Covalent Attachment of the SUMO-1 Protein to the Negative Regulatory Domain of the c-Myb Transcription Factor Modifies Its Stability and Transactivation Capacity*

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The transcription factor c-Myb is subject to several types of post-translational modifications, including phosphorylation, acetylation, and ubiquitination. These modifications regulate the transcription and transforming activity as well as the proteolytic stability of c-Myb. Here we report the covalent modification of c-Myb with the small ubiquitin-related protein SUMO-1. Mutational analysis identified two major sumolation sites (Lys499 and Lys523) in the negative regulatory domain. Interestingly, the single mutation K523R completely abolished modification of c-Myb with SUMO-1, suggesting that sumolation of Lys523 is required for modification of other lysines in c-Myb. In accordance with this observation, we found that the SUMO-1-conjugating enzyme Ubc9 interacted only with a region surrounding Lys523 (also called the PEST/EVES motif). Experiments aimed at determining the proteolytic stability of sumolated and unmodified forms of c-Myb revealed that at least two covalently attached SUMO-1 molecules dramatically increased the stability of c-Myb. However, mutations of the SUMO-1 modification sites did not alter its stability, suggesting that a mechanism(s) other than competition of ubiquitin and SUMO-1 for the same lysine is involved in the stabilization of sumolated c-Myb protein. Finally, the K523R mutant of c-Myb, entirely deficient in sumolation, was shown to have an increased transactivation capacity on a Myb-responsive promoter, suggesting that SUMO-1 negatively regulates the transactivation function of c-Myb. Thus, modification of c-Myb with SUMO-1 represents a novel mechanism through which the negative regulatory domain can exert its suppressing activity on c-Myb transactivation capacity.

The c-Myb transcription factor is a nuclear phosphoprotein that has an important function in the regulation of proliferation, differentiation, and apoptosis of immature hematopoietic cells (1). The critical role of c-Myb in normal hematopoiesis was most convincingly shown in a knockout experiment in which homozygous mutation of c-myb was embryonically lethal (2). It was originally identified as a transforming gene through its oncogenic activation of c-myb (3). The transactivation and transforming activities of Myb are modulated through the negative regulatory domain (NRD)1 (10, 11). This regulatory domain contains several specific regions such as the putative leucine zipper (12), the PEST/EVES motif (13), and other conserved sequences that specifically contribute to negative regulation of c-Myb activity through binding of several cellular proteins (14, 15).

Post-translational modifications such as phosphorylation, acetylation, and ubiquitination play an important role in the regulation of c-Myb activity and proteolytic stability (16–20). Acetylation of several lysine residues in the NRD by the histone acetyltransferases p300 and CBP was identified recently. This post-translational modification enhances binding of the CBP coactivator and increases the DNA-binding and transactivation activity of c-Myb (18, 19). In vivo, c-Myb has been shown to be phosphorylated at serines 11 and 12 by casein kinase II (16). Phosphorylation of these residues results in a lower DNA-binding affinity (16, 21). In addition, phosphorylation of serine 528 in the PEST/EVES motif, located in the NRD, down-regulates the transactivation capacity without affecting its DNA-binding affinity (22). The NRD is also subject to covalent modification with polyubiquitin chains on an unidentified lysine residue(s), and ubiquitinated c-Myb is recognized and rapidly degraded by the 26S proteasome proteolytic system (20, 23). Deletion analysis of the c-Myb NRD identified two instability regions responsible for its very short half-life (24).

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* The abbreviations used are: NRD, negative regulatory domain; SUMO-1, small ubiquitin-related modifier-1; GST, glutathione S-transferase.
addition, our recent results suggest that proteolytic stability of c-Myb is modulated through phosphorylation of the carboxyl terminus of c-Myb. Inhibitors of Ser/Thr phosphatases rapidly induce hyperphosphorylation-dependent conformational changes in the NRD, followed by accelerated proteolytic breakdown (25, 26).

In addition to ubiquitin, several ubiquitin-like (Ubl) proteins have been found to be covalently attached to target proteins (27, 28). The SUMO-1 protein belongs to the Ubl family. It was first discovered in the yeast Saccharomyces cerevisiae and named Smt3 (29). It was later identified as an interacting partner of several proteins with different functions in yeast two-hybrid screens and named Pic1 (30), sentrin (31), and Ubl1 (32). Shortly thereafter, two groups reported that a ubiquitin-related protein termed Gmp1 (33) or SUMO-1 (34) covalently attaches to RanGAP1. In recent years, there was an enormous expansion of reported SUMO-1 target proteins (28).

In contrast to ubiquitination, which usually marks protein for rapid degradation, sumolation is involved in the regulation of protein function through changes in protein-protein interaction, subcellular localization, and stabilization by antagonizing ubiquitination (27, 28).

