0026-895X/95/050798-08$3.00/0

MOLECULAR PHARMACOLOGY, 48:798–805 (1995).

Hypophosphorylation of Topoisomerase II in Etoposide (VP-16)-Resistant Human Leukemia K562 Cells Associated with Reduced Levels of β_{II} Protein Kinase C

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Received June 13, 1995; Accepted August 10, 1995

SUMMARY

We selected and characterized a 30-fold etoposide (VP-16)-resistant subline of K562 human leukemia cells (K/VP.5) that exhibits quantitative and qualitative changes in topoisomerase II, including hypophosphorylation of this drug target. The initial rate of topoisomerase II phosphorylation was reduced 3-fold in K/VP.5 compared with K562 cells, but the rate of dephosphorylation was similar. Analysis of potential topoisomerase II protein kinases revealed a 3-fold reduction in the level of the β_{II} protein kinase C (PKC) in K/VP.5 cells, whereas levels of α- and ϵ PKC, casein kinase II, $p42^{map}$ kinase, and $p34^{cdc2}$ kinase were comparable for both cell lines. The PKC activator, bryostatin 1, together with K562 nuclear extracts potentiated VP-16-induced topoisomerase II/DNA covalent complex formation in nuclei isolated from K/VP.5 cells but not from K562 cells. Bryostatin 1 effects were blocked by the PKC inhibitor 7-*O*-methyl-hydroxy-staurosporine. Bryostatin 1 also up-regulated topoisomerase II phosphorylation and potentiated VP-16 activity in intact K/VP.5 cells but had no enhancing effect in K562 cells. 4β-Phorbol-12,13-dibutyrate and 12-*O*-tetradecanoylphorbol-13-acetate did not potentiate VP-16-induced topoisomerase II/DNA complex formation in intact cells or in isolated K/VP.5 nuclei. Together, our results indicate that β_{II} PKC plays a role in modulating the VP-16-induced DNA binding activity of topoisomerase II in resistant K/VP.5 cells through a mechanism linked to phosphorylation of topoisomerase II.

The DNA binding protein topoisomerase II (M_r 170,000 isoform) is a target for several clinically effective anticancer drugs, including VP-16 (1–3). The cytotoxic effects of topoisomerase II inhibitory drugs are due to the accumulation of DNA strand breaks secondary to stabilization of a covalent topoisomerase II/DNA complex (1). A number of tumor cell lines resistant to the inhibitory effects of topoisomerase II targeting drugs have been selected after exposure to topoisomerase II inhibitors or on treatment with mutagens followed by selection in the presence of topoisomerase II inhibitors (4–14). Acquired resistance to topoisomerase II inhibitors in these cells is usually characterized by a reduction in drug-induced topoisomerase II/DNA covalent complexes in resistant compared with sensitive cells, which in turn is attributed to a reduction of enzyme levels (4–8) or to an alteration in topoisomerase II activity (8–14).

Topoisomerase II exists in cells as a phosphoprotein; this post-translational modification occurs primarily on serine residues in the carboxyl domain of the enzyme (15, 16). Studies using synchronized cells and *in vitro* phosphorylation of purified topoisomerase II have demonstrated a correlation between phosphorylation and topoisomerase II catalytic activity, suggesting that phosphorylation may be an important mechanism for regulating topoisomerase II function (17–19). For example, the extent of phosphorylation of topoisomerase II increases as cells enter G_2/M phase of the cell cycle, when activity of the enzyme is greatest (17–18). More recently, peptide mapping of topoisomerase II at various times after synchronization of Chinese hamster ovary cells revealed that the distribution of phosphorylated serine residues changes as cells progress through the cell cycle (19). Also, *in vitro* phosphorylation of topoisomerase II with casein

This work was supported by American Cancer Society Grant DHP49 (J.C.Y.) and National Institutes of Health Grants GM43186 and CA56869 (A.P.F.). M.K.R. is the recipient of a Pittsburgh Cancer Institute Postdoctoral Fellowship.

ABBREVIATIONS: topoisomerase II, DNA topoisomerase II ; VP-16, etoposide [4′-demethylepipodophyllotoxin 9-(4,6–0-ethylidene-β-D-glucopyranoside)]; mAMSA (amsacrine), 4′(9-acridinylamino)methane-sulfon-*m*-anisidide; $p42^{map}$ kinase, 42-kDa mitogen-activated protein kinase; n-HexylILV, lyngbyatoxin analogue (−)-*n*-hexylindolactam V; PKC, protein kinase C; PdBU, 4β-phorbol-12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SDS, sodium dodecyl sulfate; DMSO, dimethylsulfoxide; UCN-01, 7-*O*-methyl-hydroxy-staurosporine; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis.

kinase II or PKC increases the catalytic activity of the enzyme by increasing the rate of hydrolysis of ATP, thus escalating enzyme turnover (20–22). Finally, purified casein kinase II and yeast topoisomerase II have been shown to form a stable complex, resulting in enhanced topoisomerase II phosphorylation and a 2–3-fold increase in topoisomerase II catalytic activity (23).

It is not clear what role topoisomerase II phosphorylation plays in the induction of DNA damage by topoisomerase II inhibitors. In one study, *in vitro* phosphorylation of purified *Drosophila* topoisomerase II slightly decreased VP-16 or mAMSA-induced topoisomerase II-mediated cleavage of plasmid DNA (24). In a separate report, topoisomerase II was found to be more phosphorylated in a VP-16-resistant human KB cell line that exhibited decreased VP-16-induced DNA cleavage compared with parental KB cells (25). In another study, topoisomerase II from mAMSA-resistant HL-60 cells was shown to be translated and phosphorylated at a 2–3-fold slower rate, although the enzyme was more stable and dephosphorylated at a reduced rate compared with parental HL-60 cells (26). These findings suggest that critical phosphorylation site(s) of topoisomerase II may affect the sensitivity of cells to topoisomerase II inhibitors.

