Noncovalent SUMO-1 Binding Activity of Thymine DNA Glycosylase (TDG) Is Required for Its SUMO-1 Modification and Colocalization with the Promyelocytic Leukemia Protein

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SUMO-1 is a member of a family of ubiquitin-like molecules that are post-translationally conjugated to various cellular proteins in a process that is mechanistically similar to ubiquitylation. To identify molecules that bind noncovalently to SUMO-1, we performed yeast two-hybrid screening with a SUMO-1 mutant that cannot be conjugated to target proteins as the bait. This screening resulted in the isolation of cDNAs encoding the b isoform of thymine DNA glycosylase (TDGb). A deletion mutant of TDGb (TDGb(A11)) that lacks a region shown to be required for noncovalent binding of SUMO-1 was also found not to be susceptible to SUMO-1 conjugation at an adjacent lysine residue, suggesting that such binding is required for covalent modification. In contrast, another mutant of TDGb (TDGb(KR)) in which the lysine residue targeted for SUMO-1 conjugation is replaced with arginine retained the ability to bind SUMO-1 noncovalently. TDGb was shown to interact with the promyelocytic leukemia protein (PML) in vitro as well as to colocalize with this protein to nuclear bodies in transfected cells. TDGb(KR) also colocalized with PML, whereas TDGb(A11) did not, indicating that the noncovalent SUMO-1 binding activity of TDGb is required for colocalization with PML. Furthermore, SUMO-1 modification of TDGb and PML enhanced the interaction between the two proteins. These results suggest that SUMO-1 functions to tether proteins to PML-containing nuclear bodies through post-translational modification and noncovalent protein-protein interaction.

Post-translational modification of proteins plays important roles in the regulation of protein function and localization. Proteins are chemically modified by various molecules, including phosphate, lipids, and sugars. Modification by ubiquitin is distinct in that the modifier itself is a small protein. Ubiquitin is usually attached to a lysine residue of target proteins, resulting in the formation of a branched isopeptide chain. Such ubiquitylation serves to mark proteins for degradation by the 26 S proteasome.

SUMO-1 is a member of the ubiquitin-like protein superfamily and is post-translationally conjugated to various cellular proteins in a process that is mechanistically analogous to ubiquitylation. SUMO-1 modification is mediated by a SUMO-1-activating enzyme (E1),1 a SUMO-1-conjugating enzyme (E2), and a SUMO-1 ligase (E3) (1, 2). SUMO-1 is attached to target proteins via an isopeptide bond between the COOH-terminal glycine residue of SUMO-1 and the ε-amino group of a target lysine residue. A single E1 enzyme (the Aos1-Uba2 heterodimer) and a single E2 enzyme (Ubc9) have been identified for the SUMO-1 modification pathway in yeast and higher eukaryotes (2, 3). These two enzymes are sufficient to modify various SUMO-1 targets, including IxBo (4), RanGAP1 (5), and p53 (6) in vitro, and it had been thought that SUMO-1 modification does not require an E3 ligase. However, several E3-like factors (PIAS family, RanBP2, PC2) for SUMO-1 modification were recently identified in yeast and mammalian cells (7–10).

SUMO-1 modification has multiple roles in protein localization and stabilization, transcriptional regulation, and maintenance of genomic integrity (11). Conjugation of RanGAP1 with SUMO-1 results in its translocation from the cytoplasm to the nuclear pore complex, where it associates with RanBP2. This interaction requires SUMO-1 modification of RanGAP1 (5). In the case of IxBo, the lysine residue modified by SUMO-1 is also modified by ubiquitin, and SUMO-1 modification stabilizes IxBo by blocking its ubiquitylation (4). Various transcription factors are modified by SUMO-1, with SUMO-1 modification correlating with attenuation of transactivation activity in most instances, as has been demonstrated for the androgen receptor, the glucocorticoid receptor, Sp3, Myb, and C/EBP. Transcription factors such as p53, HSF1, and HSF2 are activated by SUMO-1 modification (11), however. Several transcription cofactors, including p300 (12), SRC-1 (13), HDAC1 (14), and HDAC4 (15), are also modified by SUMO-1. Although DNA repair proteins such as Top1 (16), proliferating cell nuclear antigen (17), and WRN (18) are modified by SUMO-1, the biological significance of this modification remains unknown in most instances.

