Overexpression of the Atypical Protein Kinase C ζ Reduces Topoisomerase II Catalytic Activity, Cleavable Complexes Formation, and Drug-induced Cytotoxicity in Monocytic U937 Leukemia Cells*

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In this study, we evaluated the influence of protein kinase Cζ (PKCζ) on topoisomerase II inhibitor-induced cytotoxicity in monocytic U937 cells. In U937-ΩJ and U937-BB cells, enforced PKCζ expression, conferred by stable transfection of PKCζ cDNA, resulted in total inhibition of VP-16- and mitoxantrone-induced apoptosis and decreased drug-induced cytotoxicity, compared with U937-neo control cells. In PKCζ-overexpressing cells, drug resistance correlated with decreased VP-16-induced DNA strand breaks and DNA protein cross-links measured by alkaline elution. Kinetoplast decatenation assay revealed that PKCζ overexpression resulted in reduced global topoisomerase II activity. Moreover, in PKCζ-overexpressing cells, we found that PKCζ interacted with both α and β isoforms of topoisomerase II, and these two enzymes were constitutively phosphorylated. However, when human recombinant PKCζ (rH-PKCζ) was incubated with purified topoisomerase II isoforms, rH-PKCζ interacted with topoisomerase IIβ but not with topoisomerase IIα. PKCζ/topoisomerase IIβ interaction resulted in phosphorylation of this enzyme and in decrease of its catalytic activity. Finally, this report shows for the first time that topoisomerase IIβ is a substrate for PKCζ, and that PKCζ may significantly influence topoisomerase II inhibitor-induced cytotoxicity by altering topoisomerase IIβ activity through its kinase function.

DNA topoisomerases II are nuclear enzymes that modify DNA topology by their ability to break and reseal both strands in concert. Topoisomerases II have important functions in DNA replication and can serve as a cancer chemotherapy target. Indeed, drugs such as etoposide (VP-16) or mitoxantrone, form drug-topoisomerase II-DNA ternary complexes referred to as “cleavable complex.” The primary cytotoxic effect of these so-called “topoisomerase II inhibitors” is not by inhibition of topoisomerase II activity but rather by stabilizing topoisomerase II cleavable complexes. This interaction prevents the DNA-resolving step normally catalyzed by topoisomerase II. The ternary complex constitutes a latent DNA-damaging state, which is ultimately converted to an irreversible DNA double-strand break (DSB). Although the mechanism by which complex formation mediates cell death is still poorly understood, it has been largely documented with few exceptions that the amount of cleavable complexes and the subsequent number of DNA breaks correlates with cytotoxicity (1). These observations suggest that abnormal intracellular distribution or a decrease in expression level, activity, and sensitivity of the inhibited topoisomerase may have major impacts on topoisomerase inhibitor clinical efficacy. This has been confirmed by the molecular characterization of the so-called atypical multidrug resistant phenotype (at-MDR) resulting from selection by topoisomerase II inhibitors. Indeed, at-MDR cells display cross-resistance to other topoisomerase II inhibitors and have been associated with a number of functional and/or structural topoisomerase II alterations, including decreased catalytic activity, abnormal interaction between topoisomerase II and nuclear matrix, reduced expression, point mutation and, finally, altered phosphorylation (2).

The role of phosphorylation on topoisomerase II function has been debated and remains controversial. Indeed, previous studies have shown that topoisomerase II contains potential serine phosphorylation sites and that this enzyme is a substrate for various serine kinases, including casein kinase II, p34cdc2 kinase, and classic protein kinase C (PKC). In a cell-free system, PKC-induced phosphorylation of topoisomerase II results in an increase in its catalytic activity by enhancing ATP hydrolysis (3, 4). In the absence of antineoplastic drugs, phosphorylation has a negligible effect on other steps of topoisomerase II catalytic cycle, including DNA binding or DNA cleavage/religation equilibrium. However, in the presence of VP-16 or amscarine, phosphorylation decreases the ability of drugs to stabilize DNA-topoisomerase II complexes, apparently by increasing the

