

The SUMO pathway is required for selective degradation of DNA topoisomerase II β induced by a catalytic inhibitor ICRF-193¹

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Abstract DNA topoisomerase I and II have been shown to be modified with a ubiquitin-like protein SUMO in response to their specific inhibitors called ‘poisons’. These drugs also damage DNA by stabilizing the enzyme–DNA cleavable complex and induce a degradation of the enzymes through the 26S proteasome system. A plausible link between sumoylation and degradation has not yet been elucidated. We demonstrate here that topoisomerase II β , but not its isoform II α , is selectively degraded through proteasome by exposure to the catalytic inhibitor ICRF-193 which does not damage DNA. The β isoform immunoprecipitated from ICRF-treated cells was modified by multiple modifiers, SUMO-2/3, SUMO-1, and polyubiquitin. When the SUMO conjugating enzyme Ubc9 was conditionally knocked out, the ICRF-induced degradation of topoisomerase II β did not occur, suggesting that the SUMO modification pathway is essential for the degradation.

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Key words: Ubiquitin-like protein; SUMO; DNA topoisomerase II; Proteasome

1. Introduction

Recent studies on post-transcriptional protein modification by proteinaceous modifiers, ubiquitin and ubiquitin-like proteins (UBLs) have opened an avenue to novel regulatory pathways in living cells [1,2]. SUMO (small ubiquitin-like modifier) is a highly conserved protein in eukaryotes and the most-studied UBL to date. Although it shares only moderate sequence identity with ubiquitin, their conjugation pathways have extensive similarities. Yeast and invertebrates have a

single SUMO (also called SMT3) but three isoforms, termed SUMO-1, SUMO-2, and SUMO-3, have been identified in vertebrates. The latter two are highly homologous to each other (94% identity) but diverge significantly from SUMO-1 (~50% identity). As compared to SUMO-1, much less information is available at present on the conjugation partners of SUMO-2/3. While selective degradation of multi-ubiquitinated proteins by the 26S proteasome has now been established, downstream events of sumoylation are less obvious. In some cases, SUMO modification occurs at the same lysine residues that are used for ubiquitination, thus inhibiting multi-ubiquitination and preventing the protein from degradation by proteasome [3]. Conversely, modification by SUMO has not yet been reported to accelerate protein degradation.

Camptothecin and VM-26, often referred to as topoisomerase poisons, are representative inhibitors of type I (topo I) and type II (topo II) topoisomerases, respectively. These drugs bind specifically to each enzyme and stabilize the topo–DNA covalent intermediates (cleavable complex) by preventing the religation of transiently cleaved DNA strands [4,5]. ICRF-193 and other bisdioxopiperazines, classified as ‘catalytic’ inhibitors of topo II, do not interfere with the DNA rejoining step but bind to the closed-clamp form of the enzyme–ATP complex to trap the DNA strands topologically between the dimeric subunits [6]. It has been shown that topoisomerases undergo proteasome-mediated degradation in cells treated with topo poisons [7,8]. Since poisoned cells eventually generate enzyme-linked DNA double strand breaks that are toxic to cells [9], the induced reduction of enzyme level was thought to be a cellular defense mechanism [10].

Two isoforms of topo II, α and β , are present in vertebrates that are encoded by separate genes [11], whereas only one form is present in lower eukaryotes including the budding yeast *Saccharomyces cerevisiae*, in which sumoylation of topo II is involved in the control of centromeric chromatid cohesion [12]. Although topoisomerase inhibitors have been reported to induce sumoylation of both topo I and topo II [13,14], its biological significance remains largely unknown. These observations suggest a connection between sumoylation and degradation but this conjecture has not been fully tested. In the present communication, we demonstrate that topo II β is selectively degraded via proteasome by exposure to ICRF-193, a catalytic inhibitor which does not damage DNA, and that protein modification by SUMO can be an early signal for the degradation.

