Doxorubicin Down-regulates Krüppel-associated Box Domain-associated Protein 1 SUMOylation That Relieves Its Transcription Repression on p21\(^{WAF1/CIP1}\) in Breast Cancer MCF-7 Cells*

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§§ The abbreviations used are: Ub, ubiquitin; SUMO, small ubiquitin-like modifier (SUMO) to proteins plays a major role in regulating cellular functions (reviewed in Ref. 1). Although ubiquitination generally promotes protein degradation, sumoylation regulates a variety of cellular processes, including nuclear transport, genome integrity, signal transduction, and transcriptional regulation (1–3). Briefly, SUMO is activated in an ATP-dependent manner by an E1 activating enzyme, transferred to the E2 conjugating enzyme, and subsequently attached covalently to the lysine acceptor site, within a particular sequence of $\Psi Xk(D/E)$ (where $\Psi$ represents hydrophobic amino acid) of SUMO-target proteins by distinct E3 ligases. Similar to phosphorylation, sumoylation is a reversible process catalyzed by de-sumoylation enzymes. The list of known SUMO targets has grown substantially in recent years, and, remarkably, over half of the presently identified SUMO targets are transcriptional factors or coregulators. In many cases, sumoylation leads to a modified protein-protein interaction of target protein(s), thus altering the biological consequences (4, 5). Recently, a SUMO-binding motif that interacts non-covalently with sumoylated proteins has been described (6), and this SUMO-binding motif is found to exist in nearly all proteins known to be involved in SUMO-dependent processes (7).

SUMO proteins, SUMO-1 through -4, form a Ub-like fold in the central region, containing an $\alpha$-helix packed on a $\beta$-sheet (8, 9). However, its N-terminal extension, which is not found in ubiquitin, is highly flexible and does not adopt any defined three-dimensional structure in solution. In addition, the amino acid residues at the protein surfaces in SUMO differ from those in ubiquitin, resulting in completely different surface charge distribution. Together, these two unique features of SUMO lead to the notion that SUMO functions as a special module that is covalently linked to target proteins and/or as a moiety to mediate non-covalent SUMO-dependent interaction with other proteins, hence modulating cellular function (2, 10, 11). An advantage of such a combined covalent modification and non-covalent interaction is to transiently and rapidly augment or attenuate protein-protein interaction and its subsequent sig-

The role of post-translational modification, such as sumoylation, in modulating the efficacy of doxorubicin (Dox) treatment remains unclear. Transcriptional cofactor KRAB domain-associated protein 1 (KAP1) has been shown to complex with the KRAB zinc finger protein, ZBRK1, to repress the transcription of target genes. Through a combination of proteomic screening and site-directed mutagenesis approaches, we have identified lysines 554, 779, and 804 as the major sumoylation sites in KAP1. We then present evidence that Dox-mediated induction of cell cycle regulator p21 expression is differentially regulated by KAP1 sumoylation status. Moreover, the KAP1 sumoylation level was transiently decreased upon Dox exposure, and transfection with the KAP1 sumoylation mimetic, SUMO-1–KAP1, desensitizes breast cancer MCF-7 cells to Dox-elicited cell death. The sumoylation-dependent stimulation of KAP1 function is achieved by enhancing the methylation of H3-K9 and attenuating the acetylation of H3-K9 and H3-K14 at the p21 core promoter. We also show that occupancy of ZBRK1 response elements located at the p21 promoter by ZBRK1-KAP1 is independent of KAP1 sumoylation. Hence, sumoylation of KAP1 represses p21 transcription via a chromatin-silencing process without affecting interaction between KAP1-ZBRK1 and DNA, thus providing a novel mechanistic basis for the understanding of Dox-induced de-repression of p21 transcription. Taken together, our results suggest that Dox-induced decrease in KAP1 sumoylation is essential for Dox to induce p21 expression and subsequent cell growth inhibition in MCF-7 cells.

Covalent attachment of ubiquitin (Ub) and small ubiquitin-like modifier (SUMO) to proteins plays a major role in regulating cellular functions (reviewed in Ref. 1). Although ubiquitination generally promotes protein degradation, sumoylation regulates a variety of cellular processes, including nuclear transport, genome integrity, signal transduction, and transcriptional regulation (1–3). Briefly, SUMO is activated in an ATP-dependent manner by an E1 activating enzyme, transferred to the E2 conjugating enzyme, and subsequently attached covalently to the lysine acceptor site, within a particular sequence of $\Psi Xk(D/E)$ (where $\Psi$ represents hydrophobic amino acid) of SUMO-target proteins by distinct E3 ligases. Similar to phosphorylation, sumoylation is a reversible process catalyzed by de-sumoylation enzymes. The list of known SUMO targets has grown substantially in recent years, and, remarkably, over half of the presently identified SUMO targets are transcriptional factors or coregulators. In many cases, sumoylation leads to a modified protein-protein interaction of target protein(s), thus altering the biological consequences (4, 5). Recently, a SUMO-binding motif that interacts non-covalently with sumoylated proteins has been described (6), and this SUMO-binding motif is found to exist in nearly all proteins known to be involved in SUMO-dependent processes (7).

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KRAB, Krüppel-associated box; KZF, KRAB domain-containing zinc finger; ZBRK1, KRAB domain 1; H3-K9, histone 3 lysine 9; Dox, doxorubicin; MS/MS, tandem mass spectrometry; GFP, green fluorescent protein; RT, reverse transcription; ChIP, chromosomal immunoprecipitation; MTT, 3-(4,5-di methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMS, methylmethane sulfonate.
Sumoylation of KAP1

naling in response to extracellular stimulation (12). Importantly, sumoylation has emerged as an imperative regulatory scheme to modify diverse cellular functions, including intracellular protein compartmentalization, DNA repair, cell-cycle regulation, transcriptional control, and hormone response (reviewed in Refs. 3 and 13).

