A Protease Pathway for the Repair of Topoisomerase II-DNA Covalent Complexes

Received for publication, May 1, 2006, and in revised form, September 13, 2006 Published, JBC Papers in Press, September 13, 2006, DOI 10.1074/jbc.M604149200

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Despite rapid advances in the field of DNA repair, little is known about the repair of protein-DNA adducts. Previous studies have demonstrated that topoisomerase II (TopII)-DNA adducts (TopII-DNA covalent complexes) are rapidly degraded by the proteasome. It has been hypothesized that proteasomal degradation of TopII-DNA covalent adducts exposes TopII-concealed DNA double-strand breaks (DSBs) for repair. To test this hypothesis, the anticancer drug, VP-16 (etoposide), was employed to induce TopII-DNA covalent complexes in mammalian cells, and the involvement of proteasome in processing TopII-DNA covalent complexes into DSBs was investigated. Consistent with the hypothesis, VP-16-induced DSBs as monitored by neutral comet assay, as well as DNA damage signals (e.g. γ-H2AX) were significantly reduced in the presence of the proteasome inhibitor, MG132. Using both top2β knock-out mouse embryonic fibroblasts and Top2β small interfering RNA knockdown PC12 cells, as well as postmitotic neurons in which TopIIα was absent, we showed that VP-16-induced DNA damage signals were attenuated upon proteasome inhibition, suggesting the involvement of proteasome in the repair-processing of both TopIIα-DNA and TopIIβ-DNA adducts. By contrast, hydrogen peroxide-induced γ-H2AX was unaffected upon proteasome inhibition, suggesting a specific requirement of the proteasome pathway in the processing of TopII-DNA covalent complexes into DNA damage.

VP-16 (etoposide), a prototypic topoisomerase II (TopII) poison which stabilizes both TopIIα- and TopIIβ-DNA covalent complexes (1), is widely used as a DNA damaging agent that induces DNA double-strand breaks (DSBs) (2). However, little is known about the mechanism by which TopII-DNA covalent complexes are transformed into DSBs. In vitro, the VP-16-stabilized TopII-DNA covalent complex presumably reflects the key covalent reaction intermediate, the reversible TopII cleavage complex in which each TopII subunit is covalently linked to the 5′-phosphoryl ends of the four-base staggered DSBs (3). In vivo, VP-16, as well as other TopII poisons, is known to induce DNA damage signals indicative of chromosomal DNA damage. For example, TopII poisons are known to cause G2 cell cycle arrest (4), elevation of sister chromatid exchanges (5, 6) and chromosomal aberrations (7), ATM autophosphorylation (at Ser-1981) (8), H2AX phosphorylation (at Ser-139) (9), NFκB activation (9, 10), and p53 stabilization (11). However, how TopII-concealed DSBs are converted into DNA damage that is recognizable by the DNA damage repair system is not clear.

A previous study has shown that TopII poisons induce proteasome-mediated degradation of TopII, a process referred to as TopII down-regulation (12). TopII down-regulation was shown to be transcription-dependent and exhibits a preference for the TopIIβ isozyme. This phenomenon of proteasome-dependent degradation of TopII is highly reminiscent of proteasome-dependent degradation of topoisomerase I (TopI) induced by the TopI poison, camptothecin (13, 14). It has been hypothesized that, similar to TopI cleavage complexes, TopII cleavage complexes are degraded by the proteasome to reveal topoisomerase-concealed DNA strand breaks for repair (12). In this study, we have tested the hypothesis that proteasome degradation of TopII cleavage complexes transforms TopII-concealed strand breaks into DSBs that are recognizable by the DNA damage signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—4,4-(2,3-Butanediyl)-bis(2,6-piperazinedione) (ICRF-193) was purchased from ICN Biomedicals. Etoposide (VP-16), carbobenzoxy-leucinyl-leucinyl-leucinal (MG132), cycloheximide (CHX), 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), aphidicolin, and 4′,6-diamidino-2-phenylindole were purchased from Sigma. Lactacystin was purchased from A.G. Scientific, Inc. The pancaspase inhibitor, benzoylcarbonyl-Val-Fluoromethyl ketone (Z-VAD-FMK), was purchased from Promega Corp. Neurobasal medium and B27 supplement were purchased from Invitrogen Corp. The anti-ATM-S1981-P antibody was purchased from Rockland Immunochemicals. Anti-γ-H2AX antibodies were purchased from Trevigen and Upstate Biotechnology. The anti-ATM-S1981-P monoclonal antibody was purchased from Cell Signaling Tech-
tology, Inc. The anti-TopIIα and TopIIβ antibodies were purchased from Santa Cruz Biotechnology, Inc.