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¹ The DT40 cell line used here (*Ubc9*^{−/−} tet*Ubc9*) will be available from RIKEN Cell Bank (http://www.rtc.riken.go.jp/CELL/HTML/RIKEN_Cell_Bank.html) by specifying the RCB number (RCB 1845) and the cell line name (delta *Ubc9*+c*Ubc9*-DT40).

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Abbreviations: SUMO, small ubiquitin-like modifier; topo I/II, DNA topoisomerase I/II; PAGE, polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Chemicals and antibodies

The following chemicals were obtained from the suppliers indicated in parentheses: ICRF-193 (Funakoshi), VP-16 (gift from Nippon Kayaku), MG132 (Calbiochem), camptothecin, leptomycin B and doxycycline hydrochloride (Sigma). Most compounds were dissolved in dimethyl sulfoxide, and stored at -20°C in small aliquots. S7 nuclease, protease inhibitor cocktail, and protein G-Sepharose were purchased from TaKaRa, Sigma, and Amersham Pharmacia Biotech, respectively.

Mouse monoclonal antibodies against human topo II α (4E12) and topo II β (3B6) were prepared as described previously [15], and used for both Western blotting and immunoprecipitation experiments. 3B6 was cross-reactive with rat and chicken antigens. Rabbit polyclonal antibody to SUMO-1 (FL-101) was purchased from Santa Cruz Biotech. The anti-SUMO-2/3 polyclonal antibody was prepared as described [16]. Mouse monoclonal antibodies against polyubiquitin (FK2) and Ubc9 (N-15) were obtained from Affinity Research Products and Santa Cruz Biotech, respectively. IgG prepared from the serum of a progressive systemic sclerosis patient was used for anti-DNA topoisomerase I antibody. Anti-matrin 3 antibody was prepared from a rabbit immunized with a synthetic peptide.

2.2. Cell culture

HeLa cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C in DMEM medium supplemented with 5% fetal calf serum (FCS) and 100 $\mu\text{g}/\text{ml}$ kanamycin. Cerebellar granule cells were prepared from the cerebellum of 8-day-old Wistar rats and processed for primary culture as described previously [15]. Wild type DT40 cells and *Ubc9*^{-/-} tet*Ubc9* cells were cultured at 39.5°C under 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated FCS plus 1% chicken serum and 60 $\mu\text{g}/\text{ml}$ kanamycin [17].

2.3. Western blotting

Subconfluent HeLa cells or cerebellar granule cells at culture day 3 were treated as described in the figure legends. After treatments, cells were lysed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (50 mM Tris–HCl: pH 6.8, 2 mM

EDTA, 1% SDS, 1% 2-mercaptoethanol, 8% glycerol, and 0.025% bromophenol blue) added directly into culture dishes. Samples were sonicated briefly and boiled for 2 min before being subjected to electrophoresis. In the experiments where VP-16 or camptothecin was used, lysates were prepared by the alkali lysis procedure followed by digestion with S7 nuclease as described [7]. Samples were run in either 6% homogeneous or 2–15% gradient polyacrylamide gels, transferred onto polyvinylidene difluoride or nitrocellulose membranes by electroblotting, and processed for immunodetection with appropriate first and second antibodies according to a standard procedure. Detection of membrane-bound antibodies was done using an ECL kit (Amersham Pharmacia Biotech). Developed films were scanned for densitometric quantification of protein bands. When successive detection with different antibodies was attempted, the bound antibodies were stripped off the membrane each time by shaking the membrane in the antibody stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8) at 60°C for 30 min.

2.4. Immunoprecipitation

Drug-treated cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing 2% protease inhibitor cocktail, at 4×10^6 cells/ml. Cell lysates were vortexed, sonicated briefly, left on ice for 30 min, and then spun at 14000 rpm for 10 min at 4°C . After pre-clearing the supernatants (1 ml) with 50 μl of 50% protein G-Sepharose slurry, the same volume of protein G-Sepharose that had been pre-coated with 10 μg of anti-topo II β monoclonal IgG was added, and incubated with gentle rotary mixing at 4°C for 1 h. The suspension was centrifuged at 6000 rpm for 1 min, and the sedimented beads were washed three times with RIPA buffer. Immunoprecipitates were resuspended in SDS–PAGE sample buffer, boiled for 2 min, and subjected to electrophoresis.