Here we report a novel post-translational modification of the c-Myb NRD by the covalent attachment of at least three molecules of the SUMO-1 protein. We have shown that sumolation modulates the proteolytic stability and transcription activity of c-Myb. Thus, these results provide another way through which the NRD affects c-Myb activity.

MATERIALS AND METHODS

Plasmid Construction—Cloning of the wild-type full-length c-Myb construct (FL-Myb) and carboxyl-terminally truncated forms (CT3-Myb118aa, CT4-Myb151aa, CT5-Myb266aa) into the eukaryotic expression vector pcDNA3.1 (+) was described previously (24). An amino-terminally truncated form of c-Myb lacking the first 74 amino acids (NT-Myb) was constructed using the QuikChange™ site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. Expression of the transfected gene was analyzed 36 h post-transfection.

Plasmid Immunoblotting—Cells were washed twice with phosphate-buffered saline and disrupted in cold lysis buffer (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5% Nonidet P-40, 0.5% SDS, and 0.5% sodium deoxycholate) supplemented with a mixture of protease inhibitors (Complete™, Roche Molecular Biochemicals) and 10 mM iodoacetamide (Fluka). Cell lysates were sonicated for 30 s and clarified by centrifugation at 13,000 rpm for 15 min at 4 °C. The c-Myb protein was immunoprecipitated with rabbit anti-c-Myb monoclonal antibody (a kind gift of Tim Bender) or anti-FLAG monoclonal antibody M2 (Sigma) and visualized with SuperSignal West Pico chemiluminescence substrate (Pierce) in accordance with the manufacturer’s instructions.

Metabolic Labeling of Proteins and Immunoprecipitation—Cells (2 × 10⁶ to 1 × 10⁷) were starved for 15 min in 1 ml of methionine/cysteine-free medium, metabolically labeled for 45 min with 400 μCi of Trasylol-slabel (ICN), washed free of radioactive amino acids (pulse), and incubated in prewarmed complete medium supplemented with 10% serum (chase). At the indicated time points, the cells were disrupted in cold lysis buffer and immunoprecipitated with rabbit anti-c-Myb polyclonal antibody against the carboxyl fusion protein (c-Myb(I)) (37). All precipitates were extensively washed with radiolabeled precipitation assay buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 1% sodium deoxycholate), electrophoresed under reducing conditions on an 8% polyacrylamide gel, and visualized by fluorography. Exposed prefixed x-ray films were quantitatively analyzed using a Model 222-020 UltraScan XL laser densitometer (Amersham Biosciences, Inc.).

Two-hybrid Assays—To identify a region located in the c-Myb NRD that interacts with murine Ubc9, the Matchmaker two-hybrid kit (CLONTECH) was used in accordance with the manufacturer’s instructions. Briefly, yeast strain PJ69-2A (MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4, gal80, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL12-αGAL12-TATA-ADE2, MEL1) was cotransformed with pGβT9-Ubc9 and with constructs carrying different regions of the c-Myb NRD fused in frame to the Gal4 activation domain of the pGADT7 vector. Interaction of Ubc9 with a specific subregion of the c-Myb NRD was identified by growth of yeast clones on selective medium lacking tryptophan, leucine, histidine, and adenine. Transformation efficiency for each experiment was evaluated by scoring the colonies on selective medium lacking tryptophan and leucine.

Transactivation Assay—COS-7 cells were plated at a density of 2 × 10⁵ cells/well in six-well tissue culture plates and grown overnight prior to transfections. Transfections were carried out using the Effectene™ transfection reagent according to the manufacturer’s instructions. To assess c-Myb transcriptional activity, COS-7 cells were cotransfected with the empty vector pcDNA3.1 (+) (Invitrogen) or expression vectors encoding wild-type c-Myb (FL-Myb) or a mutant form of c-Myb (FL-MybK523R) (0.5 μg of each) and 100 ng of pRl-TK Renilla luciferase vector (Promega). After transfection, cells were grown for 24 h and processed for both firefly and Renilla luciferase activities using the Dual-Luciferase™ reporter assay system (Promega) according to the manufacturer’s instructions. Transfection efficiency was evaluated using Renilla luciferase activity. Expression of the c-Myb protein was evaluated by Western blotting using cell extracts diluted in radiommune precipitation assay buffer. Each transfection experiment was performed in triplicate and repeated two times.
with the part of the fusion protein used for immunization of rabbits (the first 325 amino-terminal amino acids) completely blocked immunoprecipitation of both the normal-size c-Myb and slower migrating bands (data not shown). These data strongly support the contention that these novel bands are either modified forms of the c-Myb protein or its alternatively spliced forms. Slower migrating c-Myb-immunoreactive bands were also detected in erythroid and lymphoid cells (Fig. 1B), indicating that Myb-immunoreactive species are not restricted to the myeloid cell lineage.