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1 The abbreviations used are: DSB, double-strand breaks; MTT, 3-(4,5-dimethyltetrazol-2-yl)-2,5-diphenyltetrazolium bromide; at-MDR, atypical multidrug resistant phenotype; DPC, DNA protein cross-links; VP-16, etoposide; rH-PKCζ, recombinant human PKCζ; MBP, myelin basic protein; PKCζ, protein kinase Cζ; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; MAPK, mitogen-activated protein kinase.
rates of religation of DNA by the enzyme (5). Other studies have provided indirect evidences that PKC might also influence topoisomerase II function in vivo. For example, PKC inhibitors, such as suramin or staurosporine, decrease topoisomerase II phosphorylation and catalytic activity in intact cells as well as drug-induced topoisomerase II-mediated cleavage (6, 7). However, the role of topoisomerase II phosphorylation in drug resistance has been minimized on the basis of independent studies that have shown that, in at-MDR cells, topoisomerase II could be either hyperphosphorylated or hypophosphorylated (8–10).

At least 12 different isoforms of PKC have been characterized so far and have been separated into three categories based on the Ca

$^{2+}$ requirement for activation and phorbol ester binding activity. Conventional PKCs (α, β, βII, and γ) are Ca

$^{2+}$-dependent phorbol ester receptor kinases; novel PKCs (δ, ε, θ, and η) are Ca

$^{2+}$-independent phorbol ester receptor kinases; and atypical PKCs (ζ, ι, λ, and υ) are independent of both Ca

$^{2+}$ and phorbol ester. Previous studies have shown that topoisomerase II is phosphorylated in vitro by each of the conventional PKC isoforms (11). However, the influence of these PKC isoforms on cellular topoisomerase function in vivo is still largely unknown. Moreover, to the best of our knowledge, the influence of atypical PKC isoforms on topoisomerase II phosphorylation and function has not been investigated.

PKC ζ is an atypical PKC isoform, which is activated directly or indirectly by a variety of important signaling molecules, including ceramide (12, 13), phosphatidic acid (14), and diacetyl-glycerol generated from phosphatidylcholine hydrolysis (15), phosphoinositide 3-kinase lipid products (16), and p21Ras (17). PKC ζ has emerged as a critical regulator of a number of cellular functions, including proliferation, differentiation, and apoptosis inhibition (18). Despite the critical role of this enzyme in cellular signaling, its implication in the regulation of topoisomerase II function has never been examined. This study was aimed to evaluate the effect of PKC ζ overexpression on the formation of cleavable complexes and cytotoxicity induced by VP-16 in the human leukemic U937 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant PKCζ and purified PKC containing α, β, and γ isoforms were purchased from Calbiochem (San Diego, CA). Myelin basic protein (MBP) was from Sigma (St. Quentin-Fallavier, France). Anti-topoisomerase II and anti-topoisomerase II antibodies were obtained from Santa-Cruz/TEBU (Le Perray en Yvelines, France). Anti-PKCζ antibody was purchased from UBI/Euromedex (Souffelweyersheim, France). Anti-phosphoserine was obtained from Zymed Laboratories Inc. (Montrouge, France). Anti-phosphoserine was obtained from Zymed Laboratories Inc. (Montrouge, France). Anti-phosphoserine was obtained from Zymed Laboratories Inc. (Montrouge, France). Topoisomerase II was from USB (Cleveland, OH), and topoisomerase II α and β were from Dr. Y. Pom- mier (NCI, National Institutes of Health, Bethesda, MD). Kinetoplast DNA was from TopoGen (Columbus, OH). [γ-32P]ATP (7000 Ci/mmol) was purchased from ICN (Orsay, France). [methyl-3H]Thymidine (79 Ci/mmol) and an ECL detection system were from Amersham Biosciences (Les Ulis, France).

**Cell Culture**—U937 cells were transfected by electroporation at 0.25 kV and 960 farads either with 20 µg of the PKCζ plasmid (corresponding to FIG. 1. Influence of PKCζ overexpression on topoisomerase II inhibitor-induced cytotoxicity in U937 cells. U937-neo cells ○, U937-ζJ cells ( ), and U937-ζB cells ( ) were treated either with VP-16 (A, C) or mitoxantrone (B, D) for 48 h. Cell viability was assayed by MTT assay (A, B), and apoptotic cells were determined by 4′,6-diamidino-2-phenyl-indole staining (C, D). Results are means ± S.D. of three independent experiments.
PKCζ Inhibits Topoisomerase II Catalytic Activity

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...ing to full-length rat PKCζ or 20 μg of the vector without the PKCζ insert using a Bio-Rad Gene Pulser as previously described (19). For this study, two clones, U937-J and U937-B, were selected and were compared with control U937-neo cells. Cells were cultured in RPMI supplemented with 10% fetal calf serum. U937-J, U937-B, and U937-neo were cultivated with similar growth kinetics with a doubling time of about 25 h. PKCζ overexpression resulted in a 2.5-fold increase in PKCζ activity as measured by MBP phosphorylation after immunoprecipitation with anti-PKCζ antibody.

