SUMO Conjugation to the Matrix Attachment Region-binding Protein, Special AT-rich Sequence-binding Protein-1 (SATB1), Targets SATB1 to Promyelocytic Nuclear Bodies Where It Undergoes Caspase Cleavage*

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SATB1 (special AT-rich sequence-binding protein-1) provides a key link between DNA loop organization, chromatin modification/remodeling, and association of transcription factors at matrix attachment regions (MARs). To investigate the role of SATB1 in cellular events, we performed a yeast two-hybrid screen that identified SUMO-1, Ubc9, and protein inhibitor of activated STAT (PIAS) family members as SATB1 interaction partners. These proteins, working in concert, enhanced SUMO conjugation to lysine-744 of SATB1. Overexpression of SUMO or PIAS in Jurkat cells, which express high levels of endogenous SATB1, exhibited enhanced caspase cleavage of this MAR-associated protein. Sumoylation-deficient SATB1 (SATB1(K744R)) failed to display the characteristic caspase cleavage pattern; however, fusion of SUMO in-frame to SATB1(K744R) restored cleavage. A SUMO-independent interaction of inactive caspase-6 and SATB1 was noted. A subset of total cellular SATB1 localized into promyelocytic leukemia nuclear bodies where enhanced SATB1 cleavage was detected subsequent to caspase activation. These results reveal a novel sumoylation-directed caspase cleavage of this key regulatory molecule. The role of regulated proteolysis of SATB1 may be to control transcription in immune cells during normal cell functions or to assist in efficient and rapid clearance of nonfunctional or potentially damaging immune cells.

Scaffold or matrix attachment regions (MARs) are AT-rich sequences in the DNA of eukaryotic chromosomes that bind nuclear body; PIAS, protein inhibitor of activated STAT; STAT, signal transducers and activators of transcription; SUMO, small ubiquitin-like modifier; IL, interleukin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; PARP, poly(ADP-ribose) polymerase; ATRA, all-trans retinoic acid; Ni-NTA, nickel-nitrilotriacetic acid; GST, glutathione S-transferase; Ab, antibody; PBS, fetal bovine serum; CPT, camptothecin; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein.

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The abbreviations used are: MAR, matrix attachment region; SATB1, special AT-rich sequence-binding protein-1; PML, promyelocytic leukemia; NB,
Modification of proteins by SUMO (small ubiquitin-like modifier) family members, SUMO-1, SUMO-2, and SUMO-3 (12–15), plays a key role in cell growth, differentiation, and apoptosis (16). Unlike ubiquitination, which can target proteins for degradation by the 26 S proteasome, SUMO modifications mostly regulate protein targeting and protein-protein complex formation. It has been shown that SUMO modification targets proteins to promyelocytic leukemia nuclear bodies (17) or the nuclear pore complex (18–20). In a few cases, SUMO-1 conjugation occurs at the same lysine residues used for other post-translational modifications and thereby antagonizes the effects of these modifications (for example ubiquitination, which has a role in NF-κB activation (21)). A series of sequential reactions catalyzed by three enzymes, AOS1/UBA2 (E1), Ubc9 (E2), and an E3 ligase, append SUMO to substrate molecules. One of the most widely studied E3s belongs to the protein inhibitor of activated STAT (PIAS) transcription factor family. Additionally, a family of genes encodes SUMO-specific isopeptidases, which facilitate the rapid removal of SUMO from modified proteins, thus revealing sumoylation as a dynamic process that has the potential for rapid on/off responses, very likely following cellular stimuli (12, 13).

Promyelocytic leukemia nuclear bodies (PML NBs) are present in almost all mammalian cells. They are dynamic macromolecular structures that vary in size, number, and molecular content, dependent upon cellular stimuli. Sumoylation of the tumor suppressor protein PML is required for formation of these nuclear dots and for macromolecular aggregation of molecules within these structures. PML NBs contribute to a number of cellular processes including protein sequestration and release, post-translational modification, DNA repair, and apoptosis (22, 23). Evidence of a role for PML NBs in apoptosis is provided by the observation that cells derived from Pml-null mice are defective in executing cell death by different stimuli (12, 13).

Much is still unknown regarding how key regulatory proteins are targeted for caspase cleavage during cell function. Subsequent to cleavage, it is not known what the consequences of loss of that protein will be on the progression of cells into either cell differentiation or committed apoptosis. In this report, we provide evidence that SATB1 is covalently modified by SUMO at a single SUMO consensus modification site, Lys-744. SUMO conjugation to SATB1 targets this protein into punctate nuclear dots. SATB1 undergoes caspase cleavage in these subnuclear structures. This study thus presents the first report of SUMO-mediated caspase cleavage of a key regulatory protein.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—SATB1 cDNA, encoding amino acids 58–763, was excised from pECH-SATB1 (26) and inserted in-frame with the cDNA encoding the binding domain of the LexA protein in the vector pLexA (Clontech Laboratories, Inc.). Expression of the LexA-SATB1 fusion protein in yeast strain EGY48-[p80p-lacZ] was verified by Western blotting to SATB1 antibodies; this fusion protein did not autonomously activate the lacZ reporter gene in yeast (data not shown). Deletion constructs and specific site mutations of SATB1 were done using standard molecular biology techniques (27). SATB1 cDNA and deletions/mutations were subsequently inserted into pBlueScript II KS (Stratagene), pEGFP-C1 (Clontech), or pcDNA3.1/His (Invitrogen) for additional studies. Full-length SATB1 was amplified by PCR and inserted into pEGFP-C1 as described (10). To express SUMO-1 or SUMO-3 at the C terminus of SATB1, the primer pair 5′-ACAGGAGCTCTGAGTGATGGATCATTATGAAC-3′ and 5′-GTAAGATCCAGTCTTTCATAACGATTTAATGTCTG-3′ was used to PCR amplify, from a Jurkat cDNA library, a full-length SATB1 lacking a C-terminal stop codon and incorporating a BamHI restriction enzyme site for subsequent in-frame insertion of the SUMO-1 or SUMO-3 cDNAs. SUMO was PCR-amplified from the Jurkat cDNA library as described (28, 29). SUMO-1 and SUMO-3 were also expressed from the pcDNA3.1/His vector.