1 The abbreviations used are: E1, SUMO-1-activating enzyme; E2, SUMO-1-conjugating enzyme; E3, SUMO-1-protein isopeptide ligase; RARα, retinoic acid receptor α; NB, nuclear body; TDG, thymine DNA glycosylase; GST, glutathione S-transferase; HA, hemagglutinin epitope; PRS, phosphate-buffered saline; BER, base excision repair; RXRα, retinoid X receptor α; PML, promyelocytic leukemia protein; DBD, DNA binding domain; CBP, CREB-response element-binding protein.
Complementary DNA encoding SUMO(\textit{H9004})

Eagle’s medium (Sigma, St. Louis, MO) supplemented with 5% CO_2 at 37 °C in Dulbecco’s modified

and PML and thereby plays an important role in the colocalization of TDG with PML in NBs. Finally, we show that SUMO-1 modification of TDGb and PML enhances the interaction between the two proteins, including transcriptional regulation (21), DNA repair (especially repair of double-strand breaks) (22), and interferon-induced antiviral defense (23) have been suggested for PML NBs. Many proteins, including transcription factors, their regulators, and DNA repair proteins have been detected in these structures (21). Furthermore, PML appears to be responsible for targeting proteins such as Sp100, CBP, and Daxx to NBs (20). PML contains three lysine residues that are modified by SUMO-1 (24), and many NB-associated proteins also appear to be covalently modified by SUMO-1 (21). The SUMO-1 modification of PML plays an essential role in the establishment and maintenance of NBs. Thus, PML modified by SUMO-1 is localized to NBs, whereas the unmodified protein is diffusely distributed in the nucleoplasm. Other NB-associated proteins are mislocalized in PML-deficient cells and exogenous PML restores their localization to NBs (20).

Ubiquitylated proteins are recognized by effector molecules that function as receptors for ubiquitin. These effectors include S5a, Rad23, and Vps9, all of which possess ubiquitin binding domains (UBA, UEV, UIM, CUE) (25). The S5a subunit of the 19 S proteasome thus recognizes polyubiquitin chains and guides ubiquitylated proteins into the 20 S proteasome for degradation (26, 27). In contrast, molecules or motifs that interact physiologically with SUMO-1 in a noncovalent manner have not been identified to date. Such molecules or motifs, if found to exist, might be expected to play important roles in SUMO-1 function, however.

To identify proteins that might recognize SUMO-1 but are not necessarily modified by SUMO-1, we have now performed yeast two-hybrid screening with a form of SUMO-1 that lacks the COOH-terminal glycine residue and therefore cannot be conjugated to target proteins (28, 29). This screening resulted in the isolation of cDNAs for thymine DNA glycosylase (TDG), an enzyme that functions in base excision repair of DNA damage (30). TDG initiates the repair of G:U or G:T mismatched base pairs that result from spontaneous deamination of cytosine or methylated cytosine (30, 31). We also show that TDG not only binds noncovalently to, but is also covalently modified by, SUMO-1. TDG was found to bind to PML in \textit{vitro} and colocalized with PML in NBs. Furthermore, the noncovalent SUMO-1 binding activity of TDG was required for its localization to NBs. Finally, we show that SUMO-1 modification of TDGb and PML enhances the interaction between the two proteins. We thus propose that SUMO-1 functions as a molecular glue to mediate the noncovalent interaction between TDG and PML and thereby plays an important role in the colocalization of TDG with PML in NBs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK293T and HeLa cells were grown under an atmosphere of 5% CO_2 at 37 °C in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen).

**Construction of Plasmids and Site-directed Mutagenesis**—Complementary DNA encoding SUMO(\textit{ΔGG}), which lacks the two tandem glycine residues at the COOH terminus of processed SUMO-1, was generated by PCR with pGEX-SUMO-1 GG (32, 33) as the template and was subsequently cloned into pBTM116. The cloning site was selected so as to yield either DBD-SUMO(\textit{ΔGG}), in which SUMO(\textit{ΔGG}) is fused to the COOH terminus of the DNA binding domain of LexA encoded by pBTM116, or SUMO(\textit{ΔGG})-DBD, in which SUMO(\textit{ΔGG}) is fused to the NH_2 terminus of the DNA binding domain of LexA.

The cDNAs for the TDGb derivatives FL, N-1, N-2, N-3, C-1, and C-2 (see Fig. 1) were generated by PCR and cloned into pACT2 (Clontech, Palo Alto, CA) or pGEX6P (Amersham Biosciences). The TDGb(KR) plasmids pcDNA3-TDGb(KR) and pACT2-TDGb(KR) were generated by replacing the codon for Lys^{317} of TDGb with an Arg codon with the use of a QuikChange\textsuperscript{TM} site-directed mutagenesis kit (Stratagene, La Jolla, CA). To generate the TDGb(\Delta)11 mutant, we performed PCR with pACT2 or pcDNA3 (Invitrogen) containing the full-length TDGb cDNA as the template and either with a sense primer beginning at the start codon and an antisense primer beginning proximal to the SUMO-1 binding region (amino acids 305 to 315), or with a sense primer beginning distal to the stop codon at their NH_2 termini with the Myc epitope were generated by PCR and cloned into pcDNA3 for transfection experiments or into pET30 (Novagen, Darmstadt, Germany) for the production of recombinant proteins.

