SUMOylation Attenuates the Function of PGC-1α*

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Peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) is a key coordinator of gene programs in metabolism and energy homeostasis in mammals. It is highly responsive to changes in the cellular environment and physiological status of mammals and regulated by post-translational modifications: acetylation, phosphorylation, and methylation. Here, we show that PGC-1α is covalently modified by small ubiquitin-like modifier (SUMO) 1 protein, an important regulator of signaling and transcription. Conserved lysine residue 183 located in the activation domain of PGC-1α was identified as the major site of SUMO conjugation. Interestingly, the same Lys residue is also a target for acetylation. Therefore, the E185A mutation disrupting the SUMOylation consensus sequence was utilized to show that SUMOylation plays a role in the regulation of PGC-1α function. Our results show that SUMOylation does not have an apparent effect on the subcellular localization or the stability of PGC-1α, but it attenuates the transcriptional activity of the coactivator, probably by enhancing the interaction of PGC-1α with corepressor RIP140. Mutation that abolished the SUMOylation augments the activity of PGC-1α also in the context of PPARγ-dependent transcription. Thus, our findings showing that reversible SUMOylation can adjust the activity of PGC-1α add a novel layer to the regulation of the coactivator.

The coactivator peroxisome proliferator-activated receptor γ co-activator-1α (PPARGC1α or PGC-1α) was first isolated from brown fat (1). It was found to be important in the regulation of mitochondrial function and metabolism. Although the PGC-1α was initially named according to its function as a coactivator for PPAR, it is also able to interact via its N-terminal LXXLL motifs and coactivate with several other nuclear receptors, including estrogen receptor α, thyroid hormone receptor β, and glucocorticoid receptor, as well as other transcription factors, such as FoxO1 (2–7). PGC-1α is regulated by several signaling pathways and post-translational modifications (7). Protein arginine methyltransferase PRMT1 potentiates the coactivator function of PGC-1α by methylating three C-terminal amino acid residues Arg665, Arg667, and Arg669 within its RNA-binding domain (8). Distinct protein kinases exert complex regulation via targeting several sites in PGC-1α. Phosphorylation of Thr177 and Ser538 by AMP-activated protein kinase (AMPK) augments the coactivation function of PGC-1α (9), whereas intermediary protein kinase Akt2/protein kinase B-β-catalyzed phosphorylation of Ser570 can inhibit PGC-1α (10). Phosphorylation of PGC-1α at amino acid residues Thr262, Ser265, and Thr498 by p38 MAPK in turn leads to activation of the PGC-1α, which may at least in part occur via the release of repressor p160 myb-binding protein from PGC-1α (11, 12). Interestingly, the latter phosphorylation sites overlap with two Cdc4 phosphodegrons that, when further phosphorylated at Thr266 by glycogen synthase kinase 3β, can target the protein for ubiquitylation and proteasomal degradation (13). Notably, PGC-1α contains several acetylation sites, specific lysine residues, spanning the entire protein (14). General control of amino acid synthesis 5 (GCN5) acetyltransferase directly acetylates PGC-1α, thereby inhibiting its coactivator activity (15). The inhibition may be due to trafficking of PGC-1α to subnuclear domains where it interacts with transcriptional repressors, such as RIP140. Conversely, deacetylation activity of SIRT1 can keep PGC-1α in an active state and bound to chromatin (14, 16).

In addition to acetylation and methylation, lysine residues can be covalently modified by ubiquitin and ubiquitin-like proteins. Mammals contain three small ubiquitin-like modifier (SUMO) proteins, SUMO-1, -2, and -3, that are composed of ~100 amino acids and can form isopeptide linkages with ε-amino groups of lysines. SUMO-1 is only ~50% identical with SUMO-2/3, whereas SUMO-2 and SUMO-3 are nearly identical (17, 18). Although ubiquitin and SUMOs share a similar three-dimensional fold, their amino acid sequences and surface charge distributions differ considerably. In contrast to the attachment of ubiquitin, SUMO-modified lysine is often found within a minimal consensus motif ψKXE (Ψ is a large hydrophobic residue, and X is any residue) (19). SUMO-1 is not thought to form polymers, whereas SUMO-2 and -3 can form chains. The SUMO conjugation pathway is similar to that of ubiquitin, but distinct E1 (activating enzyme), E2 (conjugase), and E3 (ligase) activities are needed for both processes. SUMOs are first activated in an ATP-dependent fashion by the SAE1 and -2 dimer and subsequently conjugated by Ubc9. PIAS proteins, nucleoporin RanBP2, and polycomb protein Pc2 are able to enhance SUMOylation in a manner that resembles the action of E3 ligases (20). SUMOylation is not a static modification but...
SUMOylation has emerged as a significant regulatory mechanism in cell physiology. Especially in transcription and signal transduction, the modification can affect, in a target-specific fashion, the subcellular and subnuclear localization of the protein, its ability to interact with other proteins, and/or its activity in transcription (reviewed in Refs. 18 and 22). If the same lysine residue can be targeted either by ubiquitin or SUMO-1, the latter mono-modification can protect a protein from being degraded. In contrast, proteins containing polymeric chains of SUMO can interact with SUMO-targeted ubiquitin E3 ligase RNF4, resulting in their ubiquitylation and proteasomal degradation (23, 24). In addition to targeting a plethora of DNA sequence-specific transcription factors, SUMOylation has recently shown to regulate transcriptional coactivators, such as SRC-1, GRIP1, p300, and AIB1, and corepressors, such as NCoR and RIP140 (25–30). Here, we show that reversible SUMOylation can fine-tune the transcriptional activity of PGC-1α without having an apparent effect on its stability.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco's modified Eagle's medium (Dulbecco's modified Eagle's medium) with or without phenol red, fetal bovine serum, and penicillin-streptomycin were purchased from Invitrogen, Hyclone, and EuroClone Ltd., respectively. TransIT-LT1 transfection reagent was from MIRUS Bio Corporation. EZview™ Red Anti-FLAG® M2 affinity gel (F 2426), anti-FLAG M2 mouse monoclonal antibody (F 3165), protease inhibitor mixture (PIC), and N-ethylmaleimide (NEM) were from Sigma-Aldrich. Anti-SUMO-1 antibody (catalogue number 33-2400) and horseradish peroxidase-conjugated secondary antibody were acquired from Invitrogen. Anti-PGC-1α rabbit polyclonal (sc-13067), anti-Gal4 rabbit polyclonal (sc-577), and anti-tubulin mouse monoclonal (sc-5286) antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-HA mouse monoclonal HA.11 antibody and acetylated lysine antibody (9441) were obtained from Nordic Biosite AB and Cell Signaling Technology, Inc., respectively. Protein A-agarose was from Upstate (Millipore Corporation). The fluorescein 5-isothiocyanate-conjugated donkey anti-rabbit, the Rhodamine Red X-conjugated donkey anti-rabbit and anti-mouse secondary antibodies were from Jackson Immunoresearch Laboratories Inc. Goat anti-rabbit IgG (H&L) DyLight™ 800-conjugated secondary antibody, Goat antimouse IgG (H&L) DyLight™ 680-conjugated secondary antibody, nitrocellulose membranes, and detection reagents for ECL were from Pierce. For the in vitro assay TNT® Quick Coupled transcription/translation system and EasyTag™ LT-[35S]methionine were purchased from Promega and PerkinElmer Life Sciences, respectively.