The serine-threonine kinases, casein kinase II (15, 20), PKC (15, 21), and $p34^{cdc2}$ kinase (15) phosphorylate topoisomerase II *in vitro*. Phosphopeptide mapping of *Drosophila* and yeast topoisomerase II indicated that the pattern of *in vivo* phosphorylation of this enzyme resembles that of purified topoisomerase II phosphorylated *in vitro* by casein kinase II (15, 22, 27), suggesting that casein kinase II is the major kinase responsible for phosphorylation of topoisomerase. However, several reports suggest that PKC may also play a role in phosphorylation and/or regulation of topoisomerase II (28–30). Although these studies suggest that topoisomerase II is an important component of PKC-mediated differentiation in HL-60 cells, PKC-mediated phosphorylation of topoisomerase II has not been directly demonstrated.

PKC plays an important role in signal transduction cascades resulting from mitogenic and differentiation-inducing stimuli (31). There are three isotypes of PKC (α, β_{II}, and ϵ) expressed in K562 cells (32, 33). In K562 cells, PMA-induced differentiation and inhibition of growth resulted in an increase in α- and ϵ PKC and a decrease in β_{II} PKC levels (33). In contrast, exposure of K562 cells to bryostatin 1 did not inhibit proliferation or elicit changes in PKC isoform levels (33). Bryostatin 1 treatment of K562 and HL-60 cells results in nuclear translocation/activation of β_{II} PKC, consistent with a role for this protein kinase in supporting cell proliferation (32–34). Nuclear β_{II} PKC directly phosphorylates lamin B contributing to nuclear envelope breakdown, an event required for mitosis (35). Compatible with a function in cell cycle progression, β_{II} PKC selectively translocates from the cytosol to the nucleus during G_2/M phase (36). In addition, an endogenous nuclear membrane activation factor for β_{II} PKC has been identified (37). Because increased phosphorylation and activity of topoisomerase II are also associated with cell cycle progression into mitosis (17–19), these results invite further consideration of β_{II} PKC as a regulator of topoisomerase II activity in K562 cells.

We isolated and characterized a stable VP-16-resistant K562 cell line (K/VP.5), selected by continuous exposure to 0.5 μM VP-16 (8, 9). Compared with parental K562 cells, the K/VP.5 cell line contains reduced levels of topoisomerase II protein and exhibits decreased VP-16-induced DNA damage, more rapid dissociation of VP-16-induced covalent topoisomerase II/DNA complexes, and attenuated ATP stimulation of VP-16-induced topoisomerase II/DNA complexes (8, 9). In addition, topoisomerase II from resistant K/VP.5 cells is 2.6-fold less phosphorylated than enzyme from sensitive K562 cells (38). These observations suggest that resistance to VP-16 in K/VP.5 cells is due, at least in part, to reduced phosphorylation of topoisomerase II, which may ultimately decrease the stability of VP-16-induced topoisomerase II binding to DNA. Partial sequencing and single strand conformational polymorphism analyses of the entire topoisomerase II cDNA strongly suggest that the biochemical changes associated with topoisomerase II enzyme in K/VP.5 cells are not the result of mutations in the topoisomerase II gene (38). Thus, reduced phosphorylation of topoisomerase II is not due to mutation of a critical phosphorylation site. Alternatively, the biochemical changes in resistant topoisomerase II (in particular, reduced phosphorylation) may be attributable to a modulator of topoisomerase II, such as a protein kinase.

To investigate post-translational regulation of topoisomerase II and its effects on VP-16-induced topoisomerase II/DNA covalent complexes, we compared the rate of synthesis, turnover, and the rates of phosphorylation and dephosphorylation of topoisomerase II in K562 and K/VP.5 cells. In addition, we quantified the levels of several potential topoisomerase II protein kinases in these two cell lines. Finally, we examined the effect of PKC activators on topoisomerase II phosphorylation and on VP-16-induced topoisomerase II/DNA covalent complexes. Our results strongly suggest that changes in the levels of the β_{II} isoform of PKC affect topoisomerase II phosphorylation and, consequently, VP-16-induced topoisomerase II/DNA covalent complex formation in resistant K/VP.5 cells.

Materials and Methods

Cells. Human K562 cells and VP-16-resistant K/VP.5 cells (9) were grown in suspension in DMEM supplemented with 7% iron-supplemented newborn bovine serum (Hyclone, Logan, UT) and 2 mM L-glutamine (GIBCO-BRL, Bethesda, MD). Exponentially growing cells (4–8 $\times$ 10^5 cells/ml) were used for all experiments. K/VP.5 cells were maintained in the absence of VP-16 for at least 3 days before use in experiments.

Drugs and chemicals. VP-16 was obtained from Bristol-Myers Squibb, Co. (Wallingford, CT) and prepared as a concentrated stock solution by being dissolved in DMSO (unless specified, the final DMSO concentration in VP-16-treated and control cells was adjusted to 0.1%). Bryostatin 1 was a generous gift from Dr. W. S. May (The Johns Hopkins Oncology Center, Baltimore, MD). The lyngbyatoxin analogue n-HexylILV was provided by Dr. A. P. Kozikowski (Mayo Clinic, Jacksonville, FL). The phorbol esters PdBU and TPA were obtained from Sigma Chemical Co. (St. Louis, MO). The PKC inhibitor UCN-01 (NSC-638850) (39) was generously provided by Dr. E. Sausville (National Cancer Institute, Bethesda, MD).

Immunoprecipitation. Exponentially growing cells were pelleted, washed with saline, and then incubated with [^{35}S]methionine and [^{35}S]cysteine or [^{32}P]orthophosphate for various times as previously described (38). Resuspended cells (1 $\times$ 10^6/ml) were incubated with 10–30 μCi/ml EXPRE^{35}S^{35}S labeling mix (a mixture of [^{35}S]methionine and [^{35}S]cysteine; DuPont-NEN, Wilmington, DE) in methionine-free DMEM (GIBCO-BRL) and/or 100 μCi/ml [^{32}P]orthophos-

phate (ICN Biomedicals, Irvine, CA) in phosphate-free DMEM (GIBCO-BRL). Unless otherwise noted, cells incubated with ^{32}Pi were pooled with an equal number of ^{35}S-labeled cells. For some experiments, cells were then transferred from radiolabeled media to complete DMEM. At various times afterward, nuclear lysates were prepared from aliquots of radiolabeled cells as described previously (17). Topoisomerase II was immunoprecipitated from 50–100 μg nuclear lysate protein as previously described (38) using rabbit polyclonal anti-topoisomerase II sera produced in our laboratory (this antibody does not immunoprecipitate the 180-kDa [β] isoform of topoisomerase II). Topoisomerase II immunoprecipitated from nuclear lysates (100 μg protein) was resolved by 7% SDS-PAGE. Topoisomerase II bands (170 kDa) were localized by autoradiography using either X-ray film or phosphor screens (Molecular Dynamics, Sunnyvale, CA). Radiolabel incorporated into topoisomerase II from cells labeled with *either* ^{32}P or ^{35}S were quantified by phosphorimage analysis using ImageQuant software (Molecular Dynamics). Radiolabel incorporated into topoisomerase II from cells labeled with *both* ^{32}P and ^{35}S was detected by scintillation counting of 170-kDa bands excised from gels. For quantification of phosphorylation of topoisomerase II, ^{32}P cpm were normalized to ^{35}S cpm to account for the reduced levels of topoisomerase II in K/VP.5 compared with K562 cells (38).