**Isolation of top2β¹⁺ and top2β⁻ Mouse Embryonic Fibroblasts (MEFs)—**Embryonic day-12.5 (E12.5) mouse embryos (top2β¹⁺ and top2β⁻⁻ (15)) were dissected free of brains and livers, finely minced, and then suspended in trypsin-EDTA. The cell suspensions were incubated at 37 °C for 5 min, followed by termination with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The single cell suspensions were centrifuged to obtain cell pellets which were then re-suspended in fresh culture medium and plated in culture dishes. MEFs were then transformed with SV40 large T-antigen by co-transfection a SV40 T-antigen plasmid (pAN2) and a neo’ plasmid (at a ratio of 4:1). 48 h post-transfection, cells were selected with G418 (500 µg/ml) for 10 to 15 days. Transformated cells were then cloned.

**Comet Assay—**Wild type transformed MEFs were pretreated with 2 µM MG132 for 30 min, followed by co-treatment with 250 µM VP-16 for 1.5 h. Cells were then washed three times with fresh medium (with or without 2 µM MG132 as indicated) and incubated for additional 30 min (for reversal of topoisomerase II cleavage complexes). For comet assay, treated cells were scraped from plates and pelleted, followed by re-suspension in 1X PBS (10,000 cells/ml). 50 µL of the cell suspension was then mixed with 500 µL 0.5% low melting point agarose at 37 °C. 75 µL of the cell/agarose mixture was transferred onto glass slides. Slides were then immersed in prechilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, and 10% Me₂SO) for 1 h, followed by equilibration in 1X Tris borate-EDTA buffer for 30 min. Slides were electrophoresed in 1X Tris borate-EDTA at 1.5 volt/cm for 5 min and stained with Vistra Green (Amersham Biosciences). Images were visualized under a fluorescence microscope and captured with a CCD camera. The comet tail moment was determined using the Comet Assay IV software (Perceptive Instruments), and the mean ± S.E. of the comet tail moment was obtained from ~50 cells for each treatment group. Statistical analysis of the mean comet tail moments was performed using Student’s t test.

**TopIIβ Knockdown by Small Interfering RNA in PC12 Cells—**Based on the 643-bp partial rat TopIIβ cDNA sequence reported in the data base (GenBank™ accession number D14046), which corresponds to the N-terminal domain of the protein, the sequence 5’-GCCCCCGTTATATCTTACTAC3’ was selected for shRNA-mediated knockdown of rat TopIIβ. The duplexDNA (5’-TGGCCCGTTATATCTTACTTAAGAGAGTGAAGATATAACGGGGGCTTTTTC-3’) was made and cloned into the LentiLox 3.7 vector (obtained from Dr. Van Parijs, Massachusetts Institute of Technology, Cambridge, MA). The Top2β shRNA LentiLox 3.7 DNA was then mixed with the ViralPower™ Packaging Mix (containing pLP1, pLP2, and pLP/VSVG DNAs, which provide necessary proteins for virus production) and used for transfection into 293T cells with Lipofectamine™ 2000 (Invitrogen) to generate the lentiviral stock. Cultured PC12 cells were infected with rat Top2β shRNA lentivirus. Single colonies were isolated and characterized. The PC12-LF clone was shown to express reduced levels of Top2β mRNA and TopIIβ protein. The PC12-C4 clone was isolated from control lentivirus (vector only)-infected PC12 cells.