Several mechanisms could account for the slower migrating bands immunoprecipitated from hematopoietic cells. To determine whether at least some of the slower migrating bands could represent post-translational modifications of c-Myb, we transiently transfected plasmid encoding full-length c-Myb into COS-7 cells, metabolically labeled them, and immunoprecipitated c-Myb. In this experimental setup, there is no alternative splicing of c-myb; thus, all slower migrating Myb-immunoreactive forms represent post-translationally modified c-Myb. Slower migrating Myb-immunoreactive forms could be detected only in cells transfected with FL-Myb, but not in cells transfected with an empty vector (pcDNA3.1) or a plasmid encoding a carboxyl-terminally truncated form of c-Myb (CT6-MybΔ266aa) (Fig. 1C). This experiment indicated that an intact carboxy terminus is required for detection of the Myb-immunoreactive protein species in cells.

Several recent reports identified novel post-translational modifications of nuclear transcription factors in which the ubiquitin-like protein SUMO-1 was covalently attached to specific lysines in the target protein (28, 29). Thus, to determine whether the slower migrating Myb-immunoreactive forms were SUMO-1-modified c-Myb proteins, we analyzed COS-7 cells that were transiently transfected with constructs encoding FL-Myb and FLAG-tagged SUMO-1. c-Myb was immunoprecipitated from the transfected cells and analyzed by Western immunoblotting with anti-FLAG and anti-c-Myb monoclonal antibodies as described under “Materials and Methods.” As shown in Fig. 2A, anti-FLAG antibody recognized at least three bands in the immunoprecipitates from cells transfected with both the FL-Myb- and FLAG-SUMO-1-encoding constructs. None of these bands were detected in COS-7 cells transfected with constructs encoding either FL-Myb or FLAG-SUMO-1 alone. Anti-c-Myb monoclonal antibodies were used to confirm that a similar amount c-Myb was expressed in and immunoprecipitated from COS-7 cells transfected with the FL-Myb-encoding construct. Anti-c-Myb monoclonal antibodies also detected a SUMO-1-modified form migrating ~20 kDa above the non-sumolated parental form of c-Myb (Fig. 2A; the endogenous form of SUMO-1-modified c-Myb is marked with a closed arrowhead, and FLAG-SUMO-1-modified c-Myb is marked with open arrowheads). Transfection efficiency was evaluated by Western immunoblotting of total cellular proteins. Anti-FLAG antibodies readily detected a ladder of bands representing the SUMO-1-conjugated cellular proteins only in cells transfected with FLAG-SUMO-1. Anti-c-Myb monoclonal antibodies detected the FL-Myb protein (75-kDa form) only in cells transfected with the FL-Myb construct (Fig. 2B). Although more than one modified c-Myb species was detected with anti-FLAG antibody, only one modified form was detected with anti-FLAG antibody in the experiment depicted in Fig. 2A. This could be due to reduced sensitivity after stripping off anti-FLAG antibody. To determine whether we could detect more c-Myb-modified bands if the blot was not stripped, we performed another experiment in which Western blotting, using c-Myb-reactive antibody, was carried out on a fresh blot. This time, as shown in Fig. 2C, we detected at least two modified
Fig. 2. The c-Myb protein is modified by covalent attachment of SUMO-1 in vivo. A. COS-7 cells were transfected with constructs encoding full-length c-Myb and FLAG-tagged SUMO-1 (FL-Myb+FLAG-SUMO) or with each construct separately. Thirty-six hours post-transfection, cells were lysed, and the proteins were immunoprecipitated (IP) with anti-c-Myb antiserum (αMyb). Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose membrane, and analyzed by Western immunoblotting (WB) using anti-FLAG (αFLAG) or anti-c-Myb monoclonal antibody. The mobilities of IgGs and the c-Myb protein are indicated on the right; the positions of molecular mass standards are on the left. c-Myb proteins modified with FLAG-SUMO-1 are marked by open arrowheads. The closed arrowhead marks the endogenous SUMO-1-modified c-Myb protein. B, the transfection efficiency for the experiment shown in A was estimated by Western blotting of SDS-PAGE-separated whole cell lysates (WCE) prepared from transfected COS-7 cells using anti-FLAG or anti-c-Myb monoclonal antibody. The positions of molecular mass standards are on the left. C, COS-7 cells were transfected with constructs encoding FL-Myb or vector alone (pcDNA3.1), and lysates were immunoprecipitated with anti-c-Myb antiserum. Following electrophoretic separation and blotting as described above, the blot was incubated with anti-c-Myb antibody. D, COS-7 cells were cotransfected with FLAG-SUMO-1 and constructs encoding either FL-Myb or NT-Myb74aa or the empty vector alone (pcDNA3.1). Immunoprecipitation was performed with anti-c-Myb antiserum, and immunoblotting was carried out with anti-FLAG antibody. The blot was stripped and reincubated with anti-c-Myb monoclonal antibody to demonstrate equal loading of the immunoprecipitated c-Myb protein. NT-Myb, amino-terminally truncated c-Myb protein.
forms that had similar mobility to those detected with anti-FLAG antibody (Fig. 2A). To confirm that the slower migrating bands were indeed sumolated forms of c-Myb and not other co-immunoprecipitated sumolated proteins, we transfected COS-7 cells with the FLAG-SUMO-1 construct and either full-length (FL-Myb) or amino-terminally truncated (NT-MybΔ74aa) c-Myb. As in the previous experiment, the transfected cells were lysed, immunoprecipitated with anti-c-Myb antiserum, and analyzed by Western immunoblotting using anti-FLAG monoclonal antibody. As shown in Fig. 2D, the mobility of all Myb-immunoreactive bands detected by anti-FLAG immunoblotting correlated directly with the size of the transfected c-Myb proteins. Thus, this experiment provides strong evidence that slower migrating forms are sumolated forms of c-Myb.

