Assembly of a Polymeric Chain of SUMO1 on Human Topoisomerase I in Vitro*

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Meiluen Yang‡§, Chia-Tse Hsu¶, Chun-Yuan Ting‡, Leroy F. Liu‡, and Jaulang Hwang†§

From the ‡§ Institute of Biochemistry and Molecular Biology, School of Life Science, National Yang Ming University, Taipei 112, Taiwan, ¶ Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan, and the †§ Department of Pharmacology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854-5635

Human (h) DNA topoisomerase I has been identified as a major SUMO1 target in camptothecin-treated cells. In response to TOP1-mediated DNA damage induced by camptothecin, multiple SUMO1 molecules are conjugated to the N-terminal domain of a single TOP1 molecule. To investigate the molecular mechanism of SUMO1 conjugation to TOP1, an in vitro system using purified SAE1/2, Ubc9, SUMO1, and TOP1 peptides was developed. Consistent with results from in vivo studies, multiple SUMO1 molecules were found to be conjugated to the N-terminal domain of a single TOP1 molecule. Systematic analysis has identified a single major SUMO1 conjugation site located between amino acid residues 110 and 125 that contains a single lysine residue at 117 (Lys-117). Using a short peptide spanning this region, we showed that a poly-SUMO1 chain was assembled in this peptide at Lys-117. Interestingly, a Ubc9-poly-SUMO1 intermediate had accumulated to a high level when the sumoylation assay was performed in the absence of hTOP1 substrate, suggesting a possibility that the poly-SUMO1 chain is formed on Ubc9 first and then transferred en bloc onto hTOP1. This is the first definitive demonstration of the assembly of a poly-SUMO1 chain on protein substrate. These results offer new insight into hTOP1 polysumoylation in response to TOP1-mediated DNA damage and may have general implications in protein polysumoylation.

Human SUMO1 (small ubiquitin-related modifier) (also named UBL1, PIC1, GMP1, SMT3C, and sentrin in the literature) is a ubiquitin-like protein (1–3). It shares about 18% identity to ubiquitin (4). Human SUMO1 and its yeast homolog, Smt3p, also share a similar activation and conjugation pathway with ubiquitin but employ distinct sets of E1 and E2 enzymes (5–9). The E1 enzymes for activating human SUMO1 and yeast Smt3p are the heterodimeric proteins SAE1/SAE2 and Aos1p/Uba2p, respectively (6, 9). Ubc9 is the only E2 identified for SUMO1/Smt3p, although a dozen of E2 enzymes have been identified for ubiquitin in yeast (10–14). Recently, proteases that specifically activate SUMO1/Smt3p precursors and cleave SUMO1/Smt3p from their protein conjugates have been identified in yeast and mammalian cells (15–18). Many proteins such as RanGAP1 (19), promyelocytic leukemia protein (20), IxBe (21), RAD51, RAD52 (22, 23), p53 (24), and centromere proteins (25, 26), which have diverse functions, have been shown to interact with Ubc9/SUMO1 or be covalently modified by SUMO1. SUMO1 appears to primarily target nuclear proteins (23). Although the function of SUMO1 is still elusive, there are a few interesting observations that may shed light on the function of SUMO1. First, Pods (promyelocytic leukemia protein N-gc) domains, also called nuclear bodies or ND10 (27) and the nuclear envelope appear to be the major site of localization of SUMO1 conjugates in the nucleus (28). Second, SUMO1 and ubiquitin appear to share the same conjugation site(s) on some target proteins (e.g. IxBe and MDM2) (21). Third, stress-caused protein unfolding (e.g. heat shock) triggers SUMO (SUMO1 and SUMO2/3) conjugation to nuclear proteins, suggesting the possibility that SUMO proteins are involved in a novel protein stress response (29, 30).

Ubiquitin can be assembled into polymers with different lengths and linkages, which provides an extremely versatile means of cellular regulation (25, 26, 31–37). Because both mono- and polyubiquitination of proteins have been observed, this prompts us to speculate that sumoylation with different lengths and linkages of SUMO1 polymer may also provide various means of cellular regulation similar to ubiquitination. In the process of SUMO modification, SUMO2 and SUMO3 have been shown to possess the ability to form polymeric chains on lysine residue 11 (38), whereas the existence of polysumoylation of SUMO1 has been debated. However, Pichler et al. (39) reported that at least four poly-SUMO1 chains were formed on RanBP2. This study thus makes an effort to establish an in vitro system for studying SUMO1 conjugation, and to examine whether SUMO1 can be conjugated to protein substrates in vitro in a form of polymeric chain.