A vector for SUMO-1 or SUMO(\textit{ΔGG}) tagged with the FLAG epitope at its NH_2 terminus were generated by subcloning the SUMO-1 GG or SUMO(\textit{ΔGG}) cDNA (32, 33) into p\textbf{3}-\textit{FLAG}-CMV-7.1 (Sigma). The plasmids pGEX6P-RanGAP1-C2, pET28-Ubc9, and pET30-SUMO-1 GG were described previously (32, 34, 35). Baculoviruses encoding a glutathione S-transferase (GST) fusion protein of Uba2 and hexahistidine (His\textsubscript{6})-tagged Aos1 were kindly provided by H. Yasuda (Tokyo University).

The cDNA for human PML isoform IVa (19) was amplified by PCR from human liver cDNA (Clontech) with Taq polymerase (Takara, Tokyo, Japan) and with the primers 5’-CTTCCAGCATCCCAAAGAT-3’ and 5’-AAGCCGAAATGCACAGC-3’. The amplified DNA fragment was phosphorylated with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and cloned into pBluescript SK+ (Stratagene), and its sequence was verified. The PML cDNA was then subcloned from pBluescript SK+ into pCGN-HA (36) for transfection experiments and into pFASTBAC HTb (Invitrogen) for recombinant protein production. A cDNA for the hemagglutinin epitope (HA)-tagged mutant PML(3KR) was generated by replacing the codons for Lys\textsuperscript{665}, Lys\textsuperscript{160}, and Lys\textsuperscript{442} with Arg codons with the use of a QuikChange site-directed mutagenesis kit.

**Yeast Two-hybrid Screening**—The SUMO(\textit{ΔGG}) cDNA subcloned into pBTM116 (encoding the DNA binding domain of LexA) was used as a bait in the MATCHMAKER Two-hybrid System (Clontech). Yeast strain L40 cells (MAT\textbf{a} LYS2::lexA-HIS3 UBA::exo-lacZ trp1 leo2 his3) (Invitrogen) were sequentially transformed with pBTM116-SUMO(\textit{ΔGG}) and a cDNA library (mouse T or B cell or human brain) constructed in pACT2 (encoding the GAL4 activation domain). Approximately 5 × 10^6 cDNA clones were screened. Transformed L40 cells were grown on agar plates containing a synthetic “dropout” medium without leucine, tryptophan, histidine, lysine, and uracil but containing 25 μM 3-aminotriazole. Colonies that grew on the selection medium were transferred to a filter and assayed for β-galactosidase activity with the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Plasmid DNA was extracted from positive clones and used together with the bait plasmid to transform yeast cells to confirm binding (37).
Noncovalent Binding of TDG to SUMO-1

Production of Recombinant Proteins in Bacteria—GST fusion proteins were expressed in *Escherichia coli* strain XL-1 blue cultured in the presence of 1 mM isopropyl β-D-thiogalactopyranoside. Bacterial cells were resuspended in phosphate-buffered saline (PBS) and lysed with the use of a French press, and cellular debris was removed by centrifugation for 30 min at 15,000 × g. Glutathione-Sepharose 4B beads (Amersham Biosciences) were added to the resulting supernatant, and the mixture was rotated at 4 °C for 2 h. The beads were washed with PBS, and GST fusion proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione.

His-tagged proteins were expressed in *E. coli* strain BL21(DE3) pLys(S) (Novagen) also incubated in the presence of 1 mM isopropyl β-D-thiogalactopyranoside. The recombinant proteins were purified with the use of ProBond resin (Invitrogen). The purity of all recombinant proteins was confirmed by SDS-PAGE and staining with Coomassie Blue (data not shown).

* Baculovirus Expression System—Baculoviruses were generated in Sf21 cells with the use of Bacmid virus DNA and pFASTBAC (Invitrogen) containing the relevant cDNA. The recombinant proteins were purified from Sf21 cell lysates according to the protocol described below for lysis and immunoprecipitation in transfected cells. The plasmids pFASTBAC HTa or HTb containing the relevant cDNAs were subjected to recombination with the baculoviral genome in DH10BAC, and the resulting recombinant viral genomes were introduced into Sf21 cells by transfection to generate recombinant baculoviruses. Infected Sf21 cells were lysed and the recombinant proteins were purified by the protocol described for bacterially expressed His-tagged proteins. The purified recombinant proteins were detected by SDS-PAGE and Coomassie Blue staining (data not shown).

