A Role for Nuclear Phosphatidylinositol-specific Phospholipase C in the G_2/M Phase Transition*

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Protein kinase C (PKC) is activated at the nucleus during the G_2 phase of cell cycle, where it is required for mitosis. However, the mechanisms controlling cell cycle-dependent activation of nuclear PKC are not known. We now report that nuclear levels of the major physiologic PKC activator diacylglycerol (DAG) fluctuate during cell cycle. Specifically, nuclear DAG levels in G_2/M phase cells are 2.5–3-fold higher than in G_1 phase cells. In synchronized cells, nuclear DAG levels rise to a peak coincident with the G_2/M phase transition and return to basal levels in G_1 phase cells. This increase in DAG level is sufficient to stimulate \( \beta_{11} \) PKC-mediated phosphorylation of its mitotic nuclear envelope substrate lamin B in vitro. Isolated nuclei from G_2 phase cells contain an active phospholipase activity capable of generating DAG in vitro. Nuclear phospholipase activity is inhibited by the selective phosphatidylinositol-specific phospholipase C (PI-PLC) inhibitor 1-O-octadecyl-2-O-methylsn-glycero-3-phosphocholine and neomycin sulfate, but not by the phosphatidylcholine-PLC selective inhibitor D609 or inhibitors of phospholipase D-mediated DAG generation. Treatment of synchronized cells with 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine leads to decreased nuclear PI-PLC activity and cell cycle blockade in the G_2 phase, suggesting a role for nuclear PI-PLC in the G_2/M phase transition. Our data are consistent with the hypothesis that nuclear PI-PLC generates DAG to activate nuclear \( \beta_{11} \) PKC, whose activity is required for mitosis.

The PKC family of enzymes is involved in the transduction of external signals from many growth factors, cytokines, and hormones (reviewed in Refs. 1–3). Many of the details of the receptor-mediated signaling pathways that lead to PKC activation have been elucidated (2, 3). A common feature of these pathways is receptor-mediated activation of lipid-metabolizing enzymes that generate DAG. DAG in turn activates PKC family members. The best characterized of these pathways involves the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) activity (4). Two major classes of cellular receptor utilize the PI-PLC/PKC activation pathway. Growth factor receptors containing intrinsic tyrosine kinase activity activate PI-PLC \( \gamma \) isoforms through direct tyrosine phosphorylation and activation of the enzyme (4). Many G-protein-coupled receptors activate PI-PLC \( \beta \) isoforms through direct interaction with heterotrimeric G-proteins of the G_\_ class (4). Activation of PI-PLC enzymes leads to generation of inositol trisphosphate and DAG, two metabolites of phosphatidylinositol 4,5-bisphosphate that stimulate intracellular calcium release and activate PKC isozymes, respectively. Extracellular ligands can also stimulate activation of phosphatidylinositol-specific phospholipase C (PC-PLC) and/or phospholipase D (PLD) activities (5). These phospholipases can give rise to increased cellular DAG levels and PKC activation, including the novel, calcium-independent isozymes (3). More recently, it has been demonstrated that phosphatidylinositol 3,4,5-trisphosphate, a product of growth factor receptor-activated PI3 kinase, can directly and selectively activate the atypical PKC isozymes (6–10). Elucidation of these pathways has enhanced our understanding of how lipid metabolism and PKC activation are coupled to acute growth factor and hormone actions, including mitogenesis.

