SUMO Modification Enhances p66-Mediated Transcriptional Repression of the Mi-2/NuRD Complex

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Human p66 α and p66 β are two potent transcriptional repressors that interact with the methyl-CpG-binding domain proteins MBD2 and MBD3. An analysis of the molecular mechanisms mediating repression resulted in the identification of two major repression domains in p66 α and one in p66 β . Both p66 α and p66 β are SUMO-modified in vivo: p66 α at two sites (Lys-30 and Lys-487) and p66 β at one site (Lys-33). Expression of SUMO1 enhanced the transcriptional repression activity of Gal-p66 α and Gal-p66 β . Mutation of the SUMO modification sites or using a SUMO1 mutant or a dominant negative Ubc9 ligase resulted in a significant decrease of the transcriptional repression of p66 α and p66 β . The Mi-2/NuRD components MBD3, RbAp46, RbAp48, and HDAC1 were found to bind to both p66 α and p66 β in vivo. Most of the interactions were not affected by the SUMO site mutations in p66 α or p66 β , with two exceptions. HDAC1 binding to p66 α was lost in the case of a p66 α K30R mutant, and RbAp46 binding was reduced in the case of a p66 β K33R mutant. These results suggest that interactions within the Mi-2/NuRD complex as well as optimal repression are mediated by SUMOylation.

Methylation of cytosine at the carbon 5 position of CpG dinucleotides is an epigenetic modification that is implicated in transcriptional silencing. It has long been known that histone deacetylation is a major mechanism in DNA methylation-mediated transcriptional repression (4). In general, acetylated histone tails correlate with active gene transcription and an open chromatin conformation, whereas deacetylated histone tails correlate with inactive gene transcription and a closed chromatin conformation (41). It has been proposed that the bridge between DNA methylation and histone deacetylation is comprised of methyl-CpG-binding domain (MBD) proteins that target multiprotein repressor complexes such as Mi-2/NuRD (41). So far, five MBD proteins have been identified in vertebrates: MeCP2, MBD1, MBD2, MBD3, and MBD4. With the exception of MBD4, all of them are associated with histone deacetylases (HDACs) (39). Among the family of human MBD proteins, MBD2 and MBD3 are the most highly related. The MBD3 protein is an integral component of the multisubunit protein complex Mi-2/NuRD that contains nucleosomal remodeling activity as well as HDACs that affect chromatin conformation resulting in gene silencing (40, 42). While MBD3 of the Mi-2/NuRD complex cannot be directly recruited to methylated DNA, interaction with MBD2, which has an intrinsic affinity for methylated DNA, targets Mi-2/NuRD to methyl-CpG (5, 15). This MBD2-containing Mi-2/NuRD complex has also been called MeCP1 (9). Indeed, there seems to be an overlap for MBD2 and MBD3 binding sites (1, 24), whereas complex purification revealed the existence of distinct MBD2/NuRD and MBD3/NuRD complexes (24).

Two highly related human p66 proteins, referred to as hp66 α and hp66 β , have been shown to interact with MBD2 and with

MBD3, to enhance MBD2-mediated repression, to interact with histone tails, and to reside within the MBD2/NuRD and MBD3/NuRD complexes (6, 7, 10, 24). Both p66 proteins colocalize with MBD2 in a speckled nuclear pattern. A comparison between different species revealed two conserved regions, CR1 and CR2. CR1 of p66 β is required for interaction with MBD2, MBD3, MTA2, HDAC1, HDAC2, RbAp46, and RbAp48, whereas CR2 was reported to target p66 and MBD3 to specific nuclear loci and to mediate histone tail interaction (7, 10).

Small ubiquitin-like modifier (SUMO) is a protein of 97 amino acids that is structurally similar to ubiquitin and has been found to be covalently attached to lysine residues within specific target proteins. Four different SUMO isoforms, termed SUMO-1 to SUMO-4, have been identified in mammals to date. Of these, SUMO-2 and SUMO-3 are closely related and share about 95% amino acid sequence identity, in contrast to sharing about a 50% identity with SUMO-1 (20, 32, 35). Posttranslational modification by SUMO has been increasingly recognized as being an important regulatory mechanism in a diverse range of cellular processes. SUMO is conjugated to target proteins through a unique enzyme cascade distinct from, but analogous to, the ubiquitin conjugation pathway (18). The specific E2-conjugating enzyme Ubc9 (2, 25) is able to recognize the consensus motif ψ KXE within target proteins and to covalently link the C-terminal glycine of SUMO to the ϵ -amino group of lysine (30). Although the SUMO E1 and E2 enzymes are sufficient for SUMO conjugation to target proteins in vitro, several SUMO E3 ligases have been described that enhance the efficiency or selectivity of SUMO conjugation to target substrates in vivo (17, 19).