**Plasmid Constructions**—PGC-1α expression vectors, including phosphorylation site mutants pcDNA3.1-F-FLAG-1αT177A/S538A (9) and pcDNA3.1-F-FLAG-1αS3D (where 3D indicates T262D,S265D,T298D) (12), were acquired from Addgene. pcDNA-3.1-F-FLAG-1α (31) and pCMX-Gal4-PGC-1α (1) were point-mutated using QuikChange® II site-directed mutagenesis kit (Stratagene) on amino acids 183, 185, and 645 (numbers correspond to the mouse PGC-1α sequence). The N-terminal Gal4-PGC-1α was produced from pCMX-Gal4-PGC-1α by creation of a stop codon at amino acid 215. pCMV-Myc-SUMO-1 and pCMV-Myc-SUMO-2 were from D. Owerbach (32). pFLAG-SSIP1, pFLAG-ARIP3(PI3Axo), pFLAG-PIAS3, pFLAG-PIASy, and pERE-TATA-LUC have been described (33–35). pcDNA-FH-SENp1 and pcDNA-FH-SENp1C603S (1M) were provided by Dr. D. Bailey (36). pCMV-F-SEnPl and pFLAG-SEnPl were gifts from Drs. Chalkiadaki and Yeh, respectively (37). pcDNA3.1-FLAG-SEnPl and SENP2Ca66S (2M), SENP3C532S (3M), SENP5C713S (5M), and SENP6C1030S (6M) have been described elsewhere (38). pFLAG-SEnPl5 from Deutsches Ressourcenzentrum für Genomforschung GmbH. pSG5-HA-RIP140 was from Dr. E. Treuter. pcDNA3-MKK6b(E) and pcDNA3-Flag-p38α were from J. Han, and pCMX-ERRγ was from P. Aarnisalo. pSG5-mPARγ, pCMV-RXRα and pGL3-TK-(CPTI) were provided by Dr. S. Väisänen and described (39, 40). pG5-LUC and pCMV-β-gal were from Promega and Clontech, respectively.

**Cell Lines, Transfections, and Reporter Gene Assays**—African green monkey kidney (COS-1) cells, and HeLa cells were obtained from the American Type Culture Collection and maintained as instructed. These cells do not contain detectable amounts of endogenous PGC-1α protein. Twenty-four hours before transfection, the cells were seeded onto 6- or 12-well plates, and transfections were carried out using TransIT-LT1 according to the manufacturer's instructions. When nuclear receptors were studied, the cells received fresh Dulbecco's modified Eagle's medium without phenol red containing 2.5% charcoal-stripped fetal bovine serum 4 h before transfection, and vehicle/hormone (1 μM) was added 24 h after transfection. Nicotinamide (10 mM) treatment was added 24 h prior to collection. Forty-eight hours after transfection, the cells were harvested and lysed in reporter lysis buffer (Promega). The cleared supernatants were used for luciferase measurements with luciferase assay reagent (Promega) and a Luminoskan Ascent reader (Bio-Rad Laboratories). The relative LUC activities (i.e. luciferase light units divided by β-galactosidase values) are represented as the means ± the standard deviations. The experiments were done in triplicate and repeated at least three times. For statistical analyses a one-way or two-way analysis of variance followed by a Bonferroni's post test were used. ***, p < 0.001; **, p < 0.01; *, p < 0.05.