Western blots. Whole cell lysates were prepared from 5 × 10^6 cells by dissolving cell pellets in SDS-PAGE sample buffer (0.125 M Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol). Lysates were sonicated, and 1–10 μg protein was loaded per well onto 10% SDS-PAGE gels. Resolved proteins were electrophoretically transferred to nitrocellulose and incubated with rabbit polyclonal antisera to one of the following kinases: α, β, or ε isoforms of PKC (40), casein kinase II (Upstate Biotechnologies, Lake Placid, NY), p34^{cdc2} kinase (Oncogene Science, Uniondale, NY), or monoclonal antibody for p42^{map} kinase (a gift from Dr. Michael Weber, University of Virginia, Charlottesville, VA). Blots were then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Upstate Biotechnologies) or horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma Chemical Co.). Bound antibodies were detected using enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). Autoradiographic signals were quantified by densitometric scanning using a LKB laser densitometer (LKB Pharmacia, Piscataway, NJ).

Topoisomerase II/DNA covalent complexes. Topoisomerase II/DNA covalent complex formation in intact cells and nuclei was measured as previously described (9, 12). Before use in assays, cells were prelabeled for 24 hr with 0.5 μCi/ml [methyl-^{3}H]thymidine and 0.1 μCi [U-^{14}C]leucine. Nuclei were isolated according to the method of Filipski and Kohn (41) by lysing radiolabeled cells with 0.3% Triton X-100. Whole cells (2 × 10^6/ml in 110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 25 mM 4-[2-hydroxy-ethyl]-1-piperazineethanesulfonic acid, and 10 mM glucose, pH 7.4) were treated in the presence or absence of 100 nM bryostatin 1 for 1–4 hr, followed by 20 μM VP-16 or 0.1% DMSO for 30 min at 37°. Isolated nuclei (2 × 10^6/ml) in buffer A (1 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, and 1 mM EGTA, pH 7.4) were incubated for 30 min at 37° with 100 nM bryostatin 1 alone or together with 100 nM UCN-01, 20 μM VP-16 or 0.1% DMSO, 1 mM ATP, and 60 μg of 1 M NaCl nuclear extracts from K562 cells (10). Topoisomerase II/DNA covalent complexes (^{3}H counts) were isolated by KCl/SDS precipitation and quantified by scintillation counting. DNA in precipitates was normalized to cell number using the coprecipitated ^{14}C-labeled protein as an internal standard (9, 12).

Alkaline elution analysis of DNA damage. Drug-mediated DNA damage was assessed using the alkaline elution technique for high frequency single-strand breaks (42). K562 and K/VP.5 cells were labeled for 48 hr with [2-^{14}C]thymidine (0.02 μCi/ml), after which nuclei were isolated by lysing radiolabeled cells with 0.3% Triton X-100 (41). Nuclei were suspended in buffer A to a final density of 1 × 10^6/ml and incubated 30 min at 37° with 20 μM VP-16, 1 mM ATP, and 0 or 40 μg K562 nuclear extract. The final solvent concentration in all conditions was 0.4% DMSO. K562 cells (7.5 × 10^5) containing [^{3}H]DNA (labeled for 48 hr with 0.1 μCi/ml [methyl-^{3}H]thymidine) were irradiated with 1500 rad on ice using a ^{137}Cs source (Mark Irradiator; J. L. Sheppard and Associates, Glendale, CA). These irradiated cells were added as internal standards to drug-treated and [^{14}C]-labeled K562 or K/VP.5 nuclei, and the mixture was layered onto a polyvinyl chloride filter (pore size, 2.0 μm; Gelman Sciences, Ann Arbor, MI) and lysed with a solution of 2% SDS, 10 mM disodium EDTA, and 0.5 mg/ml proteinase K. The DNA was eluted from the filter with tetrapropylammonium hydroxide, pH 12.1, at a flow rate of 0.16 ml/min, with a fractional interval of 5 min. The frequency of VP-16-induced DNA single-strand breaks was quantified as the fraction of [^{14}C]DNA retained on the filter when 60% of the ^{3}H-labeled internal standard DNA remained. A calibration curve for relating the frequency of VP-16-induced DNA single-strand breaks to a corresponding effect of radiation (radiation equivalent DNA damage) using ^{14}C-labeled nuclei was obtained by plotting rads versus [^{14}C]DNA retention at 60% retention of the [^{3}H]DNA internal standard (not shown).

Results

Synthesis and stability of topoisomerase II in K562 and K/VP.5 cells. Topoisomerase II was immunoprecipitated from nuclear lysates of cells labeled for various times with [^{35}S]methionine and [^{35}S]cysteine (EXPRE^{35}S^{35}S). Incorporation of radiolabel into topoisomerase II increased for up to 20 hr (Fig. 1A). The level of radiolabeled topoisomerase II at 20 hr was reduced more than 2-fold in nuclear lysates of K/VP.5 compared with K562 cells (Fig. 1A), consistent with the 3-fold reduction of topoisomerase II previously observed by Western blot analysis of K562 and K/VP.5 nuclei (9). Initial topoisomerase II synthesis rates were determined from experiments in which cells were labeled for 1–8 hr with EXPRE^{35}S^{35}S (Fig. 1B). The rate of topoisomerase II synthesis was reduced 2.9 ± 0.6-fold (mean ± standard error from three experiments; p = 0.06, paired Student's t test) for K/VP.5 compared with K562 cells. We next compared the stability of ^{35}S-labeled topoisomerase II from K562 and K/VP.5 cells. For these experiments, cells were prelabeled for