MTT Assay—This assay is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into an insoluble formazan precipitate, which is dissolved in dimethyl sulfoxide and quantified by spectrophotometry. Cells (30,000) were seeded in 96-well plates and treated with cytotoxic agents for 48 h. Absorbance corresponding to MTT conversion was read at two wavelengths, 540 and 690 nm.

DNA Filter Elution Assays—Exponential growing cells were labeled with [3H]thymidine (0.02 μCi/ml) for 48 h, chased for 2 h in isotope-free medium, and exposed to VP-16 for the indicated time. Equal numbers of cells (5 × 10⁶) were loaded onto polycarbonate or PVC filters, lysed, and subjected to elution (20). Radioactivity in the DNA fractions was counted, and the fraction of the DNA retained on the filter was calculated as follows: fraction retained/[(filter plus lysis plus fraction retained) − lysis] 

Protein Analysis—For cytoplasmic protein, 1 × 10⁶ cells were washed twice in phosphate-buffered saline and lysed by resuspension in lysis buffer containing 10 mM HEPES (pH 7.8), 100 mM EDTA, 100 mM EGTA, 1 mM PMSF, 2 mM pepstatin A, 0.6 μg/ml aprotinin on ice for 10 min. Nonidet P-40 (0.3%) was then added for 2 min, and the cytoplasmic lysate (supernatant) was collected after centrifugation at 10,000 g for 2 min at 4 °C. For nuclear lysate, 1 × 10⁷ cells were washed twice in phosphate-buffered saline and lysed by resuspension in lysis buffer containing 10 mM HEPES (pH 7.8), 100 mM EDTA, 100 mM EGTA, 1 mM PMSF, 2 mM pepstatin A, 0.6 μg/ml aprotinin on ice for 10 min. Nonidet P-40 was then added at 0.3% final for 5 min, and the nuclear pellet was resuspended in 20 mM HEPES (pH 7.8), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA. Aliquots were sonicated and centrifuged at 20,000 g for 10 min at 4 °C, and supernatants containing nuclear proteins were collected. Nuclear or total cell lysates were resuspended in a denaturing loading buffer, and proteins were loaded in SDS-PAGE (7.5 or 10%), transferred onto nitrocellulose, and probed with anti-topoisomerase II and/or β or anti-PKCζ antibodies. Immune complexes were detected by using the chemiluminescence detection system.

Preparation of Nuclear Extracts—Cells (1 × 10⁶) were washed once with phosphate-buffered saline and twice with buffer A (1 mM KH₂PO₄, 5 mM MgCl₂, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.5% Triton X-100). Cells were then centrifuged and resuspended in 100 μl of buffer A. One-hundred microfilters of buffer A containing 0.55 mM NaCl was then added. After mixing and gentle rotation for 30 min at 4 °C, samples were centrifuged for 10 min at 14,000 rpm. Supernatants were used as nuclear extracts.

Topoisomerase II Decatenation Assay—Decatenation assays were carried out by incubating 0.25 μg of kinetoplast DNA with nuclear extracts or recombinant proteins in buffer B containing 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 15 μg/ml bovine serum albumin, 1 mM ATP. After 30 min at 30 °C, reactions were quenched by addition of 1% SDS, 0.5% bromphenol blue, and 30% glycerol. DNA products were resolved on 1% agarose-TBE gel at 16 V overnight. Agarose gels were stained with ethidium bromide, and fluorescence was quantified by UV imager.