In contrast to ubiquitination, which usually directs target proteins toward degradation, SUMOylation has a wide range of substrate-specific functions through multiple mechanisms. Firstly, SUMOylation can regulate the subcellular compartmentalization of target proteins. For example, SUMO conju-

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gation directs RanGAP1 protein to the nuclear pores (27). Secondly, SUMOylation and ubiquitination occur at the same lysine residues in the case of NF- κ B inhibitor I κ B α (9), and SUMOylation can stabilize target proteins through inhibiting protein ubiquitination that is followed by proteasomal degradation. Thirdly, the SUMOylation of transcription factors has been shown to have either positive or negative effects on their transcriptional activity. For instance, conjugation of GRIP1 enhances its transcriptional activity (22). On the other hand, SUMOylation of several transcription factors such as Elk-1 (43, 44), the androgen receptor (28), Sp3 (31, 33), and c-Myb (3) has been shown to down-regulate their transcriptional activity. Furthermore, SUMOylation regulates protein-protein interactions (43), protein-DNA binding activity (12), and enzymatic activity (13).

In this work we report that p66 α and p66 β proteins can be SUMOylated *in vivo*. We map two major SUMO modification sites at Lys-30 and Lys-487 of p66 α and one major SUMO modification site at Lys-33 of p66 β . SUMO-1 enhances the transcriptional repression activity of Gal-p66 α and Gal-p66 β , whereas mutation of the major SUMO modification sites or cotransfection with SUMO-1G97A or dominant negative Ubc9 (DN-Ubc9), which blocks the SUMO conjugation pathway, abolishes the SUMOylated forms of p66 α and p66 β . We also show that mutation of the major SUMO modification sites or the use of SUMO1G97A and DN-Ubc9 impairs the repressive activity of p66 α and p66 β . Furthermore, we demonstrate that HDAC1 is recruited to the N-terminal SUMO modification site of p66 α , whereas the N-terminal SUMO modification site of p66 β binds to RbAp46. In contrast, the SUMO modification sites do not influence MBD3 or RbAp48 binding to p66 α and p66 β *in vivo*.

MATERIALS AND METHODS

DNA constructs. For the construction of corresponding deletion constructs between p66 α and p66 β , specific restriction enzyme sites were introduced into cDNAs through site-specific mutagenesis PCR (see below). Primers were designed in such a manner that nucleotide mutations did not alter any amino acids. The following clones were produced at the indicated positions using this approach: pSG5-p66 β , NotI_{mut}(NotI mutation) at the position of amino acid (aa) 349, and Eco47III_{mut} at aa 479; for pSG5-p66 α , HindIII_{mut} at aa 205, Eco47III_{mut} at aa 475. The naturally occurring Eco47III site (Eco47III_{del}) at aa 14 was deleted. Gal-p66 α residues 1 to 205 (Gal-p66 α ₁₋₂₀₅), Gal-p66 α ₂₀₅₋₃₄₅, Gal-p66 α ₂₀₅₋₄₇₅, Gal-p66 α ₃₄₅₋₄₇₅, Gal-p66 α ₃₄₅₋₆₃₃ and Gal-p66 α ₄₇₅₋₆₃₃ were generated by in-frame insertions of the EcoRI/HindIII, HindIII/NotI, HindIII/Eco47III, NotI/Eco47III, NotI/BglIII, and Eco47III/BglIII fragments from pSG5-p66 α (using appropriate mutated restriction enzyme sites), respectively, into the Gal linker digested with SalI/HindIII, *Cfr*9I, Eco47III, SalI/Eco47III, SalI/BamHI, and Eco47III/BamHI. Gal-p66 β ₁₋₂₂₅, Gal-p66 β ₂₂₅₋₃₄₉, Gal-p66 β ₂₂₅₋₄₇₉, Gal-p66 β ₃₄₉₋₄₇₉, Gal-p66 β ₃₄₉₋₅₉₃, and Gal-p66 β ₄₇₉₋₅₉₃ were created by in-frame ligation of the EcoRI/HindIII, HindIII/NotI, HindIII/Eco47III, NotI/Eco47III, NotI/BamHI, and Eco47III/BamHI fragments from pSG5-p66 β (using appropriate mutated restriction enzyme sites), respectively, into the Gal linker digested with Eco47III/HindIII, *Cfr*9I, Eco47III, SalI/Eco47III, SalI/BamHI, and Eco47III/BamHI. pEGFP-hp66 α was constructed by ligating the SalI/BamHI fragment from pAB-Gal94-hp66 α into pEGFP-C1 (Clontech). The enhanced green fluorescent protein (EGFP) fusions of p66 α K30R, p66 α K487R, and p66 β K33R were generated by cutting the SalI/BamHI fragment of Gal-p66 α K30R, Gal-p66 α K487R, and Gal-p66 β K33R, followed by insertion into pEGFP-p66 α or pEGFP-p66 β cut with SalI/BamHI. pBK-CMV-FLAG vector was generated by digesting the annealed double-strand oligonucleotides (sense strand, CTAGAGCCACCATGGACTACAAGGACGACGATGACAAGGCTAGCGAATTCGCTCGAGGGGATCCGAT; antisense strand, ATCGGATCCCTCGAGCGAATTCGCTAGCCTGTGCATCGTCGCTTGTAGTCCATGGTGGCT) with SpeI/SmaI and ligating into pBK-CMV vector (Stratagene).

