DNA topoisomerase II (TOP2) cleavable complexes represent an unusual type of DNA damage characterized by reversible TOP2-DNA cross-links and DNA double strand breaks. Many antitumor drugs and physiological stresses are known to induce TOP2 cleavable complexes leading to apoptotic cell death and genomic instability. However, the molecular mechanisms(s) for repair of TOP2 cleavable complexes remains unclear. In the current studies, we show that TOP2 cleavable complexes induced by the prototypic TOP2 poison VM-26 are proteolytically degraded by the ubiquitin/26 S proteasome pathway. Surprisingly the TOP2β isozyme is preferentially degraded over TOP2α isozyme. In addition, transcription inhibitors such as 5,6-dichlorobenzimidazole riboside and camptothecin can substantially block VM-26-induced TOP2β degradation. These results are consistent with a model in which the repair of TOP2β cleavable complexes may involve transcription-dependant proteolysis of TOP2β to reveal the protein-concealed double strand breaks.

DNA topoisomerases are double-edged swords. They are essential for many important processes of DNA such as DNA replication, RNA transcription, chromosome condensation/decondensation, and chromosome segregation. However, due to their delicate act on DNA, they are also highly vulnerable to xenobiotics and physiological stresses to produce topoisomerase-mediated DNA damage, mostly in the form of topoisomerase cleavable complexes (2–5). So far, five human DNA topoisomerases, topoisomerase I (TOP1), TOP2α, TOP2β, TOP3α, and TOP3β, have been identified and characterized, and the first three have been demonstrated to be important molecular targets for anticancer drugs (1, 6–10).

Both hTOP2 isozymes have been demonstrated to be the cellular targets for many clinically useful anticancer drugs such as VP-16 (etoposide) and doxorubicin (11–13). In the presence of these TOP2-directed drugs (TOP2 poisons), TOP2 isozymes are trapped as their covalent reaction intermediates, the reversible TOP2 cleavable complexes in which each TOP2 subunit is covalently linked to the 5’-phosphoryl ends of the four-base staggered double strand breaks (14, 15). While the double strand breaks within the TOP2 cleavable complexes are normally concealed by TOP2, many of the cellular effects of TOP2 cleavable complexes are clearly indicative of DNA damage. For example, TOP2 cleavable complexes induced by TOP2 poisons are known to induce DNA damage responses (e.g. G2 arrest, elevation of sister-chromatid exchanges, NFκB activation, and p53 stabilization) (16–19). DNA repair mutant cells (e.g. ataxia telangiectasia, progeroid Werner’s syndrome, and Rad52) are also known to be hypersensitive to TOP2 poisons (20–22). However, how TOP2-concealed DNA strand breaks are converted to DNA damage signals is still unknown. Inhibitor studies have suggested that both DNA replication and RNA transcription may be important for processing TOP2 cleavable complexes into DNA damage signals (23–25).

Repair of topoisomerase cleavable complexes is conceptually challenging because of the bulkiness of the protein and the concealed nature of the breaks. However, recent studies on TOP1 cleavable complexes have suggested that both SUMO and ubiquitin pathways may be involved in repair of TOP1 cleavable complexes (26, 27). While the role of SUMO-1 conjugation to TOP1 cleavable complexes is still unclear, the role of ubiquitin conjugation to TOP1 cleavable complexes appears to trigger degradation of TOP1 via the 26 S proteasome pathway (27). Proteolytic degradation of TOP1 cleavable complexes removes the protein bulk and presumably reveals the hidden strand breaks so that the normal DNA repair process can occur (27, 28). Interestingly transcription inhibitors have been shown to block TOP1 degradation suggesting the involvement of RNA transcription in this particular repair process.2

In the current study, we show that TOP2 cleavable complexes can also trigger ubiquitin conjugation to TOP2 resulting in 26 S proteasome-mediated degradation of TOP2. Surprisingly, TOP2β is preferentially degraded over TOP2α. In addition, transcription inhibitors can substantially block TOP2β degradation. These results are consistent with a model in which repair of TOP2β cleavable complexes may involve transcription-dependant proteolysis of TOP2β to reveal protein-concealed double strand breaks.