In addition to its well-established role in acute mitogenic signaling, PKC has also been implicated in intrinsic signaling pathways, including those involved in cell cycle control (1). We recently demonstrated that activation of the \( \beta_{11} \) PKC isoform at the nucleus is both cell cycle-regulated and necessary for entry of cells into mitosis (11–16). At the nucleus, \( \beta_{11} \) PKC directly phosphorylates the nuclear envelope polypeptide lamin B at sites involved in mitotic nuclear lamina disassembly (12, 16). Inhibition of nuclear PKC activity leads to cell cycle arrest in the G_2 phase, indicating the importance of nuclear PKC in mitotic events (16). Although it is clear that nuclear PKC activity is cell cycle-regulated, the basis for this regulation is not clear. In the present study, we report that nuclear DAG levels fluctuate during cell cycle and that changes in nuclear DAG levels correlate with cell cycle progression through the G_2/M phase. Furthermore, a nuclear PI-PLC activity has been identified that is active during the G_2 phase. PI-PLC inhibitors lead to decreased nuclear PI-PLC activity and cell cycle blockade in the G_2 phase, suggesting a role for PI-PLC activity in nuclear events associated with entry into mitosis.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Cycle Synchronization, and Treatment with Phospholipase Inhibitors—The human promyelocytic (HL60) leukemia cell line was grown and maintained in Iscove’s medium supplemented with 10% calf serum as described previously (17). For cell cycle synchronization, cells were treated with aphidicolin (2 \( \mu \)g/ml) for 18 h to arrest cells in the G_1/S phase as described previously (16). Cells were released from the G_1 phase by washing four times with Iscove’s medium and resuspending the cells in Iscove’s medium containing 10% calf serum. At the indicated times, cells were either fixed and prepared for flow cytometric analysis of cell cycle progression as described previously (16) or harvested for nuclear DAG determination as described below. In some cases the phospholipase inhibitors 1-O-octadecyl-2-O-methylsn-glycero-3-phosphocholine (ET-18-OCH_3) or D609 or the cell cycle-blocking agents staurosporine or nocodazole were added to the cultures at...
the times and concentrations indicated in the figure and table legends. For determination of mitotic index, cells were stained with propidium iodide and viewed under phase fluorescence microscopy using a Nikon Axophot microscope equipped with appropriate filters as described previously (16).

Isolation of Nuclei and Assay for Nuclear Diacylglycerol and Phospholipase Activity—Highly purified nuclei were isolated from cells in the indicated phases of cell cycle as described previously (17). Isolated HL60 nuclei are largely devoid of cytoplasmic and plasma membrane contamination as revealed by phase and electron microscopic examination and the absence of immunoreactive transferrin receptor, a prominent plasma membrane constituent of HL60 cells (17). Purified nuclei were assayed for DAG levels using the DAG kinase method of Priebs et al. (18). Nuclear phospholipase activity was assayed in reaction buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 μM CaCl2, 1 mM dithiothreitol. Nuclei from 1 × 10^6 cells were resuspended in reaction buffer and incubated at 37°C for the indicated times and assayed for nuclear DAG levels as described above. The effect of the phospholipase C inhibitors ET-18-OCH3, neomycin sulfate, and D609 on nuclear phospholipase activity was determined at the concentrations indicated in the figure legends. The possible involvement of phospholipase D activity in nuclear DAG generation was assessed by addition of either 1.5% ethanol or 100 μM propranolol to the reaction buffer.

Nuclear PKC Assay—Nuclear envelopes were isolated from G1 phase cells as described previously (11). Purified nuclear envelopes were incubated with purified recombinant human βII PKC in the absence or presence of exogenous dioleoylglycerol (Avanti Polar Lipids) in PKC reaction buffer as described previously (11). βII PKC lamin kinase activity was assessed by monitoring βII PKC-mediated phosphorylation of the nuclear envelope component lamin B as described previously (11).