Calmodulin binding peptide (CBP) was amplified by PCR from pBS1479 vector (Euroscarf) and digested with XhoI/BamHI and ligated into pBK-CMV-FLAG. pBK-CMV-FLAG-p66 α /p66 β -CBP was amplified by PCR from pSG5 p66 α /p66 β , respectively. Products were then digested with EcoRI and EcoRI/XhoI and ligated into pBK-CMV-FLAG-CBP. pcDNA3-FLAG-p66 α /p66 β -CBP was generated with PCR from pBK-CMV-FLAG-p66 α /p66 β -CBP. The PCR products were digested with BamHI/XbaI and ligated into pcDNA3-FLAG-p66 α . Control plasmid pcDNA3-FLAG-CBP was constructed with PCR from pBS1479 and digested with BamHI/XbaI and ligated into pcDNA3-FLAG-p66 α . The eukaryotic expression vectors pCMV-GST-MBD3 and pCMV-GST-HDAC1 were generated by cutting the BamHI/SalI fragments of c-Myc-MBD3 (kindly provided by G. P. Pfeifer) and of pcDNA3-HDAC1, respectively, followed by insertion into pCMV-GST cut with BamHI/SalI. pCMV-GST-RbAp46 and pCMV-GST-RbAp48 were generated by cutting the NcoI/BamHI fragment of RbAp46 and the NcoI/XhoI fragment of RbAp48 (kindly provided by B. Stillman) and inserting these in frame into pCMV-GST cut with BamHI/XhoI and BamHI/NotI. The upstream activation sequence-thymidine kinase luciferase reporter construct containing four Gal binding sites (4 $\times$ UAS-TK-Luc) and expression plasmid pCMV-LacZ encoding β -galactosidase were kindly provided by A. Baniahmad. pEGFP-p66 β , Gal-p66 α , Gal-p66 β , pSG5p66 α , and pSG5p66 β have been described previously (6, 7).

Site-directed mutagenesis. Gal-p66 α with single amino acid substitutions K30R, K149R, K451R, or K487R; the double mutations K30R/K149R, K30R/K451R, K149R/K451R, or K451R/K487R; and the triple mutation K30R/K149R/K451R as well as quadruple mutation K30R/K149R/K451R/K487R and Gal-p66 β with single amino acid substitutions K33R or K454R as well as the double mutation K33R/K454R were constructed using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The last glycine (the 97th Gly residue) of SUMO1 in pcDNA3-FLAG-SUMO-1 were mutated to Ala by using site-directed mutagenesis PCR. In addition, DN-Ubc9 was mutated at the cysteine 93 position into serine in pcDNA3-HA-Ubc9. All constructs were verified by nucleic acid sequencing.

Nuclear protein extraction. 293T cells were transiently cotransfected with expression vectors (8 μ g) Gal-p66 α and/or Gal-p66 β in the presence or absence of 10 μ g of pcDNA3-FLAG-SUMO-1 and/or pcDNA3-HA-Ubc9. Cells were collected 48 h after transfection, and nuclear extracts were prepared as described previously with modification (37). Briefly, the cell pellet was resuspended in 3 volumes of buffer A (20 mM HEPES, pH 7.9, 10% glycerol, 0.2% Nonidet P-40, 10 mM KCl, 1 mM EDTA, and freshly added 0.1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol). After centrifugation at 3,000 $\times$ g for 10 min at 4°C, the supernatant (cytoplasmic fraction) was transferred into a new tube for later use. The remaining nuclear pellet was resuspended in 2 volumes of buffer B (420 mM NaCl, 20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, and freshly added 0.1 mM phenylmethylsulfonyl fluoride), followed by incubation for 40 min at 4°C on a rotator. The lysate was centrifuged at 20,000 $\times$ g for 10 min at 4°C, and the supernatant that was nuclear extract was transferred into a new tube.

Western blotting. Nuclear extracts (described above) were prepared from 293T cells after different transfection experiments. Samples containing equal protein amounts (Bradford assay) were size fractionated by 10% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane (Millipore), and blocked in 5% skim milk in phosphate-buffered saline buffer containing 0.1% Tween 20 at 4°C overnight. For the detection of the proteins, the membrane was incubated with the primary rabbit anti-Gal4 polyclonal immunoglobulin G (IgG; BAbCO) antibody at a dilution of 1:2,000, primary rabbit anti-FLAG polyclonal IgG (Abcam) antibody at a dilution of 1:2,000, or primary rabbit anti-p66 polyclonal IgG (Upstate) antibody at a dilution of 1:500; the membrane was then washed and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) at a dilution of 1:10,000. Immunoreactive proteins were visualized using an ECL kit (Amersham Biosciences) following the manufacturer's protocol.