Human TOP1 has been shown to be a major SUMO1 target in cells treated with the TOP1-specific poison camptothecin (CPT) (29, 40). Multiple SUMO1 molecules are conjugated to a single molecule of human TOP1 in cells treated with CPT (29). Studies in yeast have shown that inactivation of Ubc9 and overexpression of Smt3p lead to increased and decreased CPT sensitivity, respectively, suggesting the potential involvement of SUMO1 in the repair of TOP1 cleavage complexes (29). Systematic mutational analysis of the SUMO consensus sequences on human TOP1 has identified Lys-117 at the N-terminal domain to be the major site for sumoylation (41). However, it is unclear whether multiple SUMO1 molecules are conjugated to Lys-117. In this study, we have investigated the mechanism of SUMO1 conjugation to human TOP1 in vitro using purified protein components. Our results suggest that a poly-SUMO1 chain is formed at Lys-117 of human TOP1.

MATERIALS AND METHODS

Cell Culture—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen). The insect cell line Spodoptera frugiperda SF21 was cultured at 27 °C in

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† To whom correspondence should be addressed: Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan. Tel.: 8862-2789-9217; Fax: 8862-2782-6085; E-mail: jaulangh@imb.sinica.edu.tw.

‡ The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; h, human; Ni-NTA, nickel-nitrilotriacetic acid; GST, glutathione S-transferase; DTT, dithiothreitol; CPT, camptothecin; hTOP1, human topoisomerase I.
Hanks’ medium supplemented with antibiotics and 5% fetal calf serum for the expression of SAE1/SAE2 and full-length human topoisomerase I. Hanks’ medium was prepared from Grace’s medium (Invitrogen) supplemented with 0.33% tissue culture yeastolate (Sigma), 0.33% tissue culture lactalbumin hydrolase (Sigma).

Construction of Protein Expression Plasmids and Recombinant Baculovirus—DNA encoding for SUMO1-(1–97) was prepared by PCR using full-length SUMO1 cDNA as a template. To subclone SUMO1 cDNA into Escherichia coli expression vector pQE30 (Qiagen) in the right reading frame immediately after the His tag codon, we designed primers to carry the BamHI restriction site at the 5’-end and EcoRI restriction site at the 3’-end immediately after the stop codon of the SUMO1 cDNA. The PCR-amplified SUMO1 cDNA fragment was then digested with BamHI and EcoRI and ligated into pQE30 that was pre-cut with BamHI and EcoRI. The resulting plasmid was designated pQE30-SUMO1-(1–97). The methodology used for constructing the plasmid expressing hUbc9 was similar to that used for constructing the SUMO1 expressing plasmid described above. The GeneTailer mutagenesis kit was used to construct the plasmid for the expression of the SUMO1 mutant with all lysines changed into arginines at amino residues 7, 16, 17, 23, 25, 37, 39, 45, 46, 48, and 78 according to the manufacturer’s instruction (Invitrogen). The resulting plasmid was designated pQE30-SUMO1-(1–97) 

GST-hTOP1 fusion proteins containing various human topoisomerase I (hTOP1) fragments, GST-hTOP1-(110–125), GST-hTOP1-(1–200), GST-hTOP1-(201–400), GST-hTOP1-(401–600), and GST-hTOP1-(601–765), were prepared as described previously (42). All restriction enzymes for subcloning were purchased from MBI Fermentas.

Recombinant baculoviruses for the expression of full-length hTOP1 and SAE1/His6-SAE2 were constructed as follows. cDNAs encoding full-length hTOP1, SAE1, and SAE2 were subcloned into the baculoviral expression vector pFastBac HTa and pFastBac HTc. The bacmid constructs were prepared using the Bac-to-Bac baculovirus expression system protocol (Invitrogen). Cellfectin-mediated transfection of bacmid into Sf21 cells in 35-mm plates was applied to produce infectious recombinant baculovirus. The amplified recombinant baculovirus was then used to infect Sf21 cells in 140-mm plates for 7 days, and the supernatant was collected as a high titer viral stock.

Protein Expression and Purification—To express the recombinant proteins of His6-hUbc9, His6-SUMO1-(1–97), and His6-SUMO1-(1–97) 

plasmids pQE30-hUbc9, pQE30-SUMO1-(1–97), and pQE30-SUMO1-(1–97) 

were individually transformed into the E. coli strain TOP10 (Invitrogen). The resulting transformants were cultured in LB medium containing ampicillin (50 μg/ml) with constant shaking (200 cycles/min) at 37 °C. When A600 nm reached 0.3, isopropyl-β-D-galactopyranoside was added to a final concentration of 0.8 mM, and the culture was grown for an additional 3 h at 37 °C. The cells were centrifuged at 4,000 × g for 20 min, and then resuspended in a binding buffer (2.5 mM CaCl2, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) for 30 min at 4 °C. The supernatants were loaded onto Ni-NTA columns (Qiagen). Columns were then washed with binding buffer containing 20 mM imidazole and eluted with binding buffer containing 100 mM imidazole and 10% glycerol. Eluted fractions were pooled and concentrated. The purity of the protein was determined by electrophoresis on a 15% SDS-PAGE, followed by staining with Coomassie Blue. The purified proteins were stored at −70 °C until use. The recombinant GST fusion proteins, GST-hTOP1-(110–125), GST-hTOP1-(1–200), GST-hTOP1-(201–400), GST-hTOP1-(401–600), and GST-hTOP1-(601–765), were expressed and purified as described (42).