**Immunoprecipitation and Immunoblotting**—Cell monolayers were washed with ice-cold phosphate-buffered saline and harvested in phosphate-buffered saline containing 20 mM NEM. For immunoprecipitation, the pelleted cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 15 mM MgCl2, 5 mM EDTA, and 0.5% Triton
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X-100) with PIC and NEM protease inhibitors. The immunoprecipitation was done according to the manufacturer’s instructions for EZview™ Red Anti-FLAG® M2 affinity gel or with anti-Gal4 antibody with protein A-agarose, and the precipitates were eluted in SDS sample buffer. For direct immunoblots, the cell pellets were suspended in SDS sample buffer containing 10 mM NEM with 1:200 PIC and lysed by sonication 2 × 10 s. The samples were heated for 5 min at 95 °C and separated on 7.5% SDS-PAGE gels. The proteins were transferred onto nitrocellulose membranes and visualized by indicated antibody and horseradish peroxidase-conjugated secondary antibody using the enhanced chemiluminescence detection reagents according to the manufacturer’s instructions (Pierce). The immunoblots of immunoprecipitation samples were analyzed with a Li-COR Odyssey infrared imaging system (LI-COR Inc.) according to the manufacturer’s instructions. The kidneys were collected from adult female C57B1 mice and frozen in liquid nitrogen until sample preparation. They were homogenized in radioimmune precipitation assay buffer containing 1:100 PIC in the presence and absence of 20 mM NEM, sonicated, and centrifuged to pellet the debris. After adding 5 × SDS sample buffer, the samples were boiled and analyzed by immunoblotting.

Ni²⁺-Nitrilotriacetic Acid Pull-down—Cell pellets (collected from 2 wells of 6-well plates) were lysed in 0.5 ml of Buffer A (6 mM guanidine HCl, 0.1 mM Na₂HPO₄/NaH₂PO₄, 0.05% (v/v) Tween 20, pH 8) containing 5 mM imidazole. To enhance the lysis the samples were sonicated 1 × 20 s. After removal of the cell debris by centrifugation, 40 µl of Ni²⁺-nitrilotriacetic acid magnetic agarose beads (Qiagen) were added and incubated for 1 h at 22 °C. The washes were made in the following sequence; 0.5 ml of Buffer A, 1 ml of Buffer B (6 mM guanidine HCl, 0.1 mM Na₂HPO₄/NaH₂PO₄, 0.05% (v/v) Tween 20, pH 6.3), 0.5 ml of Buffer A, 1 ml of 1:1 Buffer A:Buffer C (50 mM Na₂HPO₄/NaH₂PO₄, 100 mM NaCl, 20% glycerol, 0.2% (v/v) Nonidet P-40, 0.05% (v/v) Tween 20, pH 8), 1 ml of 1:3 Buffer A:Buffer C, 1 ml of Buffer C, 0.5 ml of Buffer C containing 20 mM imidazole. The proteins were eluted in 2 × SDS sample buffer and incubated for 1 min; H₂O and β-mercaptoethanol were added, and the mixture was heated for 5 min at 95 °C.

In Vitro SUMOylation Assay—*In vitro* translated, [³⁵S]methionine-labeled proteins were incubated with bacterially produced glutathione S-transferase (GST) fused E1 enzyme (GST-SAE1/2) and GST-Ubc9 in the presence and absence of GST-SUMO-GG-1 at 30 °C for 2 h as described in Ref. 33. The reactions were stopped by the addition of SDS sample buffer and incubation at 95 °C for 5 min. The proteins were separated on 7.5% SDS-PAGE. The gels were fixed and treated with Amplify reagent (GE Healthcare) and visualized by fluorography.

Immunocytochemistry and Confocal Microscopy—COS-1 cells were seeded onto glass coverslips in 24-well plates. Firstly, the lowest appropriate amount of DNA was determined by titration. Twenty-four hours after transfection with 100 ng of each expression vector, the cells were treated and processed for immunocytochemical staining as described (30). Any anomalous expression patterns and overexpressing cells were discarded. Each localization experiment was repeated at least three times. The micrographs were obtained with a PerkinElmer Life Sciences UltraVIEW confocal imaging system connected to a Nikon Eclipse TE300 microscope and 100 × NA 1.3 oil immersion objective.

