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Abstract

Poly(ADP-ribose) polymerase 1 (PARP1) is a chromatin-associated nuclear protein and functions as a molecular stress sensor. At the cellular level, PARP1 has been implicated in a wide range of processes, such as maintenance of genome stability, cell death, and transcription. PARP1 functions as a transcriptional coactivator of nuclear factor B (NF-B) and hypoxia inducible factor 1 (HIF1). In proteomic studies, PARP1 was found to be modified by small ubiquitin-like modifiers (SUMOs). Here, we characterize PARP1 as a substrate for modification by SUMO1 and SUMO3, both in vitro and in vivo. PARP1 is sumoylated at the single lysine residue K486 within its automodification domain. Interestingly, modification of PARP1 with SUMO does not affect its ADP-ribosylation activity but completely abrogates p300-mediated acetylation of PARP1, revealing an intriguing crosstalk of sumoylation and acetylation on PARP1. Genetic complementation of PARP1-depleted cells with wildtype and sumoylation-deficient PARP1 revealed that SUMO modification of PARP1 restrains its transcriptional coactivator function and subsequently reduces gene expression of distinct PARP1-regulated target genes. Messner, S., Schuermann, D., Altmeyer, M., Kassner, I., Schmidt, D., Schäfer, P., Müller, S., and Hottiger, M. O. Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function.
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Simon Messner, David Schuermann, Matthias Altmeyer, Ingrid Kassner, Darja Schmidt, Primo Schär, Stefan Müller, and Michael O. Hottiger

ABSTRACT Poly(ADP-ribose) polymerase 1 (PARP1) is a chromatin-associated nuclear protein and functions as a molecular stress sensor. At the cellular level, PARP1 has been implicated in a wide range of processes, such as maintenance of genome stability, cell death, and transcription. PARP1 functions as a transcriptional coactivator of nuclear factor κB (NFκB) and hypoxia inducible factor 1 (HIF1). In proteomic studies, PARP1 was found to be modified by small ubiquitin-like modifiers (SUMOs). Here, we characterize PARP1 as a substrate for modification by SUMO1 and SUMO3, both in vitro and in vivo. PARP1 is sumoylated at the single lysine residue K486 within its automodification domain. Interestingly, modification of PARP1 with SUMO does not affect its ADP-ribosylation activity but completely abrogates p300-mediated acetylation of PARP1, revealing an intriguing crosstalk of sumoylation and acetylation on PARP1. Genetic complementation of PARP1-depleted cells with wild-type and sumoylation-deficient PARP1 revealed that SUMO modification of PARP1 restrains its transcriptional coactivator function and subsequently reduces gene expression of distinct PARP1-regulated target genes. Messner, S., Schuermann, D., Altmeyer, M., Kassner, I., Schmidt, D., Schär, P., Müller, S., and Hottiger, M. O. Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function. FASEB J. 23, 3978–3989 (2009). www.fasebj.org

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Poly(ADP-ribose) polymerase 1 (PARP1) is an abundant nuclear chromatin-associated multifunctional enzyme found in higher eukaryotes that belongs to a family of 5 “bona fide” PARP enzymes (1). PARP1 has an amino-terminal DNA-binding domain (DBD) containing 3 zinc finger motifs, as well as a central automodification domain (AMD), which functions as a target of direct covalent automodification. The carboxyl-terminal catalytic domain polymerizes linear or branched chains of ADP-ribose from the donor nicotinamide adenine dinucleotide (NAD+). ADP-ribose is mainly attached on PARP1, but also other proteins are modified (2). Together, the DBD and the automodification domain allow PARP1 to interact with genomic DNA and chromatin. Although originally characterized as a key factor in DNA single strand-break repair, a wealth of studies over the past decade have demonstrated a role of PARP1 in the regulation of gene expression under basal, signal-activated, and stress-activated conditions (1, 3). Recent studies have highlighted the role of PARP1 in distinct modes of transcriptional regulation and provided novel insight into the cellular signaling systems that interface with PARP1 in the nucleus (4).

The basal enzymatic activity of PARP1 is very low, but it is stimulated dramatically under conditions of cellular stress (2, 3). Activation of PARP1 results in the synthesis of poly(ADP-ribose) (PAR) from NAD+ and the release of nicotinamide as a reaction by-product (1). Following PARP1 activation, intracellular PAR levels can rise 10- to 500-fold (1), caused by a mechanism that remains to be resolved. Very recently, we identified 3 lysine residues in the automodification domain of PARP1 as acceptor sites for auto-ADP-ribosylation (5). PARP1 is the main acceptor for poly(ADP-ribosylation) in vivo, and automodification of PARP1 abolishes its affinity for NAD+ and DNA (5). Remarkably, the same 3 ribosylated lysines (K498, K521, K524) were previously identified as targets for acetylation by the histone acetyltransferase p300 (6). Acetylation of PARP1 has been reported to be important for its transactivation activity (6). Recently, we also highlighted the role of PARP1 as a transcriptional coactivator of hypoxia inducible factor 1-α (HIF1-α). On hypoxic induction of cells, PARP1 was shown to interact with HIF1-α and to regulate the transcriptional activity of HIF1-α-dependent genes (7).