Mammalian pull-down assay. HEK293 cells were transiently cotransfected with mammalian expression vectors (15 μ g) for pCMV-GST, pCMV-GST-MBD3, pCMV-GST-HDAC1, or pCMV-GST-RbAp46/48 with Gal, Gal-p66 α , four single mutant forms of Gal-p66 α (K30R, K149R, K451R, and K487R), Gal-p66 β , or two single mutant forms of Gal-p66 β (K33R and K454R). Cells were collected 48 h after transfection, and nuclear extracts were prepared. A total of 400 μ g of nuclear extract was incubated with 40 μ l of glutathione-Sepharose 4B beads (Amersham Biosciences) for 30 min at room temperature. The beads were washed four times with washing buffer (200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% NP-40). Proteins binding to the beads were eluted with SDS sample buffer, fractionated together with the corresponding input fractions on SDS-PAGE, and subsequently detected by Western blotting using the anti-Gal4 polyclonal IgG antibody. To remove previous primary and

secondary antibodies, membranes were submerged in stripping buffer (2% SDS, 62.5 mM Tris-HCl [pH 6.7], freshly added 100 mM 2-mercaptoethanol). Blots were subsequently probed with mouse anti-glutathione transferase (GST) polyclonal IgG (Santa Cruz Biotechnology) at a dilution of 1:2,000 and subsequently with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) at a dilution of 1:10,000. An enhanced chemiluminescence kit was used to visualize the proteins on the membrane following the manufacturer's protocol.

Cell culture and transfections. HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum containing penicillin and streptomycin at 37°C in 5% CO₂. For transfection assays, about 2×10^5 cells were seeded into each well of a six-well plate 24 h prior to transfection and then transfected using the CaPO₄ method as described previously (8). Each transfection contained 5.4 µg of total plasmid DNA that included 0.25 µg of 4×UAS-TK-Luc reporter plasmid, 0.05 µg of pCMV-lacZ encoding β-galactosidase, and various amounts of expression plasmids Gal, Gal-p66α, Gal-p66β, pairs of deletion constructs of Gal-p66α and Gal-p66β (i.e., Gal-p66α₁₋₂₀₅ and Gal-p66β₁₋₂₂₅), or the Gal-p66α or Gal-p66β mutants (i.e., K30R, K33R, or K30R/K149R). Cells were harvested 48 h after transfection and measured for luciferase and β-galactosidase activity. Transfection efficiency was normalized to β-galactosidase activity. The results are the average of at least two independent transfection experiments, and standard deviations are denoted by error bars in the figures. To determine the protein levels of wild-type Gal-p66α/Gal-p66β, deletion constructs of Gal-p66α/Gal-p66β, and various Gal-p66α/Gal-p66β mutants, immunoblotting was performed at the same time. For trichostatin A (TSA) experiments, cells were treated with 100 ng/ml TSA (final concentration) 24 h after transfection with wild-type or mutant Gal-p66α/Gal-p66β constructs. Cells were harvested after 36 h of incubation.

Stable cell transfection and in vivo SUMOylation. 293 cells were grown in six-well plates, transfected with 1.0 µg of pcDNA3-FLAG-CBP, pcDNA3-FLAG-p66α-CBP, or pcDNA3-FLAG-p66β-CBP. After 48 h cells were moved to Dulbecco's modified Eagle's medium containing 1.5 mg/ml G418. The medium was refreshed every 3 days until individual colonies appeared. Colonies were identified by reverse transcription-PCR using specific primers and by Western blotting using FLAG antibody. Positive cell clones with low-level expression were scraped off with ice-cold phosphate-buffered saline supplemented with 50 mM N-ethyl maleimide (Sigma) and Complete Mini Protease Inhibitors (Roche). Afterwards, nuclear extract was prepared as described above. All buffers contain 50 mM N-ethyl maleimide during nuclear extract preparation. A total of 40 µl of a 50% slurry of FLAG M2 Gel (Sigma) was added and incubated at 4°C overnight. The gel was washed three times with Tris-buffered saline buffer according to the instructions and eluted with FLAG peptide (Sigma). The eluted fractions were analyzed by 7.5% to 10% SDS-PAGE and Western blotting using antibody against FLAG (Abcam) and reprobed with SUMO1 antibody (Alexis Biochemicals).

Fluorescence analysis. NIH 3T3 cells were cultured to subconfluency on coverslips and transfected with pEGFP-C2, pEGFP-p66α, pEGFP-p66αK30R, pEGFP-p66αK487R, pEGFP-p66β, or pEGFP-p66βK33R. Transfection of NIH 3T3 cells was performed using jetPEI (Polyplus) reagent according to manufacturer's protocol. Images were taken by fluorescence microscopy 24 h after transfection using a 1,000-fold magnification following incubation with Hoechst DNA stain for 10 min at 37°C.

RESULTS

Transcriptional repression activity of p66α and of p66β is partially dependent on HDAC activity. p66α and p66β proteins are highly homologous in their amino acid sequences (6), and we wondered whether the transcriptional repression activity mediated by p66α or p66β proteins is similar as well. In order to assess this, we utilized the reporter plasmid 4×UAS-TK-Luc, which contains four Gal binding sites upstream of the thymidine kinase promoter that drives the luciferase reporter gene. Cotransfection with the Gal-DNA binding fusion constructs Gal-p66α and Gal-p66β resulted in a strong transcriptional repression (Fig. 1). The transcriptional repression activity of p66β was significantly lower than that of p66α. It has previously been shown that p66β-mediated transcriptional repression was partially dependent on histone deacetylation (10). We