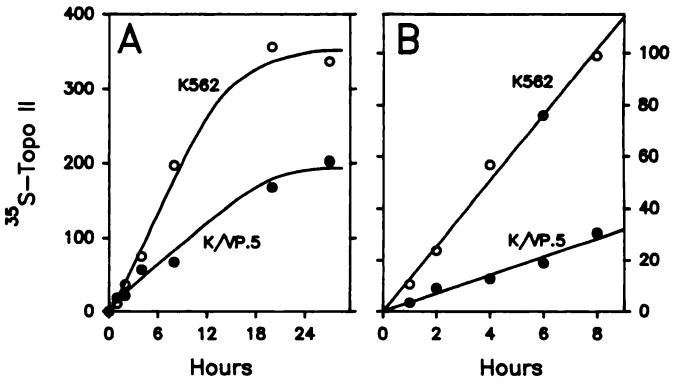

Fig. 1. Rate of synthesis of topoisomerase II in K562 and K/VP. 5 cells. Cells were incubated in the presence of 30 μCi/ml EXPRE^{35}S^{35}S labeling mix. At various times after the addition of radiolabel [1–28 hr (A) or 1–8 hr (B)], nuclear lysates were prepared from 5 × 10^6 cells. Topoisomerase II was immunoprecipitated from the lysates (50–100 μg protein) and resolved by electrophoresis, and ^{35}S signals were quantified by phosphorimage analyses of dried gels. Results are representative of three independent experiments. Ordinate values (*35S-Topo II*) are expressed as arbitrary units derived by quantifying the volume of the phosphorimager radioactive signal using ImageQuant software.

6 hr with EXPRE^{35}S^{35}S and then transferred to medium free of radiolabel. Topoisomerase II was immunoprecipitated from nuclear lysates and ^{35}S-labeled topoisomerase II remaining 3, 6, 9, 12, and 22 hr after removal of radiolabel was quantified. The half-life of ^{35}S-labeled topoisomerase II was 13.3 ± 1.7 hr for K562 topoisomerase II and 15.3 ± 0.9 hr for K/VP.5 topoisomerase II (mean ± standard error from three experiments; $p = 0.51$, paired Student's t test; not shown). Thus, the rate of turnover of topoisomerase II is comparable for both cell lines.

Phosphorylation and dephosphorylation of topoisomerase II in K562 and K/VP.5 cells. Topoisomerase II was immunoprecipitated from nuclear lysates of cells that were first prelabeled for 16 hr with EXPRE^{35}S^{35}S and then labeled for various times (1–8 hr) with [^{32}P]orthophosphate. Topoisomerase II phosphorylation was expressed as ^{32}P/^{35}S ratios to account for the different levels of enzyme in sensitive and resistant cells (Fig. 2). After accounting for the reduced levels of topoisomerase II in K/VP.5 cells, the rate of phosphorylation of topoisomerase II in these cells was reduced 2.9 ± 0.1-fold compared with K562 cells (mean ± standard error from three experiments; $p < 0.01$, paired Student's t test). The rate of dephosphorylation of topoisomerase II was determined in cells prelabeled for 6 hr with EXPRE^{35}S^{35}S and [^{32}P]orthophosphate (Fig. 3). Topoisomerase II was immunoprecipitated from nuclear lysates prepared 1, 3, 6, 9, 12, and 21 hr after removal of radiolabel, and ^{32}P and ^{35}S counts remaining were quantified. The rate of dephosphorylation of topoisomerase II was comparable in K562 and K/VP.5 cells ($t_{1/2}$ = 11.1 ± 0.6 and 11.1 ± 0.9 hr, respectively, mean ± standard error from three experiments; $p = 0.96$, paired Student's t test).

Kinase levels in K562 and K/VP.5 cells. The decreased rate of topoisomerase II phosphorylation in K/VP.5 compared with K562 cells suggested a reduction in the levels and/or activity of a kinase that phosphorylates topoisomerase II. Therefore, levels of several kinases in K562 and K/VP.5 cells were quantified by Western blot analysis of whole-cell lysates. Levels of α PKC, ε PKC (not shown), casein kinase II, p42^{map} kinase, and p34^{cdc2} kinase were comparable for both cell lines (Fig. 4). In contrast, β_{II} PKC levels were reduced 3.0 ± 0.4-fold in K/VP.5 compared with K562 cells (mean ± standard error of six determinations; $p < 0.01$, paired Student's t test). These data suggest that reduced phosphorylation of topoisomerase II and alterations in VP-16-induced topoisomerase II/DNA binding in K/VP.5 cells may be attributable to changes in the levels of β_{II} PKC in these cells.

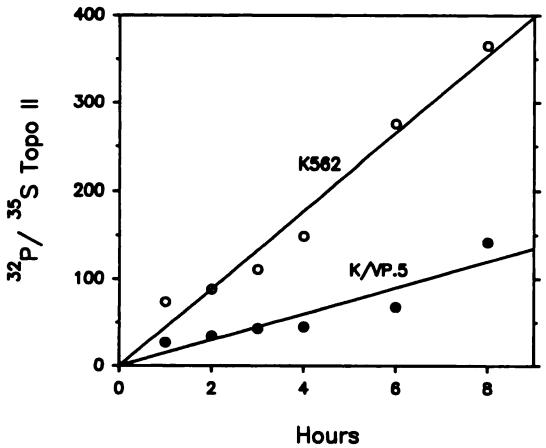

Fig. 2. Rate of phosphorylation of topoisomerase II in K562 and K/VP.5 cells. Cells prelabeled 16–18 hr with 10 μCi/ml EXPRE^{35}S^{35}S labeling mix were transferred to medium containing 100 μCi/ml [^{32}P]orthophosphate (^{32}Pi). At various times after the addition of ^{32}Pi (1–8 hr), nuclear lysates were prepared from 5 × 10^6 cells, and topoisomerase II was isolated as for Fig. 1. Topoisomerase II was localized by autoradiography, excised from gels, and quantified by scintillation counting. Results are expressed as ^{32}P/^{35}S ratios to account for the reduced rate of synthesis of topoisomerase II in K/VP.5 cells.