**Neuronal Cultures—**Cerebellar granule neurons (CGNs) were isolated as described by Levi et al. (16) from postnatal day-8 (P8) Sprague-Dawley rats. Briefly, cerebella were isolated free of meninges and blood vessels in basal medium Eagle buffered with 20 mM HEPEs, pH 7.4, at 4 °C. Cerebella were then minced and incubated in 0.08% trypsin/basal medium Eagle-HEPEs for 15 min at 37 °C. DNase I (0.05%) plus the soybean trypsin inhibitor (0.025%) were then added to halt trypsinization. The cell suspension was triturated using a fire-polished Pasteur pipette, followed by centrifugation at 300 x g for 10 min. The cell pellets were resuspended in neurobasal medium supplemented with B27 supplement (Invitrogen), 25 mM KCl, 0.5 mM glutamine, and penicillin/streptomycin (100 units/ml and 100 µg/ml, respectively), followed by passing through a 40-µm nylon filter (Sefar America, Inc.). All culture dishes and glass coverslips were precoated with 0.01% poly-L-lysine. Cortical neurons (CNs) were isolated from E17.5 mouse embryos following the same procedure as described above.

**Immunocytochemistry—**For immunocytochemistry, CGNs or CNs (1.5 × 10⁶ cells/well) were plated onto glass coverslips in a 24-well plate and cultured. Neuron cells cultured for 2 day in vitro (2 DIV), were treated with VP-16 or camptothecin for 1 h followed by fixation with 4% paraformaldehyde in PBS at 4 °C for 10 min. Fixed neurons were rinsed once with PBS and then incubated with PBS containing 10% fetal calf serum, 0.2% Triton X-100 and 0.1% sodium azide for 30 min at room temperature. For antibody staining, neurons were incubated with anti-ATM-S1981-P (1 to 500 dilution) or anti-γ-H2AX(1 to 200 dilution) antibody in PBS containing 1% fetal calf serum, 0.2% Triton X-100 and 0.1% sodium azide overnight at 4 °C. After several rinses with PBS, neurons were incubated with appropriate secondary antibody (Jackson Immuno-Research). Neurons on glass coverslips were then washed with PBS followed by counterstaining with 4',6-diamidino-2-phenylindole. The glass coverslips were mounted with an aqueous mounting medium (Gel/Mount) (Biomeda Corporation). Fluorescence images were captured using a CCD camera mounted on a Zeiss fluorescence microscope.

**Immunoblotting—**Immunoblotting was performed as described previously (17). Briefly, protein samples in SDS gel sample buffer were boiled for 10 min and then analyzed by SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes. Membranes were immersed in 5% milk for 1 h, followed by incubation with specific antibody overnight. Membranes were then washed with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated with the secondary antibody for 1 h. Bound secondary antibody was detected by either x-ray films or the Kodak Image Station 2000R (for quantification) using ECL reagents (Pierce).