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**Yeast Two-hybrid Library Screen**—Yeast two-hybrid library screening and verification of positive clones were done as described in the manufacturer’s user’s manual (Clontech Laboratories) and Yeast Protocols Handbook (Clontech Laboratories, Inc.). In brief, a Jurkat cDNA library (Clontech Laboratories), prepared in the vector pB42AD (4 × 10⁹ individual cDNAs), was amplified 2-fold. Subsequently, the cDNAs were introduced into the yeast strain EGY48-[p80p-lacZ] that previously had been transfected with pLexA-SATB1−(58–763). Colonies that expressed Leu⁺, LacZ⁺ phenotypes were picked for further analysis. True positive clones were verified by independently screening expression of the two reporter genes, leu2 and lacZ (which have different promoters), to eliminate cDNAs that interacted only weakly or transiently. In a second assay, putative positives that indiscriminately interacted with a bait protein other than SATB1 (i.e. lamin C) or with the parental pLexA vector alone were eliminated. The remaining cDNAs were sequenced from their 5′-ends and identified using the Blast search engine (NCBI).

**In Vitro SUMO-1 Conjugation Assay**—Recombinant SUMO E1 (Aos1/Uba2 heterodimer), GST-Ubc9, His-tagged PIAS1, GST-SUMO-1Δ4C, and His-tagged SUMO-3 were expressed in Escherichia coli and purified by standard protocols. SATB1 (wild type, mutated, or deleted) were labeled with [³⁵S]methionine in the T₅₅T-coupled transcription/translation system (Promega) and used directly in the *in vitro* SUMO conjugation assay (30). Reactions were analyzed by denaturing SDS-PAGE. After fixing and staining, gels were dried and exposed to Kodak BioMax MR single emulsion x-ray film.

**Antibodies**—SATB1 antibodies were prepared by injecting duplicate pairs of rabbits with recombinant SATB1-(58–763) (12–9) or with internal peptides of SATB1 (Ab 16–9, amino acid sequence KTATIERTNKPG, residues 475–489; Ab 13–9, amino acid sequence EDNGPLGRGR LGST, residues 34–48) using standard protocols. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were from Zymed Labora-
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Cell Culture and Apoptosis Induction—Jurkat cells (mature T-cells), Nalm-6 (pre-B-cells), NB4 (promyelocytic), K562 (hematopoietic), HeLa (cervical carcinoma), and MCF-7 (human breast cancer) (the latter three obtained from ATCC) were maintained under standard conditions in RPMI 1640 medium (Jurkat, Nalm-6, K562, NB4) or in Dulbecco’s modified Eagle’s medium (HeLa, MCF-7) plus 10% FBS (Hyclone). Cells were transfected with the plasmid constructs described above by standard methods: Electroporation for Jurkat, Nalm-6, K562, and NB4; lipid technologies (Effectene; Qiagen) for HeLa and MCF-7. After 24–48 h, cells were used to investigate the effects of caspase activation or for microscopic analyses (see below). Transfected suspension cells (Jurkat, Nalm-6, K562, NB4) were collected and washed once in RPMI media plus 1% FBS. Attached cells were washed on the plates with Dulbecco’s modified Eagle’s medium plus 10% FBS (Hyclone) and 100 units/ml penicillin-streptomycin (Invitrogen). Cells were transfected with the plasmid constructs described above by standard methods: Electroporation for Jurkat, Nalm-6, K562, and NB4; lipid technologies (Effectene; Qiagen) for HeLa and MCF-7. After 24–48 h, cells were used to investigate the effects of caspase activation or for microscopic analyses (see below). Transfected suspension cells (Jurkat, Nalm-6, K562, NB4) were collected and washed once in RPMI media plus 1% FBS. Attached cells were washed on the plates with Dulbecco’s modified Eagle’s medium plus 1% FBS. For cells that were inhibited with caspase-6, the inhibitor (20 μM; Calbiochem) was added 1 h prior to the addition of the inducer of apoptosis. Cellular apoptosis was induced (for times indicated in the legends for Figs. 1D, 3, 4, and 6) with either anti-Fas (100 ng/ml), camptothecin (CPT; 2 μM) for all cells except K562, which required 20 μM, actinomycin D (10 μM), or etoposide (100 μM) in medium containing 1% FBS. After treatment, cells were collected and harvested directly into SDS gel loading buffer, boiled for 10 min, and loaded onto SDS-polyacrylamide gels. For MCF-7 and HeLa cells, both the floating and attached cells were combined. Proteins were transferred to Immobilon-P membranes, and proteins of interest were detected by Western blot technologies using antibodies to SATB1, EGFP (Clontech), and poly(ADP-ribose) polymerase (PARP), caspases-3 or -6, or β-actin (Santa Cruz Biotechnology).