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Another post-translational protein modification in response to cellular stresses is the conjugation of small ubiquitin-like modifiers (SUMOs) (8). SUMOs regulate diverse cellular processes, including cell-cycle progression, genome stability, intracellular trafficking, and transcription (9, 10). In many cases, SUMO conjugation alters localization and/or activity of the substrate by providing a new protein-protein interaction interface. However, in certain cases, SUMO modification can also prevent distinct protein-protein interactions. Mammalian cells express three SUMO paralogs: SUMO2 and SUMO3, which are 96% identical and only differ by three N-terminal residues, and SUMO1, which is 45% identical to SUMO2/3. Moreover, SUMO2/3 proteins are able to form chains, which SUMO1 cannot (11). Although virtually all of the SUMO1 is engaged in conjugates, there is a free pool of the more abundant SUMO2/3 that is utilized when cells are stressed by heat shock or ethanol exposure (12). It is clear that proteins can be modified selectively by SUMO1 and SUMO2/3. Growing evidence suggests that SUMO2/3 and SUMO1 have some unique biological functions (12–14).

SUMO family proteins are conjugated to target lysines via a cascade of the E1-activating enzyme (SAE1/SAE2), the E2-conjugating enzyme Ubc9, and E3 SUMO ligases (8, 10). The SUMO E2 protein Ubc9 often recognizes the consensus sequence ΨKXE/D (where Ψ is a large hydrophobic amino acid, such as isoleucine or valine, and x is any amino acid) in the target protein and catalyzes SUMO conjugation (8). Generally, sumoylation with SAE1/SAE2 and Ubc9 only is rather inefficient, and additional proteins known as SUMO E3 ligases are often required to accelerate this reaction (10). A family of deconjugation enzymes, SENPs, is responsible for the removal of SUMO from target lysines (15), which accounts for the transient nature of this modification. In human cells, six members of this family (SENP1-3 and SENP5-7) have been identified. Importantly distinct members exhibit paralog specificity and show a characteristic subcellular localization, indicating that spatial control is an important regulatory concept of SENP activity.

Several proteomic studies to identify substrates for SUMO conjugation have been reported (16–18). In this context, PARP1 was detected to be sumoylated in HEK293 cells and in K562 cells. SUMO modification of proteins that regulate transcription has been associated with dynamic regulation of gene expression (9, 19). A large number of transcriptional regulators, including transcription factors, cofactors, and chromatin-modifying enzymes, have been found to be substrates of SUMO modification. Generally, a SUMO-modified factor exists in a dynamic distribution between the SUMO-modified and unmodified forms, and although the SUMO-modified form of a protein is often difficult to detect, it can have a great impact on transcriptional activation (9, 10). Sumoylation of transcription factors has generally been correlated with transcriptional repression (9, 10). The specific effects, however, have to be determined experimentally for each case.

In this study, we characterize the modification of PARP1 through SUMO1 and SUMO3. The modification primarily occurs at a lysine residue within the automodification domain of PARP1. The attachment site is close to hotspots of other post-translational modifications of PARP1, such as ADP-ribosylation and acetylation. This proximity led us to investigate a potential crosstalk of these modifications. Sumoylation of PARP1 inhibits its acetylation through p300, and correspondingly, a sumoylation-deficient PARP1 mutant has a higher acetylation status than wild-type PARP1. In addition, a PARP1 sumoylation-deficient cell line exhibits increased transcriptional activity of genes under the control of transcription factor HIF1-α.

MATERIALS AND METHODS

Chemicals and antibodies

Protein G sepharose and glutathione sepharose 4B were purchased from GE Healthcare (Les Ulis, France). 32P-NAD+ and 35S-methionine were from PerkinElmer (Boston, MA, USA). NAD+, trichostatin A (TSA), acetyl-coenzyme A, SAB, ATP, anti-tubulin, and anti-Flag (M2) were obtained from Sigma-Aldrich (Milan, Italy). Anti-p300 (C20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-HA antibody 16B12 from Covance (Evansville, IL, USA). Anti-myc antibody (9E10) was purchased from Roche (Basel, Switzerland), SUMO2/3 (18H8) was obtained from Cell Signaling (Beverly, MA, USA), and His antibody was from Qiagen (Valencia, Spain). Monoclonal CAIX antibody supernatant from hybridoma was a gift from D. Stehl (University of Zurich, Zurich, Switzerland). Anti-PARP1 was produced in this laboratory; anti-acetyl-PARP1 was generated in collaboration with the monoclonal antibody core facility at the EMBL Monterotondo (Monterotondo, Italy).

Cell culture and transfection

HEK293T and K562 cells were grown under standard conditions. Transfections were carried out with the calcium phosphate method. Whole-cell extracts were prepared as described previously (20) with 10 mM NEM and/or HDAC inhibitors (2 μM TSA, 5 mM NAM, 1 mM Na-butyrate). Nuclear extracts were prepared as described previously (6).

Plasmids

The baculovirus expression vectors pQE-TriSystem (Qiagen) and BacPak8 (Clontech, Mountain View, CA, USA) were used for the expression of recombinant proteins in Sf21 insect cells, as described previously (21). pCDNA-myc-SUMO1, myc-SUMO3, and myc-Ubc9 expression plasmids were kindly provided by R. T. Hay (University of Dundee, Dundee, UK). PARP1 was cloned into a pCMV-HA vector with NheI/NheI restriction enzymes. pCU vector with Ubc9 was a kind gift from R. Niedenthal (Hannover Medical School, Hannover, Germany). PARP1 was cloned into pCU with NheI/Smal restriction enzymes, generating a 15-aa linker between PARP1 and Ubc9. pCMV-Flag-p300 was used for expression in mammalian cells. Plasmids for SUMO proteases SENP1-6 were in...
pCI-Flag backbone. Short hairpin RNA was cloned and expressed in pSUPER vector.

