The Pro-Apoptotic Drug Camptothecin Stimulates Phospholipase D Activity and Diacylglycerol Production in the Nucleus of HL-60 Human Promyelocytic Leukemia Cells

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ABSTRACT

It has recently been reported (T. Shimizu et al., J. Biol. Chem., 273: 8669–8674, 1998) that the pro-apoptotic drug, camptothecin, an inhibitor of topoisomerase I, induces a protein kinase C-α-mediated phosphorylation of lamin B in HL-60 cells, which precedes both degradation of lamin B and fragmentation of DNA. In this paper, we report that, in HL-60 cells exposed to camptothecin, there is a rapid and sustained increase of protein kinase C-α activity that is due to an increase in the amount of protein kinase C-α present in the nucleus. The enhancement of nuclear kinase C-α activity is preceded by an increase in the mass of nuclear diacylglycerol. As demonstrated by its sensitivity to propranolol, the nuclear diacylglycerol mass increase is due to the activation of a phospholipase D. Indeed, inhibitors of neither phosphatidylinositol-specific phospholipase C nor phosphoinositide-specific phospholipase C blocked the rise in nuclear diacylglycerol. In vitro assays also demonstrated the activation of a nuclear phospholipase D, but not of a phosphoinositide-specific phospholipase C, after treatment with camptothecin. Propranolol was also able to block the rise in nuclear protein kinase C-α activity, thus suggesting that the increase in diacylglycerol mass is important for the activation of the kinase at the nuclear level. Moreover, propranolol was capable of drastically reducing the number of HL-60 cells that underwent apoptosis after treatment with camptothecin. Our results show the activation during apoptosis of a phospholipase D-mediated signaling pathway operating at the nuclear level. This pathway may represent an attractive therapeutic target for the modulation of apoptotic events in human disease.

INTRODUCTION

Cell death by apoptosis is a fundamental process of mammalian organisms, in which it plays a major role in development, immune system maturation, tissue homeostasis, and aging. A growing body of evidence indicates that deregulation of apoptosis is most likely to have important implications for neoplastic transformation (1, 2). Moreover, the possibility of modifying sensitivity to apoptosis through its regulatory pathways has clear implications for the treatment of cancer (3). There is increasing evidence that diverse death signals activate different pathways that converge toward a conserved execution machinery, which depends on one or more members of a family of aspartic-acid-directed cysteine proteases called caspases (4). During the apoptotic process, dramatic morphological and biochemical changes take place in the nucleus (5–8). Nuclear protein phosphorylation is likely to play an important role in the regulation of apoptosis (8). There are a few reports concerning the phosphorylation of nuclear proteins during apoptosis; in dexamethasone-treated thymocytes cultured, phosphorylation and cleavage of M, 240,000 NuMA protein are very early events, detectable after a 30-min exposure to the hormone and preceding internucleosomal DNA cleavage (9). At present, there is no indication of the protein kinase that may phosphorylate NuMA during apoptosis. On the other hand, in thymocytes exposed to the pro-apoptotic fungal toxin, gliotoxin, a protein kinase A-dependent serine-phosphorylation of histone H3 has been reported (10). Very recently, the hyperphosphorylation of replication protein A middle subunit has been described in apoptotic Jurkat cells, and it seems mediated by both DNA- and cyclin-dependent protein kinases (11).

