Role for SUMO Modification in Facilitating Transcriptional Repression by BKLF†

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Small ubiquitin-like modifier (SUMO) is a protein moiety that is ligated to lysine residues on a variety of target proteins. Many known SUMO substrates are transcription factors or coregulators of transcription, and in most cases, modification with SUMO leads to the attenuation of transcriptional activation. We have examined basic Krüppel-like factor/Krüppel-like factor 3 (BKLF), a zinc finger transcription factor that is known to function as a potent transcriptional repressor. We show that BKLF recruits the E2 SUMO-conjugating enzyme Ubc9 and can be modified by the addition of SUMO-1 in vitro and in vivo. The SUMO E3 ligases PIAS1, PIASy, PIASx α , and PIASx β but not Pc2 enhance the sumoylation of BKLF. Site-directed mutagenesis identified two lysines (K10 and K197) of BKLF as the sumoylation sites. Sumoylation does not detectably affect DNA binding by BKLF, but mutation of the sumoylation sites reduces transcriptional repression activity. Most interestingly, when mutations preventing sumoylation are combined with an additional mutation that eliminates contact with the C-terminal binding protein (CtBP) corepressor, BKLF becomes an activator of transcription. These results link SUMO modification to transcriptional repression and demonstrate that both recruitment of CtBP and sumoylation are required for full repression by BKLF.

The covalent attachment of ubiquitin-like proteins to their substrates represents an unusual posttranslational modification in that the modifier itself is a small polypeptide of around 100 amino acids (48). Ubiquitin, the founding member of the family, is well known as a modifier that directs proteins to the proteasome. Ubiquitin is also involved in other cellular processes, including the regulation of intracellular transport and gene activation (33, 67). Small ubiquitin-like modifier (SUMO) has been extensively studied recently. The enzymatic reactions involved in SUMO modification are analogous to those seen in ubiquitin modification and entail an E1-activating enzyme, consisting of an Aos1/Uba2 (SAE1/SAE2) heterodimer, the E2-conjugating enzyme Ubc9, and an E3 ligase that promotes the transfer of SUMO from the E2 enzyme to substrate proteins (29, 32). Although E1 and E2 enzymes are typically sufficient to support sumoylation in vitro, it appears that in vivo E3 ligases also play a part in the process. Thus far, the protein inhibitors of activated STATs (PIAS), the PIAS-like protein Zimp10, the polycomb protein Pc2, and the nuclear pore component RanBP2 have been identified as E3 ligases (16, 18, 19, 24, 38, 43, 52). Sumoylation is a reversible and dynamic process, and several SUMO proteases have also been described previously (30).

The functional consequences of SUMO attachment differ from substrate to substrate and in many cases are not under-

stood at the molecular level. To date, sumoylation has been reported to affect diverse cellular processes such as nuclear transport, maintenance of genome integrity, DNA repair, enzymatic activity, mitochondrial fission, signal transduction, and transcriptional regulation (11, 12, 39, 49, 50, 65, 66).

Remarkably, over half of the presently identified SUMO substrates are transcription factors or coregulators of transcription, and in most cases, modification with SUMO leads to the attenuation of transcriptional activation (49, 66). Thus, mutation of the sumoylation sites and thereby elimination of sumoylation of Sp3, p300, Elk-1, c-Jun, c-Myb, C/EBP, AP2, and diverse nuclear receptors enables them to become more potent activators (1, 2, 8, 10, 20, 31, 34, 40, 41, 46, 58, 61, 66, 68). Interestingly, the so-called synergy control motif that limits the transcriptional synergy of many transcription factors is essentially identical to the SUMO consensus sequence, further suggesting that SUMO conjugation is mechanistically involved in transcriptional attenuation (14, 15). Precisely how sumoylation causes the attenuation of activation is not yet understood, but SUMO modification has been shown to target transcription factors into repressive subnuclear structures and PML bodies and also to promote the recruitment of histone deacetylases (10, 43, 69). It is also likely that SUMO itself could act as a repressor when directed to certain promoters (14, 41, 68). Furthermore, a recent study indicated that sumoylation of histone H4 also correlates with transcriptional repression and facilitates recruitment of histone deacetylase 1 (HDAC1) and HP1 (54).

In addition to its role in limiting the activity of transactivation domains, the sumoylation of transcriptional repressors might also be required for their silencing activity (66). A number of transcriptional corepressors, such as the histone deacetylases HDAC1, HDAC4, HDAC6, and HDAC9 and the corepressor C-terminal binding protein (CtBP), have been shown to be subject to sumoylation (5, 22, 26, 36). We have

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now examined the transcriptional repressor basic Krüppel-like factor/Krüppel-like factor 3 (BKLF/KLF3) and tested the effect of sumoylation on its ability to repress target promoters. BKLF belongs to the mammalian Sp/Krüppel-like factor family, of which there are presently 24 members (Sp1 to Sp8 and KLF1 to KLF16). KLF proteins are characterized by a distinctive DNA-binding domain at the C terminus of the protein that consists of three Krüppel-like C₂H₂ zinc fingers. Outside this domain there is little homology among the known KLF proteins (17, 37).

BKLF is abundant in erythroid cells, and it has been shown to function as a strong transcriptional repressor on several target promoters (4, 62). The repression domain of BKLF has been mapped to the N-terminal region and was found to associate with the transcriptional corepressor CtBP through the short CtBP interaction motif PXDLT (62, 63). Disruption of the BKLF-CtBP interaction leads to a significant reduction of the repression potential of BKLF in cellular assays. Importantly, the elimination of the BKLF-CtBP interaction does not completely abolish the repressive properties of BKLF, suggesting that BKLF may recruit additional cofactors to regulate transcription (62).