Topoisomerase II Phosphorylation Analysis—Extracts were prepared by lysing cells (15 × 10⁶) in buffer C containing 50 mM HEPES, pH 7.0, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 10 mM NaCl, 100 mM NaF, 1 mM NaVO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, and 10 mM DTT. Cells extracts (1.5 mg) were sonicated, clarified, and immunoprecipitated with 3 μg of anti-PKCζ antibody overnight at 4 °C. Immune complexes were collected by incubation with protein G-Sepharose beads for 60 min at 4 °C. The beads were then extensively washed with buffer D. For in vitro interaction, recombinant PKCζ was preincubated with topoisomerase IIb for 1 h at 32 °C in buffer B and immunoprecipitated with 3 μg of anti-PKCζ antibody overnight at 4 °C. Immune complexes were collected with protein A-Sepharose beads for 60 min at 4 °C. The beads were then washed with buffer B. Denaturing loading buffer was added to immune complexes from in vivo or in vitro experiments. Samples were boiled for 5 min, run in SDS-PAGE (7.5%), transferred onto nitrocellulose membrane, and probed with anti-topoisomerase II antibodies. Proteins were detected by chemiluminescence.

Co-immunoprecipitation of PKCζ and Topoisomerase II—Extracts were prepared by lysing cells (15 × 10⁶) in buffer C. Cells extracts (1.5 mg) were sonicated, clarified, and immunoprecipitated with 3 μg of anti-PKCζ antibody overnight at 4 °C. Immune complexes were collected by incubation with protein G-Sepharose beads for 60 min at 4 °C. The beads were then extensively washed with buffer D. For in vitro interaction, recombinant PKCζ was preincubated with topoisomerase IIb for 1 h at 32 °C in buffer B and immunoprecipitated with 3 μg of anti-PKCζ antibody overnight at 4 °C. Immune complexes were collected with protein A-Sepharose beads for 60 min at 4 °C. The beads were then washed with buffer B. Denaturing loading buffer was added to immune complexes from in vivo or in vitro experiments. Samples were boiled for 5 min, run in SDS-PAGE (7.5%), transferred onto nitrocellulose membrane, and probed with anti-topoisomerase II antibodies. Proteins were detected by chemiluminescence.

PKCζ Activity—Cells (5 × 10⁶) were lysed in 20 mM HEPES, pH 7.4, 0.5 mM EDTA, 125 mM NaCl, 0.1% Nonidet P-40, 1 mM NaVO₃, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 0.5 mg/ml benzamidine, and 1 mM DTT. Cell extracts (300 μg) were immunoprecipitated with 3 μg of anti-PKCζ antibody overnight at 4 °C. Immune complexes were collected by incubation with protein G-Sepharose beads for 60 min at 4 °C. The beads were then extensively washed four times with lysis buffer, twice with kinase buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 25 mM NaCl) and finally incubated with a buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 1 mM DTT. For in vitro experiments, recombinant proteins were incubated in buffer B. Kinase assays were performed for 15 min at 32 °C using MBP as substrate and 10 μCi of [γ-32P]ATP. Reactions were stopped by addition of 15 μl of 2× SDS buffer and boiled for 5 min, and the samples were loaded for SDS-PAGE analysis (10%). Phosphorylated MBP levels were analyzed by autoradiography.

Phosphorylation of Topoisomerase II β by PKCζ—Recombinant PKCζ was preincubated with topoisomerase II β for 1 h at 32 °C in 20 μl of buffer B and 10 μCi of [γ-32P]ATP and 4 μg of phosphatidylinserine. Reactions were stopped by addition of 5 μl of 4× SDS buffer and boiled for 5 min, and the samples were loaded for SDS-PAGE (7.5%). Phosphorylated topoisomerase II levels were analyzed by autoradiography.

RESULTS

Influence of PKCζ Overexpression on Topoisomerase II Inhibitor-induced Cytotoxicity in U937 Cells—U937-neo cells and...
two clones overexpressing PKCζ, U937-ζJ, and U937-ζB, were treated with the topoisomerase II inhibitors VP-16 and mitoxantrone, and cell viability was evaluated 48 h later using the MTT assay. U937-ζJ or U937-ζB cells were 5-fold more resistant than U937-neo cells to VP-16 (Fig. 1A). PKCζ overexpression conferred an even more efficient protection against mitoxantrone-induced cytotoxicity (Fig. 1B). PKCζ overexpression was also found to inhibit VP-16- and mitoxantrone-induced apoptosis as evaluated by 4’,6-diamidino-2-phenyl-indole staining (Fig. 1, C and D). These results showed that PKCζ overexpression conferred resistance to topoisomerase II inhibitors. We then investigated the influence of PKCζ overexpression on topoisomerase II expression in U937 cells. Whole U937-neo, U937-ζJ, and U937-ζB cell extracts were subjected to immunoblot analysis with anti-topoisomerase IIα and anti-topoisomerase IIβ antibodies. Data are from one experiment representative of three experiments.