RESULTS AND DISCUSSION

Nuclear Diacylglycerol Levels Are Regulated during Cell Cycle—We previously demonstrated that protein kinase C is activated at the nucleus of human leukemia cells in response to a number of proliferative stimuli (11–15). Similar observations have been made in many other cell systems, indicating that nuclear PKC activation is an important physiologic response involved in proliferative signaling to a wide variety of stimuli (reviewed in Ref. 1). In human leukemia cells, we have found that nuclear PKC corresponds to the βII PKC isoform and that regions within the catalytic domain of βII PKC mediate selective translocation of this isoform to the nucleus (19, 20). At the nucleus, βII PKC directly phosphorylates the nuclear envelope polypeptide lamin B at mitosis-specific sites involved in mitotic nuclear lamina disassembly (11, 13, 15). Nuclear translocation of βII PKC is cell cycle-regulated, occurring in the G2 phase prior to mitosis (11, 16). Inhibition of nuclear βII PKC leads to cell cycle blockage in the G2 phase, demonstrating the importance of nuclear PKC activity in entry into mitosis (16). However, the mechanism by which nuclear PKC activation is coupled to cell cycle progression remains unknown.

We therefore assessed whether the nuclear levels of the major physiologic PKC activator DAG are responsive to cell cycle phase. Specifically, we wished to determine whether changes in nuclear DAG levels could account for the observed cell cycle-regulated activation of PKC at the nucleus during the G2 phase. We first compared nuclear DAG levels in cells in the G1 and G2 phase. For this purpose, cells were synchronized in the G1 phase with aphidicolin and released into medium to allow synchronous progression through S phase, G2 phase, mitosis, and the subsequent G1 phase (16). Nuclei were isolated from cells in the G1 phase or the G2 phase (8 h after release from aphidicolin) and assayed for DAG levels (Fig. 1A). G1 phase nuclei contained 10.0 ± 1.4 pmol of DAG/10^6 nuclei, whereas cells in the G2 phase contained 26.2 ± 2.2 pmol of DAG/10^6 nuclei. The nuclear DAG level in G1 phase cells is comparable with that in unsynchronized cells, indicating that the synchronization procedure itself does not affect nuclear DAG levels (data not shown). These results demonstrate that nuclear DAG levels fluctuate during cell cycle and are ≈2.5–3-fold higher in G2 phase cells than in G1 phase cells.

In contrast to the 2.5–3-fold change in nuclear DAG levels, total cellular DAG levels are only slightly elevated during the G2 phase when compared with the levels in G1 phase cells (Fig. 1B). G1 phase cells contain 170 ± 9.9 pmol of DAG/10^6 cells, whereas G2 phase cells contain 193 ± 4.1 pmol of DAG/10^6 cells, an increase of about 14%. Given the relative levels of total cellular and nuclear DAG levels, the 2.5–3-fold increase in nuclear DAG is sufficient to account for most of the observed increase in total cellular DAG in G2 phase cells. These data demonstrate that the observed cell cycle changes in DAG levels occur predominantly at the nucleus and cannot be accounted for by contamination of the isolated nuclei with DAG from other cellular sources. These results are consistent with those obtained by others in regenerating rat liver (21) and mitogen-stimulated Swiss 3T3 cells (22) where specific increases in nuclear DAG levels of 2–3-fold are observed in association with proliferation in the absence of similar changes in total cellular DAG levels.

We next determined whether nuclear DAG levels fluctuate during cell cycle progression (Fig. 2A). For this purpose, cells were synchronized in the G1 phase and allowed to progress synchronously through cell cycle. Nuclei were isolated from cells at different times after release from the G1 phase and assessed for cell cycle distribution and nuclear DAG levels. As cells progress through cell cycle, nuclear DAG levels rise to a peak at 8–9 h after release from the G1 phase. Flow cytometric and mitotic index analyses indicate that this peak of DAG coincides with the G2/M phase transition. The time course of nuclear DAG generation corresponds well with that of nuclear βII PKC activation, which also occurs at the time of the G2/M