Here we report that BKLF also interacts with the E2-conjugating enzyme Ubc9 and is consequently a target for SUMO modification. We provide evidence that PIAS family members, but not the Polycomb protein Pc2, function as E3 SUMO ligases for BKLF in vitro and in vivo. Site-directed mutagenesis and deletion analysis identified two lysines, K10 and K197, as the sumoylation sites. Mutation of these residues compromises the repression activity of BKLF. More importantly, mutations of both the sumoylation sites and the CtBP binding motif in BKLF switch it from a strong repressor to an activator of transcription. These results show that SUMO modification and CtBP recruitment act in synergy to repress transcription and link SUMO modification to transcriptional repression by BKLF.

MATERIALS AND METHODS

Plasmid constructs. For in vitro transcription and translation, the BKLF wild type and the mutant constructs K10A, K197A, and K10A/K197A were introduced by ligating EcoRI/SalI PCR fragments into EcoRI/XhoI sites of pcDNA3 plasmid (Invitrogen). Detailed primer information is available upon request. For mammalian cell expression, the previous BKLF constructs, mutations K68A, K83A, K104A/K105A, K189A, E199A, K194A/K195A, K194R/K195R, K242A, and K255A, and deletion mutants 14-344, 29-344, 60-344, 90-344, 120-344, 150-344, 180-344, 1-315, 1-300, 1-285, 1-268, 1-240, 1-268-K10A, 1-268-K197A, 1-268-E199A, and 1-268-K10A/K197A were constructed by ligating SalI/EcoRI PCR fragments into the same sites of pMT3 vector (derived from pMT2). For expression in *Drosophila* SL2 cells, the BKLF wild type and K10A, K197A, K10A/K197A, E199A, E12A/E199A, Δ DL, Δ DL-K10R, Δ DL-K197A, Δ DL-K10R/K197A, Δ DL-E12A, Δ DL-E199A, and Δ DL-E12A/E199A were cloned into the vector pPacU (3). pPacU was a gift from G. Suske (Institut für Molekularbiologie und Tumorforschung, Marburg, Germany).

SL2 cell reporter plasmids contained either three copies of a composite CACCC-glucocorticoid response element (GRE) site (tgctAGAACAATcTGTA CAgagagagCCACACCActgtg) (57) upstream of the minimal *Adh* promoter and the chloramphenicol acetyltransferase (CAT) gene in p1970 (9) or a section of the human γ -globin promoter from -383 to +48 (431 bp) inserted upstream of the CAT gene in p1970. pPac-GR has been described previously (62). pGEX-2T-SUMO-1 (7), pcDNA3-HA-SUMO-1, pcDNA3-6xHis-SUMO-1 (6), and pGEX-4T-SAE2/SAE1 (60) were kindly provided by R. Hay (University of St. Andrews, Fife, United Kingdom).

pCMV5-Myc-PIAS1, pCMV5-Myc-Miz1, and pCMV5-HA-PIASy (313-508) have been described previously (42) and were kindly provided by S. H. Lin (Hong

Kong University of Science and Technology, Hong Kong, China). pGEX-2T-SUMO-1, pGEX-2T-SUMO-2, pEGFP-C1-SUMO-1, pEGFP-C1-SUMO-2, and pEGFP-C1-SUMO-3 have been described previously (44) and were kindly provided by H. Saitoh (The Picower Institute for Medical Research, New York, N.Y.). pGEX-4T3-ARIP3 (23) was a gift from J. Palvimo (University of Helsinki, Helsinki, Finland). pCMV5-Flag-Pc2 and pcDNA3-Pc2 were kindly provided by D. Wotton (Department of Biochemistry and Molecular Genetics, Center for Cell Signaling, University of Virginia, Charlottesville, Va.) and A. P. Otte (E. C. Slater Instituut, BioCentrum Amsterdam, University of Amsterdam, Amsterdam, The Netherlands), respectively, and have been described previously (18, 51).

pcDNA3-ARIP3 was generated by digesting pGEX-4T3-ARIP3 with EcoRI and ligating into pcDNA3. pcDNA3-HA-Miz1, pcDNA3-HA-PIASy (313-508), pGEX-2T-PIAS1, and pGEX-2T-Miz1 were generated by PCR amplification of PIAS1 cDNA (forward primer, 5'-CGGGATCCATGGCGGACAGCGCGGA ACT-3'; reverse primer, 5'-GGCGAATTCTCAGTCCAATGAGATAATGTC TG-3'), Miz1 cDNA (forward primer, 5'-CGGGATCCATGCAGCAGCCGTC GCCG-3'; reverse primer, 5'-GGCGAATTCTTAGTCCAAAGAGATGATG TCA), and PIASy (313-508) cDNA (forward primer, 5'-CGGGATCCATGCG AGTGTCCCTCATCTGCCCA-3'; reverse primer, 5'-GCGAATTCTCAGCAC GCGGGCACCAGGC-3') from the vectors pCMV5-Myc-PIAS1, pCMV5-Myc-Miz1, and pCMV5-HA-PIASy (313-508), respectively, and subsequently ligated into BamHI and EcoRI sites in the vectors pcDNA3-HA and pGEX-2T.

pGBT9-BKLF (1-268) has been described previously (62). NpGBT9-SUMO-1 was generated by digesting pcDNA-6xHis-SUMO-1 with BamHI and ligating in frame into NpGBT9 (Clontech) (derived from pGBT9). pGAD10-mUbc9 was isolated from the yeast two-hybrid screening. NpGEX-2T-mUbc9 was generated by excising the mUbc9 cDNA from pGAD10-mUbc9 with EcoRI and ligating into NpGEX-2T (derived from pGEX-2T). pMT3-HA-SUMO-1 was constructed by excising HA-SUMO1 cDNA with HindIII and XhoI from pcDNA3-HA-SUMO-1, subcloning into pBlueScript-SK (HindIII and XhoI), excising again with PstI and XhoI, and ligating into pMT3.