Microscopy—For confocal microscopic studies, cells were transiently transfected with EGFP-SATB1 constructs on chamber slides, fixed for 10 min with 5% paraformaldehyde, washed three times in phosphate-buffered saline (PBS), and permeabilized in 0.5% Triton X-100 in PBS. After further washing in PBS as above, cells were blocked in 5 drops of Image-iT (Invitrogen-Molecular Probes) for 30 min at room temperature. Cells were then stained for 1 h at room temperature with rabbit-derived primary anti-PML (300 μl of 1 μg/ml anti-PML diluted in 3% bovine serum albumin-PBS; sc-966, Santa Cruz Biotechnology, Inc.). Following three, 5-min washes in 3% bovine serum albumin-PBS, the secondary antibody, Alexa Fluor 594 rabbit anti-mouse IgG, was used as described by the supplier (Invitrogen-Molecular Probes). Fluorescence signals were analyzed on a Zeiss Microimaging GmbH LSM 510 Meta (upright confocal microscope) equipped with a Zeiss C-Apochromat 63×/1.2 W cor objective. Laser excitation was 488 nm from an argon laser, 4% power for green fluorescence, and 543 nm from HeNe laser, 100% power for red fluorescence.

RESULTS

Identification of SATB1-interacting Partners—SATB1 is important for assembling various nuclear factors and for the organization and regulation of region-specific chromatin structure at MAR elements (32, 33). To better understand the role of SATB1 in cellular function, yeast two-hybrid screening of a Jurkat cDNA library identified a number of proteins with known roles in SUMO modification. SUMO-1 was encoded by almost one-third of the cDNA isolates. Additional cDNAs encoded conjugating (E2) and ligating (E3) enzymes for SUMO (ubc9, PIAS1, PIAS3, PIASy, Pc2, and Topors (28, 34–40)). Because of the importance of SUMO-1 modification in cellular processes, we have undertaken an investigation of the significance of this post-translational process for SATB1 function.

SUMO-1 and PIAS1 Display Caspase Activity in Jurkat T-cells—To examine the effect of SUMO-1 on cell function, EGFP-SUMO-1 was transiently overexpressed in Jurkat cells (mature T-cell line that expresses high levels of endogenous SATB1) for 24, 48, or 72 h, and then cells were harvested directly into SDS-PAGE loading buffer and prepared for Western blot analysis. In control cells, SATB1 was detected as a 103-kDa band by immunoblotting to anti-SATB1 (Fig. 1A, top panel, 0 h). A faster migrating band, in Jurkat cells overexpressing EGFP-SUMO-1, was detected at 70 kDa. Previously it had been demonstrated that SATB1 is cleaved by caspases during apoptotic stimulation in mouse thymocytes, Jurkat cells, and promyelocytic HL-60 cells, yielding ~70- and 30-kDa fragments (9, 10). SATB1 is also cleaved by caspase-6 under non-apoptotic conditions during B-cell stimulation (41). The 70-kDa protein detected here fractionated identically to the C-terminal product of SATB1 generated by caspase proteolysis (Fig. 1A, top panel, compare lanes 2 and 5) (9, 10, 41). This sub-band was also detected when the blot was stripped of the first probe and next immunoblotted to a C-terminal peptide anti-SATB1 (Ab 16-9, Fig. 1A, second panel) but not when immunoblotted to an N-terminal SATB1 peptide antibody (Ab 13-9; data not shown). The detection of SATB1 cleavage primarily within the first 24 h post-transfection suggested that the population of Jurkat cells transiently overexpressing EGFP-SUMO-1 was highly susceptible to elimination by apoptosis. This was corroborated by controls, which showed that cleavage of PARP (a primary indicator of apoptosis (42)), as well as activation of caspase-6 and -3, was enhanced within the first 24 h of SUMO overexpression in Jurkat cells (Fig. 1A, third, fourth, and fifth panels, respectively). We further examined transfected Jurkat cells by fluorescent microscopy at 24-h intervals post-transfection and found that, by 48 h, the majority of the EGFP-SUMO-1 was present in apoptotic and dying cells (data not shown). Next we overexpressed PIAS, which is normally present at moderate levels in cells, and which augments sumoylation of SATB1 (43), and noted the same results as above. Over-
expression of vector alone did not increase cellular caspase proteolysis of SATB1 (Fig. 1, B and C, respectively). Thus, sumoylation, either directly or indirectly, regulated caspase cleavage of SATB1 in Jurkat cells.

The SUMO pathway has been implicated in the regulation of cellular apoptosis (44). It is possible that, in Jurkat cells, overexpression of SUMO or PIAS1 enhances apoptosis, leading indirectly to cleavage of SATB1. However, SATB1 proteolysis could be mediated directly by SUMO conjugation to SATB1, for example, by directly recruiting caspase-6, which enhances its cleavage; or alternatively, SUMO modification could “tag” SATB1 leading to its relocation to nuclear areas of enhanced apoptotic proteolysis. If apoptosis induction does lead to increased sumoylation of SATB1, then the “SUMO intermediates” should be “captured” following induction of apoptosis in the presence of caspase-6 inhibitor. This was confirmed in Jurkat cells transiently overexpressing EGFP-SUMO-1. Higher molecular weight forms of endogenous SATB1 corresponding to the addition of one or more EGFP-SUMO-1 moieties were detected by Western blotting to anti-SATB1 or anti-EGFP in cells exposed to caspase-6 inhibitor but not in control cells (Fig. 1D).