**RESULTS**

**VP-16 Induces Proteasome-dependent Degradation of TopII and DNA Damage—**Previous studies have demonstrated that TopII poisons induce proteasome-mediated degradation of TopII in mammalian cells (12). Indeed, as shown in Fig. 1A, VP-16 induced a time-dependent degradation of TopIIβ (also TopIIα, but to a lesser extent) in transformed MEFs. Consistent...
with results obtained from previous studies, degradation of TopIIβ was largely prevented by co-treatment with the proteasome inhibitor MG132 (2 μM) (Fig. 1A), suggesting the involvement of proteasome in TopIIβ down-regulation. It has been proposed that proteasomal degradation of TopII cleavage complexes represents a novel repair pathway, which processes TopII-DNA covalent complexes into reparable DSBs (12). To test this possibility, we measured the amount of DSBs in VP-16-treated MEFs using neutral comet assay in the presence and absence of the proteasome inhibitor MG132. As shown in Fig. 1, VP-16 (250 μM for 1.5 h) treatment induced a statistically significant increase in average comet tail moment compared with Me2SO (0.25%, solvent control) treatment (1.9-fold, p value < 0.001, t test), suggesting induction of DSBs by VP-16. Co-treatment with the proteasome inhibitor MG132 largely abolished this VP-16 signal (p value < 0.001, t test), suggesting the involvement of the proteasome in the induction of DSBs by VP-16. In these experiments, the comet signal most likely reflected the formation of frank DSBs rather than TopII cleavage complexes, since a cleavage complex reversing procedure (incubation in fresh medium without VP-16) was applied prior to the comet assay.

We also measured the appearance of Ser-139 phosphorylated H2AX (γ-H2AX) which served as a marker for DSBs (18). As shown in Fig. 1A, γ-H2AX was induced by VP-16 treatment in MEFs as revealed by immunoblotting with anti-γ-H2AX antibody, suggesting the presence of DSBs by VP-16. Co-treatment with the proteasome inhibitor MG132 was significantly reduced by co-treatment with the proteasome inhibitor, MG132, consistent with the proposed role of proteasome in processing TopII-DNA covalent complexes into DSBs.

Involvement of Proteasome in the Processing of TopII-DNA Covalent Complex into DNA Damage—VP-16 is known to induce both TopIIα-DNA and TopIIβ-DNA covalent complexes indiscriminately (1). The DSBs induced by VP-16 could be due to the processing of either or both covalent complexes in transformed MEFs. To determine the role of the two Top II isozymes in the induction of DSBs by VP-16, γ-H2AX was measured in TopIIβ knockdown PC12 cells (PC12-I4) and the control PC12 cells (PC12-C4) treated with VP-16. As shown in Fig. 2A, VP-16 induced a similar level of γ-H2AX in PC12-C4 as compared with that in PC12-I4 cells, suggesting that the γ-H2AX signal is primarily due to the presence of TopIIα-DNA covalent complexes. This conclusion was further supported by
some inhibitor, MG132, reduced the VP-induced degradation of TopII in postmitotic CGNs. To monitor I\(\beta\) degradation, cultured CGNs (2 DIV) were treated with VP-16 (250 \(\mu\)M) for 2 DIV, 3, 5, and 5 h. The effect of various inhibitors (2 \(\mu\)M MG132, 150 \(\mu\)M DRB, or 10 \(\mu\)g/ml CHX) on VP-16 (250 \(\mu\)M)-induced I\(\beta\) degradation was monitored in CGNs following co-incubation for 5 h. Me\(\text{SO}_4\) (0.25\%) was used as the solvent control. Following treatment, cells were lysed with the alkaline lysis method followed by Staphylococcal \(\alpha\)-nuclease digestion. Digested lysates were analyzed by immunoblotting using anti-I\(\beta\) or anti-I\(\alpha\) antibody.

Studies in top2\(\beta\) knock-out MEFs. As shown in Fig. 2B, VP-16 induced a similar level of \(\gamma\)-H2AX in top2\(\beta^{+/−}\) MEFs as compared with that in top2\(\beta^{+/+}\) MEFs. Furthermore, the proteasome inhibitor, MG132, reduced the VP-induced \(\gamma\)-H2AX signal by about 40\% (in both top2\(\beta^{+/−}\) and top2\(\beta^{+/+}\) MEFs treated with 100 \(\mu\)M VP-16), suggesting that the proteasome is involved in the processing of TopII\(\alpha\)-DNA covalent complexes into DSBs.

