Sp1 is a ubiquitously expressed transcription factor that binds GC-rich cis elements. Many posttranslational modifications have been implicated in the regulation of Sp1 activity. We now provide evidence for a novel mechanism of Sp1 regulation involving the small ubiquitin-like modifier (SUMO-1). Western blot analysis revealed a high molecular mass Sp1 of 125 kDa that is stabilized by a selective SUMO hydrolase inhibitor and destabilized by a specific SUMO-1 hydrolase. The covalent modification of Sp1 by endogenous SUMO-1 and SUMO-1 that has been fused to green fluorescent protein was demonstrated using transient transfection assays. A high probability sumoylation consensus motif, VKKIE18, is located within the N-terminal negative regulatory domain of Sp1. Either arginine substitution for lysine 16 (Sp1(K16R)) or alanine substitution for glutamic acid 18 (Sp1(E18A)), abrogated Sp1 sumoylation. In vitro SUMO-1 covalently bound affinity-purified GST-Sp1, but not GST-Sp1(K16R). In vivo Sp1 was determined to be N-terminally cleaved, while Sp1(K16R) could not be cleaved indicating that sumoylation and cleavage are coupled through the key regulatory lysine 16. This coupling was evident by the demonstration of an inverse relationship between cellular SUMO-modified Sp1 and N-terminally cleaved Sp1. Compared with Sp1, sumoylation-deficient Sp1(E18A) exhibited enhanced cleavage and was a better transcriptional activator, while constitutively SUMO-1-modified Sp1 was deficient in proteolytic processing and repressed Sp1 transcriptional activity. The repressive effect of sumoylation on Sp1 activity is emphasized through the use of a GAL4 based transactivation assay. A model is proposed defining a mechanism by which sumoylation preserves the integrity of a negative regulatory domain thereby allowing for the inhibition of Sp-dependent transcription.

Sp1 was one of the first mammalian transcription factors to be cloned and biochemically characterized (1, 2). Sp1 is representative of a large Sp/KLF family (greater than 20 members) characterized by their affinity for GC-rich cis elements found in the promoters of many housekeeping genes as well as inducible genes (3, 4). Thus, Sp1 is involved in virtually all facets of cellular function. Of particular interest is Sp1 regulation of G1 phase TATA-less promoters, which govern cell growth, cell cycle regulation, differentiation, and apoptosis (5, 6).

The Sp1 molecule is organized into strong glutamine-rich activating domains (A (residues 83–262) and B (residues 263–542)) and weak activating domains (C (residues 543–610) and D (residues 709–778)). A highly conserved zinc finger region (residues 615–708) responsible for DNA binding is located between domains C and D (7). The N terminus (amino acids 1–82) endows Sp1 with repressor activity (8, 9). A stretch of conserved amino acids known as the Sp box is located at the N terminus of Sp1.8 This element contains an endoproteolytic cleavage site situated close to a region that targets Sp1 proteasome-dependent degradation in vitro (10).

The complexity of Sp1 gene regulation and function is explained in part through posttranslational modifications, which include phosphorylation, glycosylation, ubiquitinylation, acetylation, and ribosylation (11). For example, phosphorylation by cAMP-dependent protein kinase enhances Sp1 DNA binding and thereby increases its transcriptional activity (12). Sp1 phosphorylation also increases ubiquitinylation and subsequent proteolysis and thereby decreases Sp1 nuclear levels (13, 14). O-Linked N-acetyllglucosamine (O-GlcNAc) protects Sp1 from proteolysis, and since many of the same serines and threonines may be used for phosphorylation or glycosylation, there is an inverse relationship between Sp1 phosphorylation and glycosylation (15, 16). Glycosylation has been reported to enhance Sp-dependent transcription in some contexts, while in others, glycosylation interferes with Sp1/TAF factor binding resulting in transcriptional repression (17, 18). Acetylation activates Sp1 transcriptional potential by enhancing Sp1 DNA binding (19).