Another protein kinase that may be involved in apoptosis is PKC. The very first report linking nuclear PKC to apoptosis was published in 1994 by Trubiani et al. (12), who showed a redistribution of the kinase toward the nucleus in dexamethasone-treated thymocytes. In spontaneously apoptotic U937 cells, PKC-α showed a reduced nuclear level of expression while PKC-α was increased in this cell compartment (13). A direct link between PKC and apoptosis was subsequently demonstrated in HL-60 cells induced to apoptosis by camptothecin (14). Indeed, it was shown that the α isoform of PKC phosphorylated lamin B 1 h after the addition of the drug, and this phenomenon preceded both proteolytic degradation of lamin B and DNA fragmentation. It is worth recalling that in HL-60 cells, PKC-βII phosphorylates lamin B during the G2–M phase transition (15), and this PKC isoform is activated at the nucleus by DAG produced by a PI-PLC (16). In this article, we demonstrate that nuclei isolated from HL-60 cells exposed to camptothecin have more PKC-α protein than controls, and that the kinase is active. Moreover, the activation of nuclear PKC-α is preceded by a rise in nuclear DAG levels. Both the DAG rise and the activation of PKC-α at the nucleus were suppressed by propranolol (an inhibitor of PLD) but not by either ET-18-OCH3 (a specific inhibitor of PI-PLC) or D609 (a purported inhibitor of PC-PLC). Interestingly, propranolol, but not the two other inhibitors, dramatically reduced the number of HL-60 cells entering apoptosis after exposure to camptothecin.

MATERIALS AND METHODS

Materials. RPMI 1640, fetal bovine serum, 1,2-dioleyl-3-palmitoyl-glycero, dielylglycerol, CHAPS, phosphatidylserine, phosphatidic acid, normal goat serum, peroxidase-conjugated anti-rabbit, anti-goat, and antimouse IgG, camptothecin, histone H1, leupeptin, aprotinin, PMSF, benzamidine, rabbit polyclonal antibody to PKC-α, DNase I, RNase A, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Propidium iodide and phosphatidylethanol were from ICN Pharmaceuticals (Costa Mesa, CA). ET-18-OCH3, propranolol, and D609 were from Calbiochem (La Jolla, CA). Enhanced chemiluminescence detection kit and NP40 were from Roche Molecular Biochemicals (Milan, Italy). [γ-32P]-ATP, phosphatidyl[methyl-3H]choline, and...
NUCLEAR DAG IN APOPTOTIC HL-60 CELLS

Isolation of Nuclei from HL-60 Cells. This was accomplished essentially as reported by Fields et al. (17). All of the steps were executed at 4°C in buffers containing 0.1 mM Na3VO4, 20 mM NaF, 10 μM aprotinin, 10 μM benzamidine, and 1 mM PMSF. Cells were washed three times with PBS and hypotonically swelled in 50 mM of Tris-HCl (pH 7.4), 250 mM sucrose, and 5 mM MgSO4 containing 1% (w/v) 2-mercaptoethanol for 10 min at 1 × 107 cells/ml. Then, 10% (v/v) NP40 was added to a final concentration of 0.02% (w/v), and the cells were lysed with 50 strokes of a Dounce homogenizer using a B-type pestle. The lysate was layered over a cushion of 2.1 m sucrose, 50 mM of Tris-HCl (pH 7.4), 5 mM MgSO4, and 1% 2-mercaptoethanol; and the nuclei were pelleted at 70,000 × g for 60 min in a Beckman SW28 rotor.

Purity of nuclear preparation was assessed by Western blotting analysis using either goat polyclonal antibody to IGF-I receptor β chain or monoclonal antibody to histone H1.

Preparation of Whole-Cell Homogenates and Cytosolic Extracts. To obtain homogenates, cells were resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1 mM Na3VO4, 20 mM NaF, 10 μM aprotinin, 10 μM benzamidine, and 1 mM PMSF, and homogenized by 30 passages through a 25-gauge needle. Cytosolic extracts were prepared according to Shimizu et al. (18).