Influence of PKCζ Overexpression on VP-16-induced DNA Damage

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Strand Breaks in U937 Cells—U937-neo and U937-\(\zeta\)-J cells were prelabeled with \(^{3}H\)thymidine for 48 h, chased with fresh medium, and treated with VP-16 for 1 h and DSB were determined using alkaline elution (20, 21). As shown in Fig. 2, VP-16 produced significantly less DNA DSB in U937-\(\zeta\)-J cells than in U937-neo cells. VP-16-induced DPC were also compared in U937-neo and U937-\(\zeta\)-J cells. As shown in Fig. 3, whereas VP-16 induced DPC in a dose-dependent manner in both U937-neo and U937-\(\zeta\)-J cells, the levels of DPC were significantly lower in U937-\(\zeta\)-J. These results showed that PKC\(\zeta\) overexpression resulted in reduced VP-16-induced DNA damage. To rule out the possible influence of PKC\(\zeta\) on drug transport, we measured VP-16-induced DPC in isolated nuclei from U937-neo and U937-\(\zeta\)-J cells. As shown in Fig. 4, VP-16 produced a dose-dependent increase in DPC in U937-neo nuclei, whereas there was no detectable DPC formation in U937-\(\zeta\)-J nuclei. This result confirmed that PKC\(\zeta\) overexpression resulted in a significant
reduction in VP-16-induced DNA damage, which could not be explained by altered drug transport.

Influence of PKCζ Overexpression on Topoisomerase II Expression in U937 Cells—To investigate the possible influence of PKCζ on topoisomerase II expression, U937-neo, U937-ζJ, and U937-ζB cell extracts were analyzed by Western blotting with anti-topoisomerase IIα and anti-topoisomerase IIβ antibodies. As shown in Fig. 5, topoisomerase IIα and topoisomerase IIβ expression levels in U937-neo, U937-ζJ, and U937-ζB cells were comparable. This result suggests that reduced VP-16-induced DNA damage in PKCζ-overexpressing cells was not due to decreased topoisomerase II expression. For this reason, we hypothesized that PKCζ overexpression may result in reduced topoisomerase II activity.

Influence of PKCζ Overexpression on Topoisomerase II Activity in U937 Cells—Decatination of kinetoplast DNA was used as a specific assay to evaluate topoisomerase II activity of nuclear extracts prepared from U937-neo, U937-ζJ, and U937-ζB cells (22). Nuclear extracts from U937-neo cells exhibited topoisomerase II activity in a dose range comprised between 500 and 1000 ng of total nuclear protein, whereas topoisomerase II activities contained in U937-ζJ and U937-ζB preparations were dramatically reduced (Fig. 6A). Based on Western blot analysis of nuclear cell extracts, it appeared that nuclear PKCζ expression was inversely correlated with topoisomerase II activity (Fig. 6B). For this reason, we hypothesized
that PKCζ inhibited topoisomerase II activity by influencing topoisomerase II phosphorylation.

**Influence of PKCζ Overexpression on Serine Phosphorylation of Topoisomerase II in U937 Cells**—U937-neo, U937-ιζ, and U937-ιβ cell extracts were immunoprecipitated with anti-phosphoserine antibody, and topoisomerase II was immunoblotted with anti-topoisomerase IIα and anti-topoisomerase IIβ antibodies. As shown in Fig. 7, U937-ιζ and U937-ιβ cells exhibited constitutive topoisomerase IIα and β serine hyper-phosphorylation, compared with U937-neo cells, whereas anti-phosphothreonine antibody, used as control, was not reactive. This result shows that PKCζ increased phosphorylation of both isoforms of topoisomerase II.