FIG. 1. The transcriptional repression activity of p66α is stronger than that of p66β and is partially dependent on HDAC activity. 293T cells were cotransfected with a 4×UAS-TK-Luc reporter together with constructs coding for the Gal-DNA binding domain or the indicated Gal fusions in the presence or absence of TSA. Cell extracts were analyzed for reporter gene activity and protein expression. Gal-NCOR is a positive control for TSA sensitive repression (29). The increase in repression (*n*-fold) was determined relative to the Gal-DNA binding domain alone. Error bars represent variations within duplicate transfections.

therefore assumed that p66α-mediated transcriptional repression is at least partially due to histone deacetylase activity. To test this assumption the transcriptional activities of wild-type Gal-p66α/Gal-p66β were analyzed in the presence and absence of the histone deacetylase inhibitor TSA. As a control we used Gal-NCOR (where NCOR is nuclear receptor corepressor) and Gal-p66β (10, 29). As shown in Fig. 1, the transcriptional repression of both Gal-NCOR and Gal-p66β is partially released by TSA. More importantly, the strong repression mediated by Gal-p66α is sensitive to TSA as well. Several point mutants generated (see below) retained a TSA sensitivity except for Gal-p66αK30R and Gal-p66βK33R; both sites are required for the interaction with other NuRD components (see below).

Deletion constructs were generated at comparable sites in both p66 proteins in order to identify possible domains that mediate the difference in repression activity between p66α and p66β. Fusion of these deletion constructs to the Gal-DNA binding domain and transfection into 293 cells identified several domains that mediate gene repression (Fig. 2A and B). We simultaneously analyzed the protein expression of both p66α- and p66β-derived proteins by Western blotting using an anti-Gal antibody, demonstrating that similar amounts of the fusion proteins are expressed in all of the transfections (Fig. 2C). At least four domains can be distinguished for both proteins, the N-terminal region including CR1, a central region between CR1 and CR2, the CR2 region, and the C-terminal domain outside of CR2. Comparison of p66α with p66β reveals that the N-terminal as well as the central region mediates similar repression activity. In contrast, the CR2 region as well as the C-terminal region differs in repression, with the p66α

FIG. 2. Identification of a potent repressive domain in p66α. 293 cells were cotransfected with a 4×UAS-TK-Luc reporter together with vectors coding for the Gal-DNA binding domain or the indicated Gal-p66α/Gal-p66β fusion constructs. Cell extracts were analyzed for reporter gene activity and protein expression. (A and B) Schematic overview of the Gal-p66α/Gal-p66β deletion constructs and reporter gene activity expressed as an increase in repression (*n*-fold) as shown in Fig. 1. (C) The relative expression levels of each of the constructs were detected by Western blotting using anti-Gal4 antibodies.

domains being about fivefold stronger than the corresponding p66β domain. In summary, the major repression domains of p66α are within the C terminus including CR2 and within the N terminus including CR1. In contrast, only the N terminus in p66β shows a comparable activity to the corresponding region of p66α, whereas the C terminus of p66β is about fivefold less active compared to this region of p66α.

p66α and p66β can be SUMOylated in vivo. Several ubiquitin-like proteins have been shown to modify the function of target proteins upon covalent binding. One of these small ubiquitin-like modifiers is SUMO, which has been shown to affect many biological processes (14). We first examined p66α and p66β for the SUMOylation consensus sequence ΨKXE, where Ψ represents a large hydrophobic amino acid, most frequently isoleucine or valine, K is the SUMO acceptor site, and X represents any amino acid (30). Inspection of the amino acid sequence of p66α and p66β revealed four potential SUMO modification sites at Lys-30, Lys-149, Lys-451, and Lys-487 in the case of p66α and two potential SUMO modification sites at Lys-33 and Lys-454 in the case of p66β.

To examine whether p66α and p66β can be SUMOylated in vivo, 293T cells were cotransfected with Gal-p66α or Gal-p66β in the presence or absence of pcDNA3-FLAG-SUMO-1 and pcDNA3-HA-Ubc9. Expression of either or both proteins has been used in several cases to detect in vivo SUMOylated proteins. Characteristic for this modification is that only a fraction of the substrate is modified although the bulk of the substrate is functionally changed (14). We used Gal-fused p66 in order to be able to generate mutants that can be distinguished functionally from endogenous wild-type p66. SDS-PAGE separa-

tion and Western blotting using anti-Gal antibodies detected Gal-p66 as well as a higher band presumably corresponding to SUMO-modified p66α or p66β in the presence of FLAG-SUMO-1 and hemagglutinin (HA)-Ubc9 (Fig. 3A and B, arrow). To verify the involvement of SUMO-1, Gly-97, the essential amino acid of SUMO-1 required to conjugate to substrates (21, 36), was mutated to alanine. 293T cells were cotransfected with FLAG-SUMO1G97A and Gal-p66α or Gal-p66β in the presence of HA-Ubc9. As expected, no higher band was detected (Fig. 3A and D). These results confirm that p66α and p66β are indeed specifically conjugated by SUMO-1. To further confirm this SUMOylation pathway, we generated DN-Ubc9 by mutating Cys-93 of Ubc9, which has previously been shown to efficiently inhibit SUMO-1 conjugation (2, 25). Cotransfection of DN-Ubc9 with Gal-p66α or Gal-p66β in the presence of FLAG-SUMO-1 resulted in the loss of the higher band (Fig. 3A and B). To identify one or several target sites for SUMOylation, we mutated all SUMO consensus sites individually or in combination. In the case of p66α, the mutant forms K30R or K487R or any combination including one of these two sites reduce SUMOylation to below detectable levels (Fig. 3C). Thus, K30 and K487 are the major SUMOylation target sites within p66α. Apparently, both sites synergize in SUMOylation since mutation of a single site alone reduces SUMOylation dramatically. Analysis of the two consensus sites of p66β by testing the K33R or K451R mutants individually clearly showed that a single SUMOylation site at Lys 33 could be detected (Fig. 3D). Despite the presence of two SUMO sites in p66α and a single site in p66β, migration of the SUMOylated wild-type forms seems