EXPERIMENTAL PROCEDURES

Materials—ICRF-193 was purchased from ICN Biomedicals. VM-26 was kindly provided by Bristol Myers Squibb Co. Aphidicolin, cyclohex-

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2 S. D. Desai, D. Rodriguez-Rodriguez, and L. F. Liu, unpublished results.
imide, and 5,6-dichlorobenzimidazole riboside (DRB) were purchased from Sigma. Z-DEVD-FMK was purchased from Calbiochem. Staphylococcal S7 nuclease was purchased from Roche Molecular Biochemicals. Antiserum against hTOP1 was obtained from scleroderma patients. Rabbit antiserum against hTOP2α was raised against the C-terminal one-third of hTOP2α (29). The anti-human TOP2β antibodies was raised by immunization with an immunogen containing GST and seven linear repeats of the peptide fragment of human TOP2β from amino acid residues 1554 to 1565 (TOP2β1565–1565)). The construction of the DNA fragment encoding for seven repeats of TOP2β (1554–1565) and the synthesis of the immunogen GST-TOP2β (1554–1565) followed the published procedure (30). Briefly, the template-repeat polymerase chain reaction method was applied to the construct DNA fragment encoding multiple copies of TOP2β (1554–1565). We designed two oligonucleotides, oligo A and oligo B. Oligo A, 5'-ATG GAA GGC GAT TAT AAC CCT GCC AGG AAA ACA TCC, encodes the target antigen (TOP2β1565–1565), and oligo B, 5'-GTT ATA ATC GCC TTC ATT GGA TGT TTT CCT GCC AGG, is partly complementary to oligo A.

To incorporate restriction sites for subcloning at both ends of the template-repeat polymerase chain reaction products (BamHI at the 5'-end and EcoRI at the 3'-end) as well as a stop codon at the 3'-end of the coding region, a second round of polymerase chain reaction (adapter polymerase chain reaction) with two adapter primers, primer A (5'-GAT TAT AAC CCT GGC AGG AAA ACA TCC, encodes the target antigen (TOP2β1565–1565)), was introduced into XL-10 Gold, and the expressed fusion protein (GST-TOP2β1565–1565) was purified by glutathione-Sepharose 4B affinity chromatography.

**Cell Culture**—The mouse mammary carcinoma cell line ts85 (temperature-sensitive for the ubiquitin-activating enzyme, E1) (31) was cultured in a humidified atmosphere of 5% CO₂ at 30 °C in Dulbecco's minimum essential medium containing penicillin-streptomycin and 10% fetal bovine serum. FM3A, the parental cell line of ts85, was cultured under identical conditions at 37 °C. Cells were shifted to the restrictive temperature by transferring the culture dishes to a 42 °C incubator and then maintaining at 39 °C. HeLa, a human breast cancer cell ZR75-1, human lung fibroblast WI-38, and its transfectants (33). Previous studies have demonstrated that VM-26 and TOP2β cleavable complexes from DNA into cleavable protein complexes (33). To determine the amount of TOP2 cleavable complexes induced by VM-26 in HeLa cells, HeLa cells were treated with 100 μM VM-26 for different times and lysed with the alkaline lysis procedure as described under "Experimental Procedures." Lysates were analyzed by immunoblotting with anti-hTOP2α, anti-hTOP2β, and anti-hTOP1 antibodies, respectively. B, a band depletion assay to assess the amounts of TOP2 cleavable complexes induced by VM-26 in HeLa cells. HeLa cells were treated with 100 μM VM-26 for 0, 1, 2, 5, and 15 min (lanes 1–5, respectively), and cells were lysed with alkaline lysis buffer followed by neutralization. Neutralized lysates were incubated with or without S7 nuclease for 15 min (for releasing TOP2 from covalent TOP2-DNA complexes from DNA, lane 6). All samples were analyzed by immunoblotting with antibodies against hTOP2α, hTOP2β, or hTOP1.