The KRAB domain-associated protein 1 (KAP1) was first identified as a transcriptional co-repressor in several reports (14–16). These studies have shown that the N-terminal RING-B boxes-coiled-coil domain of KAP1 serves as a protein-protein interaction motif for the KRAB (Krüppel-associated box) domain. KRAB domain-containing zinc finger (KZF) proteins, characteristic of their N-terminal KRAB domain and a number of zinc fingers at their C terminus, constitute the largest single protein family of transcriptional regulators known by far (17). Although a majority of KZF proteins functions to repress RNA polymerase II-mediated transcription, their respective target genes and underlying transcriptional regulation mechanism(s) remain largely elusive. Recently, zinc finger and BRCA1-interacting protein with KRAB domain 1 (ZBRK1), a KZF protein harboring KRAB domain, was found to repress the transcription of the DNA damage-responsive gene gadd45α through a 15-bp ZBRK1 response element located on its third intron (18). Several DNA damage-responsive genes such as gadd153, p21\textsuperscript{waf1/cip1}, and bax also possess this consensus ZBRK1 response element, implying its role, and possibly its partner KAP1 as well, in regulating the transcription of these DNA damage responsive genes.

In addition to its N-terminal RING-B boxes-coiled-coil domain, there are several C-terminal domains that have been demonstrated to recruit chromatin-remodeling machinery to KAP1. For example, the PXVXL motif is involved in the binding of heterochromatin protein 1, and the PHD and Bromodomains interact with Mi-2α of the NuRD histone deacetylase complex and histone 3 lysine 9 (H3-K9) methyltransferase SETDB1 (19–24). Herein, we report that KAP1 is subjected to SUMO-1-mediated modification and the KAP1 sumoylation status correlates with its transcriptional repressive ability through ZBRK1 response element. We present evidence that KAP1 sumoylation modulates both p21-Luc reporter activation and the relative extents of H3-K9 and H3-K14 acetylation versus H3-K9 methylation at the p21 promoter in doxorubicin (Dox)-treated breast cancer MCF-7 cells. However, the ability of KAP1 to complex with ZBRK1 and the occupancy of ZBRK1 response element are not affected by KAP1 sumoylation. Moreover, Dox treatment transiently reduces KAP1 sumoylation level, and transcription with the KAP1 sumoylation mimetic, SUMO-1–KAP1, greatly enhances the cell viability against Dox treatment. Together, our results support the notion that sumoylation plays a major role in regulating the ability of KAP1-ZBRK1 to modify H3 methylation versus acetylation profile at the p21 promoter and provide a novel molecular basis for Dox to induce p21 expression and cell growth inhibition.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293 and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics at 37 °C under 5% CO\textsubscript{2}, whereas T47D cells were maintained in RPMI 1640 medium with 10% fetal bovine serum and antibiotics otherwise as the same conditions above.

Antibodies—The anti-FLAG-agarose, FLAG peptide, and anti-FLAG mouse monoclonal antibody were purchased from Sigma-Aldrich. Mouse monoclonal antibody against c-Myc was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against acetylated histone 3 and di-methylated histone 3 lysine 9 were obtained from Chemicon International (Temecula, CA).

Affinity Purification of Cellular Sumoylated Proteins—Full-length SUMO-1 in pCMV Tag 2A vector (Stratagene) was transfected into HEK293 cells, followed by the addition of 300 μg/ml gentamycin sulfate starting at the 30th h post-transfection for 2 weeks to generate the pool of FLAG-SUMO-1 stably integrated cells. Whole cell extract was prepared with radioimmune precipitation assay buffer (50 mM Tris-HCl, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) supplemented with 10 mM N-ethylmaleimide (Sigma). FLAG affinity purification was performed with anti-FLAG M2-agarose (Sigma), and nonspecific binding was washed off with twenty bed volumes of radioimmune precipitation assay buffer for three times. Immunoprecipitates were then eluted with 150 μg/ml FLAG peptide (Sigma) in phosphate-buffered saline, and concentrated with a Microcon YM-50 concentrator where FLAG peptide and FLAG-SUMO-1 were removed. Prior to subjecting to mass spectrometric analysis, eluted proteins were digested with sequencing grade-modified trypsin (Promega, Madison, WI) at 100:1 ratio twice, and the digestion mixture was desalted by passing through ZipTip C18 resins.

Liquid Chromatography-Ion Trap Mass Spectrometry—Mass spectrometric analysis of the trypsin-digested, sumoylated proteins was performed using a Thermo Finnigan LCQ Deca XP Plus mass spectrometer with reverse phase liquid chromatography implemented with an Ultra Plus II LC system (Micro-Tech Scientific) using a 150-μm × 75-μm C-18 reverse-phase column (5 μm, 300-Å particles) from Micro-Tech Scientific. Peptides were loaded onto a Michrom Bioresources peptide cap trap at 95% solvent A (2% acetonitrile, 0.1% formic acid) and 5% solvent B (95% acetonitrile, 0.1% formic acid) and then eluted with a linear gradient of 5–60% solvent B for 65 min and 60–90% solvent B for 10 min. Tandem mass spectrometry (MS/MS) spectra were acquired with Xcalibur 1.2 software. A full MS scan was followed by three consecutive MS/MS scans of the top three ion peaks from the preceding full scan. Dynamic exclusion was enabled such that, after three occurrences of an ion within 1 min, the ion was placed on the exclusion list for 3 min. Other mass spectrometric data generation parameters were as follows: collision energy, 35%; full scan MS mass range, 400–1800 m/z; minimum MS signal, 5 × 10\textsuperscript{4} counts; and minimum MS/MS signal, 5 × 10\textsuperscript{3} counts. The mass spectrometer was equipped with a nanospray ion source (Thermo Electron) using an uncoated 10-μm ID SilicaTip\textsuperscript{TM} PicoTip\textsuperscript{TM} nanospray emitter (New Objective, Woburn, MA). The spray voltage of the mass spectrometer was 1.9 kV, and the heated capillary temperature was 180 °C.