Lysines are particularly interesting sites of protein modification because of the large number of different interactions they undergo including acetylation, ubiquitinylation, methylation, or modification by various ubiquitin-like modifiers including the small ubiquitin-like modifier (SUMO)2 (20). Sumoylation is the process whereby SUMO-1, -2, or -3 is covalently and reversibly bound to specific lysines of target proteins using homologous ubiquitin-like enzymes (E1, E2, and E3). SUMO-1 is a 101-amino acid peptide sharing 18% identity to ubiquitin; however, it does not serve as a signal for protein degradation, rather sumoylation regulates protein-protein interactions resulting in modification of substrate-specific properties (21). Sumoylation has been implicated in diverse regulatory functions including subcellular compartmentalization, protein stability, chromatin structure regulation, transcription factor activity, DNA binding, and protein complex assembly. The growing list of SUMO-1 substrates includes many transcription factors suggesting a major role for sumoylation in the regulation of gene expression. Sumoylation generally represses transcriptional activity although some reports suggest that sumoylation enhances trans factor activity (22, 23).

Using several experimental approaches, we present evidence for the SUMO-1 modification of Sp1 and identify residue 16 as the SUMO-1-
Sumoylation Inhibits Sp1 Cleavage and Activity

conjugating lysine. We also demonstrate that lysine 16 regulates Sp1 N-terminal cleavage in vivo. Since the Sp1 N terminus is a co-repressor binding domain, we hypothesized that SUMO-1 modification at lysine 16 inhibits Sp1 proteolytic processing/activation. To test this hypothesis we constructed a sumoylation-deficient Sp1, Sp1(E18A), and a constitutively SUMO-1-modified Sp1. The data indicate that Sp1(E18A) is a better substrate for N-terminal cleavage and a better transactivator of transcription compared with wild-type Sp1. In accord, the constitutively sumoylated Sp1 is not a substrate for N-terminal cleavage and is a less effective transcription factor. Further evidence that sumoylation represses Sp1 activity is provided through the use of a GAL4-Sp1 fusion assay. These data suggest a novel mechanism whereby sumoylation inhibits Sp1 activity by maintaining the integrity of a negative regulatory domain. A model is proposed implicating posttranslational competition at lysine 16 in the control of Sp-dependent transcription.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF7 cells were obtained from the American Type Culture Collection. Cells were cultured in McCoy’s 5A medium (Invitrogen, Inc.) supplemented with 10% fetal calf serum in a 6% CO2 incubator at 37 °C. Phoenix cells were acquired from Dr. Nolan (Standford University, Standford, CA) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Drosophila Schneider line-2 (SL2) cells were cultured at room temperature in Schneider’s insect cell medium (Invitrogen, Inc.) supplemented with 1-glutamine and 10% fetal calf serum.

Antisera—Affinity-purified monoclonal and polyclonal anti-Sp1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-HA.11 antibody was obtained from Covance Research Products (Richardson, CA). Anti-FLAG was purchased from Sigma. Anti-SUMO-1 was purchased through Zymed Laboratories Inc.

Expression and Reporter Plasmids—pCMV-FLAG-Sp1-HA was a generous gift from Dr. Adrian Black (24). pCMV-Flag-Sp1-HA constructs carrying a substitution at lysine 16 for arginine were prepared using a site-directed mutagenesis kit (QuikChange, Stratagene, Inc., La Jolla, CA) with the oligonucleotide 5′-GCTGGTGTGTAAGATTGAAAAGGAGTTG-3′ and its complement (lowercase italicized letter indicates substituted nucleotide). Sp1 in which glutamic acid 18 was substituted with alanine was constructed with the oligonucleotide 5′-GTGGTTGAATAATGCAAGAGTTG-3′. DHR-luciferase has been described (25). pGAL4 and pGAL4-Sp1-(90–785) were digested with EcoRI to remove the truncated Sp1 gene and then inserted with a full-length Sp1 gene generated in a PCR reaction using the following primers: 5′-CGGGAATTCTAGAAGCCATCCATTGCTGATTAGAATGACAGTGTG-3′ and 5′-CGGGAATTCAAGAAGCATTGCAGTATTGAAATGATT-3′. A mammalian expression vector that directs sumoylation, UBC9, the SUMO-conjugating enzyme (25). This was cotransfected with CMV-driven expression plasmids using FuGENE 6 (Roche Diagnostics) as per the manufacturer’s instructions. Cell extracts were subjected to electrophoresis and transferred to nitrocellulose using a semidry transfer apparatus. Membranes were blocked with 5% fat-free dried milk in phosphate-buffered saline supplemented with 0.1% Tween 20 and incubated with primary antibody for 1 h at room temperature. Subsequently, membranes were incubated 1 h with secondary antibody conjugated to horseradish peroxidase. Antigen-antibody complexes were detected using a proprietary kit according to the manufacturer’s specifications (ECL, Amersham Biosciences, Inc.). LlNLN (N-acetyl-l-leucinyl-l-norleucinyl), MG132, IPTG isopropyl β-D-thiogalactopyranoside, and forskolin were purchased through Sigma Inc.

