipitated with anti-PI3K-C2β antibody, subjected to gradient gel SDS–PAGE, transferred to nitrocellulose, probed first with anti-PI3K-C2β antibody (panel A) and then stripped off antibodies and re-probed with anti-P-Tyr antibody (panel B). The position of the molecular mass marker for α_2-macroglobulin (180 kDa) is indicated on the right by the arrow.

PI3K-C2β antibody from nuclei and nuclear envelopes of the control and ATRA-treated cells and then subjected to Western blot analysis using either anti-PDGFR antibody or anti-EGFR antibody. As no immunoreactive band could be detected (results not shown) this corroborates our conclusion that the tyrosine phosphorylated protein seen in Fig. 4 is indeed PI3K-C2β and not one of the tyrosine kinase receptors such as EGFR or PDGFR.

4. Discussion

In contrast to class I PI3Ks, which have been extensively studied in response to different agonists in both the cell membrane and nuclei, little is known about the physiological role and mode of activation of PI3K-C2β. The cloning of the enzyme revealed its relatively high molecular mass (180 kDa), the presence of C2 and PX homology region at C-termini, an N-terminal extension and no binding of adapter proteins similar to those described for class I [2,3]. In vitro studies showed that the enzyme preferentially phosphorylates PtdIns, or, with much lower affinity, PtdIns(4)P but not PtdIns(4,5)P_2, and is sensitive to the inhibitory effect of wortmannin and LY 294002 [3]. The activation of PI3K-C2β has been described in response to fibrinogen-activated integrin receptors in platelets [26], PDGF and EGF-mediated receptor activation in epithelial cell and fibroblasts [29,31], but also in membrane-depleted rat liver nuclei at the peak of compensatory liver growth [11]. A possible proteolytic activation of the enzyme was suggested, as data showed that the lipid kinase activity of PI3K-C2β increased after the deletion of the C2 domain [3], and the following studies done on fibrinogen-activated platelets [26] or regenerating liver nuclei [11] showed that the activation of the enzyme is associated with a 18 kDa gel-shift which could be mimicked by the addition of calpain and prevented in the presence of calpain-inhibitor, calpeptin. Another mode of PI3K-C2β activation has recently been suggested as the interaction between the enzyme and the activated EGF receptor was shown to depend on the binding of Grb2 adapter molecule to the proline-rich motifs within the N-termini of PI3K-C2β [31]. The results of the present study demonstrated a significant increase in the activity of PI3K-C2β immunoprecipitated from the nuclei and nuclear envelopes of ATRA-differentiated HL-60 cells, together with increased level of tyrosine phosphorylation of the enzyme and a parallel increase in the level of nuclear PtdIns(3)P, suggesting that the enzyme may be activated by tyrosine phosphorylation.

Class I PI3K activity responsible for the production of PtdIns(3,4,5)P_3 in the membrane-depleted nuclei of ATRA-differentiated cells was detected to be tightly bound to nuclear matrices isolated by nuclease treatment and high salt extraction and to correspond to an increased level of nuclear p85 protein in immunolocalization studies [16]. Although the probing of the p85 immunoprecipitates with anti-Tyr antibodies showed that p85 itself was not phosphorylated, two bands of high molecular weights were tyrosine-phosphorylated and co-migrated with Vav and phospholipase C-γ, respectively [19]. On the other hand, in erythropoietin-induced erythroid differentiation of K562 cells the p85 regulatory subunit was highly phosphorylated on tyrosine residues when translocated to the nucleus, causing an increase in intranuclear synthesis of PtdIns(3,4,5)P_3 [32]. In our study, no additional phosphoproteins were identified in PI3K-C2β immunoprecipitates, suggesting that the mechanism of the activation of the nuclear PI3K-C2β does not involve a differentiation-induced association with adapter molecules. Another difference between the activation of class I enzymes and PI3K-C2β in the nuclei of HL-60 cells is the absence of the increase in the activity of PI3K-C2β in the nuclei of vitamin D_3-differentiated cells. The activation of class I enzymes is detected in the nuclei of cells induced to differentiate along monocytic lineage by the treatment with 1,25$(OH)_2D_3$ [18], but the activity of PI3K-C2β remains unchanged in the nuclei of cells induced to differentiate not only in the presence of vitamin D_3, but also after the treatment with either IFN-γ or PMA, which increase the expression of markers of mature monocytes or macrophages. It is unknown what is the possible role of the activation of PI3K-C2β in the nuclei of ATRA-differentiated cells, but the activity does not seem to correspond to granulocytic lineage commitment as no increase was observed in the nuclei of cells induced to differentiate by the treatment with DMSO or db cAMP.

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