3. Results

3.1. ICRF-193 induces proteasome-mediated degradation of DNA topoisomerase II β in different cell types

We have shown previously that topo II β , highly expressed in cerebellar granule neurons differentiating in vitro, is involved in the transcriptional induction of a subset of genes that are essential for mature neuronal functions [15]. In that study, we used the catalytic inhibitor ICRF-193 to suppress the activity of topo II β and found unexpectedly that the expression of topo II β was markedly reduced in the treated cells. Subsequent experiments have indicated that the reduction of topo II β is not accounted for by suppression of transcription or translation but is due to a specific degradation via the proteasome (data not shown).

Since granule cells cultured in vitro do not express topo II α [15], we used HeLa cells, in which both topo II isoforms are expressed, to examine whether topo II α is also affected by ICRF-193. A significant reduction in the topo II β amount was evident after the treatment with both topo II inhibitors, VP-16 (a topo poison) and ICRF-193, whereas topo II α was not affected at all under these conditions (Fig. 1A). Levels of topo I or a protein associated with nuclear matrix (matrin 3) did not change by the treatment. As in granule cells, the ICRF-induced reduction in topo II β represented its degradation by proteasome which is susceptible to MG132 (Fig. 1B). Thus, the effect of ICRF-193 on topo II β degradation is very similar to that of VM-26 [8]. Because components of the proteasome system are present in both cytoplasm and nucleus [18], cells were treated with a nuclear protein export inhibitor, leptomycin B (LMB), to see whether topo II β degradation occurs after export to the cytoplasm. LMB had little effect on the degradation, indicating that topo II β is degraded by nuclear proteasome (Fig. 1B).

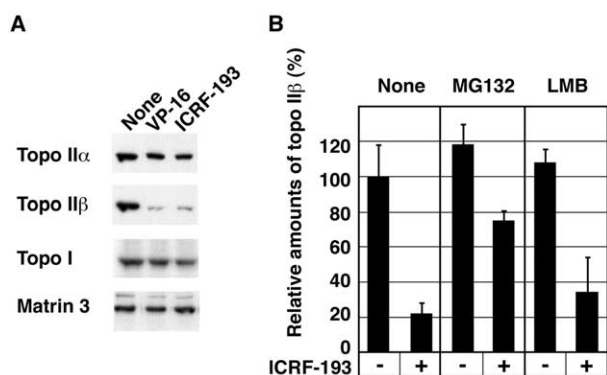


Fig. 1. Selective degradation of topo II β in HeLa cells treated with ICRF-193 and effects of inhibitors. A: Selective down-regulation of topo II β by topo II-specific inhibitors with different action mechanisms. Cell lysates prepared by alkaline solution from HeLa cells incubated for 2 h with topo II-specific inhibitors, 100 μM VP-16 or 10 μM ICRF-193, were treated with S7 nuclease and then subjected to Western blotting using antibodies against the proteins indicated. B: Effects of inhibitors on the ICRF-induced down-regulation of topo II β . HeLa cells were treated with the drugs indicated at the top for 1 h, in the presence (+) or absence (–) of 10 μM ICRF-193. These drugs were added 30 min before the addition of ICRF-193. Concentrations used: 20 μM , MG132; 10 ng/ml, LMB. Cell lysates were subjected to Western blotting to quantify topo II β . Densitometric values from film scanning were normalized to those of matrin 3, as an internal standard, whose expression level remained constant. Measurements were done on triplicate samples from separate dishes and were averaged. Amounts relative to the untreated control are shown with error bars.

Granule cells in primary culture are terminally differentiating without further proliferation whereas HeLa cells are proliferating cells. Although the ICRF-induced topo II β degradation occurs in both cell types, the rate of degradation appears to differ significantly. A time course analysis revealed that topo II β degrades much faster in HeLa cells compared to granule cells (Fig. 2). The degradation appeared to proceed in two phases, rapid and slow. The half-degradation time ($t_{1/2}$) for the rapid phase was 10 min and 53 min in HeLa cells and granule cells, respectively.