Protein purification. Glutathione S-transferase (GST) fusion proteins were produced in *Escherichia coli* (BL-21). Bacteria were resuspended in cold lysis buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40 supplemented with 0.1 mg of lysozyme/ml, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μ g of aprotinin/ml, and 5 μ g of leupeptin/ml) and sonicated to disrupt the cells. The lysates were centrifuged at 50,000 $\times$ g and 4°C for 30 min to collect soluble proteins. The lysates were incubated with glutathione Sepharose 4B beads (Pharmacia) for 30 min at 4°C, and the beads were pelleted and washed four times with 10 volumes of lysis buffer at 4°C. Bound proteins were eluted in elution buffer (100 mM Tris-Cl [pH 7.5], 120 mM NaCl, 20 mM reduced glutathione [Boehringer]). The supernatants containing the fusion proteins of interest were stored at -80°C.

In vitro transcription and translation. In vitro transcription and translation of proteins has been described previously (35).

In vitro sumoylation assay. Sumoylation reactions were performed in a total volume of 15 μ l containing 3 μ l of ³⁵S-labeled protein from in vitro transcription and translation reactions, 500 ng of E1 (SAE1/SAE2), 500 ng of E2 (Ubc9), and 1 μ g of SUMO-1 (unless otherwise stated) in sumoylation buffer (50 mM Tris-Cl [pH 7.6], 1 mM dithiothreitol, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3.5 U of creatine kinase [Sigma]/ml, 0.6 U of inorganic pyrophosphatase/ml). Reactions were incubated for 60 min at 30°C and stopped by adding sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiling for 5 min. Gels were dried, and the images were taken using a Typhoon PhosphorImager (Molecular Dynamics).

Yeast two-hybrid screen. The yeast two-hybrid screen has been described previously (62). Candidate interactors were retested by cotransfecting NpGBT9-SUMO-1 or pGBT9-mBKLF (1-268) with pGAD10-mUbc9 into the yeast strain HF7c, and transformants were selected on Trp/Leu-deficient media (-Leu -Trp). Colonies were patched onto Trp/Leu/His-deficient media (-Leu -Trp -His), and growth after 60 h at 30°C was measured.

Electrophoretic mobility shift assay. Gel retardation experiments were carried out using oligonucleotides labeled with [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs). A total of 4 μ l of nuclear extracts and labeled probes (10,000 cpm) was incubated in buffer [10 mM HEPES (pH 7.8), 50 mM potassium glutamate, 5 mM MgCl₂, 1 mM EDTA, 0.5 mM dithiothreitol, 1 μ g of poly(dI-dC), 1 μ g of bovine serum albumin, 1 mM ZnSO₄, 5% glycerol] for 20 min on ice. Then samples were subjected to electrophoresis at 4°C for 2.5 h at 12 V/cm on a native polyacrylamide gel (6% [19:1] bis:acrylamide in 0.5 $\times$ Tris-borate-EDTA).

Transfections, Western blotting, and CAT assays. COS cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum

and penicillin, streptomycin, and glutamine. Cells were transfected with 500 ng of pMT3-BKLF constructs and various amounts of vectors encoding HA-SUMO-1 or green fluorescent protein (GFP)-SUMO-1 by use of the transfection reagent FuGEN6 (Roche Diagnostics) following the manufacturer's instructions. *Drosophila melanogaster* Schneider line 2 (SL-2) cells were grown at 25°C in Schneider medium (Gibco) supplemented with 10% fetal calf serum and antibiotics. SL2 cells were transfected by the calcium phosphate method (45) with 500 ng of pPac-GR, 500 ng of p1970(GRE-CACC)₃-CAT, and 50 ng of pPacU BKLF constructs. In experiments using the human glucocorticoid receptor (GR), dexamethasone was added to achieve a final concentration of 10^{-8} M 20 h after transfection. Cells were harvested 48 h after transfection. To measure the activity of chloramphenicol acetyltransferase (CAT), the method of Sleigh (55) was used.

For nuclear extracts, transfected cells from a 10-cm-diameter petri dish were washed with cold NaCl/Pi and resuspended in 400 μ l of cold solution A (10 mM HEPES [pH 7.8], 1.5 mM MgCl₂, 10 mM KCl) supplemented before use with 1 mM dithiothreitol, 50 ng of phenylmethanesulfonyl fluoride/ml, 5 μ g of leupeptin/ml, 5 μ g of aprotinin/ml, and 25 mM *N*-ethylmaleimide. The tubes were incubated on ice for 10 min, vortex mixed for 10 s, and centrifuged for 10 s at $12,000 \times g$ to pellet the nuclei. The nuclei were resuspended in 30 to 50 μ l of solution C (20 mM HEPES [pH 7.8], 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA) supplemented as described above, incubated on ice for 20 min, and centrifuged for 3 min at $12,000 \times g$ at 4°C. The extracts were used immediately or stored at -70°C. Proteins were separated by SDS-PAGE on 10 to 12% polyacrylamide gels and transferred onto a Biotrace nitrocellulose blotting membrane (Pall Gelman Sciences, Ann Arbor, Mich.) in a TE series Transphor electrophoresis unit (Hoefer) at 50 mA overnight at 4°C.