SUMO Is Conjugated to SATB1 at a Single C-terminal Consensus Modification Site—Multiple SUMO-1 modification consensus sites (kXKXE) (45, 46) are present within SATB1 (Fig. 2B). In vitro assays detected the conjugation of GST-SUMO-1 to in vitro labeled [35S]Met-SATB1 at a single modification site (Fig. 2A, lane 4, and C, lanes 2, 4, 6, and 8). SUMO conjugation to SATB1 was greatly augmented by the addition of the E3 ligase PIAS1 (Fig. 2A, compare lanes 3 and 4 or lanes 6 and 7). SUMO-1 shares less than 50% identity with SUMO-2 or SUMO-3; however, these latter proteins are 95% identical to each other (15). SUMO-3, employed as a representative of these nearly identical proteins, modified SATB1 as a poly-SUMO chain, suggesting that SUMO-2/-3 adds multiple moieties to a single site (Fig. 2A, lanes 5–7; also see below) (29).

Of the six potential SUMO-1 consensus modification sites within SATB1 (Fig. 2B), the two N-terminal sites did not interact with SUMO-1 in the yeast two-hybrid interaction assay (data not shown). Therefore, lysines at amino acid positions 411, 486, 720, and 744 of full-length SATB1 were individually mutated to structurally similar arginines, which cannot be SUMO modified. Only mutagenesis of Lys-744, or deletion of the region encoding this lysine (residues 676–763), prevented in vitro SUMO-1 or -3 conjugation to SATB1 (Fig. 2C, lanes 10 and 12, and data not shown). Thus, PIAS1 augmented SUMO-1 and -3 conjugations to SATB1 at lysine 744.

Previous overexpression studies have shown SATB1 to be sumoylated at a single site (43); however, in vivo sumoylation has proven difficult to study (47). To trap the sumoylated form of endogenous SATB1, the hematopoietic K562 cell line (which express low levels of endogenous SATB1) was transfected with His-SUMO-1 or His-SATB1(FL), cells were harvested directly into 6 M guanidine-HCl (to inactivate proteases), and His-tagged proteins were purified by Ni-NTA pulldown (employing low stringency washes of 0 mM imidazole). Samples were fractionated on 7% SDS-polyacrylamide gels, immobilized on transfer membrane, and Western blotted (WB) to anti-SATB1 (left and right panels) or to anti-EGFP (middle panel). Relevant proteins and cleavage products are shown to the right of the panels.
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**SUMO-1 and SUMO-3 are conjugated to SATB1.** A, [35S]methionine-SATB1 was incubated with recombinant E1 (120 ng), Ubc9 (75 ng), GST-SUMO-1 (1 μg), and an ATP source at 37 °C for the times indicated above the panels. After fractionation of the products on SDS-PAGE, the gels were dried and exposed to Kodak Biomax MR single-emulsion film for up to 3 days. Input protein (SATB1) is in lane 1. Lane 2 contains GST-SUMO-1 modification under rigorous assay conditions (37 °C, 24 h). In lanes 3 and 4, [35S]Met-SATB1 was treated as described in A, except with lesser amounts of Ubc9 (15 ng) plus or minus PIASt1 (full-length; 60 ng) for 2 h. No sumoylation was detected in the absence of PIASt1 (lane 3), but significant enhancement was observed upon addition of the E3 (lane 4). Lanes 5–7 are the same as lanes 2–4, except with His-SUMO-3. B, six potential SUMO consensus modification sites within the SATB1 sequence. Their locales within the protein are indicated. C, the lysines at amino acid positions 411, 486, 720, and 744 of full-length SATB1 were individually mutagenized to encode arginines. The mutated constructs were used in the SUMO conjugation assay as described in A, wt, wild type. D, pcDNA3.1/His-SUMO-1 or pcDNA3.1/His-SATB1(FL) were transiently transfected into K562 cells. After 48 h, cells were harvested directly into 6 M guanidine-HCl and histidine-containing proteins were purified by Ni-NTA pulldown using 30 mM imidazole in the wash buffers. After fractionation of the His-tagged proteins on SDS-PAGE and immobilization on transfer membranes, the tagged proteins were detected with antibodies to SATB1 (antibody 12-9). The bottom panel is a longer exposure of the top panel. E, K562 cells were transiently transfected with pCMV-Tag2-SUMO-1 plus EGFP-SATB1(FL) (lanes 1, 2, 4, and 5) or with EGFP-SATB1(K744R) (lanes 3 and 6). After 24 h, cells were harvested directly into 6 M guanidine-HCl before purification of His-tagged proteins with Ni-NTA resin. Proteins were prepared for Western blot (WB) analysis as described in D and immunoblotted to anti-SATB1 (protein Ab 12-9; left panel) or anti-SUMO-1 (right panel).

SUMO modification of the wild type but not the mutant SATB1 (Fig. 2E, compare lanes 2 and 3 or 5 and 6). For these studies, cells were again harvested directly into 6 M guanidine-HCl followed by Ni-NTA pulldown (31). SATB1 possesses a histidine tract (His6), downstream of the MAR binding domain (Fig. 4A), that binds to the nickel resin during pulldown assays. This anomaly was exploited to select EGFP-SATB1 by performing washes under low stringency (Fig. 2E, compare lanes 1 and 2; washes contained 30 or 0 mM imidazole, high or low stringency, respectively). K562 cells expressing exogenous SUMO-1 expressed multiple forms of SATB1 including full-length SATB1 at 103 kDa and a form that migrated around 120 kDa plus higher molecular weight forms. This ladder of proteins was detected also by anti-SUMO-1, confirming that this is Flag-tagged SUMO-SATB1 (Fig. 2E, right panel; also Fig. 1C). Thus, SATB1 is sumoylated at lysine 744 both in vitro and in vivo.