The data presented in Fig. 2 confirm that slower migrating Myb-immunoreactive bands are indeed SUMO-1-conjugated c-Myb species. Despite the fact that the calculated size of SUMO-1 is ~11.5 kDa, SUMO-1-conjugated proteins run on SDS-PAGE as if they have an additional 20 kDa for each covalently attached SUMO-1 molecule (34). Unlike ubiquitin, which usually forms multimeric chains (polyubiquitin) attached to the target lysine of modified protein, SUMO-1 can be covalently attached to a single lysine only in a monomeric form (29, 38). Thus, our results suggest that at least three different lysines are modified in c-Myb by covalent attachment of three molecules of SUMO-1.

The c-Myb NRD Is Sumolated at Lysines 499 and 523—To map the target lysine residues for SUMO-1 modification of c-Myb, we tested a series of carboxyl-terminal deletion mutants in the in vivo SUMO-1 conjugation assay using COS-7 cells. Lysates from COS-7 cells transiently transfected with either FL-Myb or carboxyl-terminally truncated mutants of c-Myb and FLAG-tagged SUMO-1 were immunoprecipitated with anti-c-Myb antiserum, and immune complexes were separated on a gel and analyzed by Western immunoblotting with anti-FLAG and anti-c-Myb monoclonal antibodies. The results of this analysis showed that deletion of the last 118 amino acids from the carboxyl terminus (CT3-MybΔ118aa) entirely abrogated the modification of c-Myb with SUMO-1 (Fig. 3A), indicating that these last 118 amino acids contain all of the target lysines. Western immunoblotting with anti-c-Myb monoclonal antibodies confirmed that all the deleted forms were expressed at a level that was at least as high as the level of FL-Myb (Fig. 3B). Analysis of the entire c-Myb amino acid sequence revealed two sequences around lysines 499 and 523 that conform perfectly with the minimal SUMO-1 modification consensus sequence ϕKXE (where ϕ is a hydrophobic amino acid and X any amino acid), which is found in several nuclear protein substrates (27). A third lysine, Lys523, residing in the NRD, closely matches the SUMO-1 modification consensus sequence (Fig. 4C). Interestingly, Lys523 and Lys611 are located in the last 118 amino acids, removal of which renders c-Myb resistant to sumolation (Fig. 3A, compare FL-Myb and CT3-MybΔ118aa). To determine whether any of these three lysines are in vivo sites of SUMO-1 modification, we cotransfected COS-7 cells with FLAG-SUMO-1 and wild-type FL-Myb as well as c-Myb constructs in which each of the lysines at positions 499, 523, and 611 were changed to arginine. Lysates from transfected cells were immunoprecipitated with anti-c-Myb polyclonal antiserum and analyzed by anti-FLAG (Fig. 4A) and anti-c-Myb (Fig. 4B) Western blotting. Surprisingly, the single mutation K523R entirely abrogated modification of c-Myb with SUMO-1. In contrast, the mutation K499R led to the disappearance of the two slowest migrating bands, leaving only one band (migrating ~20 kDa above unmodified c-Myb). This band corresponds to the single SUMO-1-modified form of c-Myb (probably at Lys523). The mutation of Lys611 slightly decreased the level of the slowest migrating forms of SUMO-1-modified c-Myb, but this band was still easily detectable in our in vivo sumolation assay, suggesting that Lys611 does not serve as a conjugation site for SUMO-1 in c-Myb (Fig. 4A). Thus, Lys499 and Lys523 are the two major sites in the NRD of c-Myb modified with SUMO-1. Our results also suggest that, in addition to Lys499 and Lys523, there is at least another lysine in c-Myb undergoing SUMO-1 modification. Actually, in some experiments, we could detect up to four slower migrating bands, where the two weakest and slowest migrating bands correspond to triple and quadruple SUMO-1-modified forms of c-Myb (data not shown). The intensities of these bands was much weaker, suggesting that only a small portion of c-Myb is subsequently modified with three and four molecules of SUMO-1. As expected, mutation of both Lys499 and Lys523 also completely abolished sumolation of c-Myb in our assay (data not shown). Taken together, our results indicate that modification of lysines in the NRD of c-Myb is an orderly process, where the first modification of c-Myb with SUMO-1 takes place only at Lys523 and this step is necessary for the attachment of the second molecule of SUMO-1 to Lys499. Similarly, modification of other minor site(s) in c-Myb requires covalent attachment of SUMO-1 to both Lys499 and Lys523 (Fig. 4A).
SUMO-1 Modification of c-Myb