Analysis of MS Spectra—A Beta test version of Bioworks (Bioworks 3.1) on a nine-node (2 central processing units/node)
RNAi sequences against the mRNA of ZBRK1 and KAP1 were provided by Integrated DNA Technologies, Inc. The core molecules were designed according to a recent report (25) and a recent paper (Calbiochem). For each sample, 30 μl of lysate or immunoprecipitation of FLAG-KAP1 followed by FLAG-KAP1 and GFP-SUMO-1 in a 1:4 ratio. Sumoylated sumoylation assay was carried out with co-transfection of FLAG-ZBRK1 and Myc-KAP1 expression constructs. The p21 promoter was PCR cloned from a total DNA pool of HeLa cells by DNeasy Tissue Kit (Qiagen) and was subcloned into pGL3b reporter (Promega) as described previously (28). MCF-7 cells were co-transfected with pGL3b-p21 reporter, internal control reporter pRL-TK, ZBRK1, KAP1, or its mutants. Firefly luciferase activity was assayed with the Dual-Luciferase Reporter Assay System (Promega) and normalized against Renilla luciferase activity.

Chromatin Immunoprecipitation Assay—MCF-7 cells were cross-linked with 1% formaldehyde in growth medium for 15 min. Cross-linking was stopped by addition of glycine to a final concentration of 125 mM. Cells were rinsed with cold phosphate-buffered saline buffer and harvested, and then subsequently swelled in 1 ml of RSB buffer (10 mM Tris-HCl, 3 mM MgCl₂, 10 mM NaCl, 0.1% IGEpal CA-330, pH 7.4) supplemented with protease inhibitor mixture (Roche Applied Science). Nuclei were then collected and resuspended in nuclear sonication buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.1). Sonication was then performed to shear DNA to 500 bp or less. Samples were then diluted with 4 volumes of dilution buffer (6.7 mM Tris-HCl, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS) before being precleared with 20 μl of 50% protein A/G-agarose. Approximate 3–5 μg of antibodies against acetylated histone H3 or di-methylated H3-K9 was incubated with post-clearance supernatant overnight at 4 °C, and protein A/G- or FLAG M2-agarose that had been preincubated with 100 μg/ml tRNA and 0.1% bovine serum albumin was added to the mixture the following day and incubated for an additional 2 h. Washes were performed with LiCl buffer twice (100 mM Tris-HCl, 500 mM LiCl, 1% deoxycholate, 1% Nonidet P-40, pH 8.0), high salt buffer twice (20 mM Tris-HCl, 500 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.2% SDS, pH 8.0), and low salt buffer twice (same as high salt buffer except with an NaCl concentration at 150 mM), and 2 × TE buffer twice (20 mM Tris-HCl, 2 mM EDTA, pH 8.0). Immunoprecipitates were eluted in 2% SDS in 2 × TE buffer and subsequently treated with RNase (1 h) and protease K (Sigma) (1 h), respectively. Cross-linking was reversed by incubation at 65 °C overnight, and recovered DNA was extracted with phenol/chloroform and ethanol-precipitated. DNA of the designated amplicons was amplified with specific primer pairs (Table 1) using the My IQ real-time PCR detection system and an IQ SYBR Green supermix (Bio-Rad). Normal sera and input DNA

### Table 1

| Primer* | Purpose | Sequence |
|---------|---------|----------|
| 18 S rRNA FP | Real-time PCR | CCGCGAGCACCCATCCCAGAC |
| 18 S rRNA RP | RT and real-time PCR | GAATTCAACCTCATTPCCCGCCTC |
| p21 FP | Real-time PCR | TTTCCTCTCGTCTCCCAATOT |
| p21 RP | RT and real-time PCR | GCTGTATATTCAGCATTGTGG |
| p21 – 20 ampicon FP | ChIP (real-time PCR) | TATATCATGGGGGCGGGG |
| p21 – 20 ampicon RP | ChIP (real-time PCR) | CTTTCGGCAGCTGCTCACACCT |
| p21 – 713 ampicon FP | ChIP (real-time PCR) | TTTGCGTTGAGCTGAGTTGG |
| p21 – 713 ampicon RP | ChIP (real-time PCR) | GGAAGAGGAGATTTGGAGAG |
| p21 – 3038 ampicon FP | ChIP (real-time PCR) | CAGGCCTGTGCTTCTAAACTCC |
| p21 – 3038 ampicon RP | ChIP (real-time PCR) | GCCCTGTATATCCACCGCCTT |

* FP, forward primer; RP, reverse primer.
FIGURE 1. Transcriptional co-repressor KAP1 is subjected to multisumoylation. A, mass spectrum of fragmented KAP1 (amino acid residues 408–427). B, in vivo sumoylation of KAP1. FLAG-KAP1 and GFP-SUMO-1 were co-transfected into HEK293 cells, and immunoblotting was performed with an anti-FLAG antibody. Un-sumoylated KAP1 and SUMO-1-modified KAP1 are indicated by an arrow and asterisks, respectively. C, KAP1 is sumoylated in vitro. The reactions were carried out with purified KAP1 from HEK293 cells, sumoylation E1 (Aos1/Uba2 heterodimer), E2 (UbC9), and E3 (RANBP2) enzymes purified from Escherichia coli in ATP/Mg²⁺ buffer.
values were used to subtract and normalize the values from ChIP samples.