Cloning, expression, and purification of recombinant proteins

Wild-type hPARP1 (National Center for Biotechnology Information ID: BC037545) was cloned and expressed as amino-terminal HA-tagged and carboxyl-terminal His-tagged protein. HA-PARP1, HA-PARP1 K486R, p300, SUMO1, SUMO3, and Ubc9 proteins were purified by 1-step affinity chromatography using ProBond resin, according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA, USA). GST-SUMO3, SUMO3, SENP2 (aa 364-569), and SENP2 (aa 364-569 C548S) were cloned in pGEX-vectors, expressed from the pGEX-E1H6 vector and purified by sequential GST beads and nickel beads; GST-cleavage was performed through thrombin, and the recombinant protein was loaded and eluted from nickel beads using standard protocols.

In vitro sumoylation assay

The reaction was carried out in standard SUMO reaction buffer (50 mM Tris-HCl, pH 8.0; 50 mM NaCl; 5 mM MgCl₂; 10% glycerol; and 0.5 mM DTT). 5 mM ATP was added to start the reaction. Incubation time was 30 min at 30°C, unless otherwise indicated. The final concentration of proteins was 100 nM for SAE1/SAE2, 500 nM Ubc9, and otherwise indicated. The final concentration of proteins was 10% glycerol; and 0.5 mM DTT). 5 mM ATP was added to start the reaction. Incubation time was 30 min at 30°C, unless otherwise indicated. The final concentration of proteins was 100 nM for SAE1/SAE2, 500 nM Ubc9, 5 μM SUMO1/SUMO3, and 500 nM HA-PARP1.

Purification of sumoylated PARP1

The sumoylation reaction was 15× scaled up, and the incubation time was increased to 120 min at 30°C. Instead of SUMO3, a GST-tagged SUMO3 at a final concentration of 10 μM was used. After sumoylation, the sample was diluted with 2× the volume with SUMO-purification buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; and 1 mM DTT) and bound to glutathione sepharose beads. After 60 min of incubation on rolls at 4°C, the supernatant was washed away with the same buffer, and 2 U of PreScission protease was added to the beads and incubated 16 h at 4°C. The supernatant was used for experiments with sumoylated PARP1.

Desumoylation of PARP1 in vitro

Purified sumoylated PARP1 was subjected to active recombinant SENP2 (aa 364-569) or inactive SENP2 (aa 364-569 C548S) treatment in SUMO-purification buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; and 1 mM DTT) for 15 min at 30°C with a concentration of 10 ng SENP2/μL.

³²P-NAD automodification

Sumoylated or desumoylated PARP1 in SUMO-purification buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; and 1 mM DTT) was supplemented with 4 mM MgCl₂ and 5 pmol of annealed double-stranded oligomer (5’-GGAATTCC-3’). The reaction was started by adding ³²P-NAD⁺ at a final concentration of 100 nM NAD⁺. Automodification was allowed for 5 min at 30°C. Reactions were stopped by the addition of SDS-PAGE-loading buffer and boiling for 5 min at 95°C. Samples were subjected to SDS-PAGE, followed by detection of automodification by autoradiography.

PAR detection by silver staining

Following synthesis of PAR in the presence of 400 μM NAD⁺ and 5 pmol EcoRI linker DNA for 20 min, PAR chains were purified and separated by modified DNA sequencing gel electrophoresis, as described previously (22).

Immunoprecipitation and nickel-NTA pulldown

Sumoylated or desumoylated PARP1 was bound to protein G sepharose beads with anti-HA antibody in SUMO-purification buffer. The beads were washed and adjusted to IP buffer (50 mM Tris- HCl, pH 8.0; 100 mM NaCl; 0.25% Nonidet P-40; and 1 μg/ml protease inhibitors). Recombinant p300 (2 μg) was added to the beads and incubated for 2 h at 4°C on rolls. Washing of the beads with the same buffer removed unbound p300. Immunoprecipitation of nuclear extracts was done with HA antibody with IP-binding buffer (20 mM HEPES, 150 mM NaCl, and 0.25% Nonidet P-40; 1 μg/ml protease inhibitors, and HDAC inhibitors (2 μM TSA, 5 mM NAM, 1 mM Na-butyrate). The salt concentration was increased with 50 mM KCl for washing steps. Elution of bound proteins was done with SDS-PAGE loading buffer and boiled for 5 min at 95°C. Nickel-NTA pulldown was done as described previously (23).

HAT Assay

Sumoylated or desumoylated PARP1 was subjected to in vitro acetylation assay with recombinant p300 as described elsewhere (24).

Knockdown and complementation of PARP1 in K562 cells

Generation of viruses and transduction of cells was done as described earlier (25). shRNA was cloned into pRDI vector and transduced to K562 cells. The short hairpin RNA was designed against 5’ UTR region of PARP1 mRNA. Transduced cells were selected through puromycin resistance gene. Complementation of cells was done with pRRL-myc-PARP1 vectors containing a blasticidin resistance marker and subsequently selected with this antibiotic.