**Sumoylation Targets SATB1 for Caspase Cleavage during Apoptotic Induction**—To investigate the role of SATB1 sumoylation in caspase cleavage of target protein, EGFP-SATB1Δ57 and EGFP-SATB1Δ57(K744R) were transiently expressed for 48 h in the pre-B-cell line Nalm-6, which also expresses low endogenous levels of SATB1 and its family member SATB2 (Fig. 3, lanes 1–22, panels immunoblotted to anti-SATB1). Cells were treated subsequently with the apoptosis inducer CPT (topoisomerase I inhibitor) for up to 8 h, and whole cell extracts were prepared for immunoblotting. The N-terminal EGFP-SATB1Δ57 cleavage fragment (~54 kDa) was detected in extracts expressing exogenous wild type SATB1 but not in extracts from cells expressing mutated SATB1 (Fig. 3B, top panel, compare lanes 9–12 with lanes 14–17). The same membrane, reblotted with anti-SATB1 (antibody to SATB1(58–763)) (Fig. 3B, middle panel), detected the C-terminal cleavage product of SATB1 (both endogenous and exogenous) but not the N-terminal fragment (9, 10). Finally, the membranes were immunoblotted to anti-PARP, which confirmed that apoptosis had indeed been induced in a time-dependent manner in the transiently transfected Nalm-6 cell population (Fig. 3B, bottom panel). The SATB1 C-terminal cleavage product detected in cells expressing SATB1(K744R) was from endogenous protein, a conclusion that was corroborated by treating Nalm-6 cells alone with CPT for up to 8 h; cell extracts were immunoblotted...
to anti-SATB1 (Fig. 3A, lanes 2–6). Also, results observed for HeLa cells further confirmed that this was endogenous SATB1 (no SATB1 C-terminal fragment was detected in Fig. 3D, lane 27, immunoblotted to anti-SATB1).

To establish the universality of these observations, different mechanistic classes of drugs were utilized to induce apoptosis in diverse cell lines. The anti-neoplastic antibiotic actinomycin D inhibits RNA synthesis, and etoposide is a topoisomerase II inhibitor. Both actinomycin D and etoposide treatment of diverse cell lines. The asterisks denote nonspecific protein bands. B, either wild type (EGFP-SATB1Δ57) or mutated (EGFP-SATB1Δ57(K744R)) SATB1 was expressed transiently in Nalm-6 cells. After 48 h, cells were washed and treated with CPT followed by immunoblotting as described in A. C, as described in A, transfected Nalm-6 cells were induced to undergo apoptosis with actinomycin D and then prepared for immunoblotting; only the 0 and 8 h time points are shown. D, as described in A, transfected HeLa cells were induced to undergo apoptosis with CPT and then prepared for immunoblotting; only the 0 and 8 h time points are shown.

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**FIGURE 3. Caspase cleavage of the MAR binding protein SATB1 is dependent upon the presence of an intact SUMO modification consensus site.** A, Nalm-6 cells were suspended in RPMI 1640 medium plus 1% FBS, and caspase cleavage was induced with CPT for the times indicated above the panels. Cells were harvested into SDS-PAGE loading buffer, fractionated on 8% SDS-polyacrylamide gels, and prepared for immunoblotting (WB) to the antibodies indicated shown at the left of the panels. Relevant proteins and cleavage products are shown to the right of the panels. Protein sizes are indicated in kilodaltons. The asterisks denote nonspecific protein bands. B, either wild type (EGFP-SATB1Δ57) or mutated (EGFP-SATB1Δ57(K744R)) SATB1 was expressed transiently in Nalm-6 cells. After 48 h, cells were washed and treated with CPT followed by immunoblotting as described in A. C, as described in A, transfected Nalm-6 cells were induced to undergo apoptosis with actinomycin D and then prepared for immunoblotting; only the 0 and 8 h time points are shown. D, as described in A, transfected HeLa cells were induced to undergo apoptosis with CPT and then prepared for immunoblotting; only the 0 and 8 h time points are shown.

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SUMO-independent effects of the SATB1(K744R) mutant could account for the above results, given that lysine residues are the sites of additional post-translational modifications, including ubiquitination, methylation or acetylation. Alternatively, Lys-744 of SATB1 could direct protein-protein interactions that influence caspase proteolysis. Thus, SUMO-1 or SUMO-3 were expressed as C-terminal in-frame fusions with EGFP-SATB1Δ57(K744R) or with full-length EGFP-SATB1(K744R) (Fig. 4A) in K562 cells followed by CPT induction for 48 h. Proteolysis of all fusion SATB1-SUMO constructs was readily detected upon apoptosis induction (Fig. 4, B and C). In the middle panel (Fig. 4, B and C), immunoblotted to anti-SATB1, the SATB1-SUMO fusions are preferentially cleaved over endogenous protein (in which fragmentation was detected only upon longer exposure of this blot or after longer treatment of cells with CPT (not shown)). PARP cleavage served as an indicator that programmed cell death had indeed been induced (Fig. 4, B and C, bottom panels). We also noted that full-length SATB1, which had been mutated at the caspase-6 cleavage site (SATB1(Cs-mut)) whether SUMO-1 was appended to the C terminus or not, was not cleaved during induction of apoptosis (Fig. 4C).

**SATB1-SUMO Co-localizes with PML Nuclear Dots—**A number of transcription factors and proteins of the nuclear matrix co-localize in PML nuclear bodies (48–51), as does the MAR-binding protein Bright (52). SATB2 is present in nuclear speckles of an undefined type (47). SATB1 was recently suggested to associate with PML in nuclear dot structures in primary human lung fibroblasts as well as in HeLa cells (shown by overexpression studies) (6). These studies suggest that the N-terminal region of SATB1, which contains a putative PDZ domain, recognizes sumoylated PML; however, this region is devoid of known SUMO-binding motifs5 (also see “Discussion”). We have observed independently that, in Jurkat cells stably expressing low levels of exogenous SUMO-1, SATB1 partially co-localized into PML NBs. Cells were prepared for confocal microscopic examination by dual color fluorescence staining with SATB1 (green) and PML (red) antibodies (Fig. 5).