Transfection, Immunoprecipitation, and Immunoblot Analysis—HEK293T cells were transfected by the calcium phosphate method. After 48 h, the cells were lysed with a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, aprotinin (1 μg/ml), leupeptin (10 μg/ml), 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 0.4 mM Na3VO4, 0.4 mM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The cell lysates were incubated for 30 min at 4 °C either with mouse monoclonal antibodies to the Myc epitope (1 μg/ml, 9E10; Covance, Berkeley, CA) and protein G-Sepharose beads (Amersham Biosciences) or with mouse monoclonal antibodies to HA (0.5 μg/ml, HA.11/16B12; Babco, Richmond, CA) conjugated to NHS-activated Sepharose 4 Fast Flow (Amersham Biosciences). The resulting immunoprecipitates were washed three times with ice-cold lysis buffer, fractionated by SDS-PAGE on a 9% gel, and subjected to immunoblot analysis with mouse monoclonal antibodies to Myc (1 μg/ml, 9E10), FLAG epitope (1 μg/ml; M5, Sigma), SUMO-1 (2 μg/ml, anti-GMP-1; Zymed Laboratories, South San Francisco, CA), or HA (1 μg/ml, HA.11/16B12). Immune complexes were detected with horseradish peroxidase-conjugated rabbit polyclonal antibodies to mouse immunoglobulin (Promega, Madison, WI) and enhanced chemiluminescence reagents (Amersham Biosciences).

Immunofluorescence Staining—HeLa cells grown on glass coverslips were transfected by calcium phosphate precipitation and subsequently prepared for immunostaining. In brief, the cells were fixed for 20 min at room temperature with 4% formaldehyde in PBS and then incubated for 1 h at room temperature first with a mouse monoclonal antibody to the Myc epitope (1 μg/ml, 9E10) in PBS containing 0.1% bovine serum albumin and 0.1% saponin and then with Alexa 488-labeled goat polyclonal antibodies to mouse immunoglobulin (Molecular Probes) at a dilution of 1:2000. The cells were subsequently incubated for 1 h at room temperature first with rabbit polyclonal antibodies to HA (1 μg/ml, Y11; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 0.1% bovine serum albumin and 0.1% saponin and then with Alexa 546-labeled goat polyclonal antibodies to rabbit immunoglobulin (Molecular Probes) at a dilution of 1:2000. They were finally stained with Hoechst 33258 (Wako, Osaka, Japan), covered with a drop of GE/MOUNT (Biomedia, Foster City, CA), and examined with a confocal fluorescence microscope (Radiance 2000; Bio-Rad).

In Vitro SUMO-1 Modification Assay—Reaction mixtures (20 μl) containing 1 μg of His6-Myc-STAT3, His6-Myc-TDGb, His6-Myc-TDGb(28, 29). The DNA binding domain of LexA was fused to either the NH2 or COOH terminus of SUMO1 (GG), that lacks the two tandem glycine residues at the COOH terminus and therefore cannot be conjugated to target proteins (28, 29). The DNA binding domain of LexA was fused to either the NH2 or COOH terminus of SUMO-1 (GG), yielding DBD-SUMO-1 (GG) and DDBB-SUMO-1 (GG)-DBD, respectively. We then performed yeast two-hybrid screening with these constructs as baits. With DBD-SUMO-1 (GG) as the bait, TDG isoform b (TDGb) (two clones), Sp100 (one clone), and Uba2 (one clone) cDNAs were isolated from a mouse T cell library; Epstein-Barr virus-induced 3 (EBI3) (one clone) and Sp100 (one clone) cDNAs were isolated from a mouse B cell library; and protein inhibitor of activated STAT3 (PIAS3) (one clone) cDNA was isolated from a human brain library. With SUMO-1 (GG)-DBD as the bait, we obtained 14 clones encoding TDGb from a mouse T cell library. In the present study, we focused on the functional characterization of the noncovalent SUMO-1 binding activity of TDGb.

Identification of the TDGb Domain Required for Noncovalent Binding of SUMO-1—TDGb possesses one SUMO-1 conjuga-
tion consensus sequence, VKEE (amino acids 316 to 319), and the TDGa isoform, which is 24 amino acids longer at the NH2 terminus than is TDGb (39), has previously been shown to be modified by SUMO-1 at this site (40). To identify the region of TDGb responsible for noncovalent binding of SUMO-1, we examined the interactions of a series of deletion mutants of TDGb fused to the COOH terminus of the GAL4 activation domain with SUMO(H9004GG)-DBD in the yeast two-hybrid system; the results are summarized in the first column on the right. The ability of the TDGb derivatives to undergo SUMO-1 conjugation was also determined in transfected HEK293T cells in experiments similar to those shown in Fig. 2. The results are summarized in the second column on the right. n.d., not determined.