RESULTS

The Conserved Lysine 183 in the N-terminal Activation Domain of PGC-1α Is Subject to SUMOylation—To reveal whether PGC-1α is targeted by SUMOylation, we first searched for potential SUMO attachment lysines in PGC-1α by using SUMOpplot algorithm, a web-based tool for prediction of consensus SUMOylation sites. The *in silico* analysis revealed the presence of two lysine residues, Lys¹⁸³ and Lys⁵⁴⁵, that fulfill the minimal SUMOylation consensus (Fig. 1A). To examine whether these lysine residues serve as SUMO-1 or SUMO-2 acceptors in mammalian cells, PGC-1α was expressed in COS-1 cells without or with an excess of SUMO-1 or SUMO-2. The cell lysates prepared in the presence of NEM (a SUMO protease inhibitor) were resolved by SDS-PAGE and analyzed by immunoblotting. Expression of wild-type PGC-1α with SUMO-1 yielded one major additional, more slowly migrating form that was detected with anti-PGC-1α antibody (Fig. 1B, arrow). The formation of the additional PGC-1α form in the presence of SUMO-2 was much weaker than with SUMO-1. To investigate which residues are involved in the modification, the two lysine residues conforming to the SUMOylation consensus were separately mutated into arginine residues, and the mutants were coexpressed with and without SUMO-1 or SUMO-2. Mutation of K645 did not affect the pattern of PGC-1α forms, whereas conversion of Lys¹⁸³ to arginine (K183R) significantly diminished the amount of the PGC-1α form that was induced by SUMO-1 and abolished the PGC-1α species dependent on SUMO-2 (Fig. 1B). Because lysine residues can be targeted by several modifications, we also constructed PGC-1αE185A mutant in which the lysine residue is intact but the SUMO consensus sequence is disrupted (19, 41, 42). As shown in Fig. 1B, the effect of the E185A mutation on the SUMO-induced PGC-1α forms was comparable with that of K183R, verifying that the slower migrating PGC-1α form reflects the SUMO modification of the protein. A minor SUMO-1-induced band was still detectable with the PGC-1αK183R and PGC-1αE185A mutants, suggesting a secondary modification. The latter form was not due to modification of Lys⁵⁴⁵, because the double mutant PGC-1αK183R,K645R behaved as the PGC-1αK183R or the PGC-1αE185A mutant in the SUMOylation assays (Fig. 1B). Immunoprecipitation of Gal4 DNA-binding domain-fused full-length PGC-1α (Gal4-PGC-1α) or its N-terminal fragment containing amino acids 1–215 (Gal4-PGC-1α1–215) with anti-Gal4 antibody followed by immunoblotting with anti-SUMO-1 antibody confirmed that the slower migrating form contains, in addition to PGC-1α, SUMO-1 (Fig. 1C). Analyses of the K183R and the E185A mutants as Gal4 fusions further verified Lys¹⁸³ as the major and only SUMO acceptor site in the PGC-1α. Interestingly, based on quantification of the PGC-1α bands in immunoblots, coexpression of SUMO-1 or -2 increased the steady state level of the coactivator or its N-terminal fragment in COS-1 cells by
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FIGURE 1. Lysine 183 is the main SUMO acceptor site in PGC-1α. A, schematic presentation of the full-length PGC-1α and the N-terminal activation domain in more detail. Lys183 and Lys645 reside in the SUMOylation consensus sequence. The locations of LXXLL motifs, acetylated Lys residues, and p38 and AMPK phosphorylation sites are shown. B, SUMOylation of PGC-1α in COS-1 cells. The cells on 6-well plates were transfected with 0.5 μg of pcDNA-F-PGC-1α or pcDNA-F-PGC-1α mutants (K183R, E185A, K183R,K645R, or K645R) in the absence or together with 0.5 μg of pCMV-Myc-SUMO-1 or pCMV-Myc-SUMO-2 as depicted. Twenty-four hours after transfection, the cells were collected, and the lysates were immunoblotted with anti-PGC-1α or anti-tubulin antibody. C, immunoprecipitation-based detection of SUMO-1 in PGC-1α. The cells were transfected as in B but instead of pcDNA constructs encoding Gal4-tagged full-length PGC-1α, Gal4-tagged N-terminal PGC-1α fragment (residues 1–215) or their point-mutated versions were used as indicated. Immunoprecipitations were carried out using anti-Gal4 antibody, and the immunoprecipitates were immunoblotted with anti-SUMO-1 antibody. An asterisk denotes an unspecific band. D, SUMOylation of PGC-1α under cell-free conditions. In vitro translated [35S]methionine-labeled PGC-1α or its mutants were incubated with GST-SAE1/2 and GST-Ubc9 in the presence and absence GST-SUMO-1. The proteins were resolved by SDS-PAGE and detected by fluorography. E, mouse kidney tissue extracts were prepared in the absence or presence of 20 mM NEM as indicated and immunoblotted with anti-PGC-1α antibody. The slower migrating PGC-1α form indicated by the arrow is seen only in the presence of the SUMO protease inhibitor. F, localization of PGC-1α and its mutants by immunocytochemistry and confocal imaging. The COS-1 cells, grown on coverslips on 24-well plates, were transfected with 0.1 μg of pcDNA-F-PGC-1α or of the mutants. After 24 h of transfection, the cells were fixed, and PGC-1α was visualized by anti-PGC-1α detected by Rhodamine Red X-conjugated secondary antibody. The cells were analyzed by PerkinElmer Life Sciences UltraVIEW confocal imaging system with Nikon Eclipse TE300 microscope using the appropriate channel for rhodamine excitation at 568 nm. WB, Western blot; wt, wild type; IP, immunoprecipitation.

≥2-fold, but the effect was not dependent on the SUMOylation site of the coactivator.

To further confirm the SUMOylation of PGC-1α, we carried out assays under cell-free conditions. 35S-Labeled PGC-1α proteins were produced by in vitro translation and incubated with bacterially produced SAE1/SAE2 and Ubc9 in the presence and absence of SUMO-1. When (the mature form of) SUMO-1 was present in the reactions, the in vitro SUMOylation machinery produced an additional higher molecular mass PGC-1α band (Fig. 1D) that, because of the fusion of GST with SUMO-1, migrated considerably more slowly than the SUMOylated form in Fig. 1B. The effects of mutations of K183R and E185A on SUMOylation in vitro were comparable with the assays in intact cells. These in vitro assays thus confirm Lys183 as the main acceptor lysine of SUMO-1 in the PGC-1α. Interestingly, the SUMOylation consensus site is fully conserved from man to frog.

To investigate SUMOylation of the PGC-1α in a more physiological context, we prepared mouse kidney extracts in the presence and absence of the SUMO protease inhibitor NEM. Analysis of the tissue extracts revealed an additional slowly migrating anti-PGC-1α immunoreactive band whose occurrence required the presence of NEM in the extraction buffer, thus supporting the notion that the endogenous PGC-1α is also prone to SUMOylation.

Subcellular Localization of PGC-1α and Its SUMOylation Mutants—Because SUMOylation can influence protein targeting and subnuclear structures (27, 28), we compared the subcellular localization of wild-type PGC-1α to its SUMOylation mutants PGC-1αK183R and PGC-1αE185A by immunocytochemistry and confocal microscopy. Imaging of PGC-1α in COS-1 cells showed that the protein is predominantly localized to tiny speckles throughout the nucleoplasm (43), but the subnuclear distribution of wild-type PGC-1α did not differ from that of the SUMOylation mutants (Fig. 1F).