**RESULTS**

**The TOP2 Poison VM-26 Induces a Decrease of the hTOP2β Level in HeLa Cells**—VM-26 represents a prototypic TOP2 poison that traps both TOP2α and TOP2β into cleavable complexes (39). Previous studies have demonstrated that VM-26 induces rapid SUMO-1 conjugation to hTOP2 (32). In the current study, we show that prolonged treatment with VM-26 in HeLa cells resulted in a decrease of the hTOP2β level (Fig. 1A). In the presence of VM-26, the hTOP2β level was reduced more than 50% in 2 h. A further decrease in the hTOP2β level was observed with increasing time of VM-26 treatment (at least up to 6 h) (Fig. 1A). The decrease in the level of hTOP2β was due to the presence of VM-26 since 1% Me₃SO (solvent control) had no effect on the level of hTOP2β during the 6-h incubation (data not shown). Surprisingly, the level of hTOP2α showed very little change over the entire 6-h period (Fig. 1A). One possible explanation for the preferential decrease of the hTOP2β level could be that hTOP2β was more efficiently trapped into cleavable complexes than hTOP2α by VM-26. To examine this possibility, a band depletion assay (27) was performed to monitor TOP2 cleavable complexes in HeLa cells treated with VM-26 for 1–15 min (the short time treatment was used to avoid the complications of proteolytic degradation of TOP2 over prolonged treatment). As shown in Fig. 1B, both hTOP2α and hTOP2β are trapped by VM-26 into covalent protein-DNA complexes with equal efficiency as evidenced by their disappearance in an SDS gel (band depletion) in the absence of nuclease (S7) treatment. Treatment of the lysates with staphylococcal nuclease S7 resulted in the reappearance of the TOP2 bands due to the release of the TOP2-DNA covalent complexes from DNA into free TOP2. These results suggest that VM-26 induces the formation of both TOP2α and TOP2β cleavable complexes with equal efficiency. However, only TOP2β cleavable complexes are proteolytically degraded over time. To confirm that VM-26-induced decrease of the TOP2β level is not a unique phenomenon in HeLa cells, we have examined many other cell lines including the human lung fibroblast cell WI-38 and its transformed subline 2RA, leukemic cell lines such as U937 and CEM, and breast cancer cell lines ZR75-1 (see Fig. 2). VM-26 was shown to induce a decrease of the TOP2β level in all these cells. However, the TOP2α levels appeared not to be significantly affected in all these cells.

**VM-26 Increases the Rate of hTOP2β Degradation**—The de-
crease of the hTOP2β level induced by VM-26 could be due to an increase in the rate of degradation, a decrease in the rate of synthesis, or a combination of both. To test these possibilities, the degradation assay was performed in the presence of the protein synthesis inhibitor cycloheximide. As shown in Fig. 3 (compare lanes 5 and 6) in the presence of VM-26, hTOP2β was more than 80% degraded in 2.5 h. Co-treatment with cycloheximide had no effect on the VM-26-induced decrease of the hTOP2β level (Fig. 3, compare lanes 5 and 6 with lanes 7 and 8), suggesting that VM-26-induced down-regulation is not due to a decreased rate of TOP2 protein synthesis. As a control experiment, we showed that neither the hTOP2α nor the hTOP2β level was significantly affected by cycloheximide treatment alone, suggesting that neither isozyme was rapidly turning over in the absence of VM-26. Consequently the VM-26-induced decrease in the level of TOP2β is primarily due to an increased rate of degradation of TOP2β, and this process will henceforth be referred to as TOP2β down-regulation.

Involvement of 26 S Proteasome in hTOP2β Down-regulation—VM-26 is a potent inducer of apoptotic cell death (34). Therefore, VM-26-induced degradation of TOP2β could be due to the activation of caspases. To examine this possibility, we performed the degradation assay in the presence of the caspase inhibitor Z-DEVD-FMK (35). As shown in Fig. 4A, treatment of Z-DEVD-FMK had no effect on VM-26-induced hTOP2β down-regulation. On the other hand, treatment of cells with the proteasome inhibitor MG132 nearly completely blocked the degradation of hTOP2β (Fig. 4B, lanes 5–8). This result suggests that the 26 S proteasome rather than an apoptotic caspase may be involved in the degradation of hTOP2β. To further confirm the involvement of the ubiquitin/26 S proteasome pathway, we tested the degradation of TOP2β induced by VM-26 in a pair of mouse cell lines. The ts85 cells contain a temperature-sensitive E1 enzyme for ubiquitin conjugation. At the nonpermissive temperature, E1 enzyme is inactivated, and ubiquitin conjugation to substrates is inhibited (31). We performed the degradation assays in both ts85 cells and their parental cells (FM3A). The degradation of TOP2β was substantially blocked when cells were shifted to the nonpermissive temperature (Fig. 5A, compare lanes 1–3 with lanes 4–6). By contrast, at both temperatures, VM-26 induced equally efficient degradation of TOP2β in FM3A cells (Fig. 5B).