To express and purify the recombinant SAE1/SAE2 heterodimer, 1 ml of SAE1/baculovirus stock and 1 ml of His6-SAE2/baculovirus stock were used to infect 2 × 107 Sf21 insect cells per 140-mm plate. Infected cells were cultured at 27 °C for 3 days. Cells were collected and suspended in 4 volumes of a lysis solution containing of 10 mM HEPES, pH 7.9, 5 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 300 mM NaCl, and 10% glycerol. After incubation on ice for 20 min, lysates were centrifuged at 10,000 × g at 4 °C for 5 min. The supernatant was collected and loaded onto the Q-Sepharose column (Amersham Biosciences). After the column was washed with binding buffer (20 mM HEPES, pH 7.9, 1 mM DTT, and 10% glycerol) containing 200 mM NaCl, SAE1/His6-SAE2 was eluted with binding buffer containing 400 mM NaCl. The SAE1/His6-SAE2-containing fractions were collected and then loaded onto the Ni-NTA column, followed by successive washings with the binding buffer containing 5 mM imidazole. The column was then eluted with the binding buffer containing 100 mM imidazole. The SAE1/His6-SAE2 heterodimer containing fractions were pooled and stored at −70 °C.

[35S]Methionine-labeled full-length hTOP1 was prepared by infecting 2 × 107 Sf21 cells with 1 ml of hTOP1-baculovirus stock in methionine-free Grace’s medium containing 1 μCi of [35S]methionine. Cells were harvested 16 h post-labeling of [35S]methionine. [35S]Methionine-labeled hTOP1 was then purified as briefly described below. Infected cells were lysed with lysis buffer containing 10 mM HEPES, pH 7.9, 5 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 350 mM NaCl, 10% glycerol, and 20% PEG8000. After incubation on ice for 20 min, the lysate was centrifuged at 10,000 × g and 4 °C for 5 min. The supernatant was collected, and labeled hTOP1 was purified using the Ni-NTA column. The [35S]methionine-labeled hTOP1 was eluted from the column with 100 mM imidazole. The fractions of [35S]methionine-labeled hTOP1 were pooled and further purified on a Q-Sepharose column. After this two-step purification, the purity of [35S]methionine-labeled hTOP1 reached 95%.

Antibodies and Immunoblotting Analysis—Mouse anti-His tag antibodies and goat anti-GST antibodies were purchased from Serotec and Amersham Biosciences, respectively. Anti-SUMO1, anti-hUbc9, and anti-SEAE1/SAE2 antibodies were purchased from LAE Biotech. Co., Ltd. All the secondary antibodies were purchased from Promega. To detect SUMO1-conjugated proteins, the in vitro sumoylation reaction mixtures were analyzed by SDS-PAGE, followed by immunoblotting with appropriate antibodies as detailed in figure legends. Detection of SUMO1-conjugated proteins was achieved using either an ECL kit (Amersham Biosciences) or an alkaline phosphatase-conjugated secondary antibody, followed by staining with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium FAST substrate (Sigma).

In Vitro Sumoylation Assay—In vitro sumoylation assay was performed using purified recombinant proteins. The reaction mixture (20 μl each) contained 20 mM HEPES, pH 7.5, 5 mM MgCl2, 2 mM ATP, 1 μg of hUbc9, 1 μg of SUMO-1, 150 ng of SAE1/His6-SAE2, and 0.6 μg of substrate protein. The reaction mixture was incubated at 37 °C for 40 min. The reaction mixture was then heat-denatured with SDS-PAGE sample buffer and analyzed on SDS-PAGE, followed by immunoblotting with appropriate antibodies. All experiments were repeated at least twice, and the same results were obtained.

Isopeptidase Cleavage and Thrombin Cleavage—To remove the GST tag from GST fusion proteins, thrombin cleavage was performed at room temperature with 1 unit of thrombin (Sigma) in thrombin cleavage buffer (2.5 mM CaCl2, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) for...
The isopeptidase Ulp1 (Ub-specific protease 1) (Invitrogen) was also used to cleave SUMO1 from sumoylated substrates. The isopeptidase reaction was performed at 30 °C for 1 h in isoprotease reaction buffer (50 mM Tris-HCl, pH 8.0, 0.2% Igepal, 1 mM DTT, and 100 mM NaCl). At the end of the reaction, samples were analyzed on SDS-PAGE and immunoblotted with the appropriate antibodies.

**Plasmid DNA Relaxation Assay**—The relaxation activity of hTOP1 was measured using supercoiled plasmid DNA pGEM-5Z. Briefly, each reaction mixture had a total volume of 20 μl containing 0.2 μg of supercoiled DNA, 50 mM Tris-HCl, pH 7.5, 60 mM KCl, 2.5 mM MgCl₂, 0.5 mM EDTA, and various amounts of SUMO1-modified or unmodified hTOP1. The reaction mixture was incubated at 37 °C for 40 min. The reaction was terminated by adding 2 μl of 5% SDS and 1.5 mg/ml proteinase K at 37 °C for 1 h. Following the addition of loading buffer, DNA samples were separated on an 8% polyacrylamide gel with TBE buffer. After electrophoresis, the gel was dried onto Whatman No. 3MM chromatographic paper and autoradiographed at −80 °C using Kodak XAR-5 films.