c-Myb Interacts with the SUMO-1-conjugating Enzyme Ubc9—At present, little is known about the exact mechanism that governs the recognition of target proteins for SUMO-1 modification. However, the majority of SUMO-1-modified proteins interact directly with the SUMO-1-conjugating enzyme Ubc9 (39). A recent report showed that Ubc9 recognizes the consensus sequence $\psi$KXE, which surrounds the acceptor lysine residue in many nuclear substrates (40). Both sequences in murine c-Myb, surrounding identified SUMO-1 acceptor residues Lys\textsuperscript{499} and Lys\textsuperscript{523}, fit perfectly with this consensus sequence. Thus, to investigate the region of direct interaction of the c-Myb NRD with Ubc9, we placed several deletion mutants of the c-Myb NRD fused in frame to the activation domain of the Gal4 transcription factor and analyzed their capability to interact with murine Ubc9 fused in frame to the DNA-binding domain of Gal4 in a yeast two-hybrid system as described under "Materials and Methods." Our results identified a region around the PEST/EVES motif (surrounding Lys\textsuperscript{523}) as the only part of the c-Myb NRD capable of specific interaction with murine Ubc9 in a yeast two-hybrid system (Fig. 5). Interestingly, these results are consistent with our single lysine mutants, in which mutation of a single Lys\textsuperscript{523} completely diminished the modification of other lysines with SUMO-1 in c-Myb (Fig. 4A). Thus, this result supports our hypothesis that the covalent attachment of SUMO-1 to Lys\textsuperscript{523} is required for the subsequent modification with the second and third molecules of SUMO-1.

Sumolation of c-Myb Affects Its Proteolytic Stability—c-Myb is rapidly degraded in cells via the ubiquitin/26S proteasome proteolytic pathway (20). Deletion of the entire NRD diminishes ubiquitination of c-Myb; and consequently, it substantially increases the stability of c-Myb (20, 24). Because this deletion also abolishes the sumolation of c-Myb (Fig. 3A), we wanted to investigate the effect of SUMO-1 modification on its proteolytic stability. COS-7 cells transfected with FL-Myb and FLAG-SUMO-1 were treated with cycloheximide to block protein synthesis for the indicated times, and c-Myb was immunoprecipitated with rabbit anti-c-Myb polyclonal serum. Immunoprecipitates were separated on the gel, transferred to a membrane, and analyzed by Western immunoblotting with anti-c-Myb and anti-FLAG monoclonal antibodies. The proteolytic processing of parental c-Myb and c-Myb modified with only one molecule of SUMO-1 appeared similar. However, modification of c-Myb with at least two molecules of SUMO-1 substantially increased its stability (Fig. 6A). Because both ubiquitin and SUMO-1 are covalently attached to lysines in target proteins, we wanted to investigate whether the observed stabilization was due to competition of the protein-conjugating systems for the same lysine in the NRD of c-Myb. To test this hypothesis, we performed a pulse-chase experiment with wild-type FL-Myb, the single mutants FL-MybK499R and FL-MybK523R, and the double mutant FL-MybK499R,K523R. COS-7 cells transfected with individual constructs were metabolically labeled for 45 min with Tran\textsuperscript{35}S-label, washed free of radioactive amino acids, and chased for the indicated times in complete medium. Analysis of this pulse-chase experiment did not reveal any obvious difference in the proteolytic stability of the wild-type and mutant forms of c-Myb (Fig. 6B and data not shown). In addition, inhibition of the 26S proteasome caused accumulation of the wild-type and all mutant forms of c-Myb to a similar extent (data not shown), indicating again that SUMO-1 and ubiquitin do not compete for the same lysine in the NRD.