The sensitivity to the 26 S proteasome inhibitor and the formation of ubiquitin-TOP2 conjugates provide support of the notion that VM-26-induced TOP2β down-regulation is mediated by a ubiquitin/26 S proteasome pathway.

Involvement of RNA Transcription in VM-26-induced hTOP2β Down-regulation—Previous studies have suggested that both DNA replication and RNA transcription may be involved in the processing of TOP2 cleavable complexes into DNA damage signals (23). To test whether DNA replication and/or RNA transcription is involved in TOP2β down-regulation, we have examined the effect of various inhibitors on VM-26-induced TOP2β down-regulation. As shown in Fig. 6A, inhibition of DNA replication by aphidicolin did not affect VM-26-induced hTOP2β down-regulation. However, inhibition of transcription by DRB substantially affected hTOP2β down-regulation (Fig. 6B, compare lanes 1–3 and lanes 4–6). To confirm this result, we also tested another potent transcription inhibitor camptothecin (CPT) (36). As shown in Fig. 7A, treatment of the breast cancer ZR75-1 cells with either DRB or CPT efficiently blocked VM-26-induced down-regulation of TOP2β. To rule out the possibility that the effect is due to a reduced level of the cleavable complexes in the presence of DRB or CPT, the band depletion assay was performed. As shown in Fig. 7B, treatment of ZR75-1 cells with either DRB or CPT had no effect on the amounts of VM-26-induced TOP2β cleavable complexes. Since CPT can induce TOP1-mediated DNA damage, the blockade of

![Figure 2](http://www.jbc.org/)

**Fig. 2.** VM-26-induced decrease in the TOP2β level in different cell lines. A, U937 and CEM leukemic cells were treated with VM-26 for increasing times. Cell lysates were analyzed by immunoblotting as described under “Experimental Procedures.” The antibody used in this experiment was anti-hTOP2α that recognizes both hTOP2α and hTOP2β. B, human lung fibroblast WI-38 and its SV40-transformed subline 2RA cells were treated with VM-26. Cell lysates were analyzed by immunoblotting with anti-hTOP2α and anti-hTOP2β antibodies, respectively. C, ZR75-1 breast cancer cells were treated with VM-26. Cell lysates were analyzed by immunoblotting with anti-hTOP2α and anti-hTOP2β antibodies, respectively. The VM26 concentration used in these experiments was 100 μM.
hTOP2β degradation by CPT could also be an indirect result of a DNA damage response rather than a direct result of transcription inhibition. To rule out this possibility, we have tested the effect of another DNA damage agent, cisplatin, on TOP2 down-regulation. As shown in Fig. 7C, treatment of ZR75-1 cells with cisplatin had no effect on VM-26-induced down-regulation of hTOP2β. As a control experiment, HeLa cells were also treated with 1% MeSO (solvent control for VM-26 treatment) for 0 and 3 h in the presence (lanes 5 and 6) or absence (lanes 1 and 2) of the caspase inhibitor Z-DEVD-FMK. B, VM-26-induced down-regulation of hTOP2β is blocked by the 26 S proteasome inhibitor MG132. HeLa cells were treated with 100 μM VM-26 in the presence (lanes 5–8) or absence (lanes 1–4) of 10 μM MG132 for increasing times. Cell lysates were analyzed by immunoblotting with anti-hTOP2α, anti-hTOP2β, and anti-hTOP1 antibodies, respectively.