**RESULTS**

**Sumoylation of hTOP1 in Vitro**—To establish an in vitro sumoylation system for hTOP1, the essential components of the sumoylation reaction, recombinant SAE1/His₆-SAE2, His₆-hUbc9, and His₆-SUMO1-(1–97), were purified. As shown in Fig. 1A, the purity of these sumoylation reaction components reached more than 95% homogeneity. When purified [³⁵S]methionine-labeled hTOP1 was incubated with SAE1/His₆-SAE2, His₆-hUbc9, and His₆-SUMO1-(1–97), multiple species of hTOP1, which migrated slower than hTOP1, were revealed (Fig. 1B). The reaction mixtures were analyzed on a 7.5% SDS-polyacrylamide gel followed by autoradiography. Highly modified species of hTOP1 are marked by brackets.

**FIGURE 1. Sumoylation of hTOP1 in vitro using purified components.** A, examination of the purity of recombinant proteins SAE1/His₆-SAE2, His₆-hUbc9, and His₆-SUMO1. Two micrograms of purified recombinant proteins SAE1/His₆-SAE2, His₆-hUbc9, and His₆-SUMO1 were analyzed on a 12.5% SDS-polyacrylamide gel, followed by Coomassie Blue staining. B, sumoylation of [³⁵S]-labeled hTOP1 in vitro. The in vitro sumoylation reaction was performed with 200 ng (about 50,000 cpm) of [³⁵S]-labeled hTOP1, 1 μg of His₆-hUbc9, 1 μg of His₆-SUMO1, 2 mM ATP, and various concentrations of SAE1/His₆-SAE2 in a total volume of 20 μl at 37 °C for 40 min as described under “Experimental Procedures.” The reaction mixtures were analyzed on a 7.5% SDS-polyacrylamide gel followed by autoradiography. Highly modified species of hTOP1 are marked by brackets.
The N-terminal Domain of Human Topoisomerase I Is Extensively Modified by SUMO1 in Vitro—To determine the sumoylation site(s) on hTOP1, four different GST-fused proteins containing various hTOP1 fragments, GST-hTOP1-(1–200), GST-hTOP1-(201–400), GST-hTOP1-(401–600), and GST-hTOP1-(601–765), were used as substrates for in vitro sumoylation. GST is unlikely to contribute to the formation of SUMO1 conjugates for the GST-TOP1 fusion proteins because GST is not a SUMO1 substrate (data not shown). Although the SUMOplot™ software shows that hTOP1-(1–200), hTOP1-(201–400), hTOP1-(401–600), and hTOP1-(601–765) carry 5, 3, 1, and 3 sumoylation motifs, respectively, the results of our in vitro sumoylation reactions showed that the extent of sumoylation was not correlated with the number of sumoylation motifs among these GST-TOP1 fusion proteins. Most intriguingly, only GST-hTOP1-(1–200) formed extensive SUMO1 conjugates, whereas GST-hTOP1-(201–400), GST-hTOP1-(401–600), and GST-hTOP1-(601–765) were only weakly sumoylated (Fig. 2B), suggesting that the major sumoylation site(s) of hTOP1 reside in the N-terminal amino acid residues 1–200 of hTOP1. Notably, the number of SUMO1-modified species of GST-hTOP1-(1–200) is much more than the number of sumoylation motifs predicted by the SUMOplot™ software, suggesting the possibility of assembly of a polymeric SUMO1 chain(s) on hTOP1-(1–200).

Formation of a Polymeric Chain(s) of SUMO1 in Vitro—The formation of polymeric SUMO2/SUMO3 chains during sumoylation has been demonstrated in vitro and in vivo (38). Unlike SUMO2/SUMO3, SUMO1 has been proposed to undergo mono-SUMO1 conjugation to target proteins because of its lack of an internal SUMO consensus motif. However, this study has suggested the possibility that a poly-SUMO1 chain(s) is formed on hTOP1-(1–200). To rigorously test this possibility, we constructed a GST/hTOP1-(110–125) fusion protein, designated GST-hTOP1-(110–125), in which the 16-amino acid peptide derived from amino acids 110 to 125 of hTOP1 was fused to GST. This peptide was chosen because it contains a single SUMO motif with the highest modification probability in the N-terminal domain (1–200 amino acids) of hTOP1 (41). As a negative control, the lysine residue (Lys-117) of the SUMO motif in this fusion protein was replaced with arginine to create the mutant protein, designated GST-hTOP1-(110–125)(K117R). The results revealed that with increasing concentrations of