SUMOylation and Other Post-synthetic Modifications of PGC-1α—PGC-1α harbors several acetylation sites, one of which is interestingly Lys183 (14). To address whether there is apparent cross-talk between acetylation and SUMOylation of PGC-1α, we first compared the acetylation level of wild-type PGC-1α with its K183R and E185A mutants. The FLAG-tagged PGC-1α constructs were transfected into COS-1 cells, immu-
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The effect of SIRT1 inhibitor NAM on the SUMOylation of PGC-1α is shown. A and B, HeLa (A) or COS-1 cells (B) on 6-well plates were transfected with 1 μg of pcDNA-F-PGC-1α or corresponding mutated constructs with and without 1 μg of pcMV-Myc-SUMO-1. Twenty-four hours after transfection, the cells were treated with 10 μM of NAM for 16 h as indicated. B, acetylation level of PGC-1α was analyzed by immunoblotting of anti-FLAG-resin immunoprecipitates with anti-acetyl-lysine antibody. C, analysis of potential interplay between phosphorylation and SUMOylation sites of PGC-1α by MAPK signaling. COS-1 cells were transfected with 0.85 μg of the pcDNA-F-PGC-1α or the SUMOylation/phosphorylation site mutants with or without 0.85 μg of pcMV-Myc-SUMO-1 in the absence or presence of 0.15 μg of both pcDNA3-MK26b(E) and pcDNA-F-p38α. The lysates were immunoblotted with anti-PGC-1α antibody. The intensities of the bands were measured by Li-COR Odyssey infrared imaging system, and the amount of SUMOylated PGC-1α is shown as a percentage of the total PGC-1α. D, the effect of proteasome inhibition on PGC-1α and its SUMOylation-deficient forms in COS-1 cells. Twenty-four hours after transfection with 1 μg of the pcDNA-F-PGC-1α or the corresponding mutants and His-tagged ubiquitin-encoding vector, the cells were treated with MG132 for 2 or 4 h. Histidine-tagged proteins were isolated by Ni²⁺-Chelator affinity chromatography and analyzed by immunoblotting with anti-PGC-1α antibody. 3D, T262D,S265D,T298D; WB, Western blot; wt, wild type; IP, immunoprecipitation.

We also investigated whether AMPK or MAPK phosphorylation sites in the PGC-1α influence its SUMOylation by comparing the behavior of the phosphorylation site-mutated proteins to the wild-type protein in SUMOylation assays in COS-1 cells. However, neither the AMPK-targeting deficient PGC-1αT177A/S538A mutant nor the p38 MAPK phosphorylation site mimetic PGC-1αT262D,S265D,T298D differed from the wild-type protein in relation to their SUMOylation. To further examine whether MAPK signaling and phosphorylation influences PGC-1α SUMOylation, we coexpressed the above PGC-1α forms together with p38 MAPK and a constitutively active form of MAPK kinase 6 (MKK6E). The p38 and the MKK6E were coexpressed to achieve the maximal p38 activity and thereby force the phosphorylation of PGC-1α by the kinase. As shown in Fig. 2C, the electrophoretic mobilities of all other PGC-1α forms, except for that of the PGC-1αT262D,S265D,T298D mutant, were influenced by the forced kinase expression, confirming that Ser262, Ser265, and Thr298 are the principal sites targeted by the p38 MAPK. Interestingly, the augmented p38 MAPK signaling resulted in attenuation of the PGC-1α SUMOylation, but it had a comparable effect also on the SUMOylation of PGC-1αT262D, S265D,T298D (as well as the PGC-1αT177A/S538A mutant) (Fig. 2C). These results suggest that the MAPK activity can down-regulate the PGC-1α SUMOylation, but this is likely to occur indirectly via targeting SUMOylation machinery, not through the altered phosphorylation state of the coactivator.

To study whether the main SUMOylation site of PGC-1α influences its ubiquitination, PGC-1α and His-tagged ubiquitin were coexpressed in COS-1 cells in the presence and absence of proteasome inhibitor MG132. Isolation of His-tagged proteins by Ni²⁺-Chelator affinity chromatography under denaturing conditions verified ubiquitinylation of PGC-1α. Inhibition of

Pi31 and -3 Enhance and SExP1 and -2 Reverse the SUMOylation of PGC-1α—To study whether PIAS proteins are capable of promoting SUMO-1 modification of PGC-1α, PIAS proteins were coexpressed together with PGC-1α and SUMO-1 in COS-1 cells. Although all of the PIAS proteins promoted the SUMOylation of PGC-1α to some extent, PIAS3 and -1 had the most pronounced effects, yielding 3.5- and 2-fold enhancement of the SUMOylation, respectively (Fig. 3A). In addition to enhancing the main SUMOylated species, PIAS3 promoted formation of additional slower migrating PGC-1α forms. SUMOylation of other cryptic sites may be at least in part responsible for the latter phenomenon, because SUMO-1 is not thought to mediate chain formation in vivo (44, 45).
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SUMO-1-modified PGC-1α with Gal4-PGC-1 under the control of Gal4-binding sites (pG5-LUC) together with specific proteases SENP1 and SENP2 deconjugate SUMO from PGC-1α four hours after transfection, the cells were harvested, and the lysates were then compared the abilities of SENPs to reverse the modification. Of the SENPs 1, 2, 3, 5, and 6, only SENP1 and SENP2 were efficient in cleaving PGC-1α-SUMO-1 conjugates (Fig. 3B). The ectopic expression of SENP1 or SENP2 clearly decreased the amount of SUMO-1-modified PGC-1α forms in COS-1 cells, whereas expression of their catalytically inactive mutants slightly increased their amount. These results indicate the reversible nature of the modification.