3.2. Multiple modification of DNA topo II β in granule cells treated with ICRF-193

We used granule cells to analyze the modification status of topo II β for the following reasons. Unlike in HeLa cells, the modified forms of topo II β are readily identifiable in granule cells. The slower time course of topo II β degradation in granule cells is also advantageous to discriminate the chain of

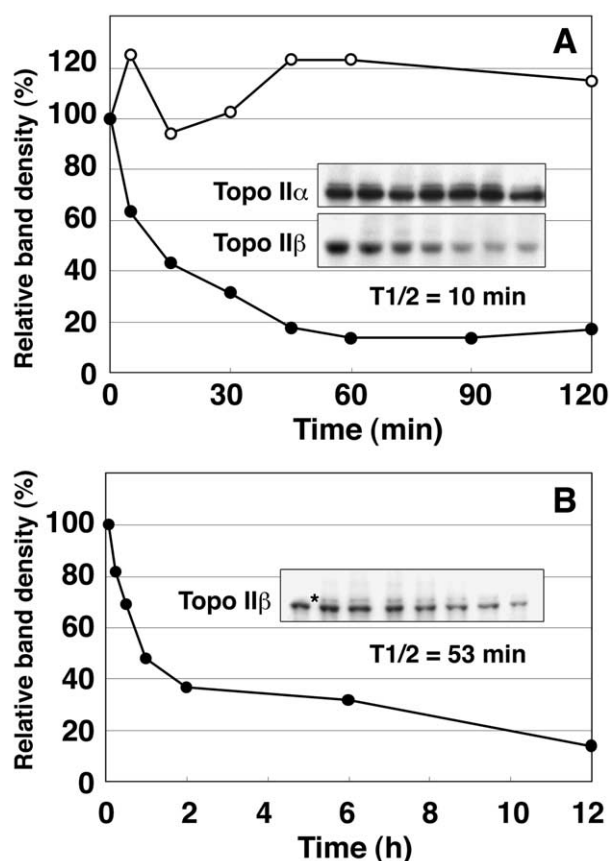


Fig. 2. Differential time course of topo II β degradation in HeLa cells and granule cells. Cultured cells treated with ICRF-193 were lysed at increasing time points and subjected to Western blotting to detect topo II isoforms (shown in insets). Band densities were quantified by densitometry and the data relative to zero time were plotted against incubation time. A: HeLa cells treated with 10 μ M ICRF-193. Open circles, topo II α ; closed circles, topo II β . Time points shown in the inset are 0, 5, 15, 30, 45, 60, and 90 min. $t_{1/2}$ for the rapid phase of topo II β degradation was estimated from a semi-logarithmic plot. B: Granule cells treated with 30 μ M ICRF-193. Closed circles designate topo II β . Time points shown in the inset are 0, 5, 15, 30, 60, 120, 360, and 720 min. Note that the upper band (marked by the asterisk) already appears after 5 min of treatment.