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In these cell nuclei, endogenous SATB1 localized in a predominantly diffuse pattern, although a fraction of the SATB1 presented as intensely stained granules in cell nuclei, as did much of the PML; as expected, the SATB1 and PML particles overlapped, with the overlap varying from partial to complete (Fig. 5A, SATB1-FL). This variation has been observed with other proteins located in PML nuclear bodies (51, 53).

We further examined SATB1 localization into PML NBs by utilizing the NB4 cell line. NB4 is a human promyelocytic leukemia cell line that exhibits the APL diagnostic t(15;17) chromosomal translocation and expresses the PML-RARα fusion protein. PML NBs are delocalized into micro-speckled NBs in these cells. Following treatment of cells with all-trans retinoic acid (ATRA), PML-RARα is degraded, and PML NBs re-form (54, 55). NB4 cells also express high levels of SATB1 (6), and following ATRA induction, a subset of the total SATB1 co-localized with newly generated PML NBs (Fig. 5A, compare cells plus and minus ATRA). Thus, endogenous SATB1 localized to PML NBs in a mature T-cell line (Jurkat) expressing low exogenous levels of SUMO-1 and in the NB4 promyelocytic cells during SUMO-enhanced regeneration of PML NBs (23).

From the above results, we suspected that sumoylation of SATB1 might play a role in its localization to PML NBs. To investigate this further, we employed transient transfection of MCF-7 cells in conjunction with antibody staining and confocal microscopy. MCF-7s are large cells that have clearly delineated nuclear and cellular compartments. MCF-7 cells express transiently introduced exogenous proteins at high levels, yet have no detectable endogenous SATB1. Furthermore, CPT-induced cleavage of SATB1 constructs within MCF-7 cells was identical to apoptotic cleavage within lymphoid and erythroid cells (data not shown). MCF-7 cells transiently expressing EGFP-SATB1 (SATB1-FL or (K744R)-SUMO-1), were fixed and stained with anti-PML (red) and examined by confocal microscopy; full-length exogenous SATB1 localized diffusely throughout the nucleus (Fig. 5B, SATB1-FL). However, a significant proportion of the nuclear dots generated upon appendage of SUMO-1 or SUMO-3 at the C terminus of SATB1 overlapped with PML-NBs (Fig. 5B, SATB1-SUMO); SUMO-3 results not shown).

During early apoptosis induced by CPT, concomitant to when chromatin begins to collapse against the nuclear periphery, the nuclear speckles also redistributed to this locale. However, SATB1-SUMO at the nuclear periphery did not, in general, co-localize with PML NBs (Fig. 5C, SATB1-SUMO), although it was still present in macromolecular aggregates. This suggested that caspase cleavage of SATB1 occurred within PML NBs prior to displacement of SATB1 from chromatin and movement of this regulatory molecule out of the nucleus (9). Other cell lines were examined for SATB1 cellular localization (K562, Nalm-6, HeLa), and they gave qualitatively identical results (data not shown).
SUMO Targets SATB1 for Caspase Cleavage

NB4 cells, which, as noted above, lack PML NBs in their undifferentiated state, were transfected with EGFP-SATB1-FL or EGFP-SATB1(K744R)-SUMO-1 and examined by PML antibody staining and confocal microscopy. Both EGFP constructs localized diffusely throughout the nucleus, not into nuclear dot structures as would be expected for EGFP-SATB1-SUMO-1, providing further support that the nuclear dots into which sumoylated SATB1 localized are exclusively PML NBs (Fig. 5B, SATB1-FL or SATB1-SUMO-1).

PML NBs Contribute to Caspase Cleavage of SATB1—The above results suggested that PML-NBs were important for caspase cleavage of SATB1. These nuclear bodies could potentially serve as “sinks” for accumulation and/or activation of factors (i.e. caspases) involved in increased apoptotic activity. To investigate the relevance of PML NBs for SATB1 cleavage, Jurkat cells were treated with agents that lead to an increase in the numbers of PML NBs (i.e. MG132, a proteasome inhibitor, or arsenic trioxide (As$_2$O$_3$)). An aliquot of the cells was taken for immunostaining 4 h after initiation of treatment and analyzed by confocal microscopy, and the remaining cells were induced to undergo apoptosis with anti-Fas. SATB1 in control cells was first detectably cleaved after 2 h of induction with anti-Fas, whereas treating cells with MG132 or As$_2$O$_3$ prior to induction of apoptosis resulted in increased sensitivity to anti-Fas. Cleavage occurred as early as 1 h after exposure of cells to MG132, but more significantly, complete cleavage of SATB1 occurred by 6 h post-induction of caspase activity for cells exposed to either MG132 or As$_2$O$_3$, whereas SATB1 in control Jurkat was cleaved only partially (Fig. 6A). Almost identical cleavage results as those described for SATB1 were observed for the control protein, PARP (Fig. 6A, second panel). This is in agreement with reports that PML NBs regulate apoptosis by regulating the subcellular availability of apoptotic signal transducers (22, 25). There was a direct correlation between SATB1 cleavage and the number of PML NBs present during the various treatments; cells treated with MG132 or As$_2$O$_3$ exhibited two to three times more nuclear dots than did untreated Jurkat cells (Fig. 6B). These results suggested that intact PML NBs are important, but not absolutely essential for SATB1 cleavage following caspase activation.