FIG. 1. Nuclear diacylglycerol levels change with cell cycle. HL60 cells were synchronized in the G1 phase as described under “Experimental Procedures” and allowed to progress synchronously through cell cycle. Cell cycle phase was confirmed by flow cytometric analysis. 1 × 10^6 cells were isolated and assayed for DAG mass (18). All data points represent the mean of triplicate determinations ± S.E. A, comparison of nuclear DAG levels in cells in the G1 phase and the G2/M phase. Cells were harvested either immediately (G1 phase) or 8 h after release from the G1 phase (G2/M phase) and isolated nuclei assayed for DAG content. B, total cellular DAG levels were determined from G1, G2/M, and G1/G2 phase cells as described above.
phase transition (16). Nuclear DAG levels subsequently drop to basal levels as the cells reenter the G1 phase. These results are consistent with the findings in regenerating rat liver hepatocytes and Swiss 3T3 cells, where a specific increase in nuclear DAG levels are seen without significant changes in total cellular DAG (21, 22).

**Increased Nuclear DAG Levels Support Nuclear PKC Activity**—The observation that nuclear DAG levels are elevated during the G2 phase of cell cycle, coupled with our previous results demonstrating that βII PKC is translocated and activated at the nucleus during the G2 phase (11), led us to determine whether the observed cell cycle-dependent changes in nuclear DAG level are capable of stimulating nuclear PKC activity. We previously demonstrated that nuclei from G2 phase cells contain detectable levels of βII PKC, whereas G1 phase nuclei do not (16). Therefore, analysis of the ability of G1 and G2 phase nuclei to support PKC activity is complicated by the difference in endogenous PKC associated with these nuclei. To overcome this problem, we isolated nuclear envelopes from G1 phase cells as described under “Experimental Procedures.” 1 × 10^7 nuclear envelopes were incubated in PKC reaction buffer containing purified recombinant human βII PKC and [γ-32P]ATP for 15 min either alone (lane 1) or in the presence of 15 pmol/10^6 nuclei of DAG (lane 2). Phosphorylation of lamin B was assessed by SDS-polyacrylamide gel electrophoresis and autoradiography as described previously (11).

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**Fig. 2.** Nuclear DAG levels rise to a peak during the G1/M phase. A, synchronized cells were harvested at the indicated times after release from G1 phase and nuclear DAG levels assessed as described under “Experimental Procedures.” Also plotted is the percentage of cells in the G2 phase (G2) and mitosis (M) as determined by flow cytometric and mitotic index analysis. Data are from a representative experiment. B, elevated nuclear DAG leads to activation of βII PKC in the absence or presence of additional βII PKC and [γ-32P]ATP for 15 min either alone (lane 1) or in the presence of 15 pmol/10^6 nuclei of DAG (lane 2). Phosphorylation of lamin B was assessed by SDS-polyacrylamide gel electrophoresis and autoradiography as described previously (11).

**Fig. 3.** A nuclear phosphoinositide-specific phospholipase C is present in G2 phase nuclei. A, time course of nuclear DAG generation. Nuclei from G2 phase cells were isolated and incubated at 37 °C. At the indicated times, aliquots were removed and assayed for nuclear DAG levels. Data points represent the mean of three determinations ± S.E. B, effect of phospholipase inhibitors on nuclear phospholipase activity. Nuclei from G2 phase cells were assayed for 2 h for phospholipase activity as described above. The inhibitors ET-18-OCH_3, neomycin sulfate, D609, ethanol, and propranolol were added at the indicated concentrations. Data points represent the mean of three determinations ± S.E. Column 1, control nuclei, 0 h; column 2, control nuclei, 2 h; column 3, +10 μM ET-18-OCH_3, 2 h; column 4, +100 μM ET-18-OCH_3, 2 h; column 5, +10 μM D609, 2 h; column 6, +30 μM D609, 2 h; column 7, +600 μM neomycin sulfate, 2 h; column 8, +1.2 mM neomycin sulfate, 2 h; column 9, +1.5% ethanol, 2 h; column 10, +100 μM propranolol, 2 h.
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**Fig. 4.** The PI-PLC inhibitor ET-18-OCH₃ causes cell cycle arrest in the G₂/M phase. HL60 cells were synchronized in the G₁ phase and released into control medium (top panel) or medium containing either 10 μM ET-18-OCH₃ (middle panel) or 30 μM D609 (bottom panel). Cells were harvested at the indicated times after release from the G₁ phase and analyzed for cell cycle phase by flow cytometric analysis.