A, full-length (FL) TDGb and a series of mutants thereof (shown on the left) were fused to the COOH terminus of the activation domain of GAL4 and tested for their ability to bind to the SUMO(H9004GG)-DBD fusion protein in the yeast two-hybrid system. The results are summarized in the first column on the right. The ability of the TDGb derivatives to undergo SUMO-1 conjugation was also determined in transfected HEK293T cells in experiments similar to those shown in Fig. 2. The results are summarized in the first column on the right. n.d., not determined.

B, recombinant His6-tagged HA-SUMO-1, Myc-TDGb, Myc-TDGb(KR), and Myc-TDGb(Δ11) were expressed in E. coli and purified with a nickel resin column. Recombinant His6-tagged Myc-STAT3 (negative control) was expressed in Sf21 cells and purified with a nickel resin column. The indicated combinations of proteins were subjected to immunoprecipitation (IP) with anti-HA, and the resulting precipitates were subjected to immunoblot analysis (IB) with anti-Myc and anti-HA. A portion (5% of the input for immunoprecipitation) of each binding mixture was also subjected directly to immunoblot analysis.

To confirm the results of the yeast two-hybrid analysis, we performed an in vitro binding assay with recombinant His6-Myc-STAT3 (negative control), His6-Myc-TDGb (FL, KR, Δ11), and His6-HA-SUMO-1. The binding mixture was subjected to immunoprecipitation with antibodies to (anti-) HA, and the resulting precipitates were subjected to immunoblot analysis with anti-Myc and anti-HA. A portion (5% of the input for immunoprecipitation) of each binding mixture was also subjected directly to immunoblot analysis.

Requirement of Noncovalent Binding of TDGb to SUMO-1 for SUMO-1 Conjugation—To examine the SUMO-1 modification of TDGb, we transiently transfected HEK293T cells with expression vectors for Myc epitope-tagged Axin (negative control), Myc-TDGb, or Myc-TDGb(KR) and either FLAG-tagged
SUMO(ΔGG) (negative control) or FLAG-SUMO-1. The transfected cells were lysed in the presence of N-ethylmaleimide, an inhibitor of SUMO-1-specific protease. Whole cell lysates and immunoprecipitates prepared with anti-Myc were subjected to immunoblot analysis with anti-FLAG and anti-Myc. Myc-TDGb was found to be conjugated with endogenous or exogenous (FLAG-tagged) SUMO-1, whereas Myc-TDGb(KR) was not (Fig. 2A), indicating that TDGb is conjugated with SUMO-1 at Lys\(^{317}\) in the VKEE consensus sequence as demonstrated for TDGa (40). Immunoblot analysis of the cell lysates with anti-FLAG also revealed that endogenous RanGAP1 was conjugated with exogenous (FLAG-tagged) SUMO-1. We next tested TDGb(Δ11), the minimal deletion mutant that was not able to bind SUMO-1 noncovalently but which retains the VKEE consensus motif, for modification by SUMO-1. HEK293T cells were transiently transfected with vectors for Myc-TDGb, Myc-TDGb(KR), or Myc-TDGb(Δ11) and subsequently subjected to immunoprecipitation with anti-Myc. Immunoblot analysis of the resulting precipitates with anti-Myc and anti-SUMO-1 revealed that, like TDGb(KR) but unlike wild-type TDGb, TDGb(Δ11) was not modified by SUMO-1 (Fig. 2B). We also examined the C-1 and C-2 mutants, which did not bind to SUMO(ΔGG) but retain the SUMO-1 conjugation consensus sequence (VKEE) and surrounding residues. These mutants were not conjugated with SUMO-1 (data not shown), making it more likely that the inability of TDGb(Δ11) to undergo conjugation is attributable to a structural alteration caused by deletion of residues adjacent to the VKEE sequence. Together, these findings suggest that the noncovalent binding of TDGb to SUMO-1 is required for covalent conjugation with SUMO-1.

In Vitro Conjugation of SUMO-1 to TDGb—TDGa was previously shown to be modified by SUMO-1 in a crude in vitro system (40). To characterize further the SUMO-1 modification of TDGb, we attempted to reconstitute the reaction in vitro with only purified recombinant proteins. In the presence of recombinant E1 (Aos1-Uba2), E2 (Ubc9), SUMO-1, and ATP, both wild-type TDGb and GST-RanGAP1-C2, the latter of which was previously shown to be modified by SUMO-1 (35) and was used as a positive control, were modified by SUMO-1, whereas TDGb(KR) and TDGb(Δ11) were not (Fig. 3). These findings demonstrate that TDGb was modified by SUMO-1 at Lys\(^{317}\) in the SUMO-1 modification consensus sequence (VKEE) and that the noncovalent binding of TDGb to SUMO-1 was required for covalent conjugation with SUMO-1.