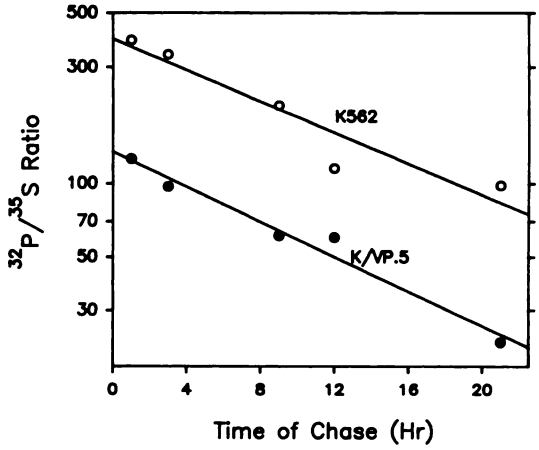

Fig. 3. Rate of dephosphorylation of topoisomerase II in K562 and K/VP.5 cells. Parallel flasks containing cells labeled for 6 hr with 30 μCi/ml EXPRE^{35}S^{35}S labeling mix or with 100 μCi/ml [^{32}P]orthophosphate (^{32}Pi) were pooled and then transferred to unlabeled medium. At various times after removal from radiolabeled media (1–21 hr), nuclear lysates were prepared from 5 × 10^6 cells, and topoisomerase II immunoprecipitated as for Fig. 1. Topoisomerase II was resolved by electrophoresis, localized by autoradiography, excised from gels, and quantified by scintillation counting. Results are expressed as ^{32}P/^{35}S ratios to account for the reduced levels of topoisomerase II in K/VP.5 cells.

Effect of K562 nuclear extract on VP-16-induced topoisomerase II/DNA complexes and VP-induced DNA breaks in K562 and K/VP.5 nuclei. VP-16-induced topoisomerase II/DNA complex formation is reduced 4-fold in K/VP.5 compared with K562 nuclei (8, 9). After normalizing for VP-16 effects in K562 and K/VP.5 nuclei, the addition of nuclear extract (40 μg of protein) from K562 cells potentiated the formation of 20 μM VP-16-induced topoisomerase II/DNA complexes 2.2-fold in K/VP.5 nuclei but did not enhance this VP-16-induced activity in K562 nuclei (Fig. 5A). In contrast, 40 μg of nuclear extract from K/VP.5 cells did not affect drug-induced topoisomerase II/DNA complex formation in nuclei from either cell line (not shown). Similarly, K562 nuclear extract (40 μg) potentiated VP-16-induced DNA single-strand breaks 2-fold in K/VP.5 but not in K562 nuclei (Fig. 5B). Thus, K562 nuclear extracts contain a modulating factor for VP-16-induced topoisomerase II/DNA binding that is absent or limiting in K/VP.5 cells. Western blot analysis indicated that levels of β_{II} PKC were reduced 2-fold in nuclear extracts of K/VP.5 compared with K562 cells (not shown), suggesting that the limiting factor is β_{II} PKC.

Effect of PKC activators on VP-16-induced topoisomerase II/DNA complexes. The effect of the PKC activators bryostatin 1, phorbol esters (TPA and PdBU), and n-HexylILV (43) on VP-16-induced topoisomerase II/DNA binding in K/VP.5 nuclei was examined (Fig. 6). Using K/VP.5 nuclei supplemented with K562 nuclear extract (60 μg of protein), only bryostatin 1, which specifically activates

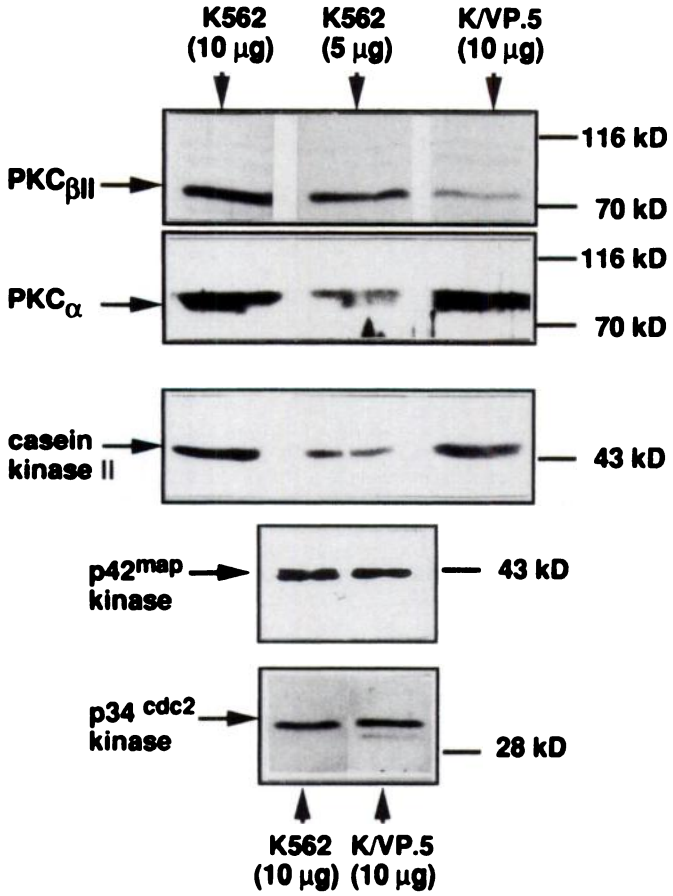

Fig. 4. Levels of protein kinases in K562 and K/VP.5 cells. Whole cell lysates were prepared from 2.5 × 10^6 cells. Proteins (5–10 μg) were resolved by SDS-PAGE and Western blotted to nitrocellulose. Immobilized proteins were incubated sequentially with a primary antibody (rabbit antisera specific for the α or β_{II} isoforms of protein kinase C, $p34^{cdc2}$ kinase, or casein kinase II or with mouse antibody specific for $p42^{map}$ kinase) and then with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit IgG or goat anti-mouse IgG). Bound antibodies were detected by enhanced chemiluminescence and quantified by densitometric scanning. The ratio of kinase levels in K562 compared with K/VP.5 cells were as follows: *βII PKC*, 3.02 ± 0.42 (nine experiments, $p < 0.01$), *α PKC*, 1.09 ± 0.05 (three experiments, $p = 0.93$), *casein kinase II*, 1.35 ± 0.16 (seven experiments, $p = 0.08$); *p34cdc2 kinase*, 0.91 ± 0.2 (seven experiments, $p = 0.66$); and *p42map kinase*, 0.92 ± 0.05 (three experiments, $p = 0.26$). Results are given as mean ± standard error; *p* values were determined with paired Student's *t* test.