In Vitro Sumoylation—An in vitro sumoylation kit was purchased from LAE Biotech and instructions were followed per manufacturer’s specifications (LAE Biotech #K007 kit). Bacterial expressed GST, GST-Sp1, and GST-Sp1(K16R) were purified using glutathione-Sepharose 4B (Pharmacia Corp.) as per manufacturer’s specifications and then used as substrates in the in vitro reaction. Topoisomerase was used as a positive control and anti-topoisomerase and anti-Sp1 Western blots were performed to visualize sumoylated proteins.

RESULTS

Sp1 Exhibits a High Molecular Mass Isoform That Is Stabilized by a Selective Inhibitor of SUMO Hydrodases and Desumoylated by a Specific SUMO Hydrodase—In a previous study, the Sp1 family member, Sp3, was determined to be sumoylated and both Sp3 and Sp1 were shown to be interacted with UBC9, the SUMO-conjugating enzyme (25). This prompted us to investigate whether Sp1 might be SUMO-1 modified. MCF7 cell lysates were immunoprecipitated with either anti-Sp1 bound to agarose or with protein A/G bound to agarose. An anti-Sp1 Western blot showed the major Sp1 protein doublet at about 100 kDa as well as minor high molecular mass proteins (Fig. 1A). Sumoylated proteins migrate in SDS-PAGE slower then unmodified forms exhibiting a larger molecular mass by about 20 kDa or higher. Typically the SUMO-modified isoform represents a small fraction of the total protein. The corresponding anti-SUMO-1 Western showed a similar banding pattern but with greater complexity suggesting that Sp1 may interact with many sumoylated proteins (data not shown). This preliminary data indicated that endogenous Sp1 maybe sumoylated, and to study this specific modification, we would need to overexpress a tagged Sp1 in the cells. In the following experiments, cells were transfected with a plasmid encoding...
The high molecular mass protein was not observed in cells transfected with pCMV-FLAG-Sp1-HA alone (lane 2), or with pCMV-FLAG-Sp1-HA and pEYEP-SUMO (lane 3). B and C, Phoenix cells were co-transfected with pCMV-FLAG-Sp1-HA and pEYEP-SUMO. Cell lysates were immunoprecipitated with anti-FLAG, and an anti-SUMO-1 Western blot (C) was performed after which the blot was stripped and reprobed for total Sp1 using anti-HA (B).

FIGURE 2. Sp1 is covalently modified by SUMO-1 in vivo. A, anti-HA Western from Phoenix cells that were transfected with pEYEP-SUMO alone (lane 1), pCMV-FLAG-Sp1-HA alone (lane 2), or with pCMV-FLAG-Sp1-HA and pEYEP-SUMO (lane 3). B and C, Phoenix cells were co-transfected with pCMV-FLAG-Sp1-HA and pEYEP-SUMO. Cell lysates were immunoprecipitated with anti-FLAG, and an anti-SUMO-1 Western blot (C) was performed after which the blot was stripped and reprobed for total Sp1 using anti-HA (B).
**Sumoylation Inhibits Sp1 Cleavage and Activity**

**FIGURE 3. Lysine 16 of Sp1 is required for SUMO-1 modification.** A, anti-HA Western blot. MCF7 cells were transfected with either pCMV-FLAG-Sp1-HA or pCMV-FLAG-Sp1(K16R)-HA and harvested in 2% SDS lysis buffer. B, anti-SP1 Western. SL2 cells transfected with pPacSp1 or pPac Sp1 (K16R).