Cell Viability Assay—MCF-7 cells were seeded into 24-well plates to obtain a confluency of 50% on the day of the experiment. The cells were treated with vehicle or 2.5 μM Dox, and medium was changed daily for 3 days. 72 h after the start of treatment, 0.2 ml of 0.1 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) in Opti-MEM I (Invitrogen) was added to each well, and the plate was incubated at 37 °C for an additional 1.5 h. After the incubation, the MTT solution was aspirated, and 0.2 ml of isopropanol was added to each well to dissolve the formazan crystals. Absorbance was read immediately at 540 nm in a scanning multiwell spectrophotometer. The results were depicted as percentage of cell viability, reported as the mean ± S.D. of three independent experiments performed in triplicates.

Statistical Analysis—p values from two-tailed t tests were calculated by using a spreadsheet program (Microsoft Excel) and assuming equal variances. The difference between two data sets is significant when p value is <0.05.

RESULTS

KAP1 Is Sumoylated in Vitro and in Vivo—In an attempt to understand the role of SUMO-1-mediated sumoylation, we utilized an affinity purification system to enrich sumoylated proteins for subsequent protein identification by mass spectrometry. To circumvent the oversumoylation by SUMO-1 overexpression from transient transfection and to facilitate the purification of sumoylated proteins, we established HEK293 cells with stable expression of FLAG-SUMO-1. To rule out non-sumoylated protein(s) also being pulled-down by non-covalent SUMO-dependent protein-protein interaction, another HEK293 line integrated with FLAG-SUMO-1a, an unconjugatable SUMO-1, was run parallel throughout the purification and the identification processes to exclude those possibilities. The immunoprecipitates were then trypsin-digested and subjected to liquid chromatography-linked tandem mass spectrometry. Approximately 30 proteins were seized with strict filtering parameters by the SEQUEST program (see “Experimental Procedures” for details), and KAP1 was selected for further analyses reported here. The mass spectrum with y-ion and b-ion assignments of KAP1 amino acid residues 408–427 following collision-induced dissociation is shown in Fig. 1A.

Next, we verified KAP1 sumoylation potential with both in vivo and in vitro sumoylation systems. Co-transfection of FLAG-KAP1 with GFP-SUMO-1 resulted in the appearance of multiple ladders with higher molecular mass other than unmodified FLAG-KAP1 visualized by an anti-FLAG antibody (Fig. 1B). The estimated increase in molecular mass between each species approximately matched with the mass of 50-kDa of GFP-SUMO-1. The intensity of KAP1-sumoylation ladders in Fig. 1B was dependent upon the input of GFP-SUMO-1 (Fig. 1B, lanes 4–6). KAP1 as a sumoylation target was further verified by in vitro sumoylation assays using purified KAP1 from HEK293 cells and sumoylation E1 (SAE1/SAE2 heterodimer) and E2 (Ubc9) enzymes purified from bacteria (Fig. 1C, lanes 5 and 6).

Mapping the SUMO-1 Acceptor Lysines in KAP1—Based on KAP1 protein motifs and domains (Fig. 2A), we hypothesized that KAP1 sumoylation could modulate either the interaction between KAP1 and its cognate KZF partner, or between KAP1 and its associated chromatin-remodeling activities. We then proceeded to engineer sumoylation-defective KAP1 to test these possibilities. Given the high probability of sumoylation occurring at the ΨKX(D/E) sequence, we scanned KAP1 amino acid sequence to search for putative sumoylation sites. Out of six putative sumoylation sites identified, five are located at the C terminus of KAP1. As shown in Fig. 2A, among these five potential sumoylation acceptor sites, two are in the N to C ter-
SUMOylation of KAP1

To map the exact SUMO-1 acceptor lysine(s), site-directed mutagenesis of these lysines to arginines individually or in combination was performed. By using in vivo sumoylation assays, KAP1 mutants carrying alterations of K554R, K779R, or K804R, but not K575R or K676R, exhibited a great reduction in their respective sumoylation capacity (Fig. 2B, lanes 3 and 6). Notably, K554R mutation in KAP1 reduced the intensities of most sumoylated species except the presumably mono-sumoylated KAP1 (Fig. 2B, lane 3). As expected, KAP1(3K/R) mutant, harboring K554R, K779R, and K804R mutations, displayed a total lack of sumoylation (Fig. 2B, lane 7). By contrast, the (2K/R) mutations at Lys-575 and Lys-676 preserved nearly all sumoylation capacity of KAP1, comparable to that of the wild-type KAP1 (Fig. 2B, lanes 8 and 2), and KAP1(2K/R) will serve as a control in the remaining studies. Therefore, we concluded that Lys-554, Lys-779, and Lys-804 are the major sumoylation sites in KAP1, and KAP1(3K/R) will be used to mimic un-sumoylated KAP1 to investigate the role of KAP1 sumoylation.