FIGURE 2. Identification of the major sumoylation site(s) on human topoisomerase I in vitro. A, the potential sumoylation sites were predicted by SUMOplot™ software. B, determination of the major sumoylation site(s) on human topoisomerase I. To identify the major sumoylation site(s) on hTOP1, four different GST-fused proteins containing various hTOP1 fragments, GST-hTOP1-(1–200), GST-hTOP1-(201–400), GST-hTOP1-(401–600), and GST-hTOP1-(601–765) were used as substrates for in vitro sumoylation. The in vitro sumoylation products were analyzed on a 7.5% SDS-polyacrylamide gel followed by immunoblotting using anti-GST antibodies. The positions of the unsumoylated substrates are indicated by asterisks, and the putative SUMO1 conjugates are marked by brackets. The same reaction products of GST-hTOP1-(1–200) were also subjected to immunoblotting with anti-SUMO1 antibodies (see the right panel).
SAE1/His$_p$-SAE2, at least three slower migrating species, corresponding to monomer, dimer, and trimer of SUMO1 conjugated to GST-hTOP1-(110–125) were observed (Fig. 3A). As expected, no SUMO1 modification species was detected when GST-hTOP1-(110–125)(K117R) was used as the substrate (Fig. 3B). Because Lys-117 is predicted to be the only SUMO1 conjugation site on GST-hTOP1-(110–125) and is the only lysine residue in hTOP1-(110–125), the formation of multiple species of GST-hTOP1-(110–125)-SUMO1 conjugates suggests that SUMO1 may form a polymeric chain(s) on hTOP1-(110–125) at Lys-117.

Because the chemical nature of the polymeric chain of SUMO1 is expected to be similar to that of SUMO2/SUMO3, the polymeric chain of SUMO1 is likely to result from the formation of an isopeptide bond between the C-terminal glycine residue of SUMO1 and the ε-amino group of an internal lysine residue of the adjacent SUMO1. Thus, if all the internal lysine residues of SUMO1 were replaced with arginine
residues, the mutated SUMO1 (lysineless SUMO1), designated as SUMO1-(1–97)(Lys→Arg), should not be able to form a polymeric SUMO1 chain(s). As shown in Fig. 3C, wild type SUMO1 formed at least two discernible SUMO1-conjugated species consistent with polysumoylation of the substrate. By contrast, only a single SUMO1-conjugated GST-hTOP1-(110–125) conjugate was cleaved with thrombin at room temperature for 1 h (lanes 3–6). The thrombin-digested products were further digested with Ulp1 at 30 °C for 1 additional hour (lane 7). All reaction mixtures were analyzed on a 15% SDS-polyacrylamide gel, followed by immunoblotting with anti-SUMO1 antibodies and the ECL chemiluminescence reagents. Asterisks indicate species in thrombin which cross-reacted with the anti-SUMO1 antibody. C, GST-SUMO1 fusion protein is sensitive to thrombin digestion. GST-SUMO1 fusion protein (0.6 μg) was incubated with 1 unit of thrombin for various times. The thrombin digestion reactions at the indicated time intervals (5, 15, and 45 min) were terminated by the addition of SDS-PAGE sample buffer, followed by incubation at 95 °C for 5 min. The digested products were then analyzed on a 15% SDS-polyacrylamide gel, followed by immunoblotting with anti-SUMO1 antibodies and detection by chemiluminescence.

To further confirm that SUMO1 is conjugated to GST-hTOP1-(110–125) in the form of a polymeric SUMO1 chain, GST-hTOP1-(110–125)-SUMO1 conjugates were processed by sequential thrombin digestion and SUMO isopeptidase Ulp1 cleavage. As illustrated in Fig. 4A, thrombin can cleave GST-hTOP1-(110–125) at the cleavage sequence LVPRGS, which resides in between the GST module and hTOP1-(110–125). If a SUMO1 polymeric chain was formed on Lys-117 of hTOP1-(110–125), we would expect to see the polymeric form of SUMO1 conjugated to hTOP1-(110–125) peptide. Indeed, and suggest the involvement of a lysine residue on SUMO1 in the formation of the polymeric chain.
as shown in Fig. 4B, the hTOP1-(110–125) species that corresponded to hTOP1-(110–125)-mono-SUMO1 and even trimeric forms of SUMO1 were produced from GST-hTOP1-(110–125)-SUMO1 conjugates upon thrombin digestion. The levels of hTOP1-(110–125)-mono-SUMO1 (Fig. 4B, labeled hTOP1-(110–125)+ 1*SUMO1) and hTOP1-(110–125)-di-SUMO1 (Fig. 4B, labeled hTOP1-(110–125)+ 2*SUMO1) conjugates were increased with increasing concentrations of sumoylated GST-hTOP1-(110–125) (Fig. 4B, lanes 3–6). This thrombin-digested product was further processed by SUMO isopeptidase Ulp1 (UbI-specific protease 1). All SUMO1-hTOP1-(110–125) conjugates were expected to be cleaved into the SUMO1 monomer and the hTOP1-(110–125) peptide, and the SUMO1 monomer was monitored by immunoblotting with anti-SUMO1 antibody (Fig. 4B, lane 7). Because the molecular weight of hTOP1-(110–125) peptide was too small, it cannot be detected on a 15% SDS-PAGE. This result again supports the notion that a polymeric SUMO1 chain was formed on hTOP1-(110–125).