**FIGURE 4. Sp1 is sumoylated in vitro.** A, bacterial expressed GST-Sp1 and GST-Sp1(K16R) were purified using glutathione-Sepharose 4B and then used as substrates in an in vitro sumoylation reaction. A high molecular mass anti-Sp1-reactive protein was detected in the presence of GST-Sp1 but not GST-Sp1(K16R). B, topoisomerase, a substrate known to be multisumoylated, was used as a positive control.

**FIGURE 5. Lysine 16 of Sp1 is required for in vivo N-terminal cleavage.** A, illustration of the Sp1 molecule tagged at the N terminus with FLAG and at the C terminus with HA. The negative regulatory domain includes a cleavage site at leucine 64 that generates an 89-kDa C-terminal product. B, MCF7 cells were transfected with pCMV-FLAG-Sp1-HA and treated either with forskolin alone (lane 2), forskolin and LLLN (lane 3), or no treatment (lane 1). The arrow in B indicates the 89-kDa Sp1 cleavage product. C, a similar blot as described for B but probed with anti-FLAG. D, MCF7 transfected with pCMV-FLAG-Sp1-HA and treated with or without forskolin (lanes 1 and 2, respectively); pCMV-FLAG-Sp1(K16R)-transfected cells were treated with or without forskolin (lanes 3 and 4, respectively). E, a similar blot as described for D but probed with anti-SUMO-1. F, in lanes 1–3, MCF7 were transfected with pCMV-FLAG-Sp1-HA. Lane 1, no treatment; lane 2, forskolin treatment; lane 3, forskolin + MG132 treatment-mediated cleavage. In lanes 4–6, cells were transfected with pCMV-FLAG-Sp1(K16R)-HA. Lane 1, no treatment; lane 2, forskolin treatment; lane 3, forskolin + MG132 treatment. The arrows in E and F indicate an ~90- and 86-kDa full-length Sp1 protein.

89-kDa cleavage product as depicted by the arrow (Fig. 5B, lane 2). Forskolin-mediated cleavage is abrogated by treatment with a proteasome inhibitor, as seen in lane 3. Confirmation that the cleaved product was generated through N-terminal cleavage was confirmed by its lack of anti-FLAG reactivity (Fig. 5C, lane 2). Our in vivo data support the in vitro demonstration that Sp1 undergoes proteolytic processing.

Analysis of the Sp1(K16R) protein profile reveals unexpected low molecular mass proteins of about 90 and 86 kDa (Fig. 5D, lanes 3 and 4, and Fig. 5E, lanes 4–6). The calculated molecular mass of Sp1 is 86 kDa. Fig. 5E, lanes 3 and 4, show that the low molecular mass Sp1(K16R) is recognized by anti-FLAG, as well as anti-HA, and therefore cannot be a cleavage product. Fig. 5F indicates that the low molecular mass Sp1(K16R), unlike the Sp1 89-kDa product, is not affected by a proteasome inhibitor or forskolin treatment. We conclude that Sp1(K16R) is not N-terminally cleaved and that its faster migrating species may be less modified isoforms (refer to “Discussion”). From these data we conclude that Sp1 exhibits in vivo proteolytic processing and that Sp1 sumoylation and N-terminal cleavage are coupled through lysine 16.

**Uncoupling the Processes of Sumoylation and Cleavage**—The results presented above indicated that the Sp1(K16R) construct would not be useful for studying the affect of sumoylation on Sp-dependent transcription, since it would not be possible to distinguish between the effects mediated by N-terminal cleavage. We reasoned that substituting the conserved glutamic acid, within the sumoylation motif, should generate a SUMO-deficient Sp1 with an intact cleavage-dependent lysine 16. The Sp1 sumoylation motif with its conserved glutamic acid (Glu-18) is seen in bold face in Fig. 6. Immediately adjacent to the glutamic acid 18 is another lysine (Lys-19). This lysine is not believed to be required for Sp1 sumoylation; however, it has been suggested that lysines in the vicinity of a sumoylation motif may influence sumoylation.