For Western blotting, the membrane was washed once in 50 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 0.05% Tween 20 (Tris-NaCl-Tween) and then incubated at room temperature in skimmed milk powder solution (5% [wt/vol] in Tris-NaCl-Tween) for 1 h. The membrane was rinsed in Tris-NaCl-Tween and incubated for 1 h with gentle shaking in 10 ml of Tris-NaCl-Tween containing 10 μ g of primary antibody. After five washes with 150 ml of Tris-NaCl-Tween, the secondary antibody solution was added and incubation was continued for 1 h. The membrane was washed for 1 h in several changes of Tris-NaCl-Tween. Detection was carried out using Renaissance Chemiluminescence Reagent Plus (NEN Life Sciences, Boston, Mass.), and the signal was detected on X-ray film (Eastman Kodak Company, Rochester, N.Y.) and developed using Kodak reagents. The anti-BKLF antibody has been described previously (4). Anti-SUMO-1 monoclonal antibody was provided by Zymed Laboratories Inc., San Francisco, Calif., and anti-HA monoclonal antibody (12CA5) was provided by Roche Corporation, Mannheim, Germany.

RESULTS

BKLF is modified by SUMO. We have previously shown that BKLF represses transcription in part by associating with corepressors of the CtBP family (64). There are two highly related CtBP proteins in mammals, CtBP1 and CtBP2, but in this work we have concentrated on CtBP2. We found earlier that BKLF retains some repression activity even when its CtBP binding site is mutated, suggesting that it associates with additional corepressors (62). To identify such proteins, we performed a yeast two-hybrid screening with BKLF and isolated the SUMO E2-conjugating enzyme Ubc9 (Fig. 1A). Interestingly, we found that Ubc9 also interacts with CtBP2 (data not shown). CtBP1 and CtBP2 have been recently shown to be modified by sumoylation (18, 26), consistent with the result that Ubc9 binds CtBP2 and suggesting that Ubc9 might functionally cooperate with BKLF and CtBP2 to effect gene repression. We confirmed that CtBP2 is sumoylated (data not shown) and set out to investigate whether BKLF was similarly modified by sumoylation.

We first tested whether sumoylation could occur in vitro. ³⁵S-labeled in vitro-transcribed and -translated BKLF was incubated in the presence of purified, bacterially expressed GST-SUMO-1, E1 (SAE1/SAE2), and E2 (Ubc9) enzymes in a buffer containing an ATP-regenerating system. As seen in Fig.

1B, additional bands of slower mobility were observed only when all proteins required for sumoylation were present, providing the first evidence that BKLF was a substrate for sumoylation. The precise nature of the modification (for instance, the number of SUMO moieties added) was difficult to deduce from this experiment as multiple bands were present, some presumably representing BKLF carrying one, two, or possibly more SUMO-1 moieties and others arising from breakdown products of these or products generated by the sumoylation of non-full-length BKLF produced during the in vitro transcription and translation process.

To clarify the situation and to establish whether BKLF was sumoylated in vivo, COS cells were transfected with an expression vector encoding BKLF in the presence or absence of an HA-tagged SUMO-1 expression plasmid. Nuclear extracts were prepared, separated on an SDS gel, and subjected to Western blotting with an anti-BKLF antibody. The characteristic doublet at ~46 kDa corresponding to BKLF can be observed in Fig. 1C, lane 3, upper panel. In nuclear extracts from cells expressing BKLF and HA-SUMO-1, at least three additional bands migrating at ~66, ~90, and ~110 kDa were detected with anti-BKLF serum (Fig. 1C, lane 4). When the membrane was stripped and reprobed with an anti-HA antibody, three bands corresponding to those detected with the BKLF antibody were again observed (Fig. 1C, bottom panel), thus confirming that these species of higher molecular mass were due to the addition of HA-SUMO-1 to BKLF.

K10 and K197 are the only sumoylation sites in BKLF. Although the pattern of new bands generated when BKLF is subjected to sumoylation is complex, it can be seen that three prominent new bands that migrate more slowly than unmodified BKLF are evident both in vivo and in vitro (Fig. 1B and C). One possibility was that these bands corresponded to the addition of one, two, and three SUMO-1 moieties. But, as explained below, this was not the case. In fact, the fastest-migrating new band represents modification at K10, the next-most-prominent band represents modification at K197, and the slowest-migrating band represents modification at both residues.

The amino acid sequence of BKLF contains K10 and K197, two lysines that fall within the sumoylation consensus motif Ψ KXE (Fig. 2A). To ascertain whether the lysines (K10 and K197) are in fact subject to sumoylation, ³⁵S-labeled constructs containing lysine-to-alanine mutations at position 10 or 197 or both were used for in vitro sumoylation assays. The K10A mutation resulted in a reduction in sumoylation (Fig. 2B, lane 4). When the K197A mutant was analyzed, a more significant reduction in sumoylation was observed (Fig. 2B, lane 6) and only a single sumoylated form of BKLF, presumably due to sumoylation of lysine 10, was observed. Mutation of both lysines was sufficient to abolish sumoylation completely (Fig. 2B, lane 8).

These observations were corroborated in a cellular context. In this experiment the results were clearer, as truncation products resulting from incomplete transcription or translation and the multiply sumoylated forms produced in vitro were not apparent. Equivalent mutant constructs were cotransfected with HA-SUMO-1 into COS cells, and the proteins were analyzed by Western blotting using anti-BKLF or anti-HA antibodies. The results obtained (Fig. 2C) confirm that both K10