RNA preparation

Total RNA was isolated from 3 biological replicates of complemented K562 cells with the Total RNA Isolation kit (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription was achieved with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

Quantitative PCR

Total reverse-transcribed cDNA from untreated or treated K562 cells was used for q-PCR with primers against carbonic anhydrase IX, LOXL2, and Pdk1. Amplification products were analyzed by SYBR Green (Quintace, London, UK), and ribosomal protein L28 was used to normalize for differences in RNA input. Rotor-Gene3000A (Qiagen, Basel, Switzerland) was used to perform the real-time PCR reactions.
RESULTS

PARP1 is sumoylated in vivo

Because PARP1 was identified as a SUMO modification target in proteomic studies, we aimed to confirm that PARP1 is indeed sumoylated in vivo. HA-tagged PARP1 was coexpressed with myc-tagged SUMO1 or SUMO3 in HEK293T cells, and extracts were analyzed by Western blot. Ectopic expression of SUMO1 or SUMO3 per se induced the modification of a multitude of proteins (Fig. 1A, bottom). Expression of SUMO induced a higher molecular form of PARP1 (depicted as Su-PARP1), which was more prominent in the presence of SUMO3 as compared to SUMO1, suggesting that PARP1 is preferentially conjugated with SUMO3 (Fig. 1A). Expressing His-tagged SUMO, we could enrich an anti-PARP1-reactive species on Ni-NTA beads, thus validating that the higher molecular form corresponds, indeed, to a covalent SUMO-PARP1 conjugate (Fig. 1B). Only one distinctive band of sumoylated PARP1 was detected, suggesting that PARP1 is monosumoylated at a single lysine residue under the tested conditions. Similar results were obtained when PARP1 was expressed as fusion protein with Ubc9/E2 conjugation protein, although the overall modification rate was clearly enhanced (Supplemental Fig. 1A). Mutation of the catalytic cysteine of the fused Ubc9 resulted in a strong reduction of the modification, indicating that the Ubc9 fused to PARP1 catalyzes the sumoylation of PARP1 (Supplemental Fig. 1B). Immunoprecipitation of this fusion protein in extracts of cells expressing myc-tagged SUMO3 and subsequent Western blot analysis using an anti-myc antibody revealed SUMO moieties on PARP1, providing additional evidence for covalent modification of PARP1 with SUMO (Supplemental Fig. 1C). To test whether PARP1 would bind to SUMO noncovalently via a SUMO-interacting motif (SIM), GST pulldowns were performed with conjugation-deficient SUMO1-4 and RelA/p65 as a positive control (Supplemental Fig. 2A–C). Although PARP1 was able to interact with RelA/p65, no interaction was detectable with GST or all tested SUMOs. Thus, we conclude that PARP1 is covalently modified by SUMO.

SENP1 and SENP3 are able to desumoylate PARP1

SUMO proteases are known to reverse sumoylation of proteins. To test whether SUMO proteases act on

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**Figure 1.** PARP1 is sumoylated in vivo. A) HA-PARP1, myc-SUMO1, or myc-SUMO3 expression plasmids were transfected in HEK293T. Whole-cell extracts were taken in presence of 10 mM NEM and resolved on SDS-PAGE and subsequently analyzed through Western blotting with the indicated antibodies. Sumoylated PARP1 is indicated as Su-PARP1. B) Denaturing nickel pulldown of whole-cell extracts from transfected HEK293T cells. Detection of sumoylated PARP1 was performed with anti-PARP1 antibody. C) HEK293T cells were transfected with a mix of HA-PARP1, His-SUMO3, and myc-Ubc9 expression plasmids and the indicated plasmids for expression of SUMO proteases SENP1 and SENP3, or the catalytically inactive mutant counterpart, respectively. Expression levels of the SENPs were monitored with anti-Flag antibody. D) HEK293T cells were cotransfected with flag-tagged expression plasmids for SENP1 or SENP3 and pSUPER vector with shRNAs against SENPs. Knockdown efficiency after 28-h expression was examined with anti-Flag antibody. E) Knockdown of SENP1 and SENP3 was achieved as in D, but in addition, a mix of HA-PARP1, His-SUMO3, and myc-Ubc9 expression plasmids and the indicated plasmids for expression of SUMO proteases SENP1 and SENP3, or the catalytically inactive mutant counterpart, respectively. Expression levels of the SENPs were
SUMO-modified PARP1, we coexpressed wild-type or catalytically inactive SENP1 and SENP3 with PARP1 and SUMO3 in HEK293T cells (Fig. 1C). This showed that coexpression of catalytically active SENP1 and SENP3 deconjugated SUMO3 from PARP1 (Fig. 1C). Correspondingly, knockdown of SENP1 and SENP3 with transiently transfected shRNAs (Fig. 1D) resulted in the accumulation of sumoylated PARP1, as compared to the control (Fig. 1E), indicating that SENP1 and SENP3 can act on PARP1-SUMO conjugates at physiological expression levels. Taken together, our results illustrate that PARP1 is preferably modified by SUMO3 and desumoylated by the isopeptidases SENP1 and SENP3. Furthermore, sumoylation of PARP1 seems thus to be a transient and reversible modification.