Preparation of Nuclear Extracts and Immunoprecipitation of PKC-α. Nuclear extracts were prepared essentially as reported previously (18), with some modifications. Nuclei were resuspended in 5 mM Tris-HCl (pH 8.0), 1 mM EGTA, 1 mM EDTA, 0.1 mM Na3VO4, 20 mM NaF, 10 μM aprotinin, 10 μM benzamidine, 1 mM PMSF, and 0.3% Triton X-100, then ruptured by 50 passages through a 25-gauge hypodermic needle, and centrifuged at 5,000 × g to remove insoluble material. Nuclear extracts (1 ml, containing 500 μg of protein) was preclarsed by adding 5 μg of normal rabbit IgG and 10 μg of 50% Protein A-Agarose, followed by incubation for 1 h at 4°C and centrifugation at 12,000 × g for 10 min at 4°C. The samples were incubated for 4 h at 4°C under constant agitation with 5 μg of polyclonal antibody to PKC-α. 50% Protein A-Agarose (10 μg) was added and incubation proceeded for 1 h at 4°C under constant agitation, then centrifuged. The beads were washed once with lysis buffer and twice with kinase buffer (50 mM Tris-HCl (pH 7.4), 10 mM NaF, 1 mM Na3VO4, 0.5 mM EGTA, 0.5 mM EDTA, 2 mM MgCl2, 5 μg/ml leupeptin, and 1 mM PMSF).

Protein Assay. This was performed using the Bio-Rad Protein Assay (detergent compatible) according to the instructions of the manufacturer.

Western Blotting Analysis. Proteins separated on SDS-PAGE (19) were transferred to nitrocellulose sheets. Sheets were saturated in PBS containing 5% normal goat serum and 4% BSA for 60 min at 37°C (blocking buffer), then incubated overnight at 4°C in blocking buffer containing the primary antibodies. After four washes in PBS containing 0.1% Tween 20, they were incubated for 30 min at room temperature with the appropriate peroxidase-conjugated secondary antibody, diluted 1:3,000 in PBS-Tween-20, and washed as above. Bands were visualized by the enhanced chemiluminescence method.

PKC Activity Assays. Immunocomplex beads were incubated in 50 μl of 20 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 10 μM ATP, 0.4 mg/ml histone H1, and 5 μCi of [γ-32P]ATP in the presence of 1.2 mM CaCl2, 40 μg/ml phosphatidylserine, 1.2 mM of CaCl2, and 3.5 μM dioleylglycerol. Incubations were carried out at 30°C for 10 min. The reactions were terminated with 15 μl of acetic acid and spotted on to Whatman p81 paper, followed by washing with 0.75 mM H3PO4. Radioactivity was measured by Cerenkov counting.

Measurement of DAG Produced in Vivo. The assay was performed according to Divecha et al. (20), using DAG kinase enzyme purified from rat brain. DAG was extracted from nuclei, whole cells, and cytosolic extracts dissolved in 20 μl of CHAPS (9.2 mg/ml) and sonicated at room temperature.

Fig. 1. Western blot analysis for either IGF-I receptor β chain or histone H1 in whole homogenates and purified nuclei of HL-60 cells. Protein obtained from 8 × 107 cells or isolated nuclei was blotted to nitrocellulose paper and probed with the appropriate antibodies. The Mv of the histone H1 was approximately 31,000 (a 11% gel was used), whereas it was approximately 94,000 for the IGF-I receptor β chain (a 8% gel was used).

Fig. 2. PKC activity assays in isolated HL-60 cell nuclei using as substrate histone H1. Cells were exposed to camptothecin for the indicated times.

Fig. 3. Western blot analysis for PKC-α in isolated nuclei (A), whole-cell homogenates (B), and cytosolic extracts (C), prepared from HL-60 cells exposed to camptothecin for the indicated times. Nuclei (5 × 107) and whole-cell homogenates and cytosolic extracts (both from 1 × 107 cells) were dissolved in electrophoresis sample buffer, and protein was separated on 8% polyacrylamide gels and transferred to nitrocellulose paper, which was then probed with a polyclonal antibody to PKC-α. The Mv of the detected band was 80,000.
for 15 s. After the addition of 80 μl of reaction buffer [50 mM Tris-acetate (pH 7.4), 80 mM KCl, 10 mM Mg acetate, and 2 mM EGTA], the assay was started by the addition of 20 μl of DAG kinase enzyme followed by 80 μl of reaction buffer containing 5 μM ATP, and 1 μCi of [γ-32P]ATP. Incubation was for 1 h at room temperature; then phosphatidic acid was extracted, chromatographed, and autoradiographed, and its radioactivity was counted in a liquid scintillation system (Betamatic IV, Kontron, Milan, Italy). Standard curves were obtained as reported by Divecha et al. (20), using 1,2-dioleoyl-3-palmitoyl-glycerol as substrate.