We also employed NB4 cells to investigate the contribution of PML NBs toward apoptotic protein cleavage. After ATRA induction of differentiation and regeneration of PML NBs, SATB1 and PARP expression was down-regulated, as detected by Western blotting (Fig. 6C, compare SATB1 +ATRA and −ATRA), although caspase-3 and -6 protein levels remained stable, as did the levels of the loading control, β-actin. ATRA treatment alone did not induce PARP cleavage or caspase activation (Fig. 6C, see 0 or 2 h time points, +ATRA or −ATRA). However, cleavage of endogenous SATB1 and PARP following induction of apoptosis with actinomycin D occurred at a significantly greater rate in ATRA-treated cells (nearly 100% proteolysis by 4 h or 8 h) as compared with control cells (∼50% or less cleaved after 8 h). Caspase-3 and -6 activation also increased significantly during induction of apoptosis by actinomycin D in ATRA-treated cells as compared with controls (Fig. 6C).
SUMO Targets SATB1 for Caspase Cleavage

FIGURE 6. Increased numbers of PML NBs lead to augmentation of SATB1 cleavage following apoptotic induction. A, Jurkat cells were treated with MG132 (10 μM) or As2O3 (10 μM) for 4 h. A control (0 time) sample was taken, and apoptosis was then induced for up to 7 h with anti-Fas (100 ng/ml). Cells were harvested into SDS-PAGE loading buffer, fractionated on 8 or 15% denaturing polyacrylamide gels, and prepared for Western blotting (WB) as indicated in the legend to Fig. 2. Blots were probed with anti-SATB1 (top panel) or anti-PARP (second panel). B, after 4 h of treatment with the above agents, samples were taken and prepared for confocal microscopy as described in the legend to Fig. 5A. Anti-PML-stained cells are shown in red, and 4,6-diamidino-2-phenylindole (DAPI) staining is blue. C, NB4 cells, either controls or those treated with ATRA for 96 h, were induced to undergo apoptosis with actinomycin D (ActD) for up to 8 h. Cells were harvested into SDS-PAGE buffer and prepared for immunoblotting as described in the legend to Fig. 2D. Proteins were immunoblotted to anti-SATB1 (12-9), top panel), anti-PARP (second panel), anti-caspase-6 (third panel), anti-caspase-3 (fourth panel), or anti-β-actin (bottom panel, loading control).

FIGURE 7. Co-immunoprecipitation of SATB1 and caspase-6. Nalm-6 cells were transiently transfected with EGFP-SATB1(K744R), and after 48 h protein extracts were prepared in radioimmune precipitation assay buffer. Reciprocal immunoprecipitations were performed in a minimal volume with 1 mg of Nalm-6 whole cell extract using standard methods. Anti-SATB1, anti-caspase-6, anti-caspase-3, or preimmune serum (pre-I) was used for immunoprecipitation (IP). Bound proteins were released into SDS sample buffer, fractionated by SDS-PAGE, and transferred to Immobilon-P transfer membrane (Millipore). Immunoprecipitated proteins were detected with anti-SATB1, anti-caspase-6, or anti-caspase-3 (Fig. 7, top, middle, and bottom panels, respectively). In Nalm-6 cells, both endogenous SATB1 and EGFP-SATB1 (FL and K744R; K744R is shown) were immunoprecipitated by anti-caspase-6, suggesting that a physical interaction occurs between inactive caspase-6 and SATB1 and that this interaction occurs in a SUMO-independent manner. These investigations were also confirmed by immunoprecipitating endogenous SATB1 and caspase-6 from Jurkat cell extracts (data not shown).

DISCUSSION

Caspases comprise a family of evolutionarily conserved cysteinyI proteases that mediate both cell death and inflammation through cleavage of nuclear substrates, leading to inactivation, activation, or destruction of target proteins (56). Proteolytic loss of these target proteins leads to DNA fragmentation, changes in cell morphology, and cytoskeletal rearrangements, as well as disrupted control of essential cell regulatory functions. However, during the past decade, many laboratories have shown that proapoptotic caspases also function at specific checkpoints during immune cell development and differentiation independently of their role in apoptosis (7, 8). Although much work has been focused on the non-apoptotic role of caspases in activation and development of T-cells (7, 57), caspases also influence development of other types of immune cells (58–61). For example, activation of normal quiescent B-cells by a variety of proliferative stimuli increases caspase-6 and -8 activities. Inhibition of these caspases, independent of each other, blocks B-cell entry into the G1 phase of the cell cycle. It was suggested that caspase-6-like activity is required for quiescent B-cells to increase expression of genes required for entry into G1, SATB1, a transcriptional suppressor, was one of the substrates cleaved by caspase-6 upon B-cell activation (41). The non-apoptotic function of caspases occurs independently of activation of an apoptotic cascade, and the proteolytic activity of these enzymes is restricted to specific substrates in target cells. How then do some caspases mediate activation in some contexts and apoptosis in others? Sequestration might be one mechanism or post-translational modification of substrate.