**Interaction between PKCζ and Topoisomerase II in U937-neo and PKCζ Overexpressing U937 Cells**—PKCζ/topoisomerase II interaction was assessed by immunoprecipitation. However, because anti-topoisomerase IIα- and β-specific antibodies used in this study were not suitable for immunoprecipitation, cellular extracts of U937-neo, U937-ιζ, and U937-ιβ were immunoprecipitated with anti-PKCζ antibody and topoisomerase IIα and β proteins were immunoblotted with relevant antibodies. In U937-neo cell extracts, we found that PKCζ interacted neither with topoisomerase IIα nor with topoisomerase IIβ, although a significant PKCζ amount was detected in the immunoprecipitates (Fig. 8). However, in U937-ιζ and U937-ιβ cellular extracts, both topoisomerase IIα and β co-immunoprecipitated with PKCζ (Fig. 8). These results suggest that, in PKCζ-overexpressing U937 cells, the enzyme may directly or indirectly interact with both topoisomerase IIα and β isoforms and that these interactions seriously interfere with topoisomerase II activity. To investigate this hypothesis, we evaluated in a cell-free system the influence of recombinant PKCζ on the activity of purified topoisomerase II preparations containing both topoisomerase IIα and β.

**Influence of PKCζ on Purified Topoisomerase II Activity in a Cell-free System**—In these experiments, recombinant human PKCζ (rH-PKCζ) (1 or 3 μg) was incubated with topoisomerase II (50 ng), and topoisomerase II activity was measured by the decatenation assay. As shown in Fig. 9A, PKCζ was found to inhibit topoisomerase II activity in a dose-dependent manner.

In contrast, a mixture containing PKCα, PKCβ, and PKCγ was found to stimulate topoisomerase II activity as previously described (Fig. 9B) (11). This result suggests that PKCζ does interfere with either topoisomerase IIα or β activities. To further investigate this finding, we evaluated the capacity of PKCζ to interact with purified topoisomerase IIα or β.

**Interaction between rH-PKCζ and Purified Topoisomerase II Isoforms in a Cell-free System**—rH-PKCζ was co-incubated with either topoisomerase IIβ or topoisomerase IIα in a molar ratio of 2:1. PKCζ/topoisomerase II complexes were immunoprecipitated using anti-PKCζ antibody, and topoisomerase isoforms were revealed by immunoblotting with specific anti-topoisomerase antibodies. Controls were provided by immunoblotting immunoextracts with anti-PKCζ (Fig. 10A). As shown in Fig. 10B, a small amount of topoisomerase IIβ nonspecifically bound to protein A-Sepharose. However, the level of topoisomerase IIβ detected following immunoprecipitation with anti-PKCζ antibody was significantly increased, suggesting that most of topoisomerase IIβ specifically bound to PKCζ. In contrast, despite many efforts, we were unable to detect topoisomerase IIα in anti-PKCζ immunoextracts (data not shown). These results showed that PKCζ was able to interact with topoisomerase IIβ but not topoisomerase IIα. The consequence of this interaction on topoisomerase II phosphorylation was also investigated. Topoisomerase IIβ (1 μg) was incubated with rH-PKCζ (1 μg) in the presence of [γ-32P]ATP and phosphatidylserine, and phosphorylated topoisomerase IIβ was revealed by autoradiography. PKCζ kinase activity was checked using MBP as a substrate (Fig. 10C). Topoisomerase IIβ was found to be constitutively phosphorylated. However, incubation with PKCζ resulted in a 2-fold increase in topoisomerase IIβ phosphorylation (Fig. 10D). Altogether, these results demonstrated that PKCζ did interact with topoisomerase IIβ and phosphorylated this enzyme.

**Influence of rH-PKCζ on Topoisomerase II Activity**—In these experiments, rH-PKCζ was incubated with topoisomerase IIβ at a molar ratio of 2:1, and topoisomerase IIβ activity was measured by the decatenation assay. As shown in Fig. 11, PKCζ was found to inhibit topoisomerase IIβ activity.
PKCζ Inhibits Topoisomerase II Catalytic Activity

DISCUSSION

This study shows that PKCζ overexpression in U937 cells resulted in inhibition of apoptosis and increased survival of U937 cells treated with VP-16 and mitoxantrone, two topoisomerase II inhibitors. Enforced PKCζ expression resulted in a marked decrease in VP-16-induced DPC and DNA DSB, whereas the level of topoisomerase IIα and topoisomerase IIβ expression was unchanged compared with control cells. These results suggest that PKCζ can interfere with topoisomerase II function. In fact, we found that PKCζ-overexpressing cells exhibited reduced topoisomerase II catalytic function as measured by the decatenation assay. Altered topoisomerase II catalytic cycle may explain reduced drug-induced DNA damage and cytotoxicity. Thus, this study shows for the first time that a specific PKC isozyme may inhibit topoisomerase II catalytic activity and VP-16-induced apoptosis and cytotoxicity by interfering with drug-induced DNA damage.