and translocates β_{II} PKC to the nucleus (32, 34), stimulated VP-16-induced topoisomerase II/DNA covalent complex formation. In separate experiments, the potentiating effect of bryostatin 1 on VP-16-induced topoisomerase II/DNA complexes was completely inhibited by 100 nM UCN-01 (Fig. 7). Pretreatment of K/VP.5 cells with bryostatin 1 for 2 or 4 hr potentiated VP-16-induced topoisomerase II/DNA covalent complex formation (Fig. 8A). In contrast, pretreatment of K562 cells with bryostatin 1 (1–4 hr) caused no further increase in VP-16-induced topoisomerase II DNA complex formation (Fig. 8A), indicating that in these cells, conditions for drug-induced topoisomerase II/DNA complex formation are already optimal. Furthermore, pretreatment of K562 or K/VP.5 cells with the PKC activators TPA and PdBU (50–200 nM for 0, 1, or 19 hr) had no effect on 20 μM VP-16-induced topoisomerase II/DNA covalent complex formation in either K562 or K/VP.5 cells (not shown). After a 4-hr incubation of K/VP.5 cells with bryostatin 1 (100 nM), there was a statistically significant increase in topoisomerase II phosphorylation (1.65 ± 0.13-fold; mean ± standard error from five separate experiments; $p = 0.043$) (Fig. 8B). In contrast, bryostatin 1 did not significantly increase topoisomerase II phosphorylation in K562 cells (1.06 ± 0.08-fold, mean ± standard error from five separate experiments, $p = 0.40$) (Fig. 8B). These results support the idea that bryostatin 1 up-regulates topoisomerase II phosphorylation through translocation/activation of β_{II} PKC, thus leading to potentiation of VP-16-induced topoisomerase II/DNA covalent complex formation. The specific effects of bryostatin 1 in K/VP.5 nuclei and cells further indicate that β_{II} PKC plays a role in modulating VP-16 activity and suggest that this PKC isoform is a determinant of resistance to VP-16 in K/VP.5 cells.

Discussion

The results of the present study suggest that a decrease in the level of β_{II} PKC contributes to the changes observed in VP-16-resistant K/VP.5 cells, particularly the reduction of topoisomerase II phosphorylation and the resultant decrease in stability of VP-16-induced topoisomerase II/DNA complexes (9, 38). Topoisomerase II in K/VP.5 cells is synthesized and phosphorylated at a decreased rate compared with parental K562 cells (Figs. 1 and 2). The decreased rate of synthesis of K/VP.5 topoisomerase II is most likely the result of reduced mRNA template for the enzyme in these cells (8). Reduced rates of synthesis and phosphorylation of topoisomerase II were also reported for mAMSA-resistant HL-60 cells (26). In contrast to this earlier study, however, we observed no decrease in the turnover (stability) or the dephosphorylation rate of topoisomerase II for resistant K/VP.5 cells compared with parental K562 cells. The relatively high stability of topoisomerase II and of topoisomerase II-associated phosphate in K562 and K/VP.5 cells is comparable to that previously reported for HeLa cell topoisomerase II (16). Furthermore, the comparable rates of dephosphorylation in sensitive and resistant cells indicate that reduced phosphorylation of topoisomerase II in K/VP.5 cells is not due to the presence of a more active topoisomerase II-associated phosphatase in these cells. Together with the observation that levels of β_{II} PKC are reduced in K/VP.5 compared with K562 cells (Fig. 3), these results suggest that a change in the levels and/or activity of this protein kinase plays a role in the decreased phosphorylation status of topoisomerase II in K/VP.5 cells.

Nuclear extracts from K562 cells, which contain β_{II} PKC, enhance VP-16-induced topoisomerase II/DNA complex formation and subsequent DNA damage in K/VP.5 nuclei (Fig. 5; see also Ref. 38). The addition of bryostatin 1 (but not TPA, PdBU, or n-hexylILV) potentiated VP-16-induced topoisomerase II/DNA complex formation in K/VP.5 nuclei supplemented with K562 nuclear extract (Fig. 6), an effect that was blocked by the PKC inhibitor UCN-01 (Fig. 7). Similarly, only bryostatin 1 pretreatment of intact K/VP.5 cells enhanced VP-16-induced topoisomerase II/DNA complex formation (Fig. 8A). It is likely that phorbol esters did not potentiate VP-16-induced topoisomerase II/DNA complex formation in cells because activation of PKC by these agents stimulates a differentiation pathway that has previously