Sumoylation of KAP1 Does Not Affect the Association between KAP1 and Its Cognate Repressor ZBRK1—Next, we examined the role of sumoylation in modulating the physical interaction of KAP1 with KZF proteins, thereby regulating KAP1 transcriptional co-repressor activity. One of the KZF proteins, ZBRK1, has recently been demonstrated to be the cognate KAP1 DNA-binding partner (29). Several DNA damage-responsive genes were also implicated to be under the transcriptional regulation of ZBRK1 through a 15-bp ZBRK1-responsive element originally identified in the third intron of gadd45b gene (18). We decided to utilize this ZBRK1-KAP1 system to investigate the functional role of KAP1 sumoylation. To mimic KAP1 sumoylation, we engineered SUMO-1-KAP1 fusion protein in which SUMO-1 was fused to the N terminus of KAP1, thus mimicking KAP1 sumoylation by being hydrolyzable by SUMO protease(s). ZBRK1 was previously reported to undergo ubiquitin-mediated degradation upon exposure to methylmethane sulfonate (MMS) (30). Consistent with previous report, co-immunoprecipitation assays demonstrated the complex of ZBRK1 with either SUMO-1-KAP1 or KAP1(3K/R) was not detected after MMS treatment (Fig. 3A, lanes 5 and 6). By contrast, co-immunoprecipitation of ectopically expressed ZBRK1 with either SUMO-1-KAP1 or KAP1(3K/R) revealed that sumoylation did not affect KAP1-ZBRK1 interactions in the presence or absence of Dox (Fig. 3A, lanes 3 and 4 versus lanes 1 and 2). Therefore, we ruled out the possibility that sumoylation of KAP1 regulates its interaction with ZBRK1 under stress and concluded that ZBRK1 and KAP1 form a complex regardless of KAP1 sumoylation status upon Dox exposure.

Cell cycle regulator p21waf1/cip1 gene, which contains three copies of ZBRK1 response elements (−318 to −332, −797 to −782, and −284 to −298), was shown to be MMS responsive in a recent study (30). We then examined whether ZBRK1 and p21waf1/cip1 gene are regulated by the same ZBRK1-responsive element. MCF-7 and T47D cells were transiently transfected with a combination of KAP1 expression constructs and ZBRK1 siRNA, and their p21 mRNA levels were assessed by quantitative real-time RT-PCR (Fig. 3B). No significant difference in p21 mRNA levels was observed when KAP1 was decreased by siKAP1 or siZBRK1. In contrast, p21 mRNA levels were not changed in either cell line even though Dox was present in the culture medium (Fig. 3B). Therefore, we concluded that the p21waf1/cip1 gene is regulated by the ZBRK1-responsive element and not by KAP1 sumoylation.
induce p21 expression (28). To ascertain that KAP1 and ZBRK1 function to repress endogenous p21 expression, we developed individual small interference RNA against KAP1 or ZBRK1 to knock down their respective expressions (Fig. 3C, panels b and d). The basal expression of p21 in MCF-7 cells was de-repressed by si-KAP1 or si-ZBRK1 but not by si-control (Fig. 3C, panels a and c), whereas the Dox-induced p21 transcription was merely affected by si-ZBRK1 and siKAP1. Thus, both KAP1 and ZBRK1 are involved in repression of p21 transcription in MCF-7 cells, and hence, the remaining experiments designed to investigate how KAP1 sumoylation affects p21 transcription were performed in MCF-7 cells.

KAP1 Transcriptional Co-repressor Activity Is Regulated by Its Sumoylation Status—Next, we assessed the relationship between KAP1 sumoylation status and its transcriptional co-repressor activity. Because KAP1 sumoylation-mimetic SUMO-1-KAP1 and sumoylation-defective KAP1(3K/R) displayed comparable interactions with ZBRK1 in the presence or absence of Dox treatment, we examined whether KAP1 sumoylation renders a differential regulation of Dox-induced p21 transcriptional activation. Transient transfection assays of pGL3b-p21, harboring 2.4 kb of p21 promoter upstream of a luciferase reporter, with individual wild-type KAP1 or its engineered mutants into MCF-7 cells were performed. As shown in Fig. 4A, SUMO-1-KAP1 potently suppressed Dox-induced p21 promoter activation (lane 5 versus lane 2). The suppression exerted by SUMO-1-KAP1 was virtually nullified upon MMS treatment (Fig. 4A, lane 6 versus lane 3). The steady-state levels of transiently transfected KAP1 and its mutants were comparable (Fig. 4B). Together with Fig. 3A, we concluded that Dox utilizes a mechanism, distinct from that by MMS, to induce p21 expression despite both mechanisms being involved in the ZBRK1-KAP1 complex.

Although the expression of KAP1(K554R) and KAP1(3K/R) de-repressed un-stimulated p21 promoter activity (Fig. 4C, lanes 3 and 4 versus lane 1), KAP1(3K/R) and KAP1(K554R) exerted a very modest, if any, enhancement of Dox-induced
Sumoylation of KAP1

A

-3083 -3013
-3038 amplicon

p21

B

IP: Anti-Flag

| DNA immunoprecipitated (% input) |
|---------------------------------|
| 6                              |
| 5                              |
| 4                              |
| 3                              |
| 2                              |
| 1                              |

Transcription Start

-20 amplicon

TATA

Dox
-3083 -3013
-3038 amplicon

-924 -910
-811 -797
-749 -695
-332 -318
-20 amplicon

ZBRK1 RE

ZBRK1 RE

ZBRK1 RE

ZBRK1 RE

TATA

-27 -24 1

C

IP: anti-FLAG-KAP1

IP: anti-FLAG-ZBRK1

DNA immunoprecipitated (% input)

-MMS +MMS

DNA immunoprecipitated (% input)

-MMS +MMS
KAP1 and its mutants exhibited a similar affinity to ZBRK1 between ZBRK1 and white columns. Dox treatment, the occupancy of ZBRK1 to induce p21 expression in MCF-7 cells. Because the disruption of occupancy by ZBRK1-KAP1 upon Dox treatment was minimal and universal among wild-type KAP1 and its various mutants, we further concluded that KAP1 sumoylation status does not affect the binding of ZBRK1-KAP1 complex to p21 promoter, therefore unaccountable for the differential effect by KAP1 sumoylation on Dox-induced p21 transcriptional activation.