Requirement of Noncovalent SUMO-1 Binding Activity for Localization of TDGb to PML NBs—Given that some proteins modified by SUMO-1 are localized in PML-containing NBs (21), we examined whether TDGb might colocalize with PML in such structures. HeLa cells were transfected with expression vectors for Myc epitope-tagged TDGb and HA-tagged PML, and the localization of the exogenous proteins was determined by immunofluorescence staining with anti-Myc and anti-HA. Wild-type TDGb was localized exclusively in the nucleus, where it was concentrated in NBs containing PML (Fig. 4A). Given that SUMO-1 modification is thought to regulate the subcellular distribution of several proteins, we investigated whether SUMO-1 modification of TDGb contributes to its localization with PML in NBs by examining cells expressing both Myc-TDGb(KR) and HA-PML. We found that TDGb(KR) colocalized with PML to an extent similar to that observed for the wild-type protein (Fig. 4B), indicating that SUMO-1 modification of TDGb is dispensable for its colocalization with PML. To investigate whether the noncovalent SUMO-1 binding activity of TDGb is necessary for its colocalization with PML, we examined cells expressing Myc-TDGb(Δ11) and HA-PML. In contrast to wild-type TDGb and TDGb(KR), TDGb(Δ11) was not concentrated in PML-containing NBs but was diffusely dispersed in the nucleoplasm (Fig. 4C). These data suggest that the noncovalent SUMO-1 binding activity of TDGb is essential for colocalization of the protein with PML.

PML is also modified by SUMO-1 at Lys\(^{65}\), Lys\(^{160}\), and Lys\(^{422}\) (19, 24), and such modification is essential for the formation of normal NBs (20). The PML(3KR) mutant, in which these three lysine residues are replaced with arginines, thus forms abnormal NBs. We investigated the subcellular localizations of Myc-TDGb and HA-PML(3KR) in transfected HeLa cells. PML(3KR) formed three to six large abnormal NBs per cell, in contrast to the 10–30 NBs formed by the wild-type protein. The distribution of wild-type TDGb overlapped partially with that of PML(3KR) at the edge of the abnormal NBs (Fig. 4D), but the colocalization of the two proteins was remarkably less pronounced than that of TDGb and wild-type PML. The distributions of TDGb(KR) and PML(3KR) did not appear to overlap specifically (Fig. 4E), suggesting that SUMO-1 modification of either TDGb or PML is required for their colocalization.

Enhanced Interaction of SUMO-1-modified TDGb with PML in Vitro—We next performed an in vitro binding assay with recombinant Myc-TDGb and recombinant PML. The binding mixture was subjected to immunoprecipitation with anti-PML, and the resulting precipitates were subjected to immunoblot analysis with anti-Myc and anti-PML. TDGb bound directly to PML in this assay (Fig. 5A). To examine whether SUMO-1 modification of TDGb affects its interaction with PML, we prepared SUMO-1-modified Myc-TDGb by an in vitro reaction and determined its ability to bind to PML in the presence of the unmodified protein. Although the PML immunoprecipitate contained both SUMO-1-modified and unmodified forms of TDGb, we found that the PML binding activity of the SUMO-1-modified form of the protein was 2.5-fold greater than that of the unmodified form (Fig. 5, B and C). The modification of TDGb by SUMO-1 thus enhanced its ability to bind PML.

Enhanced Interaction of SUMO-1-modified TDGb with PML in Vivo—To examine whether SUMO-1 modification of PML affects its interaction with TDGb, we transiently transfected HEK293T cells with expression vectors for Myc epitope-tagged Axin (negative control) or Myc-TDGb (2.5, 5, or 10 μg) and for either HA-tagged PML or HA-PML(3KR). The transfected cells were lysed in the presence of N-ethylmaleimide, and both whole cell lysates and immunoprecipitates prepared with anti-HA were subjected to immunoblot analysis with anti-Myc and anti-HA. The extent of TDGb binding to PML (containing both SUMO-1-modified and unmodified forms) was greater than that to PML(3KR) (Fig. 6). These results suggest that SUMO-1 modification of PML enhances its ability to bind to TDGb.

DISCUSSION

Ubiquitin is the prototype of a superfamily of proteins that are conjugated to other proteins and include SUMO-1, SUMO-2, SUMO-3, NEDD8, ISG15, UCRP, URM, HUB1, ATG-8, and ATG-12 (41). Whereas the 19 S proteasome has been shown to function as a receptor for polyubiquitin chains (27), receptor molecules for other members of the ubiquitin superfamily have not been identified to date, hindering progress in characterization of the biological importance of these proteins at the molecular level. In the present study, we therefore attempted to identify potential SUMO-1-binding proteins by yeast two-hybrid screening with a SUMO-1 mutant that lacks the two tandem COOH-terminal glycine residues and is thus not able to be joined covalently to target proteins (28, 29).