to be similar, suggesting that only a single site is modified at a time.

To test SUMOylation in the absence of overexpressed components, one has to purify p66 to visualize any small SUMOylated fraction. It has been shown in many cases that only a fraction of the substrate is modified although the bulk of the substrate is functionally changed (14). To efficiently purify p66, we generated stable cell lines that stably express low amounts of FLAG-tagged p66. Several colonies such as clone 54 (FLAG-CBP), clone 29 (FLAG-p66 α -CBP), or clone 87 (FLAG-p66 β -CBP), were identified by reverse transcription-PCR (data not shown) and Western blotting (Fig. 3E). There was no significant difference in the amount of p66 (Fig. 3E, input) compared to endogenous p66 (Ctrl). Nevertheless, clones C29 and C87 clearly expressed FLAG-p66 α or FLAG-p66 β . Nuclear extracts were immunoprecipitated with the FLAG M2 gel, and the resulting elution fractions were subjected to Western blotting with anti-FLAG antibody and anti-SUMO1 antibody (Fig. 3E, IP). As shown in Fig. 3E, the FLAG antibody detected, in addition to the major FLAG-p66 protein, a small fraction with a higher molecular weight in the case of the C29 and C87 clones. By using the SUMO1 antibody, the higher-molecular-weight band was identified to rep-

represent the SUMOylated form. These results indicated that endogenous p66 α and p66 β are modified by SUMO1 *in vivo*.

SUMO modification sites of p66 α and p66 β are required for maximal repression. We next used Gal-p66 fusions in reporter gene repression assays in order to test for possible functional differences of p66 mutants that cannot be SUMOylated. We compared the transcriptional repression activity of wild-type Gal-p66 α and Gal-p66 β and various SUMOylation-deficient mutants. HEK293 cells were cotransfected with the reporter plasmid 4 $\times$ UAS-TK-Luc together with Gal-p66 fusion constructs. Expression levels of wild-type and mutant Gal-p66 α and Gal-p66 β proteins were normalized using Western blot analysis (Fig. 4A and B). The results show that all SUMOylation-deficient mutants, Gal-p66 α K30R, Gal-p66 α K487R, or Gal-p66 β K33R, are severely impaired in their transcriptional repression activity. In addition, Gal-p66 α K149R, which has no effect on SUMOylation (see above) but, rather, abolishes binding to MBD2 (7), reduces repression. We went on to test double mutants, triple mutants, and the quadruple mutants. In general, any combination of individual mutations that impair SUMOylation also shows a strong loss of repression. This is dramatically demonstrated by the quadruple mutation of p66 α that results in a 20-fold reduction of repression compared to

FIG. 4. Loss of SUMO modification impairs p66α- and p66β-mediated repression. 293 cells were cotransfected with a 4×UAS-TK-Luc reporter together with vectors coding for Gal constructs. Shown is the transcriptional repression of the indicated Gal-p66α variants (A) and of the indicated Gal-p66β variants (B). The increase in repression (*n*-fold) was determined relative to the Gal-DNA binding domain. Error bars represent variations within duplicate transfections. Anti-Gal4 Western blotting shows expression levels of wild-type Gal-p66α/Gal-p66β or mutant forms. (C) Transcriptional repression of GAL-fusions after coexpression FLAG-SUMO1, FLAG-SUMO1G97A, HA-Ubc9, or HA-DN-Ubc9.

wild-type p66α (Fig. 4A). This mutant cannot be SUMOylated (sites K30 and K487 are mutated), nor can it bind to the Mi-2/NuRD component MBD2 (data not shown). In contrast, there was almost no difference in transcriptional repression activity in the case of Gal-p66αK451R or Gal-p66βK454R, the wild-type sites of which are not SUMOylated (see above). These results indicate that SUMOylation influences transcriptional repression of p66α and p66β. To further confirm the effect of SUMOylation on repression, we utilized the SUMO mutant FLAG-SUMO1G97A as well as the dominant negative mutation of the E2 ligase HA-DN-Ubc9 in the transcriptional repression assay (Fig. 4C). Transcriptional repression mediated by either p66α or by p66β can be increased by the wild-type FLAG-SUMO1 and Ubc9, and this repression can be relieved by about twofold by the FLAG-SUMO1 mutant and the Ubc9 mutant. Thus, mutation of the two SUMOylation sites of p66α and the single site of p66β and expression of defective SUMO and Ubc9 impair the repression activity of both p66 factors.