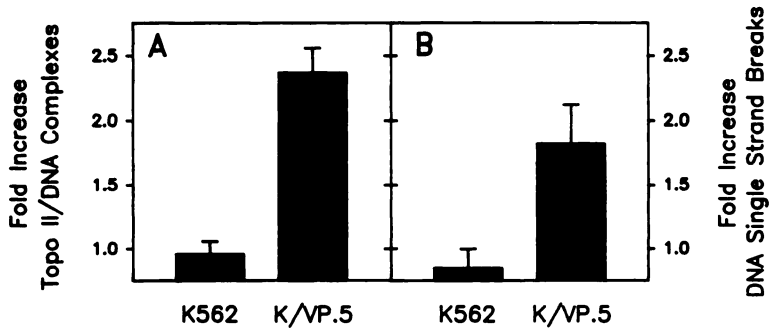

Fig. 5. Effect of K562 nuclear extract on VP-16-induced topoisomerase II/DNA covalent complexes (A) and DNA single-strand breaks (B) in K562 and K/VP.5 nuclei. Isolated nuclei from K562 and K/VP.5 cells, prelabeled 24 hr with 0.5 μCi/ml [methyl-^{3}H]thymidine and 0.1 μCi/ml [U-^{14}C]leucine (A) or 48 hr with 0.02 μCi/ml [^{14}C]thymidine (B), were incubated 30 min with 1 mM ATP and 20 μM VP-16 in the absence or presence of 40 μg K562 nuclear extract. A, Topoisomerase II/DNA covalent complexes were isolated by KCl/SDS precipitation and quantified by scintillation counting as described in Materials and Methods. Results are expressed as -fold increase in VP-16-induced topoisomerase II/DNA covalent complexes for nuclei incubated in the presence compared with the absence of nuclear extract. Data shown are the mean ± standard error of 10–19 experiments. For K562 nuclei, there was no significant potentiation of VP-16 activity in the presence of nuclear extract (p = 0. 96, Wilcoxon signed-ranks test). For K/VP.5 nuclei, nuclear extracts caused a statistically significant increase in VP-16 activity (p = 0.001, Wilcoxon signed-ranks test). B, DNA single-strand breaks were quantified by alkaline elution as described in Materials and Methods. Bars represent the mean ± range or standard error from two (K562 nuclei) to five (K/VP.5 nuclei) independent experiments. For K/VP.5 nuclei, there was a statistically significant potentiation of VP-16-induced DNA damage in the presence of nuclear extract (p = 0.04, Wilcoxon signed-ranks test).

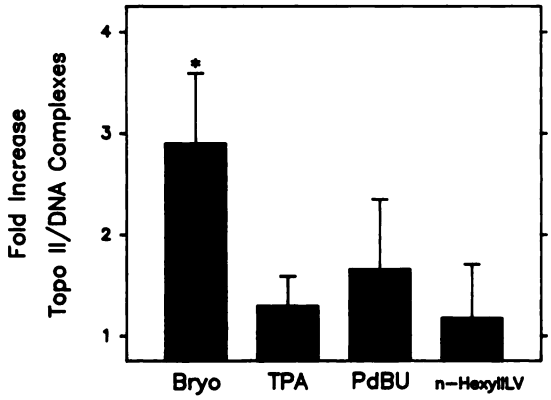

Fig. 6. Effect of PKC activators on VP-16-induced topoisomerase II/DNA covalent complexes in K/VP.5 nuclei in the presence of K562 nuclear extracts. Isolated nuclei from K/VP.5 cells (prelabeled for 24 hr with [methyl-^{3}H]thymidine and [U-^{14}C]leucine) were incubated 30 min with 1 mM ATP, 20 μM VP-16, and 60 μg K562 nuclear extract in the absence or presence of 100 nM bryostatin 1 (*Bryo*), TPA, PdBU, or n-HexylILV. Topoisomerase II/DNA covalent complexes were isolated by KCl/SDS precipitation and quantified by scintillation counting as described in Materials and Methods. Results are expressed as -fold increase in VP-16-induced topoisomerase II/DNA covalent complexes in nuclei treated in the presence compared with the absence of PKC activators. Results are given as mean ± standard error from five to nine separate experiments [p = 0.04 (bryo), p = 0.42 (TPA), p = 0.50 (PdBU), and p = 0.85 (n-HexylILV); Wilcoxon signed-ranks test].

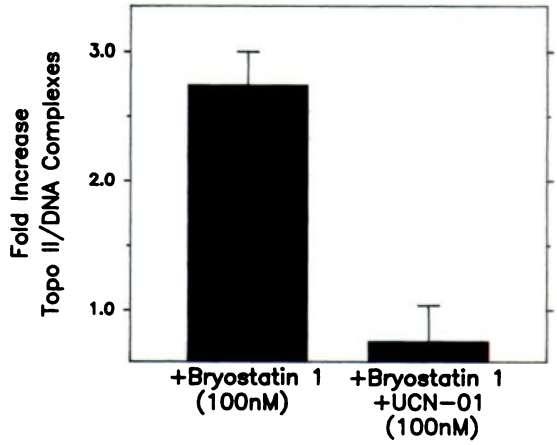

Fig. 7. Effect of the PKC inhibitor UCN-01 on bryostatin 1 potentiation of VP-16-induced topoisomerase II/DNA complex formation. Isolated nuclei from K/VP.5 cells (prelabeled for 24 hr with [methyl-^{3}H]thymidine and [U-^{14}C]leucine) were incubated 30 min with 1 mM ATP, 20 μM VP-16, 100 nM bryostatin 1, and 0 or 60 μg K562 nuclear extract in the absence or presence of 100 nM UCN-01. Topoisomerase II/DNA covalent complexes were isolated by KCl/SDS precipitation and quantified by scintillation counting as described in Materials and Methods. Results are expressed as -fold increase in VP-16-induced topoisomerase II/DNA covalent complexes in nuclei treated in the presence compared with the absence of K562 nuclear extract. Results are given as mean ± standard error from five independent experiments [p = 0.04 (bryostatin 1), p = 0.69 (bryostatin 1 + UCN-01); Wilcoxon signed-ranks test].

been associated with down-regulation of topoisomerase II (28–30). In contrast, bryostatin 1 stimulates the translocation and activation of β_{II} PKC to the nucleus, an event associated with proliferation in K562 cells (32–34). Nuclear extracts from K562 cells contain 2-fold more β_{II} PKC than K/VP.5 extracts (not shown). Together with results that demonstrate the potentiating effect of K562 (but not K/VP.5) nuclear extracts on VP-16-induced topoisomerase II/DNA binding (Fig. 5), these observations are consistent with the hypothesis that β_{II} PKC is involved in regulating topoisomerase II/DNA interactions and is a determinant of VP-16 resistance in K/VP.5 cells. This hypothesis is further supported by the demonstration that bryostatin 1 up-regulates topoisomerase II phosphorylation and potentiates VP-16-induced topoisomerase II/DNA covalent complex formation in K/VP.5 cells (Fig. 8).