KAP1 Sumoylation Differentially Regulates Lysine Acetylation and Methylation of Histone H3 at p21 Promoter upon Dox Exposure—It appeared that Dox utilized a regulatory mode distinct from that by MMS to induce p21 expression. Because all identified SUMO-1 acceptors of KAP1 are located at its C terminus (Fig. 2), which is known to be associated with a number of chromatin-remodeling activities, we postulated that KAP1 sumoylation may regulate the local chromatin structure where ZBRK1-KAP1 stable complex binds at the p21 promoter.

It has been established that H3-K9 and H3-K14 acetylation facilitates the transcriptional activation by de-condensing transcriptionally inactive chromatin, whereas H3-K9 methylation silences transcription in the opposite manner (18, 31–33). We therefore investigated whether the suppression of Dox-induced endogenous p21 transcription by SUMO-1-KAP1 and the derepression of un-stimulated p21 transcription by KAP1(3K/R) are associated with the changes in H3 acetylation versus dimethylation at the p21 promoter. By using ChIP assays with antibodies against acetylated histone H3 or di-methylated H3-K9, the H3-K9 and H3-K14 acetylation versus H3-K9 dimethylation profiles at the endogenous p21 proximal promoter were determined. As shown in Fig. 6A, Dox treatment induced a marked increase of H3-K9 and H3-K14 acetylation at the p21 proximal promoter (−20 ampiclon (4.5-fold, black column 5 versus black column 1) in MCF-7 cells transfected with wild-type KAP1. Substitution of wild-type KAP1 with SUMO-1-KAP1 drastically suppressed this inducible acetylation (Fig. 6A, black column 6 versus black column 5), whereas KAP1(3K/R) elicited a 2-fold increase of H3-K9 and H3-K14 acetylation at the p21 promoter in the absence of Dox (Fig. 6A, black column 3 versus black column 1). In addition, H3-K9 and H3-K14 acetylation profiles at the −20 ampiclon were comparable between MCF-7 cells transfected with KAP1 or KAP1(2K/R) in the absence or presence of Dox treatment (Fig. 6A, black columns 1 and 5 versus black columns 4 and 8), thus supporting the notion that the observed modulation on H3-K9 and H3-K14 acetylation was indeed as a consequence of the change in KAP1 sumoylation capacity. By contrast, the acetylation profile at the distal p21 5′-flanking region was unaffected, as assessed at the −3038 ampiclon (Fig. 6A, white columns).

Then, we employed the same approach to evaluate the relationship between H3-K9 di-methylation and KAP1 sumoylation in MCF-7 cells. Due to the low level of endogenous H3-K9 di-methylation, we co-expressed a H3-K9-specific methyltransferase ESET/SETDB1 to enhance global H3-K9 di-methylation. Opposite to the H3-K9 and H3-K14 acetylation, Dox treatment diminished H3-K9 methylation at the −20 ampiclon of the p21 proximal promoter by ∼75% (Fig. 6B, black column 1 versus black column 5). Moreover, SUMO-1-KAP1 almost blocked this Dox-induced de-methylation (Fig. 6B, black column 6 versus closed black column 5) without affecting proximal promoter di-methylation in the absence of Dox (Fig. 6B, black column 2 versus black column 1). Intriguingly, KAP1(3K/R) reduced basal H3-K9 di-methylation at the −20 ampiclon (Fig. 6B, black column 3 versus black column 1) without affecting Dox-induced profile (Fig. 6B, black column 7 versus black column 1).

Functional Consequence of Dox-induced Down-regulation of KAP1 Sumoylation—We have demonstrated that KAP1 de-sumoylation correlates with the transcriptional activation at the p21 promoter, suggesting that KAP1 de-sumoylation is required for the optimal p21 transcriptional induction by Dox. As shown in Fig. 7A, the sumoylation level of ectopically expressed KAP1 in MCF-7 cells was modulated by Dox treatment. It appeared that the steady-state levels of triply and doubly sumoylated KAP1 were reduced to one-half and three-fifths, respectively, of those in vehicle-treated cells at 4 h post treatment. KAP1 sumoylation then gradually returned to its

**FIGURE 5.** Sumoylation-mimetic and sumoylation-deficient KAP1 mutants exhibit similar affinity to ZBRK1 response elements at the p21 promoter. A, diagram of the p21 promoter indicates the relative position of transcription start site, TATA box, and ZBRK1 binding elements, and amplicons used in the ChIP assay. ZBRK1 RE depicts ZBRK1 response element. B, the binding of KAP1, SUMO-1-KAP1, KAP1(3K/R), and KAP1(2K/R) to p21 promoter in MCF-7 cells was assessed by ChIP assays in the absence or presence of Dox treatment. Cells were transiently transfected with individual KAP1 wild-type and its engineered expression constructs, as indicated. Dox treatment (1 μM) was applied to cells at 30 h post-transfection for 3 h. ChIP was carried out as described under “Experimental Procedures.” After genomic DNA was recovered, individual amplicons were quantified by real-time PCR, and values are expressed as a percentage of input DNA immunoprecipitated. Results represent means ± S.D. from three independent immunoprecipitations. *p = 0.52; **p = 0.176; and †p = 0.244 between corresponding samples. C, the p21 promoter occupancy by exogenous ZBRK1 and KAP1 upon MMS treatment was assessed by ChIP assays. MCF-7 cells received 3 μM MMS 30 h post-transfection for 4 h, followed by ChIP and the quantitation of the −713 ampiclon by real-time PCR.
pretreatment level afterward, marking this as a transient process.