**Table I**

| Treatment       | G₁ | S   | G₂ | M   |
|-----------------|----|-----|----|-----|
| No addition     | 65 | 22  | 2  | 11  |
| Nocodazole      | 20 | 30  | 2  | 48  |
| Staurosporine   | 14 | 28  | 48 | 12  |
| ET-18-OCH₃      | 40 | 28  | 23 | 9   |
| D609            | 67 | 22  | 2  | 9   |

Cell cycle distribution of HL60 cells after treatment with various cell cycle inhibitors. HL60 cells were synchronized in the G₁ phase as described under “Experimental Procedures.” Synchronized cells were washed and resuspended in control medium (no addition) or medium containing nocodazole (40 ng/ml), staurosporine (150 nM), ET-18-OCH₃ (10 μM), or D609 (30 μM). The cells were incubated at 37 °C for 12 h, at which time the cells were fixed and assessed for cell cycle distribution by flow cytometric and mitotic index analysis as described under “Experimental Procedures.”

**Fig. 5.** ET-18-OCH₃ inhibits nuclear PI-PLC activity. Cells were synchronized in the G₁ phase and released into medium containing either 10 μM ET-18-OCH₃ (+Et-18-OCH₃) or diluent (Control). Cells were harvested at 8 h (peak of the G₂ phase), and isolated nuclei were assayed for nuclear PI-PLC activity as described under “Experimental Procedures.” Results represent the mean of three determinations ± S.E.

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shown). Furthermore, aphidicolin (2 μM), the agent used in the synchronization of these cells, has no effect on nuclear DAG generation (data not shown). These results indicate that nuclear DAG comes from an intrinsic nuclear phospholipase activity and argue against the translocation of DAG, produced in other cellular membranes, to the nucleus.

We next determined the nature of the nuclear phospholipase responsible for generating nuclear DAG. For this purpose, we assessed the effect of various phospholipase inhibitors on nuclear DAG generation (Fig. 3B). Incubation of nuclei for 2 h at 37 °C leads to accumulation of DAG to levels 3–5-fold that of starting G₁ phase nuclei (Fig. 3B, columns 1 and 2). Inclusion of the PI-PLC-selective inhibitor ET-18-OCH₃ leads to dose-dependent inhibition of nuclear DAG generation (40% inhibition at 10 μM (Fig. 3B, column 3) and 85% inhibition at 100 μM (Fig. 3B, column 4)). Likewise, the nonselective phospholipase inhibitor neomycin sulfate leads to inhibition of nuclear phospholipase activity (Fig. 3B, columns 7 and 8). The data are in good agreement with the published IC₅₀ values of these compounds (IC₅₀ ET-18-OCH₃ = 10 μM (23), neomycin sulfate = 65 μM (24)). In contrast, addition of the PC-PLC inhibitor D609 at 10 μM and 30 μM (Fig. 3B, columns 5 and 6) had no effect on nuclear DAG generation.

To assess the possible contribution of PLD activity to nuclear DAG generation, we determined the effect of ethanol on nuclear DAG production. In the presence of 1.5% ethanol, PLD generates phosphatidylethanol rather than phosphatidic acid (25). Since phosphatidylethanol is not a substrate for phosphatidic acid phosphohydrolase, the enzyme responsible for conversion of phosphatidic acid to DAG, 1.5% ethanol effectively blocks PLD-mediated generation of DAG (25). Inclosure of 1.5% ethanol in the nuclear phospholipase assay had little effect on nuclear DAG generation (Fig. 5B, column 9). Likewise, 100 μM propranolol, a phosphatidic acid phosphohydrolase inhibitor (26), had no effect on nuclear DAG generation (Fig. 5B, column 10). Taken together, these data indicate that the major phospholipase activity responsible for generating nuclear DAG in G₂ phase nuclei is a PI-PLC activity. Although it is possible that other phospholipase activities may contribute to nuclear DAG levels, the inhibitor studies clearly demonstrate that the major nuclear phospholipase activity present in G₂ phase nuclei is PI-PLC.