PARP1 is sumoylated at K486 in vitro and in vivo

The consensus sumoylation site sequence is ΨKxE/D (8). As determined by the SUMOsp analysis program (http://sumosp.biocuckoo.org), the highest score matched to lysine 486 in human PARP1 (Fig. 2A), which is located in proximity to previously described sites of acetylation and ADP ribosylation. To confirm PARP1 sumoylation in vitro and to map the modification site, we established an in vitro sumoylation system reconstituted with recombinant human E1 (SAE1/SAE2 heterodimer), E2 (Ubc9), wild-type SUMO1, or SUMO3 and wild-type PARP1 (Fig. 2B). PARP1 sumoylation was efficiently reconstituted in vitro with purified proteins: reactions containing all components produced slower migrating PARP1 forms, consistent with conjugated SUMO moieties. Attachment of a single moiety was detected with low E2 concentrations (running at ~140 kDa), whereas multiple SUMO moieties were attached only at higher E2 concentrations (Fig. 2B). One additional band between 120 and 140 kDa was observed at elevated Ubc9 concentrations and likely represents the modification of a degradation product of PARP1 by SUMO3. To test for putative sumoylation sites in PARP1, lysine 486 of PARP1 was substituted with arginine (PARP1 K486R) and analyzed in vitro. Substitution did completely prevent the sumoylation of PARP1 in vitro with SUMO1, SUMO3, or GST-tagged SUMO3, as monitored by Western blot analysis (Fig. 2C, D), indicating that K486 is the major SUMO acceptor site of PARP1.
To verify sumoylation of PARP1 at K486 \textit{in vivo}, we coexpressed wild-type or the K486R mutant of PARP1 with myc-tagged SUMO3. Sumoylation of wild-type PARP1 could be detected but not of the K486R mutant (Fig. 2E), confirming K486 as the main sumoylated residue \textit{in vivo}.

**Sumoylation of PARP1 does not affect its ADP ribosylation activity**

To explore a potential interplay of PARP1 sumoylation with PARP1 function, we modified the established \textit{in vitro} sumoylation system to purify sumoylated PARP1. A large-scale sumoylation reaction was performed with GST-tagged SUMO3, E1–E2 enzymes, and HA-PARP1, followed by GST affinity purification and subsequent protease digestion to remove the GST tag and to purify Su-PARP1, specifically modified at K486 (Fig. 3A). On purification, only Su-PARP1 could be detected, indicating that no unmodified PARP1 was in the purified complex (Fig. 3B). Because PARP1 was described to form a homodimer, this result suggests that both subunits are equally accessible for SUMO-conjugation. PARP1 was also efficiently sumoylated in the presence of double-stranded DNA ends, suggesting that binding of PARP1 to DNA does not affect its sumoylation \textit{in vitro} (Supplemental Fig. 3A). Similarly, Su-PARP1 was still able to bind specifically to DNA fragments that mimic damaged DNA (Supplemental Fig. 3B).

To determine whether sumoylation regulates the intrinsic ADP-ribosylation activity of PARP1, mono-ADP-ribosylation of purified Su-PARP1 was measured using an \textit{in vitro} ADP-ribosylation assay in the presence of 100 nM $^{32}$P-NAD$^+$. Sumoylation of PARP1 still allows its mono-ADP-ribosylation activity (Fig. 3B, lane 1). To compare the extent of ADP ribosylation, Su-PARP1 was either desumoylated by recombinant SENP2 (aa 364-569) before or after ADP ribosylation took place (Fig. 3B, lanes 2 and 3). Quantification of the detected radioactivity confirmed that both proteins were modified to the same extent. Similar experiments were repeated with 400 \mu M NAD$^+$, a concentration that allows detection of poly(ADP-ribosyl)ation of PARP1. PAR polymers synthesized by Su-PARP1 and desumoylated PARP1 were isolated and analyzed with silver-stained PAGE (Fig. 3C). Neither the amount nor the distribution of freshly synthesized PAR was altered by sumoylated PARP1, indicating that SUMO modification neither alters the ability of PARP1 to initiate nor to extend PAR synthesis. In addition, overexpression of SUMO3 in HEK293T cells \textit{per se} did not stimulate PAR formation (Supplemental Fig. 4A), although PARP1 is sumoylated under these conditions (see Fig. 1A, last lane). Furthermore, H$_2$O$_2$-treated cells showed PAR formation (Supplemental Fig. 4B), which was independent of SUMO3 levels, indicating that SUMO modification of PARP1 does not enhance its poly(ADP-ribosyl)ation activity. To test the possibility of differential localization of PARP1 upon sumoylation, we overex-

![Figure 3. Sumoylation of PARP1 does not affect its ADP-ribosylation activity.](image-url)
pressed SUMO3 in cells and monitored PARP1 localization by immunofluorescence. However, we did not observe differential localization of PARP1 within the nucleus upon ectopic SUMO3 expression (Supplemental Fig. 4C).

We recently reported that PARP1 is auto-ADP-ribosylated at several lysines adjacent to the identified sumoylation site (26). To exclude that ADP-ribosylation would affect sumoylation, we mono-ADP-ribosylated PARP1 in vitro with radioactive NAD$^+$ and subsequently sumoylated the labeled PARP1 fraction (Fig. 3D). PARP1 was sumoylated in an ATP-dependent manner independent of its ADP-ribosylation. Consistently, in vivo treatment of cells with the PARP inhibitor 3-amino-benzamide (3AB) did not affect sumoylation (data not shown). Taken together, this suggests that although the sumoylated and ADP-ribosylated lysines are rather close within the PARP1 amino acid sequence, their modifications do not interfere with each other.