Because the amount of the GST-hTOP1-(110–125)-mono-SUMO1 (Fig. 4B, labeled hTOP1-(110–125)+ 1*SUMO1) conjugate was much higher than that of the GST-hTOP1-(110–125)-di-SUMO1 (Fig. 4B, labeled hTOP1-(110–125)+ 2*SUMO1) conjugate in the sumoylated GST-hTOP1-(110–125) products (Fig. 4B, lane 2), it was surprising that the amount of the hTOP1-(110–125)-mono-SUMO1 conjugate was less than that of the hTOP1-(110–125)-di-SUMO1 conjugate after thrombin digestion. This apparent contradiction could be explained by the result presented in Fig. 4C. When GST-SUMO1 was processed with thrombin under the same conditions, the GST-SUMO1 was almost completely degraded to become undetectable within 5 min of thrombin digestion (Fig. 4C). The sensitivity of GST-SUMO1 to thrombin may suggest that monomeric SUMO1 is more sensitive than dimeric SUMO1 to thrombin digestion. Consequently, it seemed possible that most of the hTOP1-(110–125)-mono-SUMO1 conjugate was degraded by thrombin, whereas the hTOP1-(110–125)-di-SUMO1 conjugate was more resistant to thrombin digestion (Fig. 4B). As a result, the intensity of the hTOP1-(110–125)-mono-SUMO1 conjugate underestimates its true amount (Fig. 4B).

Formation of a Polymeric Chain(s) of SUMO1 on hUbc9 in the Absence of hTOP1—SUMO1 could be either sequentially added to the polymeric chain on hTOP1 or added as a polymeric block by hUbc9 (E2) to hTOP1. To distinguish between these possibilities, we investigated the mechanism of sumoylation in vitro in the absence of hTOP1 substrate. To demonstrate that the conjugates did form a thioester bond, the conjugates were analyzed under reducing as well as nonreducing conditions.
conditions. Recombinant SAE1/His₆-SAE2, His₆-hUbc9, and SUMO1 were incubated at 37 °C for 40 min in the absence of hTOP1 substrate. One set of the reaction mixtures was analyzed under reducing conditions and the other under nonreducing conditions by SDS-PAGE, followed by immunoblotting with anti-hUbc9 and anti-SUMO1 antibodies, respectively. The high molecular weight hUbc9-SUMO1 conjugates were observed when samples were analyzed under nonreducing conditions, and the levels of the high molecular weight species became more prevalent with increasing SAE1/His₆-SAE2 (Fig. 5). In addition, the high molecular weight hUbc9-SUMO1 conjugates were detected, irrespective of whether they were immunoblotted with anti-hUbc9 (Fig. 5A) or anti-SUMO1 antibodies (Fig. 5B). By contrast, the high molecular weight hUbc9-SUMO1 conjugates were almost undetectable, whereas the polymeric SUMO1 chains containing dimer and trimer of SUMO1 were observed when samples were analyzed under reducing conditions (Fig. 5). The residual lower molecular weight conjugates observed in Fig. 5A could be due to the formation of SUMO1-hUbc9 isopeptide bonds, whereas the residual low molecular weight species observed in Fig. 5B could be the SUMO1 dimer. These results suggest that hUbc9 forms a thioester bond with a polymeric SUMO1 chain(s) and support the notion that Ubc9 may transfer a polymeric chain to hTOP1.

Conjugation of Monomeric SUMO1 on SAE2 in the Absence of hUbc9 and hTOP1 Substrate—The formation of a polymeric chain of SUMO1 on hUbc9 could be due to the formation and subsequent transfer (to hUbc9) of a polymeric chain of SUMO1 on the SAE1-His₆-SAE2 complex. To answer this question, SUMO1 was incubated with SAE1/His₆-SAE2 at 37 °C for 40 min in the absence of hUbc9 and hTOP1 substrate. The reaction products were analyzed under nonreducing conditions in SDS-polyacrylamide gel (to detect the thioester bond of the SUMO1-conjugated SAE1/His₆-SAE2), followed by immunoblotting with anti-His tag or anti-SUMO1 antibodies. With the presence of an increasing concentration of SAE1/His₆-SAE2, only monomeric SUMO1 conjugated to His₆-SAE2 was detected, and the level of the His₆-SAE2-mono-SUMO1 conjugate was increased in parallel with increasing concentrations of SAE1/His₆-SAE2 (Fig. 6). No polymeric form of SUMO1 conjugated to His₆-SAE2 was observed even in the presence of 80 μg/ml of SAE1/His₆-SAE2. This result suggests that only monomeric SUMO1 is conjugated to SAE2. Our results so far support the notion that the SAE1-SAE2-SUMO1 complex transfers the activated SUMO1 sequentially to Ubc9. After the assembly of poly-SUMO1 on hUbc9, the Ubc9-poly-SUMO1 conjugate then transfers the entire polymeric chain of SUMO1 to the substrate protein.