Using site-directed mutagenesis, glutamic acid 18 was substituted with alanine (Sp1(E18A)), and lysine 19 was substituted with arginine (Sp1(K19R)). To enhance observation of Sp1 sumoylation, cells were co-transfected with plasmids encoding the Sp proteins and pEYEp-SUMO-1. Anti-HA Western showed that Sp1 (Fig. 6A, lanes 2 and 6) and Sp1(K19R) (lane 4) were substrates for sumoylation, while Sp1(K16R) and Sp1(E18A) were not (lanes 3 and 5). We next determined whether the sumoylation-deficient Sp1(E18A) retained the potential to be N-terminally cleaved. Cells were transfected as above and treated with or without forskolin and analyzed by Western blot (Fig. 6B). As observed in lane 6, Sp1(E18A) is a good substrate for the process of N-terminal cleavage. The processes of sumoylation and cleavage are uncoupled in the point mutant Sp1(E18A) thereby allowing us to study the affects of sumoylation on Sp1 function.

**Sumoylation Inhibits N Terminus Cleavage**—If sumoylation and N-terminal cleavage are coupled through Sp1 lysine 16, then an inverse relationship should exist between the cellular concentrations of SUMO-Sp1 and 89-kDa cleaved Sp1. Sp1 was co-expressed with increasing amounts of SUMO-1 in cells treated with forskolin overnight. Anti-HA
Western blot shows that the 89-kDa Sp1 product decreases with the concomitant increase in SUMO-modified Sp1 suggesting that these two activities are mutually exclusive (Fig. 7, A and B).

Our ability to study the effect of sumoylation on Sp1 activity is greatly limited because only a small fraction of total Sp1 is SUMO-modified. To facilitate a functional analysis of Sp1 sumoylation, we designed a constitutively SUMO-modified Sp1. Using a PCR approach, the SUMO-1 gene (minus the last 6 amino acids, which include the SUMO hydrolase cleavage site) was fused to the 5′ end of the Sp1 cDNA (Fig. 7C). To determine whether sumoylation influences the cleavage of Sp1, cells expressing wild-type Sp1, Sp1(E18A), and constitutively SUMO-modified Sp1 were treated with forskolin followed by Western blot analysis (Fig. 7D). Constitutively SUMO-modified Sp1 could not be cleaved, as indicated by the lack of an 89-kDa product (Fig. 7D, lane 3). In accord with these data, SUMO-deficient Sp1 was a significantly better substrate for N-terminal cleavage (lane 2), compared with Sp1 (lane 1), thereby reaffirming that sumoylation inhibits Sp1 cleavage.

**Sumoylation Negatively Regulates Sp1-dependent Transcription**—The N terminus of Sp1 is a NRD/co-repressor binding domain, and if sumoylation maintains the integrity of this domain, then SUMO-modified Sp1 would repress Sp-dependent transcription. The following set of luciferase reporter assays was performed to test the effect of sumoylation on Sp1 transcriptional activity. To determine whether sumoylation affects endogenous Sp1 activity, MCF7 cells were transfected with a mutant DHFR reporter containing only Sp1 binding sites. Increasing amounts of pSENP1, encoding a de-sumoylating enzyme, was co-transfected. As observed in Fig. 8A, there is a direct relationship between the amount of enzyme expressed and the increase in Sp-dependent transcription. As expected, these data indicate that sumoylation negatively influences endogenous Sp-activity.

MCF7 cells were transfected with the p21 reporter and plasmids encoding the Sp proteins. Mammalian cells show robust endogenous Sp1 expression resulting in a high background signal and ectopic Sp1 only mediates a 2-fold increase above this background. The high background made it difficult to observe differences between the activities of Sp1 and Sp1(E18A). Therefore, as seen in Fig. 8B, only Sp1 is compared with SUMO-modified Sp1, and the endogenous Sp1 luciferase activity was subtracted from the activity mediated by ectopic Sp proteins. The results showed that SUMO-SP1 repressed Sp1 transcriptional activity by about 6-fold, and the Western blot insert indicated that the Sp protein levels were comparable.