Most of the proteins identified by such screening have been shown to have a functional relation with SUMO-1, supporting the validity of this approach. Uba2 is thus one of the subunits...
FIG. 2. In vivo modification of TDGb by SUMO-1. A, HEK293T cells were transiently transfected with expression vectors for Myc epitope-tagged Axin (negative control), Myc-TDGb, or Myc-TDGb(KR) and either FLAG-tagged SUMO(ΔGG) (negative control) or FLAG-SUMO-1, as indicated. Cell lysates were subjected to immunoprecipitation with anti-Myc, and the resulting precipitates were subjected to immunoblot analysis with anti-Myc and anti-FLAG. A portion (3% of the input for immunoprecipitation) of the cell lysates was also subjected directly to immunoblot analysis. The signal at an apparent molecular size of ~86 kDa (asterisk) corresponds to Myc-TDGb conjugated with FLAG-SUMO-1. B, Myc epitope-tagged TDGb, Myc-TDGb(KR), or Myc-TDGb(Δ111) was expressed in HEK293T cells, immunoprecipitated with anti-Myc, and subjected to immunoblot analysis with anti-Myc or anti-SUMO-1. IgH, immunoglobulin heavy chain.
of the E1 enzyme for SUMO-1 (Uba2-Aos1) (3). Sp100 is modified by SUMO-1 and is one of the constituents of PML-containing NBs (42). PIAS3 is a member of the PIAS family of proteins, several of which appear to function as E3 ligases for SUMO-1 (1). TDG contributes to the base excision repair (BER) of DNA. It thus initiates the repair of G:U or G:T mismatched base pairs, which are generated as a result of spontaneous deamination of cytosine or methylated cytosine, by removing the incorrect (uracil or thymine) bases (30, 43). A SUMO-1-modified form of TDG was found not to act on G:T mismatches but its ability to process G:U was markedly enhanced (40). Although covalent SUMO-1 modification of TDG was shown to

Fig. 3. In vitro modification of TDGb by SUMO-1. Recombinant His6-tagged SUMO-1, Ube9 (E2), Myc-TDGb, Myc-TDGb(Δ11), and Myc-TDGb(KR) were expressed in E. coli and purified with a nickel resin column. Recombinant His6-tagged Myc-STAT3 (negative control) and both GST-Uba2 and His6-Aos1 (E1) were expressed in Sf21 cells and purified with a nickel resin column. GST-RanGAP1-C2 (positive control) was expressed in E. coli and purified with glutathione-Sepharose 4B beads. Myc-STAT3, Myc-TDGb, Myc-TDGb(Δ11), Myc-TDGb(KR), or GST-RanGAP1-C2 were incubated with E1, E2, and SUMO-1 in the absence or presence of a reaction mixture containing ATP, as indicated. The reaction mixtures were then subjected to immunoblot analysis with anti-SUMO-1, anti-Myc, and anti-GST. The asterisk indicates the modification of wild-type TDGb by SUMO-1 in the presence of E1, E2, and ATP.

Fig. 4. Colocalization of TDGb and PML. HeLa cells were transiently transfected with expression vectors for Myc-TDGb and HA-PML (A), Myc-TDGb(Δ11) and HA-PML (B), Myc-TDGb(KR) and HA-PML (C), Myc-TDGb and HA-PML(3KR) (D), or Myc-TDGb(KR) and HA-PML(3KR) (E). After 36 h, the cells were fixed and immunostained with anti-Myc (left panels) and anti-HA (center panels). Merged images are also shown (right panels).
FIG. 5. Enhancement of the binding of TDGb to PML by SUMO-1 modification. Recombinant PML and Myc-TDGb were expressed as His6-tagged proteins in Sf21 cells and E. coli, respectively, and both proteins were purified with a nickel resin column. PML was mixed with Myc-TDGb (A) or with Myc-TDGb and SUMO-1-modified Myc-TDGb (B) in vitro, as indicated, and the binding mixtures were then subjected to immunoprecipitation with anti-PML. The resulting precipitates, as well as a portion (10%) of the original binding mixtures, were subjected to immunoblot analysis with anti-Myc and anti-PML. The amounts of SUMO-1-modified and unmodified TDGb in both the PML immunoprecipitate (lane 4) and the original binding mixture (lane 8) in B were quantified by scanning densitometry of the immunoblots, and the ratio of the amount of each protein in the immunoprecipitate to that in the binding mixture was calculated and expressed relative to the value for unmodified TDGb (C).
increase its activity in BER, the function of the noncovalent SUMO-1 binding activity of TDG is unclear. Two isoforms of TDG, TDGa and TDGb, are generated as a result of alternative RNA splicing, with TDGb lacking the 24 NH₂-terminal amino acids of TDGa (39). Given that this NH₂-terminal region does not contain any known functional domain and that the expression patterns of TDGa and TDGb are similar (39), there may be no functional difference between the two proteins. We used mouse TDGb isolated in our screening for subsequent experiments in this study.