**PLD in Vitro Activity Assay.** This was accomplished in one of two ways:

(a) isolated nuclei were resuspended at 4°C in reaction buffer [50 mM Hepes-NaOH (pH 7.2), 2 mM EDTA, 0.5 mM EGTA, 5 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM Na3VO4]. Nuclear protein (150 μg in 400 μl of buffer) was incubated with 100 μl of a Triton X-100 (6.25 mM), phosphatidyl[methyl-3H]choline (2.25 mM at 29 μCi/μmol) mixed micelle (3:1, Triton X-100:PC). The reaction mixture (a total reaction volume of 500 μl) was incubated at 37°C for 1 h, and the released water-soluble headgroups were separated by ion pairing with tetraphenylboron and quantified by liquid scintillation counting (21); or

(b) cells were labeled for 20 h in the presence of [3H]palmitic acid (5 μCi/ml). Nuclei were isolated and incubated (50 μg per assay in 200 μl final volume) for 30 min at 37°C in 25 mM Hepes-NaOH (pH 7.4), 100 mM KCl, 3 mm NaCl, 5 mM MgCl2, 1 μM CaCl2, 1 mM PMSF, 10 μM benzamidine and leupeptin, and 1.5% ethanol (22–23). Total lipids were extracted (24), and the radioactivity that was incorporated was quantified at this time by scintillation counting. Phosphatidylethanol and phosphatidic acid were resolved from nuclear lipids by TLC according to standard methods (21–24). Spots of interest were identified by comparison with authentic standards, scraped from the plates, and counted by scintillation counting.

Values were expressed as percentage of radioactivity in either phosphatidylethanol or phosphatidic acid with respect to total nuclear phospholipid.

**PI-PLC Activity Assay.** The procedure outlined by Martelli et al. (18) was followed. Briefly, assays (100 μl) contained 100 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 6.7), 150 mM NaCl, 0.06% sodium deoxycholate, 3 nmol [3H]PIP2 (specific activity: 30,000 dpm nmol-1), and 10 μg of nuclear protein. Incubation was for 30 min at 37°C. Hydrolysis was stopped by adding chloroform-methanol-HCl, and PIP2 hydrolysis was quantified by liquid scintillation counting.

**Statistical Analysis.** Data are the mean from three different experiments and are expressed as mean ± SD. The asterisk indicates significant differences (P < 0.01) in a Student’s paired t test. All of the other differences were found to be not significant with P > 0.01.

**RESULTS**

PKC-α Activity and Protein Level in Isolated Nuclei after Treatment of HL-60 Cells with Camptothecin. Because a very critical issue in the present study was the purity of nuclear preparations, we addressed it by Western blotting analysis. As shown in Fig. 1, our preparations of nuclei, when compared with whole cell homogenates, were highly enriched in histone H1 and devoid of the β chain of IGF-I receptor (which is expressed by these cells; see Ref. 25).

Then, we assayed PKC-α activity in isolated nuclei prepared from HL-60 cells treated for various times with the pro-apoptotic chemical camptothecin. Because various PKC isoforms have been reported to be present in HL-60 cell nuclei (26–27), we prepared nuclear extracts and immunoprecipitated PKC-α using a specific polyclonal antibody. The immunoprecipitates were then tested for PKC-α activity using histone H1 as substrate. As shown in Fig. 2, nuclear PKC-α activity started to increase 30 min after the addition of camptothecin and peaked at 60 min, when an almost 4-fold increase was seen. After 150 min, the enzyme activity returned to basal levels.