Sumolation Decreases the Transactivation Capacity of c-Myb—To assess the potential functional consequences of the conjugation of SUMO-1 to c-Myb, we decided to compare the transactivation capacity of wild-type FL-Myb with that of the sumolation-deficient mutant FL-MybK523R. We used COS-7 cells lacking endogenous c-Myb. Cells were cotransfected with the reporter plasmid p5xMRE-A-luc (containing five copies of the mim-1A MRE ligated to a minimal herpes simplex virus thymidine kinase promoter-driven luciferase gene) and either the reporter plasmid pCMV-hr luciferase gene expression above the basal level (pcDNA3.1). However, FL-MybK523R activated transcription ~5-fold more strongly than wild-type FL-Myb (Fig. 7). Because this single mutation (K523R) completely destroys sumolation of c-Myb, our results suggest that covalent modification of the c-Myb NRD negatively regulates the transactivation activity of the protein. Furthermore, we did not observe any differences in the DNA-binding activities of wild-type FL-Myb and FL-MybK523R (data not shown), suggesting that a mechanism other than DNA binding is involved in the observed differences in transactivation activity.
SUMO-1 Modification of c-Myb Is Independent of Ser<sup>528</sup> Phosphorylation—Similar to ubiquitination, sumolation of some proteins is accomplished through a phosphorylation-dependent process. We localized the crucial sumolation site at Lys<sup>523</sup>, which lies in the PEST/EVES region. This region plays an important role in the regulation of c-Myb activity, and it was shown previously that phosphorylation of Ser<sup>528</sup> negatively regulates the transactivation of c-Myb (17). To determine whether the phosphorylation of Ser<sup>528</sup> has any influence on the conjugation of SUMO-1 to c-Myb, we subjected two mutant forms of c-Myb (FL-MybS528A and FL-MybS528D) to our in vivo sumolation assay. The results of this experiment showed that both mutant forms of c-Myb, one that cannot be phosphorylated at Ser<sup>528</sup> (FL-MybS528A) and one that mimics phosphorylation of this serine (FL-MybS528D), had similar transcriptional activity, transactivation activity, or proteolytic stability of c-Myb. We also provide experimental evidence that the sumolation of c-Myb can alter its proteolytic stability and decrease its transactivation capacity.

Our data suggest that full sumolation of c-Myb is an ordered process that begins with the modification of Lys<sup>523</sup>. To our knowledge, this mechanism of coordinated attachment of three SUMO-1 molecules to c-Myb is novel and has not been described for any other SUMO-1 target protein. Support for this has come from the demonstration that mutation of Lys<sup>523</sup> alone abolishes sumolation of the entire c-Myb protein. We have shown that sumolation of Lys<sup>523</sup> is necessary for covalent attachment of a second molecule of SUMO-1 to Lys<sup>499</sup>, and subsequent modification of both Lys<sup>523</sup> and Lys<sup>499</sup> is required for conjugation of the third molecule of SUMO-1 to another, as yet unidentified lysine in c-Myb. Presently, very little is known about the precise mechanism that is responsible for the specific recognition of the protein targets subject to sumolation. However, many proteins modified with covalent attachment of SUMO-1 interact directly with the SUMO-1-conjugating enzyme Ubc9 (27). More recently, the SUMO-1 consensus sequence ψKXE in target proteins was identified as a major determinant of direct interaction with the SUMO-conjugating enzyme Ubc9 (40). Interestingly, we were able to detect specific interaction of Ubc9 within a region surrounding Lys<sup>523</sup> using a yeast two-hybrid assay. Thus, we hypothesize that covalent conjugation of SUMO-1 to Lys<sup>523</sup> changes the conformation of the carboxyl terminus, allowing modification of Lys<sup>499</sup>. Conformational changes in the target protein were described for RanGAP1, in which SUMO-1 conjugation was shown to induce structural changes that allow the binding of modified RanGAP1 to RanBP2 (41, 42).