**DISCUSSION**

VM-26 is a potent inducer of TOP2 cleavable complex formation on chromosomal DNA. Different from other DNA damages, these TOP2 cleavable complexes are highly reversible. In addition, the DNA strand breaks are protein-linked and protein-concealed (14, 15). It has been suggested that the DNA-damaging effect of TOP2 cleavable complexes is dependent upon the cellular processing of TOP2 cleavable complexes into DNA damage (12). DNA replication, RNA transcription, helicase activity, and proteolysis have been speculated to be potentially capable of converting TOP2 cleavable complexes into DNA damage (23–25). However, the demonstration that any of these cellular processes is actually involved in processing TOP2 cleavable complexes into DNA damage *in vivo* is still lacking. Our current results suggest that both proteolysis and RNA transcription may be involved in the processing of hTOP2β cleavable complexes into DNA damage. In addition, proteolysis via the ubiquitin/26 S proteasome pathway and transcription appear to be coupled.

VM-26-induced down-regulation of TOP2β appears to be quite similar to CPT-induced down-regulation of TOP1. Both processes are dependent on the formation of topoisomerase cleavable complexes and involve ubiquitin/26 S proteasome (27). In addition, both processes appear to be transcription-dependent. CPT-induced down-regulation has been suggested to be triggered by collision between TOP1 cleavable complexes and RNA polymerase elongation complexes. Down-regulation of TOP1 presumably reveals the TOP1-concealed single strand breaks so that repair can occur. By analogy, we propose that TOP2β cleavable complexes can also collide with RNA polymerase elongation complexes resulting in transcription arrest (see Fig. 8). Proteolysis of TOP2β cleavable complexes by ubiquitin/26 S proteasome results in exposure of the protein-concealed double strand breaks (Fig. 8). The exposed double strand breaks can then be repaired by either homologous recombination or nonhomologous end joining (37, 38). Alternatively unrepaired double strand breaks can trigger apoptotic cell death.
abolishes VM-26-induced down-regulation of hTOP2β. A, the mouse cell line ts85 with temperature-sensitive mutant E1 was treated with 100 μM VM-26 for different times at both the permissive (30 °C) and nonpermissive (39 °C) temperatures. Cell lysates were analyzed by immunoblotting with anti-hTOP2α and anti-hTOP2β antibodies, respectively.

The preferential degradation of TOP2α is mediated by multiple mechanisms, and induction of double strand breaks. However, VM-26-induced apoptosis is likely to be mediated by multiple mechanisms, and degradation of TOP2β cleavable complexes may represent only one of these mechanisms.

The preferential degradation of TOP2β over TOP2α in VM-26-treated cells is intriguing. Apparently preferential degradation is not due to more efficient trapping of TOP2β cleavable complexes by VM-26. It appears that TOP2β cleavable complexes can be more efficiently recognized by the ubiquitin/26 S proteasome pathway than are TOP2α cleavable complexes. The preferential recognition by the ubiquitin/26 S proteasome pathway may reflect the functional difference between the two isoforms. These two isoforms are encoded by distinct genes but share about 72% identity in their primary sequences. Immunohistochemical studies have shown that TOP2α is only present in proliferating tissues including tumors, while TOP2β is present in all tissues including terminally differentiated tissues. The two isoforms are regulated very differently in cells. The TOP2α level peaks in G2/M phase, while the TOP2β level is not significantly changed throughout the cell cycle.
breakpoint cluster region (56, 57). Recent studies have demonstrated that TOP2 can specifically form TOP2 cleavable complexes within the mixed lineage leukemia breakpoint cluster region, suggesting a direct role of TOP2 in mixed lineage leukemia gene translocations (58). Our current demonstration that TOP2 cleavable complexes can be efficiently degraded into translocation-competent double strand breaks within the transcribed regions may suggest the involvement of TOP2 cleavable complexes in chromosomal translocations. It remains to be determined whether TOP2 and TOP2α cleavable complexes may play different roles in their anti-tumor and carcinogenic activities.

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**FIG. 8.** A working model for VM-26-induced down-regulation of TOP2α. In the presence of VM-26, TOP2α is trapped as covalent TOP2-DNA cleavable complexes on DNA. These TOP2α cleavable complexes block transcription and trigger ubiquitin/26S proteasome-dependent degradation of TOP2α. The consequence of TOP2α degradation is the revelation of the DNA double strand break. The double strand break can either be repaired by homologous recombination and/or non-homologous end joining or induce apoptotic cell death. Ub, ubiquitin.
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