**Sumoylation counteracts p300-induced acetylation of PARP1**

As p300 is critical for PARP1 transcriptional coactivation and acetylates PARP1 at distinct lysines (6), we first examined whether acetylated PARP1 would still be sumoylated in vitro. Acetylation of PARP1 was monitored with a specific anti-acetyl PARP1 (E4) antibody (Supplemental Fig. 5A), while sumoylation was assessed by the migration difference between unmodified and Su-PARP1. Acetylation with the indicated control and subsequent addition of sumoylation enzymes, followed by the sumoylation reaction, revealed that similar to the mono-ADP-ribosylated PARP1, acetylated PARP1 could also be efficiently modified with SUMO (Fig. 4A, lane 4).

Moreover, we tested whether sumoylation of PARP1 would affect acetylation. Purified Su-PARP1 or de-sumoylated PARP1 by recombinant SENP2 was both incubated with p300 and acetyl-CoA in vitro. Western blot analysis using the specific anti-acetyl PARP1 (E4) antibody revealed that PARP1 is acetylated only when PARP1 was desumoylated prior to acetylation (Fig. 4B, lane 2), suggesting that the SUMO-modification inhibits p300-mediated PARP1 acetylation. To substantiate this, we examined protein interactions with p300, PARP1, or Su-PARP1. p300 could interact efficiently with PARP1 but not with Su-PARP1, as demonstrated by coimmunoprecipitation of p300 (Fig. 4C). Thus, the absence of detectable acetylation of Su-PARP1 (see Fig. 4B) suggests that SUMO modification at K486 prevents p300-mediated acetylation of PARP1, likely because of steric hindrance of the bulky SUMO conjugate blocking p300 binding and acetylation at the adjacent lysine residues. To explore whether the inhibitory effect of PARP1 sumoylation on acetylation is also observed in vivo, we coexpressed wild-type PARP1 or the sumoylation-deficient PARP1 mutant (K486R) with p300 and monitored acetylation with the E4 antibody on Western blots. This revealed lower levels of acetylation for the wild-type PARP1 compared to the sumoylation-defi-

**Figure 4. Sumoylation counteracts p300-induced acetylation of PARP1.** A) In vitro acetylation of PARP1 through p300 was done in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of acetyl-CoA. Proteins necessary for sumoylation (E1, E2, SUMO3) were added after the acetylation reaction. Proteins were separated with SDS-PAGE and analyzed with anti-PARP1 and anti-acetyl-PARP1 (E4) antibody. B) In vivo acetylation reaction was done with p300 and sumoylated PARP1 (lane 1) or PARP1 desumoylated prior to the acetylation reaction (lane 2). After acetylation reaction, sumoylated PARP1 was desumoylated with SENP2 (lane 1), which allowed direct comparison of acetylation status of equal amounts of PARP1. C) Coimmunoprecipitation of p300 with PARP1 was carried out on protein G sepharose beads, using HA antibody to capture HA-tagged PARP1, either sumoylated (Su-PARP1) or desumoylated (deSu-PARP1). As control, only the HA antibody was bound to the matrix (no PARP1). Beads were incubated with purified p300, and the unbound fraction was removed by extensive washing. D) HEK293T cells were transfected with either HA-PARP1 wt or HA-PARP1 K486R mutant along with p300 expression plasmid. Cells were incubated with HDAC inhibitors 2 h prior to lysis, and HDAC inhibitors were present at all steps of manipulation. Nuclear extracts were taken and subjected to immunoprecipitation using an HA antibody. After SDS-PAGE, proteins were detected by anti-acetyl-PARP1 (E4), anti-HA, or anti-p300 antibody.
cient PARP1 mutant (Fig. 4D). Together, these results provide evidence for a crosstalk between these modifications.

The sumoylation-deficient K486R PARP1 mutant exhibits higher coactivator function compared to wild-type PARP1

To explore a possible mechanism by which sumoylation affects PARP1-dependent transcriptional coactivator function in vivo, we first knocked down endogenous PARP1 protein levels in K562 cells with an shRNA approach directed against the untranslated region of PARP1’s mRNA and subsequently complemented these cells with wild-type or sumoylation-deficient K486R PARP1 mutant (Fig. 5A, B). The expression levels of the complemented cells were comparable to the endogenous wild-type counterpart. Hypoxia treatment of these cells for 28 h and subsequent profiling of the gene expression of defined hypoxia-inducible genes revealed that certain genes, such as CAIX, LOXL2 or Pdk1, are dependent on PARP1, but only a subset was affected by the sumoylation-deficient K486R mutation (Fig. 5C). Similar results were obtained when PARP1−/− mouse lung fibroblasts were complemented with wild-type or sumoylation-deficient K486R PARP1 mutant and stimulated by the hypoxia-mimicking drug ciclopiroxolamine (Supplemental Fig. 5B). Sumoylation-deficient K486R PARP1 mutant not only enhanced CAIX mRNA levels in K562 cells, but also CAIX protein levels in vivo (Fig. 5D). Furthermore, hypoxia treatment of K562 cells very strongly correlated with protein sumoylation and enhanced SUMO modification of PARP1 in HEK293T cells (Supplemental Fig. 5C, D). Thus, we conclude that sumoylation of PARP1 reduces its coactivator activity and thus regulates gene expression in vivo.