A number of DNA damage-responsive genes possess consen-
sus ZBRK1 response elements, therefore subjecting them to the
transcriptional regulation of ZBRK1

To understand the functional consequences of the transcription of all
these genes under the differential control of SUMO-1-KAP1 or
KAP(3K/R) in the context of Dox treatment, we evaluated their
respective effect on Dox-induced growth inhibition in breast
cancer MCF-7 cells. As expected, SUMO-1-KAP1 desensitized
MCF-7 cells to Dox-induced cell death (Fig. 7B, lanes 2 and
4 versus lanes 1 and 3), whereas vehicle-treated cells did not
exhibit a significant difference in their respective viability (Fig.
7B, lane 3 versus lane 1). Notably, KAP1(3K/R) partially
reduced vehicle-treated cell viability by /H11011

30% (Fig. 7B, lane 5 versus lane 1) and enhanced the efficacy of Dox treatment by
50% (Fig. 7B, lane 6 versus lane 2). By contrast, MCF-7 cells
transfected with sumoylation-competent KAP1(2K/R)
behaved comparably to those with wild-type KAP1 toward
Dox treatment.

Taken together, we concluded that 1) H3-K9 and H3-K14
acetylation increases and H3-K9 methylation decreases at the
p21 proximal promoter when KAP1 is de-sumoylated, leading
to the de-repression of the p21 transcription upon Dox treat-
ment, and constitutive KAP1 sumoylation renders the opposite
effect via an inverse H3-K9 and H3-K14 acetylation

FIGURE 6. Modulation of H3-K9 and H3-K14 acetylation and methylation
at the p21 promoter by KAP1 sumoylation or not. A, breast cancer MCF-7
cells were transiently transfected with a combination of expression con-
structs as indicated. Dox treatment and quantification of amplicons by real-
time PCR are the same as described in Fig. 5B. H3-K9 and H3-K14 acetylation at
the −20 amplicon of p21 proximal promoter was assessed by ChIP with an
anti-acetylated H3-K9 antibody, whereas distal −3038 amplicon upstream of
the p21 promoter was immunoprecipitated as a control. Result represents
means ± S.D. from three independent ChIP assays. *, p = 0.0002; **, p =
2.84e−5; †, p = 0.046; and †, p = 0.9 between corresponding samples. B, H3-K9
methylation at the −20 amplicon of p21 proximal promoter was assessed by
ChIP with an anti-di-methylated H3-K9 antibody, whereas distal −3038
amplicon upstream of p21 promoter was immunoprecipitated as a control.
Result represents means ± S.D. from three independent immunoprecipita-
tions. *, p = 0.28; **, p = 0.005; †, p = 0.025; and †, p = 0.035 between
corresponding samples.

FIGURE 7. The KAP1 sumoylation-mimetic, SUMO-1-KAP1, desensitizes
Dox-induced breast cancer MCF-7 cell death. A, MCF-7 cells were tran-
siently transfected with FLAG-KAP1 and GFP-SUMO1 and were treated with 1
µM Dox for different time periods, as indicated. Immunoprecipitation and
immunoblotting were then performed with an antibody against FLAG tag.
Quantitation was done with Bio-Rad Quantity One volume report program.
After normalization with transfection efficiency, the relative KAP1 sumoyla-
tion level is calculated by designating the individual level of sumoylated KAP1
prior to DOX treatment as 1. B, MCF-7 cells were transiently transfected with
various KAP1 expression constructs, as indicated. These transfected cells
were treated with 2.5 µM Dox in growth medium for 3 days. Cell survival was
then measured by MTT assays. Results represent means ± S.D. from three
independent experiments. *, p = 4.5e−5; **, p = 0.0065; †, p = 0.13; and ‡,
p = 1.83e−7 between corresponding samples.
H3-K9 methylation, and 2) the decrease in KAP1 sumoylation is conceivably required for Dox to elicit cell growth-inhibitory effect.

DISCUSSION

The presented studies expanded our understanding of KAP1 biology, as well as perhaps the role of KAP1 (de)sumoylation in regulating Dox-induced p21 expression. Herein, we report that KAP1 is a sumoylation target, and its sumoylation level is transiently decreased upon the exposure to chemotherapeutic agent Dox. Our findings suggest that de-sumoylation or sumoylation of KAP1 defines the extent to which Dox induces the expression of cell cycle regulator p21 via ZBRK1-KAP1 response element and subsequent breast cancer MCF-7 cell death. We further demonstrate that sumoylated KAP1 decreases H3-K9 and H3-K14 acetylation and augments H3-K9 methylation at the p21 promoter, and de-sumoylated KAP1 renders an inverse methylation and acetylation profile. In addition, this represents the first demonstration, to our knowledge, that (de)sumoylation governs KAP1 function and modulates, at least in part, the effect Dox treatment in MCF-7 cells.

We first identified and verified that KAP1 is subjected to sumoylation modification (Fig. 1). By using site-directed mutagenesis and in vivo sumoylation assays, we determined that Lys-554, Lys-779, and Lys-804 are the prime sumoylation sites in KAP1 (Fig. 2). Among these lysines, although K779R or K804R mutations of KAP1 result in a decrease of KAP1 overall sumoylation, K554R mutation not only reduces high molecular mass sumoylation species but specifically abrogates the putative di-sumoylated KAP1 (Fig. 2B). The exact mechanism underlying this observation is still unclear. We speculate that the sumoylation of lysines other than Lys-554 is dependent on that of Lys-554. Alternatively, the conjugation of SUMO-1 to Lys-554 is capable of being poly-sumoylated. Although no sumoylation consensus motif can be found in SUMO-1, it has been reported that SUMO-1 could form polymeric sumoylation chain (34), supporting this possibility. Another interesting observation is that sumoylation can take place on consensus sequences in both orientations; Lys-554 is in the N to C termini direction of ΨKX(E/D), where Lys-779 and Lys-804 are positioned in a reverse orientation of ΨKX(E/D).