FIG. 1. BKLf interacts with Ubc9 and is modified by SUMO-1 in vitro and in vivo. (A) mBKLf interacts with mUbc9. The results of yeast two-hybrid assays using mUbc9 as prey are shown. The interaction of mUbc9 with hSUMO-1 (positive control) and with mBKLf (1–268) is indicated by growth on –Leu –Trp –His plates. Control cells were transfected with empty bait vector. (B) In vitro sumoylation of BKLf. [³⁵S]-methionine-labeled BKLf was expressed in rabbit reticulocyte lysate (lane 1), and SUMO modification was assayed in the presence or absence of purified GST-E1 (SAE1/SAE2), GST-E2 (Ubc9), GST-SUMO-1, or GST alone as indicated. Apparent molecular mass markers (in kilodaltons), full-length BKLf (double arrow), and BKLf-SUMO-1 conjugates (thick arrows) are indicated. Protein bands marked with asterisks appear to correspond to SUMO-modified forms of BKLf translation products of lower molecular mass produced in the in vitro reaction. (C) In vivo sumoylation of BKLf. COS-1 cells were transfected with constructs encoding full-length BKLf and HA-tagged SUMO-1 (500 ng). At 48 h posttransfection, nuclear extracts were prepared in the presence of *N*-ethylmaleimide (NEM) to inhibit desumoylating enzymes. Proteins were separated on SDS–12% polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were subsequently incubated with BKLf-specific (αBKLf, top panel) or HA (αHA, bottom panel) antibodies as indicated. Thick arrows point to the covalently modified wild-type BKLf protein. The asterisk depicts an endogenous sumoylated protein(s) (possibly RanBP2/Nup358) that was detectable with the HA antiserum but not with the BKLf antibody.

and K197 are targets of SUMO-1 modification in vivo. Importantly, site-directed mutagenesis of the two consensus lysines (and of E199, which disrupts the consensus and abolishes sumoylation at K197) (Fig. 2C, lane 5) clearly indicates that the fastest-migrating sumoylated form of BKLf (running at ~66 kDa) corresponds to modification of K10, the next-fastest-migrating form (running at ~90 kDa) carries a SUMO-1 group at K197, and the slowest-migrating form at ~110 kDa corresponds to BKLf modified at both K10 and K197 (Fig. 2C). The relative intensities of the three bands suggest that K197 is the major site of sumoylation, with a lower proportion of BKLf being modified on K10 alone or being sumoylated on both K10 and K197.

The site of modification by SUMO-1 influences the migration of the protein on SDS-PAGE. The observation that the form of BKLf modified at K197 migrates significantly more slowly than the K10-modified form was perplexing. One pos-

sibility was that a chain of two SUMO-1 residues linked by an isopeptide bond was added to K197, but there is little evidence that polysumoylation occurs with SUMO-1 (although it is possible with SUMO-2 and SUMO-3 additions). Another possibility was that modification at K197 unmasked a new site for sumoylation and that the slow-migrating band thus represented a form of BKLf carrying SUMO-1 groups at K197 and at another unknown lysine residue. To exclude this possibility, we systematically carried out a lysine replacement strategy across the entire molecule but found no evidence for any additional site of sumoylation (see Fig. S1 and S2 in the supplemental material).

Our results are most consistent with the interpretation that a single SUMO-1 residue is added at only two sites, K10 and K197, but that the K197-sumoylated form migrates more slowly than the K10-sumoylated species (the K10-sumoylated species runs at the expected molecular weight of ~66 kDa,

FIG. 2. Identification of amino acids essential for SUMO-1 modification of BKLF. (A) Schematic representation of wild-type BKLF protein and the BKLF mutants used in this study. The black box indicates the repression domain of BKLF, the grey box indicates the PXDLT motif that recruits the corepressor mCtBP2, and three black arcs represent the zinc fingers of the DNA-binding domain of BKLF. K₁₀ and K were mutated to 197 or alanine (A) or arginine (R). E199 was mutated to alanine. The mutant proteins were subjected to in vitro and in vivo SUMO modification. Only results obtained with lysine-to-alanine mutations are shown. (B) Mouse BKLF is sumoylated on residues 10 and 197. In vitro sumoylation was performed as described for Fig. 1B. Thick arrows indicate the major SUMO-1-modified forms of BKLF. (C) K197 is the major SUMO-1 acceptor site in BKLF. COS cells were cotransfected with the indicated BKLF mutants and a plasmid expressing HA-SUMO-1 (500 ng). Nuclear extractions and Western blotting were performed as in Fig. 1C. Thick arrows indicate the three SUMO-1-modified forms of BKLF.

whereas the K197-sumoylated form migrates more slowly, with an apparent weight of ~90 kDa). One simple explanation for this might be that modification at K197 near the middle of the protein (BKLF comprises 344 residues) creates a branched protein that migrates more slowly than the K10-modified form, which would be essentially linear, as K10 is very near the N terminus.

To investigate this possibility further, deletion analysis was carried out to, in effect, move the K197 site towards a terminus and see whether the migration rate of the K197-modified form approached that of the K10-modified form (see Fig. S1 and S2 in the supplemental material). Deleting N- or C-terminal regions did indeed cause the K197-sumoylated form of BKLF to progressively shift towards the band that corresponds to BKLF modified at K10, suggesting that the creation of a branched protein was contributing to the aberrant migration. For instance, sumoylation of a construct containing residues 1 to 268 generates only two major slower-migrating bands—one band that corresponds to modification at K10 or at K197—the two species now comigrate and show combined modification at both K10 and K197 (see Fig. S1 and S2 in the supplemental material).

Although branching is likely to contribute to the aberrant migration of the K197 form, there is also evidence that the charge of the region around K197 significantly affects the mi-

gration (and most likely the conformation) of the protein. We carried out further experiments on full-length BKLF and mutated residues K189 and K194/K195 to alanine. These changes did not affect the migration of unmodified BKLF or of BKLF sumoylated at K10 but significantly affected the migration of the K197-sumoylated BKLF. On the other hand, when the same two lysines (K194/K195) were mutated to arginine, the migration profile reverted to the wild-type appearance (see Fig. S2B, lanes 5 to 7, in the supplemental material). We conclude that K197 lies in some critical domain in the protein and that alteration of this by sumoylation or mutation significantly affects how the protein behaves on SDS-PAGE and may also influence its activity in vivo (see below).