The biological consequences of the two processes, sumolation and ubiquitination, are quite different despite the following facts. SUMO-1 shares some identity with ubiquitin; both SUMO-1 and ubiquitin use lysine residues for the covalent conjugation to targets; and the steps of enzymatic machinery leading to sumolation and ubiquitination are mechanistically very similar (27, 28). Ad-
Modification of c-Myb with at least two molecules of SUMO-1 increases its proteolytic stability. A. COS-7 cells transfected with constructs encoding full-length c-Myb and FLAG-tagged SUMO-1 (FL-Myb+FLAG-SUMO) were treated with cycloheximide (15 μg/ml) for the indicated times, lysed in lysis buffer, and immunoprecipitated (IP) with anti-c-Myb antiserum (αMyb). Immune complexes were separated by SDS-PAGE and analyzed by Western immunoblotting (WB) using anti-c-Myb (αMyb), or anti-FLAG (αFLAG) monoclonal antibody. The mobilities...
The results are representative of two independent experiments performed in triplicate. Expression of c-Myb (from a representative experiment) was evaluated by immunoprecipitation and Western immunoblotting in normalized cell extracts using anti-c-Myb antibody and is shown inside the plot area.

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FIG. 7. The sumolation-deficient K523R mutant of c-Myb has increased transactivation activity. COS-7 cells were transfected in triplicate with an empty expression vector (pcDNA3.1 (+)) or with expression constructs encoding wild-type FL-Myb or FL-MybK523R along with c-Myb-responsive reporter plasmid p5xMRE-A-luc (encoding the firefly luciferase gene) and plasmid pRL-TK (encoding the Renilla luciferase gene; used as a control for transfection efficiency). Luciferase activities were measured in cell lysates as described under "Materials and Methods." Firefly luciferase activity was normalized using Renilla luciferase values. Each bar represents a relative value, where the activity of wild-type FL-Myb was assigned 1. Error bars represent S.E. The results are representative of two independent experiments performed in triplicate. Expression of c-Myb (from a representative experiment) was evaluated by immunoprecipitation and Western immunoblotting in normalized cell extracts using anti-c-Myb antibody and is shown inside the plot area.
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FIG. 8. Sumolation of c-Myb is independent of Ser\textsuperscript{528} phosphorylation. COS-7 cells were cotransfected with constructs encoding either FL-MybS528A (mimicking unphosphorylated c-Myb at Ser\textsuperscript{528}) or FL-MybS528D (mimicking phosphorylated c-Myb at Ser\textsuperscript{528}) and FLAG-SUMO-1. The c-Myb protein was immunoprecipitated (IP), and the expression of c-Myb species as well as the level of their SUMO-1 modification were analyzed with anti-FLAG (αFLAG) or anti-c-Myb (αMyb) monoclonal antibody as described in the legend to Fig. 2. The mobilities of FLAG-SUMO-1-modified c-Myb proteins (open arrowheads) are indicated on the right. WB, Western blotting.
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In our study, a region covering the PEST/EVES motif was identified as a region of direct interaction of Ubc9 with c-Myb. PEST sequences are commonly found in proteins that are rapidly degraded via the ubiquitin/26S proteasome degradation pathway (47); however, deletion of this region does not alter the intrinsic proteolytic instability of c-Myb (24). Instead, it appears, from data presented here, that this PEST/EVES motif is involved in protein-protein interactions. Actually, PEST regions have been identified in a large number of bona fide SUMO-1 protein substrates (27). Similar to our results, Ubc9 directly interacts with the PEST region of another nuclear SUMO-1 target protein, HIPK2 (48). However, in many other sumolated proteins, PEST sequences do not overlap with the identified Ubc9-interacting regions (42, 45, 49). PEST sequences are rich in SP/TP motifs and are often recognized and phosphorylated by proline-directed Ser/Thr protein kinases (47). Phosphorylation of the target proteins can allow or prevent conjugation of SUMO-1. This was shown for several SUMO-1 targets, including c-Jun (50), p53 (50), IκBα (45), and Mdm2 (46). Although Ser\textsuperscript{528} in the PEST sequence of c-Myb is phosphorylated in vivo (17), we were not able to show dependence of SUMO-1 conjugation on the phosphorylation status of Ser\textsuperscript{528}.

Sumolation of transcription factors has been shown to have diverse effects on their activity. For example, whereas sumolation decreases the transactivation potential of c-Jun (50) and the androgen receptor (51), sumolation of the tumor suppressor p53 (49, 50, 52) and heat shock factor-2 (53) increases transactivation capacity. The results of our trans-
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SUMO-1 Modification of c-Myb 9007
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of IgGs, FLAG-SUMO-1-modified c-Myb proteins (open arrowheads, with numbers indicating one, two, or three molecules of FLAG-SUMO-1 attached to a single c-Myb molecule), and endogenous SUMO-1-modified c-Myb (closed arrowhead) are indicated on the right. Quantitative analysis was performed on a Model 222-020 UltraScan XL laser densitometer. αMyb represents unmodified protein; αMyb(SUMO)\textsubscript{1-3} represents c-Myb modified with one to three molecules of SUMO-1, B, COS-7 cells were transfected with an expression construct encoding either wild-type FL-Myb (c-Myb\textsubscript{wt}) or a mutant form of c-Myb in which the two major sumolation sites (Lys\textsuperscript{499} and Lys\textsuperscript{523}) were mutated to arginine (FL-MybK499R,K523R, FL-Myb2K/R, c-Myb2K/R)). The stability of both forms of c-Myb was evaluated by pulse-chase experiments as described under "Materials and Methods." Exposed x-ray films were quantitatively analyzed on a Model 222-020 UltraScan XL laser densitometer and are shown underneath the autoradiogram.