We next wanted to determine whether the increase in nuclear PKC-α activity might be due to an increase in the protein amount...
Table 1 Nuclear PLD activity in HL-60 cell nuclei after treatment with camptothecin

<table>
<thead>
<tr>
<th>Exposure to camptothecin (min)</th>
<th>Phosphatidylethanol</th>
<th>Phosphatic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.41 ± 0.08</td>
<td>0.255 ± 0.09</td>
</tr>
<tr>
<td>30</td>
<td>1.29 ± 0.19*</td>
<td>0.797 ± 0.11*</td>
</tr>
<tr>
<td>45</td>
<td>1.86 ± 0.24*</td>
<td>1.068 ± 0.15*</td>
</tr>
<tr>
<td>120</td>
<td>0.98 ± 0.12*</td>
<td>0.634 ± 0.10*</td>
</tr>
<tr>
<td>150</td>
<td>0.77 ± 0.10</td>
<td>0.273 ± 0.09</td>
</tr>
</tbody>
</table>

* values that were statistically different from control (0 time).

Table 2 Nuclear PI-PLC activity in HL-60 cell nuclei after treatment with camptothecin

<table>
<thead>
<tr>
<th>Exposure to camptothecin (min)</th>
<th>nmoles PIP₂ hydrolyzed/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.4 ± 1.5</td>
</tr>
<tr>
<td>30</td>
<td>10.9 ± 1.6</td>
</tr>
<tr>
<td>45</td>
<td>10.2 ± 1.5</td>
</tr>
<tr>
<td>120</td>
<td>9.9 ± 1.3</td>
</tr>
<tr>
<td>150</td>
<td>10.2 ± 1.7</td>
</tr>
</tbody>
</table>

Fig. 6. PKC-α activity in isolated HL-60 cell nuclei. Cells were exposed for the indicated times to camptothecin only (no pretreatment) or incubated for 10 min with the DAG production inhibitors and then exposed to camptothecin. Nuclei were then isolated and solubilized. PKC-α was immunoprecipitated, and its activity was assayed using histone H1 as substrate.

Fig. 7. A. Western blot analysis for PKC-α in isolated nuclei prepared from HL-60 cells exposed to camptothecin for 45 min and pretreated with various chemicals interfering with DAG generation. Protein from 5 × 10⁶ nuclei was separated on 8% SDS-PAGE and transferred to nitrocellulose paper, which was then probed with a polyclonal antibody to PKC-α. Lane 1, camptothecin only; Lane 2, camptothecin plus D609 (30 μM); Lane 3, camptothecin plus ET-18-OCH₃ (100 μM); Lane 4, camptothecin plus propranolol (100 μM).

B. The same as A, except that no camptothecin was used. Lane 1, control nuclei; Lane 2, D609 (30 μM); Lane 3, ET-18-OCH₃ (100 μM); Lane 4, propranolol (100 μM).

**PI-PLC Activity in Isolated HL-60 Nuclei.** As a further control, we measured PI-PLC activity in isolated HL-60 nuclei after treatment with camptothecin. The results are shown in Table 2. No enhancement of basal activity was measured at any time after incubation with the pro-apoptotic drug.

**Activation of Nuclear PKC-α Is Dependent on DAG Levels.** To test whether activation of PKC-α was dependent on the levels of DAG...
present in isolated nuclei, we measured the isozyme activity in nuclei prepared from cells that had been pretreated for 10 min with the inhibitors and then had been exposed to camptothecin. The results from these experiments are presented in Fig. 6. It is clear that whereas neither ET-18-OCH₃ nor D609 blocked nuclear PKC-α activation, propranolol did block it. Moreover, as shown in Fig. 7A, Western blotting analysis revealed that, in nuclei obtained from cells that were pretreated with propranolol and exposed to camptothecin for 45 min, the amount of PKC-α protein was dramatically reduced in comparison with nuclei prepared from control cells (no pretreatment) or from cells pretreated for 10 min with either ET-18-OCH₃ or D609. In contrast, in nuclei obtained from cells exposed to the inhibitors but not to camptothecin, the nuclear PKC-α levels did not show changes (Fig. 7B).