PIAS family members act as E3 ligases for BKLF. PIAS proteins have been shown to act as SUMO E3 ligases; as such, they significantly enhance the sumoylation of substrates. It has been proposed that in vivo E3 ligases may work in conjunction with the E2-conjugating enzyme to confer substrate specificity (30, 47). To examine whether PIAS1, PIASxα/ARIP3, or PIASxβ/Miz1 proteins could act as specific SUMO E3 ligases for BKLF, ³⁵S-labeled BKLF was first subjected to SUMO-1 modification in vitro with limiting amounts of recombinant SUMO-1 and Ubc9 (1/10 of the amount used in the experiments described for Fig. 1B and 2B). Under these conditions, SUMO conjugation to BKLF was almost undetectable except

FIG. 3. PIAS family members stimulate SUMO conjugation to BKLF. (A) E3 activity in vitro. ³⁵S-methionine-labeled BKLF was sumoylated in vitro as described for Fig. 1B but using only 100 ng of GST-SUMO-1. A total of 500 ng of purified GST-PIAS1, GST-ARIP3, or GST-Miz1 was added as indicated. Molecular mass markers (in kilodaltons), full-length BKLF (double arrows), and BKLF-SUMO-1 conjugates (thick arrows) are indicated. Protein bands marked with asterisks appear to correspond to SUMO-modified forms of BKLF translation products of lower molecular mass or to a multiply sumoylated form. (B) E3 activity in vivo. (Left panel) COS-1 cells were transfected with 500 ng of BKLF and HA-SUMO-1 expression vectors (lane 1) or 50 ng of HA-SUMO-1 expression vector (lanes 2 to 7) and 2 μ g of expression vector for Miz1, PIASy, ARIP3, PIAS1, and Pc2 as indicated. The proteins were detected by Western blotting using anti-BKLF antibody. (Right panel) Experiments were also performed with a mutant BKLF that prevents CtBP2 binding (Δ DL).

for the major site of modification at K197 (Fig. 3A, lane 2). However, addition of bacterially expressed GST-PIAS1, GST-ARIP3, or GST-Miz1 allowed for efficient conjugation of SUMO-1 to BKLF (Fig. 3A, lanes 3 to 5).

We also examined whether PIAS members can act as SUMO E3 ligases for BKLF in vivo, this time limiting the amount of the HA-tagged SUMO-1-expressing vector. The results obtained (Fig. 3B) reflect those observed in the in vitro assays and confirm that Miz1, ARIP3, and PIAS1 but also PIASy act as E3 ligases for SUMO-1 conjugation to BKLF. These data also suggest that ARIP3 was the most efficient E3

ligase for BKLF in our assays. Interestingly, we preferentially detected an increase in sumoylation of K10 and the double sumoylation of K10 and K197 (Fig. 3A and 3B), consistent with the view that the presence of E3 proteins potentiates sumoylation of sites that are suboptimal in the absence of E3 proteins (Fig. 2B and 2C).

Notably, Pc2, the E3 ligase for CtBP sumoylation (18), did not exert any effects on the sumoylation of BKLF in vivo (Fig. 3B, lane 7), supporting the idea that E3 SUMO ligases display substrate specificity (30). Furthermore, it has been shown that CtBP proteins interact with Ubc9 and are themselves sub-

FIG. 4. SUMO-modified BKLf retains DNA-binding activity. A gel mobility shift experiment using BKLf or mutants BKLf K10A, K197A, or K10A/197A and GFP-SUMO-1 coexpressed in COS-1 cells shows that SUMO-modified BKLf binds a CACCC-box element. Lanes 1 to 15 contained COS-1 cell nuclear extracts prepared from cells transfected with the following expression vectors: lane 1, empty vector; lanes 2 to 3, BKLf-expressing vector; lanes 4 to 15, BKLf or indicated BKLf mutants and GFP-SUMO-1. The asterisk depicts an endogenous protein that binds to the probe. Antibodies were used as follows: anti-BKLf (lanes 3, 5, 8, 11, and 14) or anti-SUMO-1 (lanes 6, 9, 12, and 15). The arrows indicate the sites of migration of the BKLf-DNA complex, the SUMO-modified BKLf-DNA complexes supershifted with anti-SUMO-1 antisera, and the BKLf-DNA complex supershifted with anti-BKLf antisera, as indicated. The free probe is not shown.

strates for sumoylation (18, 26). We therefore tested whether CtBP2 might function as an E3 by recruiting additional Ubc9 to BKLf but found no evidence that either the overexpression of CtBP2 or the mutation of BKLf to prevent CtBP2 binding (Δ DL) influenced the sumoylation of BKLf (Fig. 3B, lanes 8 to 14, and data not shown). Interestingly, the sumoylation of this BKLf mutant is enhanced by the PIAS E3 ligases as efficiently as that of wild-type BKLf, further indicating that CtBP is not acting as a bridging factor between BKLf and the sumoylation machinery. Thus, BKLf and its corepressor CtBP appear to be differentially sumoylated via separate E3 ligases, suggesting that distinct pathways lead to BKLf and CtBP modification.

Sumoylated BKLf retains DNA-binding activity. The major site of SUMO-1 modification K197 in BKLf is located close to the zinc finger DNA-binding domain (Fig. 2A). Thus, it seemed possible that conjugation of SUMO-1 to BKLf would affect the binding of BKLf to DNA. To analyze the capacity of sumoylated BKLf to bind to DNA, we employed electrophoretic mobility shift assays with nuclear extracts from COS cells cotransfected with various BKLf constructs and a GFP-SUMO-1-expressing vector. Wild-type BKLf and BKLf derivatives that are refractory to sumoylation are supershifted in a similar manner by an anti-BKLf antibody (Fig. 4, lanes 3, 5, 8, 11, and 14). An anti-SUMO-1 antibody clearly supershifted a fraction of the bound complexes (Fig. 4, lanes 6, 9, and 12), indicating that the SUMO-conjugated forms of BKLf bind DNA. As expected, the double mutant K10A/K197A is not supershifted by the anti-SUMO-1 antibody, further demonstrating that these mutations abolished the sumoylation of BKLf (Fig. 4, lane 15). Interestingly, the protein-DNA complexes obtained with the SUMO-BKLf fraction migrated to a position similar to that seen with unmodified BKLf (Fig. 4; compare lanes 2 and 4). This is somewhat unexpected, as

addition of GFP-SUMO-1 to K10 and K197 (~100 kDa) does not seem to have significantly altered the migration of the BKLf-DNA complex.