**Nuclear PI-PLC Is Involved in Cell Cycle Progression into Mitosis**—To assess the potential physiologic role of nuclear PI-PLC, we determined the effect of PI-PLC inhibition on cell cycle progression through the G₂/M phase transition. For this purpose, cells were synchronized, released from the G₁ phase, and assessed for cell cycle progression by flow cytometric analysis as described previously (16). Using this protocol, HL60 cells progress synchronously through the G₂/M phase and return to the G₁ phase by 12 h after release (Fig. 4, top panel). However, when 10 μM ET-18-OCH₃ is included in the culture medium, cells progress through the S phase but exhibit a blockade in the G₂/M phase (Fig. 4, middle panel). This blockade is not seen with the PC-PLC inhibitor D609, indicating that it is selective for PI-PLC inhibition (Fig. 4, bottom panel). To assess the level of cell cycle inhibition, we compared the cell cycle distribution of ET-18-OCH₃-treated cells with those treated with other cell cycle inhibitors. The spindle poison nocodazole was used to arrest cells in M phase, and the nonselective protein kinase inhibitor staurosporine was used to arrest cells in the G₂ phase (16, 27) as positive controls for complete arrest in these cell cycle phases (Table I). From this...
nuclear PI-PLC activity in vitro provided evidence implicating nuclear PI-PLC activities in the G1 progressive induction of apoptosis, an effect of ET-18-OCH₃ that has been well documented (28–31).

Concomitantly with the G₂ phase cell cycle arrest, treatment with 10 μM ET-18-OCH₃ led to a 35% reduction of nuclear DAG levels when compared with untreated G₂ phase cells, in line with the level of cell cycle arrest induced by ET-18-OCH₃. In addition, ET-18-OCH₃ treatment leads to an 83% reduction in nuclear PI-PLC activity in vitro (Fig. 5). A similar G₂ phase cell cycle arrest has been reported in colony-stimulating factor 1-dependent cells after treatment with ET-18-OCH₃ (32). Our data demonstrate that ET-18-OCH₃ leads to inhibition of nuclear PI-PLC activity concomitant with cell cycle arrest in the G₂ phase, providing a plausible mechanism for the cell cycle effects of ET-18-OCH₃.

In conclusion, our data provide direct evidence that nuclear DAG levels are cell cycle-regulated and that they rise to a peak corresponding to the G₂/M phase transition. The major phospholipase responsible for the generation of nuclear DAG during the G₂ phase of cell cycle is a PI-PLC activity. Nuclear PI-PLC activity appears to be important for the G₂/M phase transition of cell cycle, since when this activity is inhibited, cell cycle arrest in the G₂ phase is observed. Recent studies have provided evidence implicating nuclear PI-PLC activities in the G₁ to S phase transition and in DNA replication (33–36). Our present data indicate an additional, novel role for nuclear PI-PLC during the G₂/M phase of cell cycle. We hypothesize that the DAG generated by nuclear PI-PLC activity is responsible for activating nuclear PKC during the late G₂ phase, which is required for entry of cells into mitosis (16). Here, we provide direct evidence that the elevated nuclear DAG levels observed during the G₂ phase of cell cycle are sufficient to stimulate nuclear βII PKC-mediated phosphorylation of its physiologic nuclear substrate lamin B. Future studies will focus on identifying which PI-PLC isoform(s) correspond to nuclear PI-PLC in G₂ phase cells, determining the molecular mechanisms by which this nuclear PI-PLC activity is regulated during cell cycle and further assessing its role in the G₂/M phase transition.

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