Involvement of Proteasome in the Process of TopII\(\beta\)-DNA Covalent Complex into DNA Damage—To determine whether TopII\(\beta\)-DNA covalent complexes can also be processed by proteasome into DSBs, CGNs were used for our studies. As shown in Fig. 3A, in vitro culturing of CGNs on poly-L-lysine plates induced neuronal differentiation as evidenced by neurite outgrowth (data not shown) and a rapid decline of TopII\(\alpha\) levels. After culturing for 1 day in vitro (1 DIV), the TopII\(\alpha\) level in CGNs decreased to a non-detectable level (Fig. 3A). In these post-mitotic CGNs (2 DIV), VP-16 (1–5 h of treatment) was shown to induce degradation of TopII\(\beta\) (Fig. 3B). VP-16-induced TopII\(\beta\) degradation in postmitotic CGNs was abolished by co-treatment with the proteasome inhibitor, MG132 (Fig. 3B), suggesting the involvement of the proteasome in degrading TopII\(\beta\) cleavage complexes.

DRB (150 \(\mu\)M), but not CHX (10 \(\mu\)g/ml), was shown to block TopII\(\beta\) degradation, suggesting the involvement of transcription but not new protein synthesis in TopII\(\beta\) degradation, consistent with previous findings in HeLa cells (12).

VP-16 also induced DNA damage signals such as ATM autophosphorylation (at Ser-1981, ATM-S1981-P) and \(\gamma\)-H2AX in postmitotic CGNs (2 DIV) as revealed by immunoblotting using their respective phospho-specific antibodies (Fig. 4). ATM-S1981-P autophosphorylation was abolished by co-treatment with MG132 or DRB but not with CHX (or aphidicolin), suggesting that VP-16-induced DNA damage signals requires the proteasome and transcription but not protein synthesis.

Similar results were obtained on the VP-16-induced \(\gamma\)-H2AX signal in postmitotic CGNs. As shown in Fig. 4B, VP-16-induced \(\gamma\)-H2AX in postmitotic CGNs was abolished by co-treatment with ICRF-193 (a TopII catalytic inhibitor known to block the formation of TopII-DNA covalent complexes), DRB and MG132. These results are consistent with the notion that TopII\(\beta\)-DNA covalent complexes are degraded by the proteasome in a transcription-dependent manner. In addition, the formation of ATM-S1981-P and \(\gamma\)-H2AX DNA damage foci was also monitored by immunocytochemistry in VP-16-treated postmitotic CGNs (2 DIV) using their respective phospho-specific antibodies. As shown in Fig. 5 (A and B), VP-16 induced both ATM-S1981-P and \(\gamma\)-H2AX foci in CGNs. The merged images of ATM-pS1981 and \(\gamma\)-H2AX foci (Fig. 5C) showed co-localization of both foci. Consistent with our immunoblotting results, the formation of both ATM-S1981-P and \(\gamma\)-H2AX foci was abolished by ICRF-193 and MG132. In addition to MG132, lactacystin (a specific proteasome inhibitor) was also shown to block ATM-S1981-P foci induced by VP16 (Fig. 5A), confirming the involvement of proteasome in the activation of DNA damage signals.

To confirm that VP-16-induced DNA damage signals is due to the formation of TopII\(\beta\) covalent complexes, CNs isolated from top2\(\beta^{+/+}\) and top2\(\beta^{−/−}\) embryos were used. As shown in Fig. 5, D and E, VP-16 induced \(\gamma\)-H2AX foci in CNs (2 DIV) isolated from top2\(\beta^{−/−}\) but not top2\(\beta^{+/−}\) mouse embryos. Immunoblotting using anti-\(\gamma\)-H2AX antibody also demonstrated that VP-16-induced \(\gamma\)-H2AX signal was TopII\(\beta\)-dependent (Fig. 5E). Together, these results support the notion...
that TopIIβ-DNA covalent complexes are degraded by the proteasome to generate DNA damage signals.