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SUMO-1 Modification of c-Myb

activation assay indirectly suggest that SUMO-1 conjugation has a negative role on c-Myb transcription because the SUMO-1-deficient mutant K523R is transcriptionally more active than the wild-type protein. Covalent conjugation of SUMO-1 to Lys\(^{499}\) and Lys\(^{523}\), which maps just two residues upstream of the Myb EVES motif (PLLKKIK\(^{523}\)QEVESP), could potentially influence the flexibility of the carboxyl terminus. This could cause it to adopt a different conformation that would affect its transactivation potential. In support of this notion, the PEST/EVES region was shown to adopt a different conformation in response to p100 binding (13). Furthermore, as we already mentioned, SUMO-1 has been shown to alter the conformation of RanGAP1 (41, 42).

Modulation of the level of c-Myb acetylation is another possible way by which conjugation of SUMO-1 could potentially repress the transactivation activity of c-Myb. It was shown that the histone acetylases p300 and CBP can acetylate c-Myb at five lysine residues (Lys\(^{438}\), Lys\(^{441}\), Lys\(^{471}\), Lys\(^{480}\), and Lys\(^{485}\)) located in the NRD (18, 19). Transactivation assays with c-Myb acetylation site mutants revealed that acetylation of c-Myb at each of these five sites can synergistically enhance activity, most likely through the increased affinity of acetylated c-Myb for the coactivator CBP (19). Despite the fact that the major SUMolation sites (Lys\(^{499}\) and Lys\(^{523}\)) are not the same lysines modified by acetylation, there is the possibility that SUMO-1 conjugation can negatively influence acetylation of several lysines because some of them (Lys\(^{471}\), Lys\(^{481}\), and Lys\(^{485}\)) lie in close proximity to the sumolated lysines. Thus, competition between acetylation and SUMolation of c-Myb can provide another explanation for the decreased transactivation capacity of sumolated c-Myb.

Interestingly, the region surrounding Lys\(^{523}\) (PLLKKIK\(^{523}\)QEVESP) conforms perfectly to the consensus sequence \(\text{PEX}_{7-11}\text{K/Q}/\text{T/S/L/E/P/PEX}_{7-11}\text{P}\) identified recently in the NRDs of several transcription factors and named the synergy control motif (54). It was hypothesized that this motif, which is frequently found in NRDs of DNA-binding regulators, restrains the higher order interactions among transcription factors. Mutations of the conserved Lys or Glu residues within the synergy control motif of the glucocorticoid receptor, for example, significantly increase the transcriptional activity. However, this occurs only when the reporter has multiple recognition sites (54). What role, if any, conjugation of SUMO-1 to Lys\(^{523}\) (or phosphorylation of Ser\(^{528}\)) has on the activity of c-Myb as defined by these studies of the synergy control motif is not known at present.

SUMolation has been shown to be responsible for the sub-nuclear translocation of SUMO-1-modified proteins into detergent-insoluble nuclear clots (35, 48, 55). These are called PML nuclear bodies because the sumolated PML (promonocytic leukemia) protein was identified as a major component of these structures (55–57). We also observed that, unlike unmodified c-Myb, the sumolated Myb protein was insoluble in a Nonidet P-40-based lysis buffer, suggesting that conjugation of SUMO-1 may target c-Myb to detergent-resistant structures similar to PML nuclear bodies. The sequestration of SUMO-1-conjugated c-Myb could create a privileged pool of the c-Myb protein that has unique activities and functions in the cell.

In summary, our data demonstrate a novel post-translational modification of c-Myb at two lysines, Lys\(^{499}\) and Lys\(^{523}\), which are located in a conserved region and which are absent following oncogenic activation (3, 4). It was shown previously that deletion of the carboxyl terminus of c-Myb increases DNA-binding affinity (58), proteolytic stability (24), and transactivation and transforming capacity (10, 59). Our work reports a new way through which the NRD can exert its negative effect on c-Myb function.

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Covalent Attachment of the SUMO-1 Protein to the Negative Regulatory Domain of the c-Myb Transcription Factor Modifies Its Stability and Transactivation Capacity
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