Based on the kinase function of PKCζ, we hypothesized that PKCζ overexpression might result in abnormal topoisomerase II phosphorylation. In fact, we found that, in PKCζ-overexpressing cells, PKCζ was not only found to interact with topoisomerase IIα and topoisomerase IIβ but also that these two topoisomerase II isoforms were heavily phosphorylated on serine residues. These results suggest that, in PKCζ-overexpressing cells, PKCζ not only directly or indirectly interacts with the two topoisomerase II isoforms but also phosphorylates these enzymes. However, using a cell-free system, we described that only topoisomerase IIα is a substrate for PKCζ and that PKCζ inhibits topoisomerase IIβ activity. This result suggests that, in PKCζ-overexpressing cells, PKCζ interacts directly with topoisomerase Iβ and inhibits topoisomerase Iβ catalytic activity. This hypothesis is consistent with the role of this topoisomerase Iβ form in the cytotoxicity of topoisomerase II inhibitors (23, 24).

With regard to topoisoerase IIα, the fact that this enzyme was found to interact in situ but not in vitro, with PKCζ suggests that, in PKCζ-overexpressing cells, PKCζ/topoisomerase IIα interaction involves one or several other proteins required for the constitution of this complex. Moreover, the fact that, in PKCζ-overexpressing cells, topoisomerase IIα was found to be constitutively phosphorylated whereas, in vitro, PKCζ was unable to phosphorylate this enzyme, suggests that topoisomerase IIα is phosphorylated by another PKCζ-regulated kinase. In this perspective, it is interesting to note that in a recent study topoisomerase IIα was found to be phosphorylated in intact cells by ERK2, the effector serine kinase of the classic MAPK module (25). Based on previous studies, which have documented that PKCζ is a downstream target of MAPK (26, 27), topoisomerase IIα phosphorylation could result from PKCζ-mediated ERK2 activation in PKCζ-overexpressing cells. The fact that, in these cells, ERK2 was found to be constitutively activated and accumulated in the nucleus (data not shown) supports this hypothesis.

The role of atypical PKC isoforms, including PKCζ, in cell survival has been previously documented. Indeed, it has been described that the blockade of PKCζ or PKCζ/ is with dominant-negative mutants or antisense oligonucleotides is sufficient to promote apoptosis (28, 29). The inactivation of PKCζ by caspase-dependent proteolysis during apoptosis induced by UV (30) or by cisplatin (31) strengthens the role of PKCζ in the cellular protection against genotoxic stress. The mechanism by which atypical PKC isoforms exert their anti-apoptotic effect has received a great deal of attention. These studies strongly suggested that NF-κB signaling pathways could play an important role in PKCζ-induced inhibition of apoptosis (32). Indeed, NF-κB is a negative regulator of apoptosis induced by genotoxic agents, including topoisomerase II inhibitors (33, 34). Therefore, we cannot rule out that PKCζ overexpression may result in the activation of anti-apoptotic signals that interfere with the post-damage apoptotic response and, therefore, contribute to drug resistance.

To conclude, we propose a model in which, upon PKCζ accumulation in the nucleus, this enzyme interacts with and phosphorylates nuclear topoisomerase IIβ. Topoisomerase IIβ hyperphosphorylation reduces catalytic function and decreases formation of ternary complexes and drug-induced cytotoxicity. If so, nuclear PKCζ accumulation might function to regulate topoisomerase II function. Although very little is known about expression and subcellular localization of PKCζ in tumor cells, PKCζ may translocate to the nucleus upon stimulation by differentiating agents (35), growth factors (36, 37), cytokines (38), or hypoxia (39). Whether PKCζ alters topoisomerase II function in these conditions will be the subject of further investigations.

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