**VP-16, but Not Hydrogen Peroxide, Induces Proteasome-dependent Phosphorylation of γH2AX in Postmitotic Neurons**—The proteasome requirement for the induction of DNA damage signals was further investigated in CNs treated with either VP-16 or H2O2. As shown in Fig. 6A, both VP-16 and H2O2 induced γH2AX, indicative of the presence of DSBs in treated CNs. However, MG132 specifically abolished γH2AX induced by VP-16, but not by H2O2, suggesting that TopIIβ-DNA covalent complexes but not oxidative DNA damages were specifically processed by the proteasome to induce DNA damage signals (Fig. 6A).

**DISCUSSION**

To date, little is known about the molecular mechanism for the repair of covalent protein-DNA adducts. Studies of topoisomerase and Spo11 (a TopII-like protein involved in initiation of meiotic recombination (19–22)) have suggested that nucleases as well as TDP1, a phosphodiesterase, are involved in their removal from the covalent protein-DNA adducts (23–26).

Previous studies have suggested that a protease mechanism may be involved in the repair/processing of TopII-DNA covalent adducts. It has been proposed that proteasomal degradation of TopII-DNA covalent complexes represents a novel repair mechanism that transforms TopII-concealed DSBs into protein-free DSBs (12). To test this possibility, we have evaluated the role of proteasome in the induction of DNA damage in VP-16-treated cells. Using neutral comet assay, we have monitored the formation of DSBs in VP-16-treated MEFs in the presence and absence of MG132. In the presence of MG132, VP-16-induced DSBs was largely abolished, suggesting the involvement of proteasome in the induction of DSBs in VP-16-treated cells. Additionally, we have monitored the induction of the DNA damage signal γH2AX which is widely used as a signal for DSBs (18). Indeed, the VP-16-induced γH2AX signal in MEFs is reduced in the presence of MG-132, suggesting the involvement of proteasome in induction of DNA damage by VP-16.

The two TopII isozymes are known to be regulated very differently (27–30) and their covalent DNA adducts could be repaired by different pathways. TopIIα, which is only expressed in proliferating and tumor cells, has been located in replication foci in S phase possibly through its interaction with proliferating cell nuclear antigen (PCNA) (31). In addition, TopIIα (identified as the major chromosome scaffold protein Sc1), together with condensin, form the structural scaffold for mitotic chromosomes (32). It appears that TopIIα plays an important role in cell cycle events such as DNA replication, chromosome condensation/decondensation, and sister-chromatid segregation (33–39). By contrast, TopIIβ is present in all cells including terminally differentiated cells and has been shown to be involved in transcription (15, 27, 29, 40–43). We have evaluated the role of proteasome in the processing of TopII isozyme-DNA covalent complexes into DNA damage. The TopII isozyme responsible for the VP-16-induced γH2AX signal in MEFs is primarily TopIIα, since the similar level of VP-16-in-

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**FIGURE 5. VP-16 induces DNA damage foci in postmitotic neurons.** Cultured rat CGNs (2 DIV) were treated with VP-16 (3 μM) in the presence or absence of various inhibitors (75 μM ICRF-193, 2 μM MG132, 10 μM lactacystin). Immunocytochemistry using anti-ATM-S1981-P antibody (A) and anti-γH2AX antibody (B) was performed as described under “Experimental Procedures.” C, co-localization of ATM-S1981-P and γH2AX foci in postmitotic CGNs. CGNs (2 DIV) cultured on glass coverslips were treated with VP-16 (3 μM) for 1 h. CGNs were then fixed and co-stained with anti-ATM-S1981-P (red) and anti-γH2AX (green) antibodies. D, mouse E17.5 cortical neurons (2 DIV) were treated with 20 μM VP-16 for 1 h and then analyzed by immunoblotting using anti-γ-H2AX or α-tubulin antibody.
Repair of TopII-DNA Covalent Complexes

A - + - - - + VP-16 (20 μM) - - + + + + H2O2 (100 μM) - - + + + + MG132 (4 μM)
- γ-H2AX - α-Tubulin