Sp-deficient SL2 cells were transfected with a p21 luciferase reporter and plasmids encoding the Sp proteins. It was observed that SUMO-SP1 protein levels were consistently low in the insect cell and therefore this construct could not be used in this experiment. However, as seen by the Western blot inserted in Fig. 8C, Sp1 and Sp1(E18A) were found in equal concentrations. Lane 1 shows a low background signal from cells transfected with reporter promoter alone. Sp1 activity seen in lane 2 is nearly 10-fold greater than the background and Sp1(E18A) activated almost 2-fold greater than Sp1 (lane 3). Since only a small fraction of total Sp1 is SUMO-1-modified, the transcriptional differences between Sp1 and Sp1(E18A) suggest that sumoylation plays an important role in the attenuation of Sp1-dependent transcription. In the final functional experiment, Ga4DBD-Sp fusion constructs were co-expressed along with the Ga4 luciferase reporter gene. Compared with the widely used
Gal4-Sp1-(83–778), Gal4-Sp1 (full-length) exhibits much less activity (Fig. 8D, lanes 2 and 3). Gal4-Sp1(E18A) activity is 3-fold more than that of Gal4-Sp1 (compare lanes 3 and 4). These data confirm that the Sp1 N terminus is a negative regulatory domain and implicate the SUMO-1 modifier as a major player in this repression.

Proposed Model—Taken together these data suggest that lysine 16 is a key regulatory switch controlling Sp1 activation and repression. Upon ubiquitinylination at lysine 16 the proteasome is recruited mediating the N-terminal cleavage of a co-repressor binding domain and thereby generating a more active Sp1 transactivator. If SUMO-1 out-competes for the covalent binding of lysine 16, then the NRD integrity is maintained allowing the recruitment of co-repressors and subsequent repression of Sp-dependent transcription.

DISCUSSION

External cues from the environment promote cell signaling that mediates reversible posttranslational modifications. Protein modifications regulate the activity of promoter-specific trans factors resulting in dynamic changes in gene expression. Ubiquitous transcriptional activator, Sp1, is extensively modified by numerous processes including phosphorylation, glycosylation, acetylation, ubiquitination, and, as we presently report, sumoylation.

It has been reported that Sp1, unlike Sp3, is not a substrate for SUMO-1 modification (26); however, the Sp1 used to conduct these experiments lacked the N terminus and thus did not contain the key sumoylation site identified in this study. Using molecular genetic and immunobiochemical approaches we present much evidence for Sp1 sumoylation. Sp1 physically interacts with UBC9, an E2 SUMO-conjugating enzyme (25). An anti-Sp1-reactive 125-kDa protein is stabilized in the presence of a selective SUMO-1 hydrolase inhibitor and abrogated in the presence of a specific SUMO hydrolase. Anti-SUMO-1 Western blot analysis from cell lysates co-expressing Sp1 and GFP-SUMO-1 demonstrate that SUMO-1 covalently binds to Sp1. Sp1 includes a sumoylation consensus motif, which is located within an N-terminal negative regulatory domain. Substitution of arginine for the SUMO-conjugating lysine abrogated Sp1 sumoylation. A commercially available sumoylation kit was used to confirm that purified GST-Sp1 was SUMO-1 modified, whereas GST-Sp1 (K16R) was not. Immunostaining assays revealed no differences in subcellular localizations of Sp1(K16R) compared with Sp1 nor did we observe differences in promoter binding using an Active Motif Sp1 ELISA kit. These negative data are not shown in this study, but the immunostaining patterns observed are similar to those previously reported in an investigation of Sp3 sumoylation (25).