Notably, the intensity of the complexes supershifted with anti-SUMO-1 appears to correlate well with the proportion of BKLf modified by SUMO-1 detected *in vivo* (Fig. 2C). In particular, a reduction of the SUMO-conjugated form bound to DNA is observed when K197 is mutated (Fig. 4, lane 12), further indicating that this residue is the major site of sumoylation in BKLf. Taken together, the above-described results show that SUMO-1 conjugation of BKLf does not abolish or detectably alter the DNA-binding capacity of BKLf. In addition, the presence of DNA containing a cognate BKLf binding site does not enhance or inhibit the degree of sumoylation observed for BKLf *in vitro* (data not shown).

Sumoylation of BKLf facilitates transcriptional repression activity. Having established that sumoylation did not significantly alter the DNA-binding properties of BKLf (Fig. 4) we proceeded to assess the potential functional consequences of the conjugation of SUMO-1 to BKLf. We carried out transactivation assays to compare the transcriptional repression activity of wild-type BKLf with that of the sumoylation-deficient mutants. We used *Drosophila* SL2 cells, since these are used conventionally for the study of CACCC-box factors, given that they do not contain significant amounts of ubiquitous CACCC-binding proteins (such as Sp3, which is also modified by SUMO) (41, 46), which complicate interpretations of experiments in mammalian cell lines. We previously reported that BKLf strongly represses a composite CACCC box-GRE-driven promoter in these cells (62).

Indeed, wild-type BKLf potently repressed GR-activated transcription to basal levels. Mutation of K10 (BKLf-K10A) reduced repression only slightly, as did mutation of the K197 site (Fig. 5A); however, mutation of both sumoylation sites

FIG. 5. SUMO modification sites are required for BKLF-mediated transcriptional repression. (A) A schematic diagram of the reporter construct used is shown at the top of the panel. A total of 50 ng of BKLF and the mutants indicated was cotransfected with 500 ng of glucocorticoid receptor expression plasmid and 500 ng of the CAT reporter into SL-2 cells. The addition of dexamethasone (Dex.) is indicated by plus signs. CAT activity was determined as described in Materials and Methods. Severalfold activation values were calculated from CAT activity levels in relation to glucocorticoid receptor activity, which has been given the arbitrary value of 100. CAT activity values are representative of at least three independent experiments. Adh, alcohol dehydrogenase promoter. (B) DNA-binding activity of BKLF mutants. Nuclear extracts from transfected SL-2 were used for a gel mobility assay using a CACCC-element-containing probe. The arrows indicate the BKLF/DNA complex. The free probe is not shown. C, untransfected SL-2 cells. The asterisk indicates an endogenous protein that binds to the probe.

FIG. 6. Influence of the promoter context on BKLf repression activity. A schematic diagram of the γ -globin reporter construct used is shown at the top. Transient transfections into SL-2 cells were performed as described for Fig. 5.

(K10A/K197A) significantly impaired the repression activity of BKLf (Fig. 5A). To ascertain that the effects of the mutations are due to a lack of SUMO-1 modification rather than due to other potential lysine modifications such as acetylation or methylation, we disrupted the sumoylation sites by mutating residues E12 and E199 to alanine. Notably, the double mutation E12A/E199A was found to reduce repression activity of BKLf to a level comparable to that obtained with the K10A/K197A mutant (Fig. 5A, upper left and bottom panels).

BKLf repression activity is also linked to the recruitment of the corepressor CtBP2 (62). Consistent with previous results, elimination of CtBP2 recruitment by the introduction of a mutation in the CtBP2 contact motif, PVDLT to PVAAT (Δ DL), reduced repression about sevenfold (Fig. 5A, upper right panel). Unexpectedly, combining this mutation with mutations in the sumoylation sites had a significant impact. When sumoylation and CtBP binding were both prevented by mutation, BKLf became an activator rather than a repressor of transcription (Fig. 5A). It should be noted that all mutants were expressed at similar levels and bound DNA with indistinguishable levels of affinity (Fig. 5B).

To further decipher the regulation of BKLf activity by sumoylation, the transcriptional-repression properties of BKLf mutants were tested in the context of a natural promoter (Fig. 6). The γ -globin promoter is activated by the erythroid-specific transcription factor GATA-1 (28). The promoter contains multiple CACCC elements, and we have previously found that BKLf can potently repress GATA-1-mediated activation (64). BKLf derivatives that are refractory to sumoylation, however, displayed the same transcriptional repression ability as the wild-type protein (Fig. 6). This observation suggests that the modulation of BKLf activity by SUMO modification depends on the promoter context. Of interest, in contrast to the results seen with the GR-activated promoter, the mutation of BKLf that abolished CtBP2 recruitment completely eliminated the repression activity of BKLf on γ -

globin GATA-1-mediated activation in this case, suggesting that this is a CtBP-dependent promoter and that sumoylation of BKLf is not required in this context. Taken together, these data are consistent with previous reports (27, 59) about the central importance of the promoter context when investigating the effects of sumoylation.