Propranolol but not ET-18-OCH₃ nor D609 Blocks Camptothecin-dependent Apoptosis. Finally, we evaluated whether or not the chemicals used to inhibit phospholipases could block the induction of apoptosis caused by camptothecin. As shown in Table 3, propranolol, but not ET-18-OCH₃ nor D609, dramatically reduced the number of apoptotic HL-60 cells.

DISCUSSION

Proteolysis of lamin B has long been recognized as one of the most distinctive features of apoptotic nuclear changes (5, 30). Very recently, Shimizu et al. (14) demonstrated that in camptothecin-treated HL-60 cells a PKC-α-dependent lamin B phosphorylation preceded its proteolytic degradation as well as chromatin fragmentation. It is important to recall that in HL-60 cells lamin B phosphorylation also occurs at the G₂-M phase transition of the cell cycle. The protein kinase responsible for the phosphorylation of lamin B at the G₂-M phase transition was identified as the -β₁₁ isozyme of PKC. In this case, however, phosphorylation causes a solubilization of lamin B followed by a breakdown of the nuclear envelope (31). Therefore, PKC-mediated lamin B phosphorylation during apoptosis may also affect nuclear and chromatin structure. Interestingly, Sun et al. (17) have reported that, in HL-60 cells during the G₂-M phase transition, there is an increase in the levels of nuclear DAG, which is responsible for the activation of PKC-β₁₁. Because of these results, we sought to determine whether in the nuclei of camptothecin-treated HL-60 cells, there also might be an increase in DAG mass that might explain the activation of PKC-α. Indeed, an increase in nuclear DAG linked to translocation and/or activation of PKC-α has been demonstrated to occur in other experimental models, mostly during cell proliferation (20, 32, 33).

We first demonstrated that in nuclei isolated from HL-60 cells treated with camptothecin, there is an increase in PKC-α activity. The activity enhancement started at 30 min after the initiation of treatment and returned to basal levels at approximately 150 min. This is in good agreement with the results of Shimizu et al. (14), which showed that in vivo phosphorylation of lamin B in camptothecin-exposed HL-60 cells was first detectable at 30 min after starting incubation and declined at 180 min. They also showed an induction of PKC-α activity in whole HL-60 cells that peaked around 60 min after the incubation of camptothecin and that was largely gone after 3 h. It may be that this increase in total-cell PKC-α activity reflects the enhancement of nuclear PKC-α activity that we have demonstrated to occur on a similar time scale. The same group (34) had previously reported that changes in soluble PKC-α activity in this experimental model were seen at later times and were due to a hyperphosphorylation of the kinase.

In contrast, the results of our Western blotting analysis showed that isolated HL-60 nuclei that were prepared from cells exposed to camptothecin retained more PKC-α protein. However, this increase is likely to be due to an insolubilization of PKC-α already resident in the nucleus because, by immunofluorescent staining, we did not see a translocation of cytoplasmic PKC-α to the nucleus after treatment with camptothecin, in contrast to the behavior of PKC-β₁₁ in bryo-33, sta-tin-treated HL-60 cells (35).