SATB1 and Caspase-6 Co-localize in Nalm-6 and Jurkat Cells—Next, to investigate the interaction of SATB1 and caspase-6, reciprocal immunoprecipitation studies were undertaken. Nalm-6 cells were transiently transfected with EGFP-SATB1(K744R). Reciprocal immunoprecipitations were performed in a minimal volume with 1 mg of Nalm-6 whole cell extract using standard methods. Anti-SATB1, anti-caspase-6, anti-caspase-3, or preimmune serum was used for immunoprecipitation. Bound proteins were released into SDS sample buffer, fractionated by SDS-PAGE, and transferred to Immobilon-P transfer membrane (Millipore). Immunoprecipitated proteins were detected with anti-SATB1, anti-caspase-6, or anti-caspase-3 (Fig. 7, top, middle, and bottom panels, respectively). In Nalm-6 cells, both endogenous SATB1 and EGFP-SATB1 (FL and K744R; K744R is shown) were immunoprecipitated by anti-caspase-6, suggesting that a physical interaction occurs between inactive caspase-6 and SATB1 and that this interaction occurs in a SUMO-independent manner. These investigations were also confirmed by immunoprecipitating endogenous SATB1 and caspase-6 from Jurkat cell extracts (data not shown).
SUMO-Dependent Caspase Cleavage of SATB1 in Specific Subnuclear Structures during Apoptosis—In this study we have demonstrated a novel mechanism for caspase cleavage of the critical regulatory protein SATB1. Post-translational conjugation to the ubiquitin-like protein SUMO targeted SATB1 for caspase cleavage. Whether caspase cleavage of SATB1 is normally associated with activation of cell development or, alternatively, with apoptosis is not currently known. It is also possible that SATB1 cleavage normally functions in an activating capacity, but when stress pathways are induced beyond a certain point, then cell death occurs. In these studies, sumoylation did not recruit caspase-6 to SATB1. Indeed, caspase-6 does not harbor a SUMO-binding motif (30), although it does possess strong SUMO consensus conjugation sites, which might facilitate SUMO-SATB1/caspase-6 localization into PML NBs. PML-NBs are suggested to be sites at which sumoylated caspase-7 and -8, as well as caspase-2, are targeted (62–64). Such localizations could function to concentrate apoptotic molecules leading to enhanced SATB1 cleavage. This premise was further supported by amplification or induction of PML NBs in Jurkat or NB4 cells, which led to augmentation of programmed cell death-mediated SATB1 cleavage (Fig. 6). Previously, caspase activation was noted to be markedly inhibited following Fas stimulation in PML-deficient cells (22) leading one to speculate whether PML itself, or the NBs that PML plays a critical role in assembling, regulate programmed cell death. The nuclear speckles or dots into which SATB1-SUMO aggregated (Fig. 5) are similar to other protein complexes that have multiple functions, including DNA condensation and fragmentation associated with apoptosis (25, 65). For example, the first helix of the caspase recruitment domain of caspase-2 is critical in mediating its ability to localize in dot-like filament-like structures (66).

The SUMO-regulated caspase cleavage of SATB1 was not dependent upon the specific inducer used; four mechanistically diverse agents (anti-Fas, CPT, actinomycin D, and etoposide) all converged in a common pathway, resulting in exclusive proteolysis of the sumoylated form of SATB1. Additionally, SUMO-regulated fragmentation of this protein occurred in diverse cell lines (lymphoid, erythroid, promyeloid, cervical, and breast), suggesting that SUMO-regulated proteolysis is not limited to the properties of a particular cell line.

SUMO Regulation of SATB1 Caspase Cleavage—Much still remains unknown regarding the mechanistic details and time frame of SUMO participation in SATB1 cleavage and whether this is related to apoptosis or to non-apoptotic developmental processes. SATB1 is present in cells within at least two separate cellular compartments: associated with chromatin or the nuclear matrix and also as a soluble fraction (10, 67, 68). This suggests that SATB1 may have distinct cell functions as has been noted previously for a number of proteins, including SAF-A, which binds with chromatin but also with heterogeneous nuclear ribonucleoprotein (hnRNPs) complexes; apoptotic cleavage disrupts the association of only the former function of SAF-A (1). The subset of SATB1 that binds to specific MAR regions has been suggested to interact as a homodimeric complex via dimerization mediated through an N-terminally localized PDZ domain (9). Supposedly, caspase cleavage of SATB1 that occurs after the VEMD caspase recognition sequence disrupts the homodimeric SATB1 and releases the 70-kDa C terminus of SATB1 (which encodes the MAR-binding domain and homeodomain) from DNA, thereby disrupting chromosomal structure and function (9, 10). This mechanism of interaction has not, however, been confirmed in a mammalian system; indeed, a relatively well conserved tetrapeptide in PDZ domains (GLGF) is not present within the N terminus of SATB1. Another possibility is that the region of SATB1 encompassing amino acids 224–278 (overlapping the caspase recognition site at residue 254), which has recently been identified as a nuclear matrix targeting sequence (68), is disturbed during caspase cleavage, leading to release of SATB1 from the nuclear matrix and subsequent disruption of protein expression or of chromatin structure. Regardless of the mechanism, such a scenario has been described for other MAR-associating proteins (1). Because SATB1 and other key regulatory molecules orchestrate an intricate balance between normal cell function and cell death, protein fragmentation must be strictly regulated. The SUMO conjugation/deconjugation system is a plausible candidate for mediating such regulation. SUMO conjugation to SATB1 facilitates protein-protein interactions, either through aggregation of apoptotic proteins, which leads to SATB1 proteolysis and subsequently to cellular apoptosis, or by redistribution of SATB1 to a more favorable cleavage site. SUMO conjugation to SATB1 is likely regulated in a temporal and/or spatial fashion related to T-cell development. Further investigations are required to delineate the time line of events in SATB1 sumoylation and apoptosis. However, it is clear that once SATB1 is covalently SUMO-modified, its function at the bases of chromatin loop domains is immediately and irreversibly altered by cellular relocation and caspase cleavage.

This is the first report of SUMO conjugation to a key regulatory protein serving as a signal for that molecule to undergo caspase cleavage. Future investigations will shed light on the intricate balance of protein-protein interactions mediated via SUMO conjugation that transform SATB1 from its primary role of maintaining cellular integrity to either a key participant in the ordered process of cell death or, alternatively, to a regulator in the activation and development of immune cells.

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