SUMO modification sites of p66α and p66β are required for specific Mi-2/NuRD complex interactions. It has been shown that SUMO modification can regulate the subcellular localization of target proteins. To test whether SUMO modification affects nuclear localization of p66α and p66β, we compared the subcellular localization of wild-type pEGFP-p66α and pEGFP-p66β to the localization of the corresponding SUMOylation-defective forms pEGFP-p66αK30R, pEGFP-p66αK487R, and pEGFP-p66βK33R as detected by fluorescence microscopy. All mutant forms of pEGFP-p66α and pEGFP-p66β showed a speckled nuclear pattern similar to that observed with wild-type pEGFP-p66α or pEGFP-p66β (Fig. 5). These results suggest that SUMO modification or loss of modification of p66α and p66β does not lead to a change in the subcellular or subnuclear localization. Another mechanistic role for SUMO modification of p66 might be in the protein-protein interaction within the Mi-2/NuRD complex. Therefore, we tested four of

the known components of the Mi-2/NuRD complex in their binding to p66α or p66β and looked for a possible effect of the SUMO-defective p66 mutants.

MBD3 has previously been shown to be part of the Mi-2/NuRD complex (10, 15, 24) and to interact with p66α and p66β in vitro (6). We cotransfected vectors expressing pCMV-GST or pCMV-GST-MBD3 together with Gal, Gal-p66α, Gal-p66β or the corresponding point mutant forms in 293T cells. After nuclear extract preparation, the input fraction and purified

FIG. 5. Subnuclear distribution of GFP-p66α and GFP-p66β is not changed in the SUMOylation mutants. NIH 3T3 cells were transfected with the indicated EGFP-fused hp66 proteins. Phase-contrast (left column) and fluorescent images were taken 24 to 48 h posttransfection after incubation with Hoechst DNA stain (center column). Mutant forms of pEGFP-p66α and pEGFP-p66β revealed similar nuclear speckle patterns as observed with wild-type pEGFP-p66α and pEGFP-p66β (right column). EGFP control expression resulted in a diffuse whole-cell distribution.

FIG. 6. Mutation of the N-terminal SUMO modification site inhibits HDAC1 binding to p66α but not to p66β in vivo, whereas MBD3 binding is not affected. HEK293 cells were harvested 48 h after transfection with various combinations of DNA constructs as indicated above the figure. Nuclear protein fractions were prepared and purified using glutathione Sepharose beads. Bound proteins (purified) were analyzed by Western blotting using an anti-Gal antibody compared to input, which was analyzed with the anti-GST antibody as well. (A and B) Purification of GST-MBD3 and bound proteins. (C and D) Purification of GST-HDAC1 and bound proteins.

GST-bound proteins were analyzed by Western blotting using an anti-Gal antibody. We observed that the GST-MBD3 containing sample retained Gal-p66α, Gal-p66β, and all single mutant forms (Fig. 6A and B). The results indicate that mutation of the SUMO modification sites does not influence MBD3 binding to either p66α or p66β.

Previous results showed that HDAC1 is a component of the Mi-2/NuRD complex (24, 45). This is in agreement with functional results (see above) that p66α-mediated transcriptional repression is at least partially due to histone deacetylation. Therefore, we expressed the vectors pCMV-GST or pCMV-GST-HDAC1 together with Gal, Gal-p66α, Gal-p66β, or their respective mutants (Fig. 6C and D). Again, we observed that GST-HDAC1 retained wild-type Gal-p66α, Gal-p66β, and most of the mutant forms with one exception: Gal-p66αK30R, which destroys the major SUMO modification site of p66α (see above) and which is insensitive to TSA treatment (Fig. 1), is not bound to GST-HDAC1. The results indicate that HDAC1 binding to p66α and TSA sensitivity of repression require an intact SUMO modification site at K30 of p66α. In contrast, mutation of a paralogous site in p66β, K33R, does not interfere with HDAC1 binding (Fig. 6D). Nevertheless, this mutant is resistant to TSA, indicating that other components of HDAC complexes are bound at this site (see below).

Other components of the Mi-2/NuRD complex, the RbAp46 and RbAp48 proteins, have been shown to function as histone escort proteins in various histone-related complexes (26). We

tested the binding of these factors to p66α or p66β and to the respective mutant forms. Both RbAp proteins are found to bind to both p66 proteins (Fig. 7). All of the p66α and p66β mutant forms showed RbAp48 binding similar to the wild-type p66 proteins (Fig. 7A and B). In contrast, the SUMOylation mutant p66βK33R was clearly impaired in binding to RbAp46. The p66α mutants did not interfere with RbAp46 binding. Thus, the related RbAp46/48 proteins and the related p66α/p66β proteins are highly specific in protein-protein interaction. Within these four combinations, only the interaction between RbAp46 with p66β requires the intact SUMOylation site of p66β.