Figure 5. Sumoylation-deficient K486R PARP1 mutant exhibits higher coactivator function compared to wild-type PARP1. A) Stable knockdown of PARP1 in human chronic myelogenous leukemia cell line K562 using a lentiviral system. shRNA against PARP1 targets the 5′ untranslated region of PARP1 mRNA. B) Stable complementation of PARP1 with either pRRL-empty vector, myc-tagged wild-type PARP1, and sumoylation-deficient PARP1 K486R mutant. C) Hypoxic induction of complemented K562 cells at 0.2% O2 for 28 h. RNA was isolated, and the quantitative PCR was done using primers for carbonic anhydrase IX (CAIX), lysyl oxidase-like 2 (LOXL2), and pyruvate dehydrogenase kinase, isoenzyme 1 (Pdk1) and normalized by using L28 ribosomal protein. Normoxic samples were set to 1; fold induction of hypoxic samples is depicted. Three biological replicates are shown. Statistical analysis was done with unpaired t test between biological replicates. Data are represented as means ± se. D) Complemented K562 cells were exposed for 29 h to 0.2% O2 hypoxia. Whole-cell extracts were prepared and Western blotted with monoclonal anti-CAIX antibody and anti-tubulin antibody.
DISCUSSION

The aim of this study was to characterize and investigate the role of PARP1 sumoylation in the cellular context. We provide biochemical and cellular evidence for SUMO modification of PARP at lysine 486 within its automodification domain. Mutation of K486 enhances the transcriptional activity of PARP1, suggesting that sumoylation restrains its transcriptional activity.

PARP1 is covalently sumoylated

Noncovalent interactions of proteins can occur through SUMO interaction motifs (SIMs) (27). Proteins like the DNA repair enzyme TDG and the tumor suppressor PML were shown to interact with SUMOs via SIMs, and such interactions were associated with important biological activities (28–30). Although PARP1 exhibits several putative SIMs, we did not observe any direct noncovalent interaction of PARP1 with SUMOs, indicating that the interaction of PARP1 with SUMO is exclusively covalent. Pulldown experiments of sumoylated PARP1 under denaturating conditions and site-directed mutagenesis revealed that sumoylation of PARP1 is indeed a covalent and site-specific modification. A possible involvement of SUMO E3 ligases for the sumoylation of PARP1 needs further investigations. Although PIAS family members are attractive candidates, overexpression of different PIAS proteins did not enhance PARP1 sumoylation (data not shown).

Notably, we observed only monosumoylation of PARP1 in vivo, but do not exclude that under specific conditions, PARP1 may also be polysumoylated. In support of this idea, heat shock has been reported to induce a pattern of PARP1 sumoylation, which would be consistent with polysumoylation (18). Understanding the balance between monosumoylation and poly-sumoylation of PARP1, as well as their functional differences will remain an exciting issue.

Crosstalk between sumoylation and other post-translational modifications

The crosstalk of post-translational modification systems is an emerging concept (31). Sumoylation of target proteins can be regulated through crosstalks with other post-translational modification events. Phosphorylation, for instance, was shown to regulate SUMO conjugation through a highly conserved motif, which is called phosphorylation-dependent sumoylation motif (PDSM) (32). The PDSM motif, which contains a SUMO consensus site and an adjacent proline-directed phosphorylation site (ΨKxExxSP, where Ψ represents large hydrophobic residue and x is any amino acid), regulates phosphorylation-dependent sumoylation of multiple transcription factors (33–35). Lysine residues are targeted by several other post-translational modifications, including ubiquitination, acetylation, methylation, and ADP-ribosylation. It has been documented that SUMO conjugation can occur on the same lysine residue as ubiquitination or acetylation in some proteins. For example, the competition between sumoylation and ubiquitination of the same lysine regulates the stability of IkBα (36), whereas in other cases, sumoylation acts as a recognition signal for a ubiquitin ligase (37). An interplay between sumoylation and acetylation has been observed in the regulation of proteins, such as MEF2, the core histones, and hypermethylation in cancer 1 (HIC1) (38, 39). In the case of MEF2, the sumoylation-acetylation switch is regulated by phosphorylation (40). These studies demonstrate the importance of signaling crosstalk in the regulation of protein sumoylation.