Because we observed the formation of the SAE2-SUMO1 conjugate, it would be interesting to know whether SAE2 alone could activate SUMO1 to form the SAE2-SUMO1 conjugate. As shown in Fig. 6B, SAE2 alone failed to activate SUMO1 to form the SAE2-SUMO1 conjugate. To activate SUMO1, the SAE1/SAE2 heterodimer was apparently required.

SUMOylation of hTOP1 Does Not Alter Its Enzymatic Activity and Camptothecin Sensitivity—It has been suggested that sumoylation of hTOP1 may evoke a cellular defense mechanism against TOP1-mediated DNA damage induced by camptothecin (29). It has been speculated that sumoylation of hTOP1 may alter its catalytic activity, stability, or relocalization. To answer the question on the possible alteration of the catalytic activity of hTOP1 by SUMO1 conjugation, we performed hTOP1 sumoylation in vitro and then measured the DNA relaxation activity of sumoylated hTOP1. The immunoblotting result indicated that at least 80% of hTOP1 was sumoylated (Fig. 7A). As shown in Fig. 7B, the DNA relaxation activity of hTOP1 was not affected after hTOP1 sumoylation. We also examined whether SUMO1 modification of hTOP1 affects the DNA cleavage activity and camptothecin sensitivity. As shown in Fig. 7C, the DNA cleavage activity in the presence of various concentrations of camptothecin was not affected after hTOP1 sumoylation. These results suggest that the significance of SUMO1 modification of hTOP1 is neither linked to the DNA relaxation activity nor to the sensitivity of camptothecin-induced DNA cleavage.

DISCUSSION

CPT, which induces TOP1 cleavable complexes, is known to rapidly induce the formation of multiple TOP1-SUMO1 conjugates (29, 40). The formation of these multiple SUMO1-TOP1 conjugates has been suggested to represent a novel repair response to TOP1-mediated DNA damage (14). In this study, we have established an in vitro system for studying SUMO1 conjugation to TOP1. Our in vitro system appears to mimic in vivo TOP1 sumoylation in that multiple SUMO1 molecules are conjugated to a single hTOP1 protein. Because hTOP1 contains multiple potential sumoylation sites, the multiple TOP1 conjugates may result from either polysumoylation at a single sumoylation site or monosumoylation at multiple sumoylation sites (multisumoylation), or a combination of both. Although monosumoylation for SUMO1 conjugation has been proposed to be the major mechanism for protein sumoylation (2), our current results support the notion that a poly-SUMO1 chain is conjugated to hTOP1 at Lys-117. This conclusion is based on the study of a single SUMO1 consensus motif-containing TOP1 peptide (amino acid residues 110–125). This peptide contains Lys-117, which has been identified previously as the major TOP1 conjugation site in vivo (41). This in vitro study has demonstrated that a polymeric SUMO1 chain is assembled at Lys-117 in the wild type pep-
tide but not Lys-117 to Arg-117 mutant hTOP1 peptide, suggesting that a polymeric SUMO1 chain is formed at Lys-117 in vivo upon CPT treatment. The function of this polymeric chain of SUMO1 in the repair of TOP1-mediated DNA damage remains to be determined.

Upon careful examination of the three-dimensional structures of hTOP1, we found that some of the potential sumoylation sites predicted by the SUMOplot™ software are buried inside the folded hTOP1. Because the accessibility of the sumoylation sites is a determining factor for sumoylation, the sumoylation sites buried inside of hTOP1, such as lysine residues 328 and 354, are expected not to be used for sumoylation. However, the lack of accessibility of these internal sites cannot fully explain why the major sumoylation sites reside at the N-terminal 200 amino acid residues of hTOP1 but not at other regions of hTOP1. One possibility is that sumoylation may prefer the unstructured region of proteins because the N-terminal region of hTOP1 is known to be unstructured (43, 44). The observation that protein unfolding can cause massive sumoylation is consistent with this interpretation (45).

Sumoylation of TOP1 has been suggested to be a cellular defense mechanism to TOP1-mediated DNA damage. Sumoylation of TOP1 could result in alteration of the catalytic activity, stability, or relocalization of sumoylated hTOP1. We found that the DNA relaxation activity and the sensitivity of camptothecin-induced DNA cleavage of hTOP1 were not affected by SUMO1 conjugation. Thus, the function of the hTOP1 N-terminal sumoylation may be linked to cellular localization, stability, or interaction to other protective proteins. It is interesting to note that the N-terminal region of hTOP1, which contains the nuclear localization signal (46), is known to mediate protein-protein interactions (47). It seems possible that SUMO1 conjugation to the N-terminal domain of hTOP1 could affect hTOP1 functions mediated through the N-terminal domain.