Based on reports from several proteomic studies on the global sumoylation, a large portion of sumoylation substrates are found to be located in the nucleus or nucleolus (35–38). Further examination of these substrates reveals that many of them are transcription factors or cofactors. In most cases, sumoylation of these transcription factors and cofactors enhances their transcriptional repression capability on their target genes. Currently, the one or more molecular mechanisms by which conjugated SUMO elicits transcriptional repression remain largely unclear. Recent studies of the transcription co-activator p300 and transcription factor Elk-1 revealed that sumoylation mediates the recruitment of histone deacetylase 6 and histone deacetylase 2 to the cell-cycle-regulated domain 1 of p300 and the repression domain of Elk-1, respectively, leading to SUMO-dependent transcriptional repression (5, 39, 40). In this study, we further demonstrate that sumoylation of KAP1 leads to an enhanced H3-K9 methylation (Fig. 6B).

SETDB1, possessing H3-K9 methylase activity, has been reported to interact with KAP1 (23). Neither SETDB1 nor KAP1 binds to specific DNA sequences, suggesting that the observed increase in p21 de novo H3-K9 methylation is a result of protein-protein interaction via ZBRK1. Although the interaction between ZBRK1 and KAP1 is independent of KAP1 sumoylation (Fig. 2), the ZBRK1-KAP1-mediated histone methylation is, at least in part, dependent upon KAP1 sumoylation. In addition, SETDB1 is reported to interact with histone deacetylases 1/2 (41), consistent with the level of H3-K9 and H3-K14 acetylation correlating inversely with H3-K9 methylation level (Fig. 6).

Data base and literature searches suggested the presence of conserved ZBRK1-KAP1 binding motifs in a number of genes other than p21, including gadd45α, bax (18), puma, and noxa,4 which are involved in cell cycle regulation or cell death pathways. Therefore, it is not surprising to note that the transfection of a de-sumoylase-resistant SUMO-1-KAP1 results in a marked reduction of Dox-induced cell growth inhibition (Fig. 7B). Presumably, SUMO-1-KAP1 fusion protein, which mimics sumoylated KAP1, could have inhibited the transcriptional induction of all these genes, thus desensitizing cell death, whereas KAP1(3K/R), analogous to de-sumoylated KAP1, might have de-repressed the transcription of all these genes, therefore curtailing cell viability, even in the absence of Dox treatment. We have attempted to provide evidence that endogenous KAP1 is sumoylated in cells prior to Dox treatment, thus silencing the transcription of those DNA damage-responsive genes. Unfortunately, we realized that the detection of KAP1 sumoylation by endogenous SUMO-1 could be difficult without overexpression of SUMO-1. Obviously, elucidating the fraction of endogenous KAP1 is sumoylated in different cell contexts will be another intriguing future work.

Taking together these results from p21-reporter assays and ChiP experiments, we conclude that the optimal activation of p21 transcription by Dox, but not by MMS, requires the down-regulation of KAP1 sumoylation level. Given that Dox is a known topoisomerase II inhibitor that induces DNA double strand breaks, whether this modulation of KAP1 sumoylation process is mediated by the effectors of DNA damage signaling pathway responding specifically to DNA double strand breaks warrants further investigation. Conceivably, the reduction of KAP1 sumoylation level by Dox treatment (Fig. 7A) could be a result of activation of de-sumoylase and is currently under investigation.

In addition, DNA CpG methylase DNMT3 also interacts with SETDB1 (42), making it possible that KAP1 sumoylation also promotes DNA methylation in the p21 promoter. Whether sumoylated KAP1 could interact with additional gene-silencing machinery through SUMO-dependent protein-protein interaction remains to be tested.

It is worth noting that Waf1/MDM2 and its colleagues (28) have reported that the activation of p21 transcription is largely dependent on p53 activity. The lack of p21 induction by Dox in p53-defective T47D cells (Fig. 3B) supports this notion. How-

4 Y. K. Lee and D. K. Ann, unpublished observation.
ever, it was intriguing to observe that the activation of p21 transcription by Dox, which activates p53 expression, is abrogated by SUMO-1-KAP1 fusion protein in MCF-7 cells. Our approach utilizing si-KAP1 or si-ZBRK1 (Fig. 3C) provided additional support that KAP1-ZBRK1 complex suppresses the basal p21 expression. By the same token, it is tempting to speculate that SUMO-1-KAP1 represses Dox-induced p21 expression by inhibiting p53 transcription factor activity but not p53 expression through, for example, the de-acetylation of p53. Alternatively, the de-sumoylation of KAP1 could be a downstream event of p53 activation, and SUMO-1-KAP1 functions in a dominant-negative manner on Dox-mediated p21 induction.

In summary, we have demonstrated that the ZBRK1 response elements at the p21 promoter are occupied by KAP1-ZBRK1 complex, regardless of KAP1 sumoylation status. Functionally, sumoylation is required for KAP1 full transcription repressor activity. Taken together, our results suggest a more detailed scenario regarding Dox-mediated p21 transcriptional induction. In this process, the ZBRK1-KAP1 complex is first recruited to p21 promoter through ZBRK1 response elements. Histone tails are then subjected to repressive modifications, and p21 transcription is repressed, presumably through H3K9me3 recruitment to sumoylated KAP1. Upon Dox exposure, de-sumoylated KAP1 accumulates (without affecting the occupancy of ZBRK1 response elements) and histone acetylation is recruited to the p21 promoter, leading to the activation state of p21 transcription. It will be of interests to examine whether KAP1 sumoylation is enhanced in cancer or other (patho)physiological conditions or regulated by other therapeutic agents.

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