Ly3783 Negatively Regulates the Transcription Activation Function of PGC-1α—We next assessed the potential effects of SUMOylation on the function of PGC-1α as a transcriptional activator. HeLa cells were transfected with a reporter gene under the control of Gal4-binding sites (pG5-LUC) together with Gal4-PGC-1α. As shown in Fig. 4A, expression of increasing amounts of Gal4-PGC-1α resulted in a robust and dose-dependent activation of transcription. Interestingly, both Gal4-PGC-1αK183R and Gal4-PGC-1αE185A had a higher activation potential than the wild-type protein, although the K183R mutant was more active than the E185A mutant. We next investigated how the SUMOylation site influences the activation function of PGC-1α in isolation (in the absence of repression and RNA-binding domains). To that end, the first 215 N-terminal amino acids of the wild-type PGC-1α and the corresponding SUMOylation mutants were analyzed as Gal4-DNA-binding domain fusions. In keeping with the notion that PGC-1α harbors separate and independent activation and repression domains (7), the PGC-1α1-215 fragment was more active than the full-length PGC-1α. Also in this context, both the K183R and E185A mutations significantly enhanced the transcription function, but the latter SUMOylation-specific mutation enhanced the function more than the lysine-disrupting mutation (Fig. 4B). We also carried out the transcription assays with the wild-type and the point-mutated N-terminal fragments in the presence of the SIRT1 inhibitor NAM. The addition of NAM to the assay decreased the transcriptional activity of all N-terminal constructs, but interestingly only the activity of PGC-1α1-215E185A differed significantly from that of the wild-type fragment in the presence of NAM. Taken together, these results strongly suggest that SUMOylation at Lys3783 plays a specific and repressive role in the regulation of PGC-1α activity.

Ly3783 Modulates the Activity of PGC-1α on PPARγ and ERRγ-dependent Transcription—To study the importance of the SUMOylation site of PGC-1α in the context of nuclear receptor signaling, HeLa cells were transfected with a luciferase reporter containing binding sites for PPARs together with constructs for PPARγ and RXRα along with the wild-type PGC-1α or PGC-1α mutants. As reported previously, PGC-1α acted as a potent transcriptional coactivator for PPARγ (Fig. 5A) (1). Interestingly, the PGC-1αE185A mutant showed modestly enhanced activity in this assay, whereas the PGC-1αK183R mutant was slightly less active than the wild-type coactivator. The PGC-1α mutants had similar effects on the activity of ERRγ (Fig. 5B). Because the K183R mutation and the E185A mutation had interestingly dissimilar consequences on the function of the coactivator on nuclear receptors, these results suggest that acetylation and SUMOylation of Lys3783 (perhaps also methylation and ubiquitylation) play different roles in fine-tuning the activity of PGC-1α in nuclear receptor signaling.

SUMOylation of PGC-1α Modulates Its Sensitivity to Repression by RIP140—Co-repressor RIP140 has been shown to interact with and repress the function of PGC-1α (15, 46). Because SUMOylation can provide substrate proteins with additional interaction surfaces, we investigated whether SUMOylation of PGC-1α plays a role in the repression mediated by RIP140. We first checked whether the N-terminal fragment of PGC-1α is capable of interacting with RIP140. To that end, Gal4-PGC-1α1-215 was coexpressed with SUMO-1 and HA-tagged RIP140. Immunoblotting of anti-Gal4 immunoprecipitates with anti-HA antibody revealed interaction between the coactivator and the corepressor (Fig. 6A). However, the K183R or the E185A mutant did not markedly differ from the wild-type coactivator with respect to their interaction with the corepressor (Fig. 6A). To further investigate the role of SUMOylation in the repression of PGC-1α by RIP140, HeLa cells were cotrans-
SUMO-1 Modification of PGC-1α

![Diagram](image)

**Figure 4. SUMOylation represses the coactivator function of PGC-1α.** A, HeLa cells on 12-well plates were transfected with 200 ng of pG5-LUC and 20 ng of pCMV/β together with 1, 2.5, 5, or 10 ng of pCMX-Gal4-PGC-1α (wt) or corresponding K183R or E185A mutant constructs. B, HeLa cells were transfected with pG5-LUC and 20 ng of pCMV/β together with 1, 2.5, 5, or 10 ng of pCMX-Gal4-PGC-1α (wt) or corresponding mutant constructs K183R or E185A in the absence and presence of 10 mM NAM. Reporter gene activities were measured as described under “Experimental Procedures,” and the relative luciferase activities are represented as the means ± S.D. The activity of pG5-LUC in the absence of Gal4-PGC-1α was set as 1. The experiments were done in triplicate and repeated at least three times. For statistical analyses a one-way or two-way analysis of variance and Tukey–HSD test were used. ***, p < 0.001; **, p < 0.01; *, p < 0.05. C, the protein expression levels of the Gal4-fused PGC-1α and the mutants were checked by immunoblotting with anti-PGC-1α antibody. wt, wild type.