The N terminus of Sp1 undergoes endoproteolytic cleavage in vitro resulting in an unstable 89-kDa C-terminal product (10). The N terminus of Sp1 is a negative regulatory domain, which binds co-repressors (9). Since sumoylation has been implicated in both substrate stabilization and in the recruitment of co-repressors, we hypothesized that sumoylation may regulate one or more of Sp1 N-terminal activities. An in vitro investigation by Kudlow and colleagues (10) demonstrated that the first 64 amino acids of Sp1 is a recognition site for proteasome-dependent N-terminal cleavage. The cAMP activator, forskolin, was used to induce Sp1 cleavage into an 89-kDa product. In accord with the in vitro results, our in vivo data demonstrated that forskolin facilitated the production of an 89-kDa C-terminal fragment in a proteasome-dependent manner. Interestingly, we observed that Sp1(K16R) could not be N-terminally cleaved in vivo. These data indicate that aside from regulating sumoylation, lysine 16 is also required for proteasome-dependent cleavage. The observed lower molecular mass forms of Sp1(K16R) are likely to be less glycosylated Sp1 that are stabilized by the absence of the proteasome recruiting lysine 16. As an aside, lysine 19 was neither required for sumoylation nor N-terminal cleavage; however, in its absence there is a significant increase in Sp1 cleavage (Fig. 6B, lane 8).

Many functional studies on sumoylated proteins utilized a point mutant of the SUMO-conjugating lysine. However, this lysine may compete for multiple modifiers, any of which may be responsible for the observed point mutant phenotype. Having determined that Sp1 lysine 16 is a key molecular switch controlling the processes of sumoylation and N-terminal cleavage, it was necessary to design a sumoylation-deficient Sp1 that was uncoupled to proteolytic processing. This was accomplished through mutation of the conserved glutamic acid 18 found in the Sp1 SUMO-motif, which resulted in a sumoylation-deficient yet cleavage proficient Sp protein (Sp1E18A). The SUMO-modified form of a protein typically represents a small fraction of the total protein, and SUMO binds in transient thereby adding to the difficulty of functional analysis. In an effort to overcome these difficulties, we generated a constitutively SUMO-modified Sp1 by ligating the SUMO-1 gene, minus its C-terminal cleavage site, to the 5’ end of the Sp1 gene. Similar approaches have been used involving covalent attachment of ubiquitin to the VP16 activation domain and SUMO-1 to a truncated Sp3 protein to expedite functional studies (30).

Equipped with a constitutively SUMO-modified Sp1 and SUMO-deficient Sp1 (Sp1(E18A)), we began a functional analysis. Forskolin could not induce cleavage of a constitutively SUMO-modified Sp1; however, Sp1(E18A) showed enhanced N-terminal cleavage compared with wild-type Sp1 (Fig. 7). Further evidence that sumoylation inhibits Sp1 cleavage is provided by a demonstration showing the inverse relationship between cellular concentrations of SUMO-modified Sp1 and cleaved Sp1 (Fig. 7, C and D). This Western blot also hints that it is phosphorylated Sp1 that may preferentially undergo proteolytic cleavage. It is possible that phosphorylation precedes ubiquitinylination in this proteasome-dependent event. Interestingly, Sp1 proteolytic processing is similar to that observed in forskolin-induced activation of NF-κB. To test the role of sumoylation in Sp-dependent transcription, we overexpressed a luciferase reporter promoter with increasing amounts of the de-sumoylating enzyme, SENP1. As predicted, sumoylation exerted a negative regulatory effect on Sp-dependent transcription. However, there may be several indirect effects contributing to these data. In the next luciferase assay, the DHFR reporter promoter and Sp proteins were co-expressed in MCF7 cells. Mammalian cells have significant endogenous Sp1 (as observed in the previous experiment), which results in high...
background signals that mask the activity of ectopic Sp1. This may explain why ectopic Sp1 exerted only a 2-fold difference above background activity. The data are presented with endogenous activity subtracted to show only ectopic Sp1 activity. The results confirm that constitutively SUMO-modified Sp1 was a poorer transactivator compared with Sp1. No differences were detected between Sp1 and Sp1(E18A) (data not shown). The insect cell, SL2, is reportedly devoid of endogenous Sp1 and therefore is the preferred cell line for studying small differences in Sp1 activity. The Sp1 and Sp1(E18A) were found in comparable protein levels in SL2, while SUMO-SP1 protein levels were found in lower concentrates and therefore could not be included in this assay. In insect cells, ectopic Sp1 exhibited 10-fold activity over background activity (Fig. 8, lanes 1 and 2). Sp1(E18A) activated luciferase reporter genes almost 2-fold greater than wild-type Sp1 (lanes 2 and 3). Similar 2-fold differences in trans activity have been observed between SUMO-1-modified and unmodified forms of other transcription factors including c-Jun, androgen receptor, and Sp3 (25, 32, 33). Considering that only a small fraction of total Sp1 is SUMO-1-modified, this difference in activity suggests that sumoylation plays an important role in Sp-dependent transcription. To generate more significant differences between wild-type and sumoylation-deficient Sp1, we utilized Gal4DBD-Sp fusion constructs along with the 5X-Gal4 binding site luciferase reporter. To our knowledge, this is the first time a Gal4 luciferase assay has been performed with full-length Sp1 (including Sp1 NRD). The significant difference between N-terminally truncated Sp1 and full-length Sp1 suggests that much of our knowledge of Sp1 as a potent transcriptional activator may need to be reassessed, since most functional analysis of Sp1 has been performed using Gal4-SP1-(90–785). These data confirmed the repressor function of the Sp1 N terminus and demonstrated that Sp1 sumoylation serves a major role in this repression.