B

Proteasome

DNA Damage Signals

DSB

FIGURE 6. A, Proteasome is specifically required for VP-16-induced, but not H2O2-induced, γ-H2AX. Mouse E17.5 cortical neurons (2 DIV) were treated with VP-16 (20 μM) or H2O2 (100 μM) in the presence or absence of MG132 (4 μM) for 1 h, followed by analysis with immunoblotting using anti-γ-H2AX and α-tubulin antibody. B, a model for proteasomal degradation of TopII-DNA covalent complexes. TopIIα- and TopIIβ-DNA covalent complexes, which are stabilized by TopII poisons, are degraded by the proteasome. Following proteasomal degradation, TopII-concealed DSBs are exposed and subsequently recognized by the DNA damage repair system, resulting in the activation of DNA damage signals.

duced γ-H2AX signal was observed in top2β−/− and top2β+/− MEFs. Consequently, we conclude that proteasome is involved in the processing of TopIIα-DNA covalent complexes into DSBs. However, inhibition of proteasome with MG132 only led to a 40% reduction of the VP-16-induced γ-H2AX signal, suggesting the possible existence of a proteasome-independent pathway(s) for processing TopIIα cleavage complexes into DNA damage. TopIIα-DNA covalent complexes may be processed by another mechanism due to its predominant role in cell cycle events such as DNA replication. TopIIα-DNA covalent complexes could undergo collisions with replication forks (44) and trigger a proteasome-independent mechanism for repair. Clearly, further studies are necessary to establish the mode of repair of TopIIα-DNA covalent complexes.

We have also demonstrated that proteasome is involved in the processing of TopIIβ-DNA covalent complexes into DNA damage. Using postmitotic neurons where only TopIIβ is expressed, we have shown that VP-16 induces proteasome-dependent degradation of TopIIβ. Parallel to TopIIβ degradation in VP-16-treated neurons, accumulation of ATM-S1981-P and γ-H2AX, as well as the formation of DSB foci were observed and shown to be proteasome-dependent. The proteasome dependence for the generation of the DNA damage signal, γ-H2AX, appears to be specific for VP-16 since hydrogen peroxide-induced γ-H2AX is independent of proteasome, suggesting that proteasome is specifically required for processing of TopIIβ-DNA adducts (Fig. 6A).

Our results could best be explained by the model shown in Fig. 6B. In this model, VP-16 stabilizes both TopIIα- and TopIIβ-DNA covalent complexes in the form of reversible TopII cleavage complexes. Proteasomal degradation of TopII cleavage complexes exposes TopII-concealed DSBs which are then recognized by the DNA damage repair system(s). At the present time, it is unclear whether proteasome is directly or indirectly involved in processing TopII-DNA covalent complexes. However, the specific requirement of proteasome for VP-16-induced, but not hydrogen peroxide-induced, DNA damage in postmitotic neurons suggests a possible direct role of proteasome in processing TopIIβ-DNA adducts.

VP-16-stabilized TopII-DNA covalent complexes are reversible (45). It has been suggested that the reversibility of these TopII-DNA covalent complexes requires fixation through their interactions (collisions) with DNA helix-tracking processes such as advancing DNA replication forks, transcribing RNA polymerase elongation complexes, and translocating helicases. Previous studies have demonstrated that degradation of TopIIβ is transcription-dependent (12), consistent with its predominant role in transcription. It seems possible that proteasomal degradation of TopIIβ-DNA covalent complexes could be triggered by their collisions with the transcription elongation complexes within the actively transcribed genes (12). However, the triggering event for proteasomal degradation of TopIIβ-DNA covalent complexes remains unclear. Further studies are necessary to establish the molecular mechanism for the repair of these TopII isozyme-specific DNA adducts.

Acknowledgment—We are grateful to Dr. J. C. Wang for providing the top2β+/− mouse strain.

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