It is unclear how phosphorylation regulates drug-induced topoisomerase II/DNA covalent complexes. Phosphorylation of *Drosophila* topoisomerase II *in vitro* with PKC and/or casein kinase II slightly decreased VP-16-induced topoisomerase II-mediated cleavage of purified pBR322 DNA (24). In contrast, our earlier work indicated that in K/VP.5 cells, topoisomerase II was hypophosphorylated (38) and VP-16-induced topoisomerase II binding to DNA was less stable than K562 cells (9). It is possible that *in vivo*, drug-induced binding of topoisomerase II to chromatin DNA may be more sensitive to changes in the phosphorylation status of this

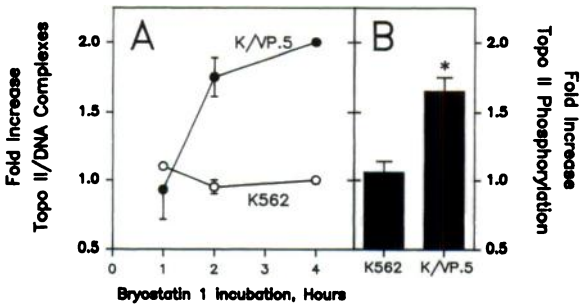

Fig. 8. Effect of bryostatin 1 (100 nM) on VP-16-induced topoisomerase II/DNA covalent complex formation (A) and topoisomerase II phosphorylation (B) in K562 and K/VP.5 cells. A, Cells (prelabeled for 24 hr with [^{3}H]thymidine and [^{14}C]leucine) were incubated with 100 nM bryostatin 1 or 0.1% DMSO for 1–4 hr at 37°, after which VP-16 (20 μM) was added for an additional 30 min. Topoisomerase II/DNA covalent complexes were isolated by KCl/SDS precipitation and quantified by scintillation counting as described in Materials and Methods. Results are expressed as -fold increase in VP-16-induced topoisomerase II/DNA covalent complexes isolated from cells incubated in the presence compared with the absence of bryostatin 1. Data shown are the mean ± standard error from one to six independent experiments. For the 2-hr bryostatin 1 pretreatment, there was a statistically significant increase in VP-16-induced topoisomerase II/DNA covalent complexes in K/VP.5 cells (p = 0.02; Wilcoxon signed-ranks test, averaging six experiments). The data shown for the 4-hr bryostatin 1 pretreatment was derived from a single experiment during which results were obtained after 1-, 2-, and 4-hr bryostatin 1 pretreatment. B, Cells were incubated for 4 hr at 37° with 2.5 mCi/ml of [^{32}P]orthophosphate in the presence or absence of 100 nM bryostatin 1. Topoisomerase II was immunoprecipitated from from 2–3 × 10^6 lysed cells as described (38). Topoisomerase II phosphorylation was quantified by phosphorimage analysis of [^{32}P]orthophosphate from dried gels or by excision of topo II bands and scintillation counting. Results are expressed as -fold increase in topo II phosphorylation in the presence compared with the absence of bryostatin 1 in both K562 and K/VP.5 cells. Results are given as mean ± standard error from five separate experiments. *, p = 0. 04, Wilcoxon signed-ranks test.

protein. Alternatively, the extent and/or sites of phosphorylation of topoisomerase II may affect the distribution of enzyme in the nucleus or its association with the nuclear matrix, which in turn may affect VP-16-induced topoisomerase II/DNA covalent complexes. Topoisomerase II associated with the nuclear matrix has been demonstrated to be reduced in CCRF-CEM cells selected for resistance to teniposide (VM-26), suggesting that nuclear matrix binding of topoisomerase II is an important determinant of drug sensitivity (44). Also, a recent report demonstrated that *Drosophila* topoisomerase II binding to DNA is regulated by phosphorylation (45). Topoisomerase II phosphorylation in *Drosophila* also regulates multimeric topoisomerase II forms, which may be critical for nuclear matrix binding (45). In light of these studies, changes in the phosphorylation status of topoisomerase II might be expected to reduce the ability of the enzyme to stably associate with the nuclear matrix, subsequently decreasing its availability as a target for the inhibitory effects of VP-16 and ultimately contributing to resistance to topoisomerase II inhibitors.

The amino acid sequence of human topoisomerase II (46) reveals a number of consensus phosphorylation sites: 28 for PKC (S*/T*-X-K/R), 10 for p34^{cdc2}kinase (X-S*/T*-P-X), and 6 for casein kinase II (S*/T*-X-(2)D/E). Previous studies in yeast, *Drosophila*, and HeLa cells suggested that casein kinase II is most likely the primary topoisomerase II kinase (15, 47–49). Our data do not challenge this concept. However, the data presented in the present study suggest that β_{II} PKC also regulates topoisomerase II phosphorylation and VP-16-induced topoisomerase II/DNA complex formation. Because recent evidence demonstrates that casein kinase II activity is increased subsequent to phosphorylation of its β subunit by PKC (50), β_{II} PKC-mediated phosphorylation of casein kinase II may indirectly influence topoisomerase II phosphorylation. Therefore, reduced β_{II} PKC levels and/or activity in K/VP.5 cells may result in hypophosphorylation of topoisomerase II as a consequence of less-efficient phosphorylation and activation of casein kinase II. Preliminary results indicate that nuclear casein kinase II activity is increased 1.4 ± 0.1-fold (mean ± standard error from seven experiments) after 30-min incubation of K/VP.5 cells with 100 nM bryostatin 1, suggesting that translocation/activation of β_{II} PKC initiates a signaling pathway involving casein kinase II. The greater time requirement for bryostatin 1 potentiation of VP-16-induced topoisomerase II/DNA complexes in intact K/VP.5 cells (2 hr; Fig. 8) compared with nuclei (30 min; Fig. 6) is also consistent with a cascade mechanism involving nuclear β_{II} PKC translocation/activation followed by direct or indirect (through casein kinase II) phosphorylation of topoisomerase II and, finally, stabilization of VP-16-induced topoisomerase II/DNA complexes.

Finally, it is conceivable that topoisomerase II is phosphorylated by both β_{II} PKC and by casein kinase II and that phosphorylation at any one site influences either nuclear localization or stabilization of drug-induced topoisomerase II/DNA binding. Clearly, to understand the role of phosphorylation in regulating VP-16-induced topoisomerase II/DNA complex formation, it will be necessary to determine whether β_{II} PKC phosphorylates topoisomerase II directly and/or indirectly through activation of casein kinase II or another protein kinase. Therefore, studies are under way to identify the primary topoisomerase II kinase(s) in K562 and K/VP.5 cells by comparing phosphopeptide maps of trypsin-digested topoisomerase II from ^{32}P-labeled cells (treated in the presence or absence of bryostatin 1) with tryptic maps of topoisomerase II phosphorylated *in vitro* with β_{II} PKC and/or casein kinase II. A better understanding of the post-translational regulation of topoisomerase II will be useful for characterizing mechanisms of acquired resistance to VP-16 in K562 cells.

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