In this study, we have characterized the transcriptional coactivator PGC-1α and show that the lysine residue 183 that is located in the transcription activation domain of PGC-1α is subject to reversible SUMOylation. The lysine conforms to the SUMO attachment consensus sequence, and the site is evolutionarily conserved from *Homo sapiens* to *Xenopus laevis*. The coactivator PGC-1α also harbors another lysine, Lys^645^ that fulfills the consensus, but the latter site was not prone to modification by SUMOs. Interestingly, Lys^183^ is also among the residues in PGC-1α that are acetylated by GCN5. Because PGC-1α contains altogether 13 acetylation sites, an individual acetylation site seems to only contribute to a limited extent to the overall repressive effect of acetylation (14). Moreover, previous studies have not addressed the individual role of Lys^183^ acetylation for the function of the coactivator. Interestingly, however, mutation of Lys^183^ to Arg, which prevents both acetylation and SUMOylation, had a significant enhancing effect on the transcriptional activity of PGC-1α. Mutation of the critical glutamate residue at the −2 position in the SUMOylation consensus (E185A), which disrupts SUMOylation but is thought to preserve acetylation and potentially also methylation and ubiquitination, allowed us to distinguish the regulatory role of SUMOylation from the other Lys^183^ lysine modifications in the full-length PGC-1α or its mutants (K183R and E185A) were coexpressed with RIP140 in COS-1 cells, and the proteins were analyzed by immunocytocchemistry and confocal microscopy. Imaging of the cells clearly showed that RIP140 alters the nuclear distribution of the coactivator in that a considerable portion of PGC-1α adopted a more dispersed nuclear distribution in the presence of the corepressor, which resulted in colocalization of the proteins in these nuclear granules (Fig. 6C). Also, the PGC-1α E185A mutant showed colocalization with the corepressor, but there was a tendency to a more dispersed nuclear distribution with the E185A mutant than with the wild-type PGC-1α or the K183R mutant. These results imply that SUMOylation of PGC-1α may modulate the interaction of PGC-1α with the corepressor RIP140.

**Discussion**

In this study, we have characterized covalent modification of the transcriptional coactivator PGC-1α and show that the lysine residue 183 that is located in the transcription activation domain of PGC-1α is subject to reversible SUMOylation. The lysine conforms to the SUMO attachment consensus sequence, and the site is evolutionarily conserved from *Homo sapiens* to *Xenopus laevis*. The coactivator PGC-1α also harbors another lysine, Lys^645^ that fulfills the consensus, but the latter site was not prone to modification by SUMOs. Interestingly, Lys^183^ is also among the residues in PGC-1α that are acetylated by GCN5. Because PGC-1α contains altogether 13 acetylation sites, an individual acetylation site seems to only contribute to a limited extent to the overall repressive effect of acetylation (14). Moreover, previous studies have not addressed the individual role of Lys^183^ acetylation for the function of the coactivator. Interestingly, however, mutation of Lys^183^ to Arg, which prevents both acetylation and SUMOylation, had a significant enhancing effect on the transcriptional activity of PGC-1α. Mutation of the critical glutamate residue at the −2 position in the SUMOylation consensus (E185A), which disrupts SUMOylation but is thought to preserve acetylation and potentially also methylation and ubiquitination, allowed us to distinguish the regulatory role of SUMOylation from the other Lys^183^ lysine modifications in the full-length PGC-1α or its mutants (K183R and E185A) were coexpressed with RIP140 in COS-1 cells, and the proteins were analyzed by immunocytocchemistry and confocal microscopy. Imaging of the cells clearly showed that RIP140 alters the nuclear distribution of the coactivator in that a considerable portion of PGC-1α adopted a more dispersed nuclear distribution in the presence of the corepressor, which resulted in colocalization of the proteins in these nuclear granules (Fig. 6C). Also, the PGC-1α E185A mutant showed colocalization with the corepressor, but there was a tendency to a more dispersed nuclear distribution with the E185A mutant than with the wild-type PGC-1α or the K183R mutant. These results imply that SUMOylation of PGC-1α may modulate the interaction of PGC-1α with the corepressor RIP140.
PGC-1α function. Interestingly, the E185A mutant was also transcriptionally more active than the wild-type coactivator. In the context of isolated N-terminal activation domain, the E185A mutant displayed even more robust transcriptional activity than the wild-type activation domain. Similarly, disruption of the SUMOylation consensus of PGC-1α enhanced its capability as a coactivator for PPARγ- and ERRγ-dependent transcription. Because the K183R mutant showed contrastingly somewhat weakened coactivator activity in the context of nuclear receptors, these results suggest that acetylation and SUMOylation have different roles in the regulation of PGC-1α function in the nuclear receptor signaling. Moreover, we cannot totally rule out the possibility that the disruption of the SUMOylation consensus (E185A mutation) has consequences beyond the covalent attachment of SUMOs. It is also possible that Lys183 is targeted by other lysine modifications, such as methylation and ubiquitylation, and there may even be a sequential relationship or a cascade between various lysine modifications. Although there was no clear interplay between the major phosphorylation sites and the SUMOylation site of PGC-1α, interestingly, MAPK signaling was able to attenuate the SUMOylation level of PGC-1α. This occurred most likely indirectly via modulation of the SUMOylation machinery.

The N-terminal domain of PGC-1α contains two PEST-like motifs (43), the C-terminal one of them partly overlapping with one of the Cdc4 phosphodegrons (13). The SUMOylation site of PGC-1α resides between the PEST-like motifs, but our studies did not reveal differences in the stability or turnover of the

**FIGURE 5.** SUMOylation modulates the ability of PGC-1α to coactivate PPARγ- and ERRγ-dependent transcription. A, HeLa cells were cotransfected with 200 ng of pGL3-TK-(CPTI)4-LUC and 20 ng of pCMVβ together with 20 ng of both pSG5-mPPARγ and pCMV-RXRα, and 100 ng of pcDNA-F-PPAR-1α (wt), pcDNA-F-PPAR-1αK183R (KR), or pcDNA-F-PPAR-1αE185A (EA) as indicated. Twenty-four hours after transfection, the cells were treated with 1 μM of troglitazone (TGZ) for 16 h. B, HeLa cells were cotransfected with 200 ng of pERE3-TATA-LUC, 20 ng of pCMVβ, and pCMX-ERRγ, together with 50 ng of pcDNA-F-PPAR-1α or the mutants. The experiments were done at least three times with triplicate samples. The relative luciferase activities are represented as the means ± S.D. values.