Previous in vitro studies concluded that an 89-kDa Sp1 C-terminal product was highly unstable under high cAMP and low glucose levels and an intermediate product in the degradation of Sp1. Biochemically active proteins are typically unstable compared with their less active counterparts suggesting a link between protein activation and degradation. We propose a model in Fig. 9 whereby the 89-kDa cleavage product is a more efficient transcription factor under the negative control of SUMO-1.

SUMO-1 is emerging as a versatile modifier of diverse substrates influencing numerous biological processes. Among these substrates is a growing list of transcription factors, indicating the importance of sumoylation in gene regulation. The reversible conjugation of SUMO-1 has been reported to affect transactivation through transcription factor stabilization, intracellular relocation, DNA binding, conformational changes that alter other posttranslational modifications, and/or protein-protein interactions. As proposed in this work, sumoylation may also regulate proteolytic processing. It is postulated that sumoylation negatively regulates synergy at compound response elements (33) and that sumoylation mediates the recruitment of co-repressors to the promoter. For example, sumoylated p300 and sumoylated histone 4 exert a negative regulatory effect through the recruitment of the co-repressor, HDAC (34, 35). A negative regulatory protein, p74, binds the Sp1 N terminus, and this binding correlates with attenuation of Sp-dependent transcription (8). Recently it has been determined that the co-repressors SMRT, NcoR, and BcoR interact with the Sp1 N terminus (9). It is possible that SUMO-modified Sp1 has a greater affinity for co-repressors.

Multiple human Sp1 mRNAs have been observed, and mouse cells express an Sp1 mRNA encoding a protein of 778 amino acids (36). Multiple Sp1 mRNAs may be explained by multiple translational initiation start sites as has been observed to occur with Sp3, which results in four Sp3 isoforms (31, 37). Amino acid analysis of the recently identified Sp3 N terminus indicates that Sp3 possesses a similar Sp1 N-terminal sumoylation site and N-terminal cleavage site. It is plausible that the first initiating methionines for Sp3 and Sp1 may regulate the expression level of N-terminal SUMO-1-modified Sp proteins. This regulation would be absolute for Sp3, since its second initiating methionine excludes the potential N-terminal sumoylation site.

Sp1 is the model transcription factor for a large family of proteins and is the first mammalian transcription factor to be identified and extensively characterized; yet, there is still much to learn about Sp1 gene regulatory functions. Our data demonstrate that Sp1 can be N-terminally cleaved in vitro. We identify Sp1 as a substrate for SUMO-1 modification and suggest that sumoylation may repress Sp1 transcription by inhibiting the cleavage of a N-terminal negative regulatory domain. These data provide new insights into the complexity of Sp-dependent gene regulation, emphasize the importance of sumoylation in Sp-dependent gene regulation, and suggest a novel molecular mechanism underlying the repressor function of Sp1.

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