Mechanisms of SUMO-mediated repression of PARP1 coactivator function

First, sumoylation may directly affect PARP1’s binding to DNA by promoting its dissociation from specific chromatin regions. This possibility seems unlikely, since sumoylation of PARP1 did not alter its ability to recognize and bind damaged DNA in vitro. Second, SUMO modification could also affect enzymatic activities of PARP1, which is important for gene expression. Also, this seems unlikely, since we have shown that SUMO modification of PARP1 does not interfere with DNA-dependent ADP-ribosylation activity in vitro. In addition, increased SUMO3 levels do not correspond to elevated poly(ADP-ribosyl)ation in cells on hydrogen peroxide-induced DNA damage, suggesting that sumoylation of PARP1 does not have a stimulatory effect on its enzymatic activity. However, it was shown that poly-(ADP-ribosyl)ation is not required for NF-κB-dependent gene expression (41). Neither the enzymatic activity of PARP1 nor its binding to DNA was required for full activation of NF-κB in response to various stimuli in vivo when tested on transiently transfected reporter plasmids (21, 42). Consistently, the enzymatic activity of PARP1 was not required for full transcriptional activation of NF-κB in the presence of the histone acetyltransferase p300 (6). Because sumoylation of PARP1 inhibits its acetylation at adjacent lysine residues and because these residues are also targets of ADP-ribosylation, a potential acetylation-ADP-ribosylation switch, which is controlled through sumoylation of PARP1, is very likely. Third, the SUMO modification could promote or inhibit protein-protein interactions through protein complex formation. This scenario seems to be the most relevant for PARP1, since the interaction of PARP1 with p300 and subsequent PARP1 acetylation was impaired after sumoylation of PARP1 at K486. This lysine residue lies within the domain of PARP1, which contributes to most protein-protein interactions such as XRCC1 (6). However, we did not observe a general SUMO-dependent inhibition of protein interactions in this region since HIF1-α and XRCC1 binding does not seem to be affected by sumoylation of PARP1 (Supplemental Fig. 5E and unpublished results). In addition to the inhibition of p300
binding. SUMO modification of PARP1 may facilitate the recruitment of a transcriptional corepressor. Currently, several chromatin-modifying enzymes and chromatin-binding proteins have been implicated as effectors of SUMO-mediated repression. For example, SUMO modification of the transcription factor Elk-1 promotes recruitment of HDAC2, associated with histone deacetylation and transcriptional repression of the c-fos promoter (43). Very recently, CoREST1 and Mi2 were identified as SUMO-dependent corepressors, and evidence was provided that CoREST1 binds directly and noncovalently to SUMO2/3, but not to SUMO1 (44, 45). Notably, the aforementioned interaction of PARP1 with PIAS family members could contribute to gene silencing.

Desumoylation of PARP1 by SENP1 and SENP3

We observed that SENP1 and SENP3 are able to catalyze PARP1’s SUMO deconjugation. The nucleoplasmic SENP1 relieves SUMO-dependent repression of Ets1, c-Jun, and the androgen receptor, the latter effect being through desumoylation of histone deacetylase 1 (46). Recent data also implicate the nucleolus in dynamic cycles of sumoylation and desumoylation. For example, nucleolar SENP3 is able to catalyze desumoylation of various proteins in this compartment, with specificity to SUMO2/3 (15, 46). In addition, it seems that SENPs regulate SUMO paralog preference of substrate proteins by deconjugation of specific SUMOs, as shown for RanGAP1 (47). This could also explain the higher steady-state level of SUMO3-modified than SUMO1-modified PARP1.

Only a subset of PARP1-dependent genes are affected by sumoylation

Analyses of the role of SUMO in transcriptional regulation have mainly relied on the use of protein overexpression and transiently transfected reporter genes, which may not give a true reflection of the physiological situation. Therefore, we have established a system where we complement cells depleted from endogenous PARP1 with sumoylation-deficient PARP1 or wild-type PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1 and analyzed the expression of endogenous PARP1. Known HIF1-α-dependent genes with a high induction upon hypoxia were tested. Of these, CAIX and LOXL2 showed increased transcript levels in sumoylation deficient K486R mutant cell line, whereas other genes were solely dependent on PARP1, but not on its sumoylation. Consistent with the qPCR data, the expression levels of CAIX were increased in cells expressing sumoylation-deficient PARP1. Previous studies on PARP1’s coactivator function revealed that this function is heavily dependent on its acetylation through p300. Here, we showed that acetylation is abrogated if the SUMO moiety is present on PARP1. Consistently, the sumoylation-deficient mutant showed a higher acetylation status, which corresponded to higher gene expression status for some genes. Collectively, these data support the mechanistic studies performed in vitro, unraveling an important role of sumoylation in regulating PARP1-dependent transcriptional coactivation through regulation of its acetylation. It is largely accepted that post-translational modifications fine tune and regulate the requirement of certain transcriptional cofactors for gene expression by transcription factors and might thus influence only a subset of genes (48).

Hypoxia-induced gene expression is affected by PARP1 sumoylation

The role of sumoylation in the regulation of hypoxia-induced gene expression and HIF-1α stability is controversial (49). Hypoxia can induce the expression of SUMO1 (50) and an RWD-containing sumoylation enhancer (RSUME) that functions as a promoter of protein sumoylation (51). RSUME expression is induced by hypoxia, which leads to the enhanced sumoylation and stabilization of HIF-1α. Alternatively, a recent study indicates that the hypoxia-induced sumoylation of HIF-1α targets HIF-1α for degradation through the von Hippel–Lindau (VHL) protein-mediated ubiquitin-proteasome pathway (37). Clearly, further studies are needed to clarify these controversial findings on the role of sumoylation in the regulation of HIF-1α stability during hypoxia. In this study, we have investigated the regulation of the known HIF1-α-dependent genes CAIX and LOXL2 and provide novel insights to understand the complex transcriptional regulation of these emerging tumor markers. The expression of these genes is restrained through SUMO modification of the transcriptional coactivator PARP1, indicating that sumoylation of PARP1 dampens HIF1-α signaling for these genes. Thus, this pathway may have an important regulatory role in the regulation of intracellular pH and hypoxia-induced metastasis (52, 53). Moreover, it remains to be elucidated whether sumoylation of PARP1 is also affecting transcription mediated by other transcription factors and whether SUMO modification is associated with the role of PARP1 in several pathophysiological disease models. Further studies of the sumoylation of PARP1 will determine the role of SUMO modification/deconjugation in these pathological states.

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