DISCUSSION

SUMO modification has recently emerged as a mechanism for modulating transcriptional activators (66). Interestingly, sites of SUMO modification are often located within previously defined inhibitory domains of transcriptional activators such as Sp3, Elk-1, diverse nuclear receptors, and other transcription factors such as c-Myb. In this context, mutation of the acceptor lysine negates the function of the inhibitory domain and unmasks a stronger activation potential. Although there are many examples where sumoylation attenuates the activity of transactivators, the role of sumoylation in potentiating the gene-silencing activity of repressors is less well established. It is known, however, that the corepressor CtBP is sumoylated and that this modification is important for its activity (18, 26). Here, we have shown that BKLf, a DNA-binding protein that represses transcription by recruiting CtBP, also requires sumoylation for full repression activity, at least on some promoters.

In this work we have shown that BKLf binds the SUMO E2-conjugating enzyme Ubc9 and is modified by the addition of SUMO-1 in vitro and in vivo. Sumoylation of BKLf is potentiated both in vitro and in vivo by known E3 SUMO ligases such as PIAS1, PIAS γ , PIAS α /ARIP3, and PIAS β /Miz1 (Fig. 3). Importantly, the E3 SUMO ligase Pc2, which acts in the modification of BKLf's partner CtBP (18), did not enhance sumoylation of BKLf. CtBP binds the SUMO E2-conjugating enzyme Ubc9 (as well as the SUMO E3 ligase Pc2) (18, 26), but the association of CtBP with BKLf does not affect BKLf sumoylation (Fig. 3), arguing against a simple model in which recruitment of enzymes involved in sumoylation is sufficient to bring about the modification.

BKLf contains K10 and K197, two lysine residues which lie within typical consensus motifs for sumoylation Ψ KXE. Our results indicate that both of these lysines are modified by the addition of a single SUMO-1 moiety, and we found no evidence of any additional sumoylation sites within the molecule. K197 is the major site of modification (Fig. 2; also see Fig. S1 and S2 in the supplemental material). Interestingly, sumoylation at K197 or mutation of the nearby residues K189 and K194/K195 to alanine significantly affects the migration of the modified protein on SDS-PAGE. Sumoylation of BKLf does not appear to alter its DNA-binding activity (Fig. 4), but mutations that prevent sumoylation impair the transcriptional repression activity of BKLf (Fig. 5). The residual repression activity observed can be attributed to the binding of BKLf's corepressor CtBP. When CtBP binding is prevented (by mutating the CtBP contact site in BKLf) and the sumoylation sites are also mutated, BKLf no longer represses transcription but becomes an activator. These results show that both CtBP binding and sumoylation are critical for the ability of BKLf to function as a repressor. However, the finding of a sumoylation-independent repressive activity of BKLf on the γ -globin

promoter suggests that sumoylation is tightly controlled, depending on the promoter and cellular contexts. Previous studies have already reported a direct link between differential sumoylation activity and the nature of the responsive element (in particular, the number of response elements) (14, 15, 27, 59). We thus propose that BKLF targets may be classified into at least two different categories: (i) CtBP-dependent and SUMO-independent promoters and (ii) CtBP- and sumoylation-dependent promoters. The existence of SUMO-dependent and CtBP-independent BKLF targets remains to be assessed and will be the subject of future investigations.

Other important dynamic aspects should also be taken in consideration, as sumoylation processes may participate in the regulation of target protein stabilization or nucleocytoplasmic shuttling. For example, mutation of the sumoylation site in CtBP1 causes its relocalization to the cytoplasm and thus inhibits its ability to repress transcription (26). We have investigated the nuclear localization of BKLF but have found no evidence that sumoylation influences its nuclear localization (data not shown). Sumoylation may alter the function of proteins by several other mechanisms (49, 66). PCNA, Smad4, and I κ B α are all examples of substrates with which a single lysine residue can be either sumoylated or ubiquitinated, and competition between these modifications may therefore play a role in regulating protein stability (6, 13, 25, 46, 56). We have not observed significant differences in the steady-state levels of BKLF proteins carrying wild-type or SUMO mutant sequences, suggesting that sumoylation is unlikely to significantly alter the stability of BKLF (Fig. 2). In addition, the observation that the repressive activity of BKLF is compromised upon disruption of the sumoylation sites (E12 and E199 mutations) (Fig. 5) argues against destabilization on the basis of competing lysine modifications.

CtBP proteins have been shown recently to be part of a corepressor complex containing the histone deacetylases HDAC1 and HDAC2, the histone methyltransferases G9a and Eu-HMTase1, and the E3 SUMO ligase Pc2 (53). Both CtBP and HDAC1 are known substrates of sumoylation (5, 18, 26). CtBP also binds HIPK2, a kinase that binds Ubc9 and was one of the first substrates of sumoylation to be identified (21, 70). Our finding that BKLF recruits Ubc9 and is also a substrate for sumoylation further strengthens the idea of a link between sumoylation and gene repression. Most recently, it has been found that the recruitment of Ubc9 and sumoylation of histone H4 can facilitate gene silencing (54). The recruitment of Ubc9 by BKLF may also contribute to gene repression. However, the observation that a K197R mutant form of BKLF (that can still bind Ubc9 but is not sumoylated at this residue) is impaired in its repression activity (data not shown) suggests that sumoylation of BKLF per se rather than recruitment of Ubc9 is the most important factor in this aspect of BKLF activity. How sumoylation alters the activity of BKLF is not yet known. It is likely that SUMO-1 modification of BKLF may alter its conformation and thus regulate the interactions of binding partners. In some cases the recruitment of HDACs has been shown to depend on the sumoylation of partner proteins (10, 69), but we have not found evidence that BKLF-mediated repression depends on HDAC association. Experiments are presently under way to establish whether sumoylated BKLF may specifically recruit new coregulatory partners.

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