**FIGURE 6.** Repression of PGC-1α activity by RIP140. A, coimmunoprecipitation of PGC-1α1-215 and RIP140. COS-1 cells were transfected with 650 ng of pCMX-Gal4-PGC-1α1-215 or its mutants, pSG5-HA-RIP140 and pSG5-His-SUMO-1. The cells were lysed in radiimmune precipitation assay buffer and immunoprecipitated with anti-Gal4 antibody, and the immunoprecipitates were immunoblotted with anti-HA antibody. B, HeLa cells were transfected with 200 ng of pG5-LUC and 20 ng of pCMVβ with 5 ng of pCMX-Gal4-PGC-1α1-215 or its mutant forms along with increasing amounts of pSG5-HA-RIP140 (0, 100, 200, 300, and 500 ng). The analyses were carried out as in Fig. 4. The bars indicate percentages of repression of PGC-1α activity by RIP140. C, subnuclear localization of PGC-1α relative to RIP140 was analyzed by immunocytochemical staining and confocal microscopy of COS-1 cells. The upper panel shows subnuclear localizations of wild-type PGC-1α, PGC-1αK183R, and PGC-1αE185A in the absence of coexpressed RIP140, whereas RIP140 expressed alone is shown on the left. PGC-1α was visualized by anti-PGC-1α and fluorescein 5-isothiocyanate-labeled secondary antibody, and RIP140 was detected by anti-HA antibody and Rhodamine Red X-labeled secondary antibody in the coexpression assay. The cell images were captured separately by using a PerkinElmer Life Sciences UltraVIEW confocal imaging system connected to Nikon Eclipse TE300 microscope for each channel (fluorescein 5-isothiocyanate at 488-nm excitation and rhodamine at 568-nm excitation) and merged as indicated. IP, immunoprecipitation; WB, Western blot; wt, wild type.
SUMOylation-deficient PGC-1α forms compared with the wild-type protein. Treatment of cells with proteasome inhibitor MG132 results in stabilization of PGC-1α protein and formation of intranuclear aggregates of PGC-1α (43). We observed a similar phenomenon in COS-1 cells, but the effect of the proteasome inhibitor on the intranuclear aggregate formation did not differ between the wild-type and the SUMOylation-deficient PGC-1α forms. Our confocal imaging analyses showed that PGC-1α normally (in the absence of proteasome inhibitors) localizes in small speckles in the nucleoplasm, which is in accordance with the two recent reports (15, 43). Disruption of the SUMOylation site of PGC-1α did not alter the nuclear distribution pattern under these conditions.

Previous examples of proteins in which the same lysine residue can be targeted either by acetylation or SUMOylation include transcription factors Sp3, MEF2, and HIC1 (47–49). In the case of HIC1 (hypermethylated in cancer 1), there is an inverse correlation between the levels of these two lysine-targeting modifications in that the activation of SIRT1 that deacetylates HIC1 Lys314 results in enhanced SUMOylation of the same lysine residue (49). Similar interplay between acetylation and SUMOylation was not evident with PGC-1α, because inhibition of the SIRT1 activity by NAM treatment did not show any apparent effect on the SUMOylation of PGC-1α.

Several coactivators, including p300/CBP, SRC-1, and PRMT1, are known to interact with the N-terminal activation domain of PGC-1α (7, 50). Because SUMO is a bulky modification, the observed repressive effect of the SUMOylation could be due to the fact that the modification sterically hinders coactivators from interacting with the activation domain. However, we did not observe differences between the wild-type PGC-1α and its SUMOylation mutant with respect to their response to SRC-1 and GRIP-1 (SRC-2) in our transcription assays. On the other hand, SUMOylation could regulate interactions of PGC-1α with corepressors, such as RIP140, which has recently been reported to repress PGC-1α and colocalize with it in the same subnuclear structures (15, 46). In support of the latter corepressor context, we found that disruption of the SUMOylation consensus (the E185A mutation) rendered the transcription activation function of PGC-1α significantly less sensitive to the repression by RIP140. Furthermore, our confocal imaging analyses suggest that the modification can play a role in trafficking and recruitment of RIP140 to the PGC-1α-containing subnuclear sites. Interestingly, RIP140 is also covalently modified by SUMO-1 (30), but its SUMOylation does not appear to play a role in the PGC-1α-RIP140 interactions.

Recent proteomic analysis of PGC-1α-binding proteins and complexes has revealed association with SUMO-specific protease SENP3 (15). According to our results, SENP1 and -2, but not SENP3, are the likely deconjugases responsible for reversing the SUMOylation of PGC-1α. Nevertheless, SENP3 may be the likely deconjugase responsible for reversing the SUMOylation of PGC-1α (30), but its SUMOylation does not appear to play a role in the PGC-1α-RIP140 interactions. In conclusion, our demonstration that the N-terminal domain of PGC-1α is subject to reversible SUMO modification that regulates its capacity to function as a transcriptional coactivator adds a novel level to the regulation of the PGC-1α activity. The cells may utilize SUMOylation as a backup means to down-regulate PGC-1α activity under conditions where sustained cellular SIRT1 activity renders the acetylation-based inhibition inefficient.

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