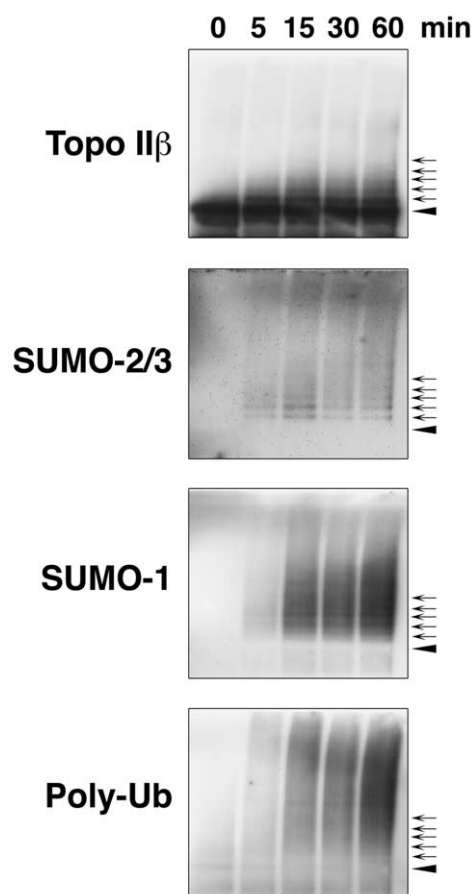


Fig. 3. Distinct modification patterns of topo II β in granule cells treated with ICRF-193. Granule cells treated with 30 μ M ICRF-193 were lysed with RIPA buffer at increasing time points and subjected to immunoprecipitation with anti-topo II β antibody. Immunoprecipitates were run in SDS-PAGE gradient gel, and then subjected to Western blotting to detect the proteins indicated on the left with corresponding antibodies. The same membrane was used throughout by stripping off antibodies between detections (see Section 2). Modified protein bands in the high-molecular-weight region are labeled on the right (arrows). The unmodified topo II β band is indicated by arrowheads. Incubation times are shown at the top.

events. Topo II β immunoprecipitates prepared at various times of incubation with ICRF-193 were separated in a gradient gel to attain a better resolution in the high-molecular-weight region. After blotting, the same membrane was probed with antibodies to topo II β , SUMO-2/3, SUMO-1, and polyubiquitin. These antibodies revealed a ladder of discrete bands overlying on smears (Fig. 3). The bands right above the topo II β band that are detected with anti-SUMO-2/3 antibody appeared first as early as after 5 min of ICRF treatment and decreased after 15 min. Bands reactive to anti-SUMO-1 increased abruptly at 15 min and continued to increase afterwards. The smearing polyubiquitinated bands followed a similar time course although they reached a higher molecular weight region. It should be noted that these smears are hardly discernible in the top panel (topo II β) because of the much lower epitope density. Since the anti-ubiquitin antibody used here reacts specifically with polymerized ubiquitin, the modified topo II β detected by the antibody is likely to be targeted directly to proteasome.

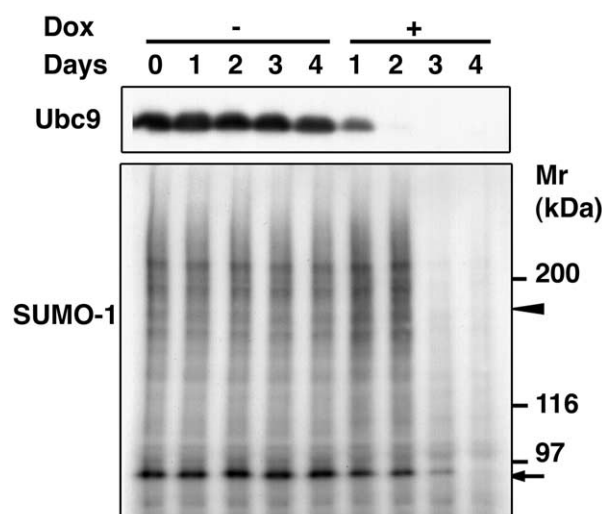


Fig. 4. Down-regulation of Ubc9 and sumoylation in a Ubc9-deficient chicken DT40 cell line containing an exogenous *Ubc9* gene that can be turned off by doxycycline. The mutant cells (*Ubc9*^{-/-}-tet*Ubc9*) were incubated in the presence (+) or absence (-) of 10 ng/ml doxycycline (Dox) for the days indicated, and the expression of Ubc9 or proteins modified with SUMO-1 was analyzed by Western blotting using specific antibodies. Positions of topo II β (arrowhead), RanGAP1 (arrow), and molecular size markers are indicated.