In this study, we have demonstrated the formation of a polymeric chain of SUMO1 on a small hTOP1 peptide containing a single sumoylation site in the in vitro sumoylation reaction. Surprisingly, we also observed the formation of a polymeric chain(s) of SUMO1 on Ubc9 in the absence of the TOP1 substrate. The simplest explanation for our results is that Ubc9-poly-SUMO1 directly transfers the entire poly-SUMO1 chain to hTOP1. In the absence of the TOP1 substrate, the steady-state level of the Ubc9-poly-SUMO1 intermediate accumulates to a high level. Although this possibility seems reasonable and interesting, other possibilities, albeit less likely, cannot be ruled out. For example, the formation of the polymeric chain of SUMO1 on hTOP1 is achieved through a stepwise transfer of monoSUMO1 from Ubc9-monoSUMO1 to hTOP1, and the presence of Ubc9-poly-SUMO1 is an in vitro artifact. Clearly, further in vitro studies are necessary to establish the precise molecular mechanism for the assembly of the poly-SUMO1 chain on substrate.

In the course of our study, we found that there are two types of control was performed under the identical conditions except that the sumoylation reaction was performed in the absence of SAE1/His6-SAE2. The sumoylated hTOP1 was then checked on a 7.5% SDS-polyacrylamide gel, followed by immunoblotting using anti-TOP1 antibodies. Notably, more than 80% hTOP1 was sumoylated in the in vitro sumoylation reaction, whereas no sumoylation of hTOP1 was detected in the absence of SAE1/His6-SAE2 (A). The reaction mixtures were then applied for DNA relaxation assay (B). Various volumes (8, 4, 2, 1, 0.5, 0.25, and 0.125 µl) of sumoylated and unsumoylated hTOP1 reaction mixtures were directly applied for DNA relaxation assay as described under “Experimental Procedures.” The reaction mixtures were separated on a 0.8% agarose gel, followed by EtBr staining. To examine the effect of hTOP1 sumoylation on camptothecin-induced DNA cleavage, another batch of sumoylated and unsumoylated hTOP1 was prepared as described previously in A. The hTOP1-containing sumoylation reaction mixtures were subjected to DNA cleavage assay with various concentrations of camptothecin (1, 3, 10, 30, and 100 µM) as described under “Experimental Procedures.” The reaction mixtures were analyzed on an 8% polyacrylamide gel with TBE buffer followed by autoradiography (C).
chemical bonds on the polymeric chain of SUMO1 conjugated to Ubc9. One type of chemical bond is likely to be the thioester bond (sensitive to reduction by DTT), which presumably is due to SUMO1 C-terminal glycine conjugating to the terminal cysteine residue of Ubc9, whereas the other type is likely to be the isopeptide bond (insensitive to reduction by DTT) formed between two SUMO1 molecules. A question was then raised of how these two different chemical bonds were formed. We prefer to believe that the thioester bond formation is through the transfer of SUMO1 from SAE1/SAE2-SUMO1 to Ubc9 (pink arrow, thioester bond formation). This Ubc9-SUMO1 conjugate can further accept another SUMO1 transferred from another Ubc9-SUMO1 to form a Ubc9-poly-SUMO1 conjugate (light blue arrow, isopeptide bond formation). In the presence of substrate, this SUMO1 modifier or the polymeric SUMO1 chain can be transferred en bloc from Ubc9-SUMO1 or Ubc9-poly-SUMO1 conjugates to the substrate protein, respectively (deep blue arrow, isopeptide bond formation). Hence, the substrate protein can be monosumoylated or polysumoylated. Alternatively, Ubc9-poly-SUMO1 may repeatedly transfer a single SUMO1 molecule to substrate protein until a polymeric SUMO1 chain is formed on substrate protein.

Based on our present data, we propose the mechanism of SUMO1 polysumoylation as diagrammatically shown in Fig. 8. The full-length SUMO1 peptide (101-amino acid residues) initially undergoes maturation through cleavage by a SUMO protease (such as Ulp protease), which yields SUMO1 with the exposed C-terminal glycine residue. The SAE2 subunit of the SAE1-SAE2 heterodimer complex (E1) forms a thioester bond with the C-terminal glycine of SUMO1 in the presence of ATP (green arrow). The SAE1-SAE2 complex then transfers SUMO1 from the SAE2 subunit to Ubc9 (pink arrow, thioester bond formation). This Ubc9-SUMO1 conjugate can further accept another SUMO1 transferred from another Ubc9-SUMO1 to form a Ubc9-poly-SUMO1 conjugate (light blue arrow, isopeptide bond formation). In the presence of substrate, this SUMO1 modifier or the polymeric SUMO1 chain can be transferred en bloc from Ubc9-SUMO1 or Ubc9-poly-SUMO1 conjugates to the substrate protein, respectively (deep blue arrow, isopeptide bond formation). Hence, the substrate protein can be monosumoylated or polysumoylated.
likely possibilities. We are currently carrying out more detailed analysis
in vitro to elucidate the molecular mechanism of polysumoylation.

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