DISCUSSION

p66α and p66β are two closely related proteins with similarly conserved regions CR1 and CR2. The sequence similarity as well as their ubiquitous expression suggests that they have similar properties and exert similar functions (6). In this report we asked whether both p66 proteins are functionally equivalent or whether each may have unique molecular features. Here we show that the transcriptional repression of p66α is much stronger than that of p66β. In addition, we demonstrate that p66α contains two major repression domains, whereas in p66β only the N-terminal domain is functionally equivalent to p66α (Fig. 8). Since p66 has been shown to be part of the NuRD complex, which harbors HDAC1 and HDAC2 (10, 24),

FIG. 7. Mutation of the N-terminal SUMO modification site inhibits RbAp46 binding to p66β but not to p66α in vivo, whereas RbAp48 binding is not influenced. HEK293 cells were harvested 48 h after transfection with various combinations of DNA constructs, as indicated above the figure. Nuclear extracts were prepared, and GST-RbAp48 (A and B) or GST-RbAp46 (C and D) was purified using glutathione Sepharose beads. Bound proteins (purified) were analyzed by Western blotting using an anti-Gal antibody compared to input, which was analyzed with the anti-GST antibody as well.

we tested p66-mediated repression in the presence of TSA. As expected, inhibition of histone deacetylase by TSA relieved the repression. In comparing p66α with p66β, no qualitative difference in TSA sensitivity was observed.

Another mechanism to control repression is modification by SUMO. Emerging evidence indicates that SUMOylation negatively regulates the transcriptional activity of several tran-

scriptional factors (3, 28, 31, 33, 43, 44). Here we show that both p66α and p66β can be SUMOylated in vivo. Blocking the SUMO pathway with SUMO1G97A or with DN-Ubc9 prevents generation of SUMOylated p66α and p66β and impairs repression. Similarly, mutational analysis of the SUMO modification sites in p66α and p66β resulted in a loss of SUMO modification sites as well as a reduction of repression by p66α and p66β. Detailed inspection and mutation analysis revealed another difference between p66α and p66β. For p66α we identified two SUMOylated sites (K30 and K487). Mutation of either site reduced SUMOylation of p66α to undetectable levels. This suggests that both sites synergize in SUMO modification. In contrast, the p66β factor, with only one major repression domain, harbors a single SUMOylation site (K33).

SUMOylation of transcription factors has been reported to have different effects on transcriptional activity in diverse pathways. In some cases a SUMOylation consensus motif is located within an inhibitory or repressive domain. For instance, Sp3 and Elk-1 contain an inhibitory domain responsible for the repressive function of the transcription factor (34, 44). Mutation of the critical lysine dramatically enhances the transcriptional activity of the target protein, indicating that SUMOylation is indeed implicated in repression function. Two models have been presented that might explain the repression function of SUMO. One model proposes that SUMOylated transcription factors are targeted to specific subnuclear domains, as

FIG. 8. The major repression domains, relevant amino acid positions, and SUMOylation sites of p66α and p66β. SUMOylation sites affecting interaction with HDAC1 and RbAp46, as well as the CR1 and CR2 regions that are conserved between different species (6, 10), are also indicated.

documented for the PML nuclear body. PML itself and several other components of the PML bodies are SUMOylated (14). SUMO conjugation is required for the formation of these nuclear bodies (16, 23, 46). SUMOylation or inhibition of SUMO conjugation results in a redistribution of nuclear components within the nucleus. This type of SUMO function does not seem to play a role in the context of p66. p66 colocalizes with MBD2 and MBD3 in nuclear speckles (6, 10) that are clearly different from PML bodies and that are found at replication foci (38). Furthermore, mutation of the SUMO modification sites in p66 α or p66 β does not lead to a redistribution in comparison to wild-type p66 (Fig. 5).

Another model for SUMO function is exemplified by p300 and by Elk-1. In both cases SUMOylation allows HDAC binding and mediates repression by histone deacetylation (11, 43). This is reminiscent of the situation we find with p66. Mutation of the SUMO modification site (K30) abolishes the interaction between HDAC1 and p66 α and leads to a loss of TSA sensitivity. This suggests that SUMO modification at this site is required for the interaction. In the case of p66 β , we observed that mutation of the N-terminal SUMO modification site (p66 β K33) abolishes the interaction between p66 β and RbAp46, which is also a component of the Mi-2/NuRD complex. This mutation led to TSA insensitivity, probably caused by the loss of RbAp46 binding which itself may be bound to HDACs. Again, this was specific for p66 β and not found for p66 α .

These results lead to a model in which certain interactions within the many interactions of a multiprotein complex, such as the Mi-2/NuRD complex, are favored by SUMOylation. The functional integrity of the established complex is probably not affected by SUMOylation because of the many other interactions within such a complex. Rather, one might envisage that SUMOylation may only be required during the transient phase of complex formation. Such a phase may rely on initial dual protein interactions that are subsequently replaced by multiple interactions that generate large interaction surfaces within the multiprotein complex later on during complex formation. The beauty of this model is that it could explain the "SUMO enigma" (14). This enigma is described by the fact that in most systems analyzed the proportion of a particular protein found to be SUMOylated is rather small. If SUMOylation is indeed only required during a transient phase of complex formation, the amount of protein that needs to be modified at any given time point would then only be a small fraction of the total amount of this protein present in the cell.

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