3.3. The SUMO conjugating enzyme Ubc9 is essential for the degradation of DNA topo II β

Ubc9 is the only enzyme identified so far to conjugate SUMO isoforms to target proteins [19]. To test whether sumoylation is an essential modification for ICRF-induced topo II β degradation, we used a mutant chicken DT40 cell line which lacks the endogenous *Ubc9* gene but contains a *Ubc9* transgene under the control of a tetracycline-repressible promoter (*Ubc9*^{-/-}-tet*Ubc9* cells). When the *Ubc9* mutant cells were treated with doxycycline for 2 days, Ubc9 expression was reduced markedly as expected, and abolished completely after 3 days (Fig. 4). The sumoylated proteins detected by anti-SUMO-1 antibody started to decrease later and became almost undetectable on day 4 of doxycycline treatment, including the 90 kDa protein which is most likely RanGAP1 modified by SUMO-1 [20]. These results are consistent with the original character of *Ubc9*^{-/-}-tet*Ubc9* cells reported previously [17].

Wild type DT40 cells and *Ubc9*^{-/-}-tet*Ubc9* cells cultured for 4 days in the presence or absence of doxycycline were next treated with ICRF-193 and levels of topo II β or sumoylated proteins were determined by immunoblotting (Fig. 5A). In wild type DT40 cells, as in HeLa cells and granule cells, ICRF-193 induced degradation of topo II β with a concomitant increase in the high-molecular-weight proteins recognized by antibody to SUMO-1. The topo II β degradation was also susceptible to MG132 (not shown). In doxycycline-treated *Ubc9*^{-/-}-tet*Ubc9* cells, however, ICRF-193 induced neither topo II β degradation nor an increase in sumoylated proteins. The result was confirmed by densitometric quantification using triplicate samples (Fig. 5B). To see whether the ubiquitin–proteasome pathway is intact in the absence of Ubc9 expression, the camptothecin-induced degradation of topo I was examined (Fig. 5C). The induced reduction of topo I, which was suppressed by MG132, occurred to the same extent even