This close association of PKC-α with the nucleus may be due to an increase in nuclear DAG levels. In fact, it has been previously hypothesized that PKC may be continuously cycling in and out of the nucleus and may become fixed in the nucleus by an increase of DAG, which can activate it (20). Using mass assays, we showed that an increase in the level of nuclear DAG preceded activation of PKC-α at the nucleus. Next, we tried to determine the source of nuclear DAG, because at least two different types of phospholipase activities have been shown to operate at the nuclear level and generate DAG, PI-PLC, and PLD (18, 28, 36–38). To this end, we used various inhibitors that have been reported to be specific for these enzymes (16, 33). The increase in nuclear DAG mass was not affected by either ET-18-OCH₃, a specific inhibitor of PI-PLC, or by D609, a purported inhibitor of PC-PLC. In this connection, it should be recalled that the existence of PC-PLC in mammalian cells is controversial (39), although some evidence points to the presence of such an enzyme in isolated nuclei (21, 40). In contrast, propranolol, a well-established blocker of PLD-mediated DAG generation, almost completely inhibited the DAG rise. It should be recalled that our nuclei were prepared according to a protocol that allows the conservation of the nuclear envelope (17). Thus, our results are suggestive of events that take place at the nuclear envelope level in agreement with the “NEST” hypothesis proposed by Raben et al. (41). However, there is also evidence that HL-60 nuclei—deprived of their envelope—still retain a PLD activity (29). Activation of PLD after treatment with camptothecin was demonstrated in isolated nuclei using two different enzymatic assays (42–44). Both of the assays demonstrated an approximately 4-fold increase in nuclear PLD activity. In contrast, we did not detect significant changes in nuclear PI-PLC activity. Furthermore, exposure of cells to propranolol, but not to either ET-18-OCH₃ or D609, prevented the rise in nuclear PKC-α activity and protein levels. An important role played by nuclear PLD in the control of apoptotic events elicited by camptothecin in HL-60 cells was also suggested by the fact that, of the three inhibitors we used, only propranolol was able to significantly reduce the number of apoptotic HL-60 cells measurable at various times after incubation with camptothecin.

Thus, a model could be envisioned in which nuclear PI-PLC and PKC-β₁₁ are necessary for HL-60 cell proliferation and survival because PKC-β₁₁ is essential for the transition from G₂ to M-phase of the cell cycle (45), and ET-18-OCH₃, which inhibits PI-PLC, induces apoptosis (16). In contrast, both nuclear PLD and PKC-α play a role in camptothecin-mediated apoptosis.

A. M. Martelli et al., unpublished observations.
It may be that the differences in the composition of fatty acids between DAG generated by PI-PLC and those generated by PDL are important for attracting and/or activating different PKC isoforms at the nuclear level. Our results also demonstrate that after the camptothecin-treatment of HL-60, a rise occurred only in nuclear DAG, whereas DAG levels in other cell fractions did not change. However, we were unable to induce apoptosis in these cells by the use of membrane-permeant DAG analogues such as dioctanoyl-c1r-glycerol (data not shown; see Ref. 46). Therefore, it seems that DAG is necessary but not sufficient to induce the apoptotic process in camptothecin-exposed HL-60 cells. We can speculate that lamin B, once it is phosphorylated by DAG-activated PKC-α, becomes more susceptible to proteases, which are then required—together with nuclease—for the completion of the execution phase of apoptosis. This is consistent with the observation that overexpression of mutated lamins A or B resistant to caspase cleavage delayed DNA fragmentation, a fact that could indicate the lamins are the only caspase substrates known to be directly involved in the execution phase of apoptosis (14).

It is still unclear how a nuclear PDL may become activated during apoptosis. Baldassare et al. (21) have demonstrated that a nuclear translocation of RhoA mediates the mitogen-induced activation of PDL involved in nuclear envelope signal transduction in IIC9 cells exposed to α-thrombin. In this connection, it should be emphasized that there are some reports hinting at an important role played by RhoA in the induction of apoptosis (for example, Ref. 47). Nevertheless, RhoA has also been demonstrated to counteract apoptosis, for example, by increasing Bcl-2 expression (48, 49). In any case, we think that involvement of RhoA in the activation of nuclear PDL activity during apoptosis should be further investigated.

An important role played by PKC-α during apoptosis has been suggested by Rusnak and Lazo (50) in DU-145 human androgen-independent prostatic carcinoma cells exposed to either etoposide or melphalan. However, other investigators came to opposite conclusions by studying COS cells (51). Because these differences may be due to the diverse cell types studied, future investigations entailing the use of transfected cDNAs for both PKC isoforms in sense or antisense orientations by studying COS cells (51) may be necessary but not sufficient to induce the apoptotic process in camptothecin-exposed HL-60 cells.

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