PML contains a SUMO-1-interacting motif similar to that present in PIAS family proteins (44). It is possible that the region of PML that contains this motif recognizes the SUMO-1 moiety conjugated to TDGb. We propose that the noncovalent SUMO-1 binding activity of TDGb plays two roles in intramolecular and intermolecular interactions (Fig. 7). First, such noncovalent binding is required for covalent conjugation of SUMO-1 to TDGb. The TDGb(KR) mutant is not susceptible to SUMO-1 modification but retains noncovalent SUMO-1 binding activity. In contrast, none of the deletion mutants examined that had lost the ability to bind SUMO-1 noncovalently underwent covalent conjugation to SUMO-1. The noncovalent interaction of SUMO-1 with TDGb might thus lead to conjugation of the associated SUMO-1 molecule to TDGb (Fig. 7A). Second, the reciprocal SUMO-1 binding activity may be required for the intermolecular interaction between TDGb and PML (Fig. 7B). The TDGb(Δ11) mutant lacked the ability to colocalize with PML in NBs and was instead diffusely dispersed in the nucleoplasm. Similarly, the ability of the PML(3KR) mutant, which cannot be modified with SUMO-1, to colocalize with TDGb was greatly impaired.

FIG. 7. Model for the role of noncovalent SUMO-1 binding activity in the interaction between TDGb and PML. A, noncovalent binding of SUMO-1 to TDGb appears to be required for SUMO-1 conjugation. SUMO-1 may be tethered to TDGb in a noncovalent manner before becoming conjugated to TDGb. The affinity between TDGb and PML appears be determined by the interaction of the noncovalent SUMO-1 binding site of each protein with SUMO-1 conjugated to the other. A hierarchy of binding affinities between TDGb and PML derivatives is proposed.
Noncovalent Binding of TDG to SUMO-1

On the basis of our immunofluorescence and biochemical data, we propose that SUMO-1 modification of each of TDG and PML enhances the interaction between the two proteins. In vitro modification of TDG with SUMO-1 enhanced its binding to PML, suggesting that wild-type TDG binds to PML with higher affinity than does TDG(KR) in transfected cells. We were not able to achieve the sumoylation of the recombinant PML in vitro, whereas RanGAP1 (positive control) was sumoylated under the same conditions (Supplementary Materials Fig. S1), probably because the sumoylation of PML requires an unidentified E3 and/or because other modifications are prerequisite for sumoylation. Wild-type PML bound to TDG with higher affinity than did PML(3KR) in transfected cells, indicating that SUMO-1 modification of PML enhances its binding to TDG. Consistent with these results, colocalization of TDG with PML(3KR) was partially defective, whereas wild-type TDG or TDG(3KR) colocalized to similar extents with wild-type PML. Colocalization of TDG(3KR) and PML(3KR) was not apparent in cells expressing these two mutant proteins, suggesting that the interaction between these molecules is weaker than that between wild-type TDG and PML(3KR). TDG(Δ11) did not colocalize with wild-type PML; this mutant likely does not bind to the SUMO-1 moiety conjugated to PML and is itself resistant to SUMO-1 conjugation. Two-way interaction, one-way interaction, or no interaction between SUMO-1 and SUMO-1 binding sites may thus underlie the relative affinities and colocalization of the various TDG and PML binding domains (Fig. 7B).

PML functions as the organizer of NBs. These structures participate in various cellular activities including apoptosis, transcriptional regulation, and DNA repair. The many factors associated with PML-containing NBs include Sp100, p53, Daxx, CBP, and Bloom, and many of these proteins have been shown to be modified by SUMO-1 (20). SUMO-1 modification of these proteins is not clear. Both RAD51 and RAD52, which play an important role in homologous recombination repair of DNA damage, bind to SUMO-1 in a noncovalent manner, although, again, the significance of these interactions remains unclear (47). Proteins that contribute to homologous recombination repair, such as BLM, RAD51, and the Mre11-RAD51-NBS1 complex, also colocalize with PML in NBs in response to the induction of DNA double-strand breaks by ionizing radiation (22, 48, 49). It is thus possible that PML-containing NBs constitute sites of BER. PML might serve to recruit BER factors and regulate their activity as a result of changes in its binding affinity for these proteins mediated by SUMO-1 modification and noncovalent SUMO-1 binding activity.

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Noncovalent SUMO-1 Binding Activity of Thymine DNA Glycosylase (TDG) Is Required for Its SUMO-1 Modification and Colocalization with the Promyelocytic Leukemia Protein

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