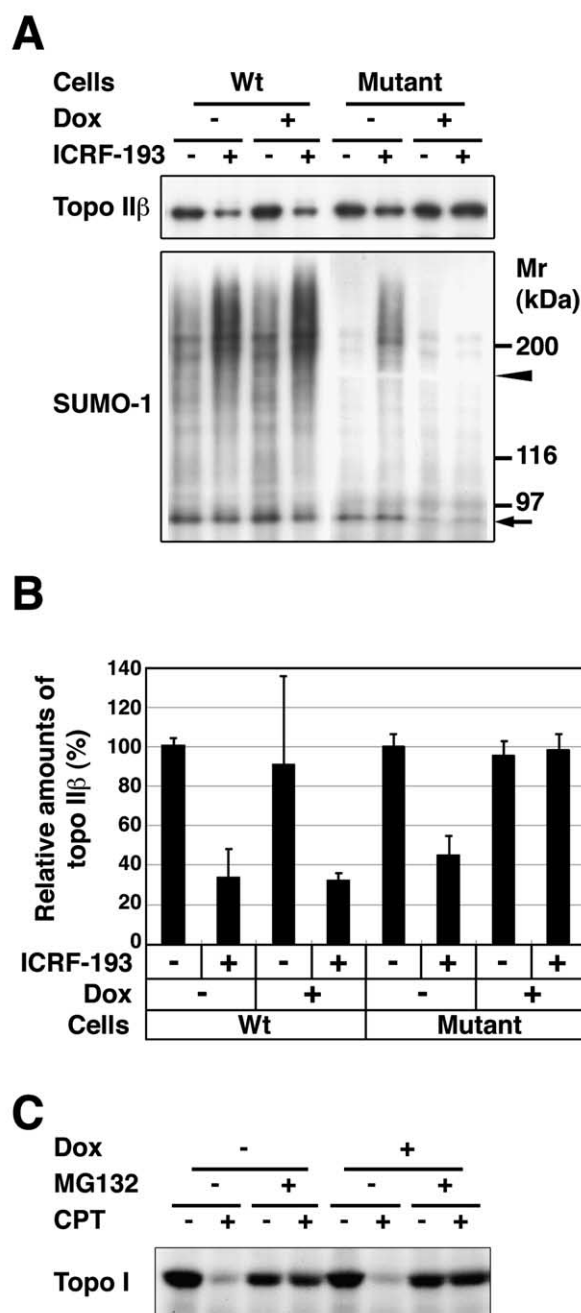


Fig. 5. Ubc9 is essential for the degradation of topo II β induced by ICRF-193. A: After incubating the wild type DT40 cells (wt) or *Ubc9*^{-/-}-tet*Ubc9* cells (mutant) in the presence (+) or absence (-) of 10 ng/ml doxycycline for 4 days, cells were incubated with (+) or without (-) 10 μ M ICRF-193 for 2 h. Levels of topo II β and sumoylated proteins were analyzed by Western blotting using specific antibodies. B: Three independent reactions were done under the same experimental conditions shown above. After normalization to an internal standard, densitometric readings for topo II β from triplicate samples were averaged and the amounts relative to untreated controls are shown with error bars. C: *Ubc9*^{-/-}-tet*Ubc9* cells treated as above were incubated for 4 h in the presence (+) or absence (-) of 25 μ M camptothecin (CPT). MG132 (30 μ M) was added 30 min prior to the start of incubation. Cell lysates were prepared by the alkali lysis procedure followed by S7 nuclease digestion and then topo I was detected by Western blotting.

in the presence of doxycycline, indicating that the proteolytic system itself is still unimpaired under these conditions. Therefore, it is now clear that sumoylation is a prerequisite for ICRF-induced topo II β degradation.

4. Discussion

We have shown in the present study that topo II β degradation can be induced by the catalytic inhibitor ICRF-193 which does not damage DNA. Since the topo II protein clamp on DNA induced by the drug could be an obstacle against various DNA transactions, the same consequence, namely the accelerated degradation that is caused by topo II poisons, may be expected also for catalytic inhibitors. A similar observation was made by others recently [21].

The ICRF-induced multiple modification of topo II β appeared to occur in a cascade-like manner (Fig. 3). SUMO-2/3 conjugation may be particularly important because it preceded that of SUMO-1 and polyubiquitin. Consistent with this view is a rapid and extensive conjugation of SUMO-2/3 to high-molecular-weight proteins following various protein-damaging stresses such as high temperature, oxidative agent, and ethanol [16]. It is an interesting possibility that SUMO-2/3 conjugation to denatured proteins somehow triggers subsequent events toward their disposal through the proteasome system. Unlike SUMO-1, SUMO-2/3 has a consensus motif for autSUMOylation and can form polymeric chains on protein substrates [22]. Formation of heteromultimers of different SUMO isomers is also feasible, where SUMO-1 may serve as a chain terminator. Whether topo II β is modified by polymeric chains of SUMO or whether the same molecule of topo II β is simultaneously modified with SUMO and ubiquitin remains to be shown. The SUMO moiety might be removed by deconjugating isopeptidase before ubiquitination as suggested by the transient nature of SUMO-2/3 conjugates (Fig. 3).

Analysis of Ubc9-deficient DT40 cells demonstrated unambiguously that SUMO modification is essential for ICRF-induced degradation of topo II β (Fig. 5). Although our data do not exclude the possible involvement of other sumoylation targets, the selective degradation of topo II β may imply that potential sumoylation sites are different between topo II isoforms. The topo II β –inhibitor–DNA ternary complex may be a preferential target of Ubc9. Whether SUMO conjugation facilitates the dissociation of topo II β clamps from DNA is still unclear. An interesting discovery in this regard is that the affinity of thymine–DNA glycosylase to abasic site DNA is significantly reduced when the enzyme is modified by both SUMO-1 and SUMO-2/3, resulting in an increased enzymatic turnover [23]. In contrast to topo II β , it appears that SUMO modification plays little role in camptothecin-induced topo I degradation which occurs independent of Ubc9 (Fig. 5C). This is consistent with the previous report showing that the rate of degradation was not altered even when all the

major sumoylation sites of topo I were disrupted by mutation [24].

As for the relationship between sumoylation and ubiquitination, our results imply that these modifications are cooperating, instead of antagonizing [3], to direct the stalled topo II β to destruction via proteasome, suggesting a possible cross-talk between these pathways in the repair of damaged chromatin.

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