SUMOylation Modulates the Transcription Repressor Function of RIP140*

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Miia M. Rytinki and Jorma J. Palvimo

From the Institute of Biomedicine/Medical Biochemistry, University of Kuopio, FI-70211 Kuopio, Finland

RIP140/NRIP1 (receptor-interacting protein 140) functions as a corepressor of nuclear receptors. It plays an important role in the transcriptional control of energy metabolism and female fertility. RIP140 contains four distinct repression domains (RD1–RD4), and the repressive activity of RIP140 involves complex mechanisms. The function of both RD1 and RD2 is linked to recruitment of histone deacetylases and C-terminal binding protein, respectively, but the mechanism of repression for RD3 and RD4 has remained elusive. Because covalent modification by small ubiquitin-like modifiers (SUMO-1, -2, and -3; SUMOylation) is often associated with transcriptional repression, we studied whether SUMOylation is involved in the repressive activity of RIP140. We show that two conserved lysines, Lys756 and Lys1154, located in RD3 and RD4, respectively, are subject to reversible SUMOylation, with SUMO-1 being more efficiently conjugated than SUMO-2. Interestingly, mutations of the RIP140 SUMOylation sites compromised the transcription repressor function of RIP140 and blunted its capacity to repress estrogen receptor α-dependent transcription. Conjugation of SUMO-1 also influenced the subnuclear distribution pattern of RIP140. In sum, our demonstration that the function of RIP140 repression domains 3 and 4 can be modulated by reversible SUMO modification thus adds a novel level to the regulation of RIP140 activity, which may have ramifications in the control of gene networks exerted by RIP140.

RIP140/NRIP1 (receptor-interacting protein 140) is a transcriptional corepressor for nuclear receptors. It appears to be capable of interacting with several, maybe most, members of the nuclear receptor (NR)2 superfamily, including estrogen receptor (ER), estrogen-related receptors, peroxisome proliferator-activated receptors, glucocorticoid receptors, and thyroid receptors (1–5). RIP140 has an important role in the control of gene networks regulating female fertility and energy homeostasis. Studies with mice lacking RIP140 indicate that the corepressor is necessary for oocyte release during ovulation and for maintaining normal fat accumulation (6–9). The corepressor function of RIP140 is mediated by four distinct repression domains (RD1–RD4) that are conserved in vertebrates (10, 11). The repression by RD1 involves recruitment of histone deacetylases (HDACs) (11, 12), and RD2 harbors interaction motifs for C-terminal binding protein (CtBP) that in turn may also recruit HDACs (10, 11, 13). However, the function of individual RDs and the role of HDAC activity in the context of whole RIP140 molecule appear to be complex. The HDAC enzymatic activity has been reported to be both dispensable and indispensable for the repressive activity of full-length RIP140 (10–12, 14). RIP140 binds to nuclear receptors through its LXXLL motifs (NR boxes), and because several NR coactivators also possess and utilize the NR boxes, RIP140 may compete with the coactivators of the same binding sites on the ligand-bound receptors (2, 9).

RIP140 has recently been shown to be subject to phosphorylation, arginine methylation, and acetylation (13, 15–17). Phosphorylation of several sites at the N-terminal region of RIP140 was reported to increase its transrepression activity because of enhanced recruitment of HDACs (15). In contrast, arginine methylation at Arg749, Arg650, and Arg948 was shown to weaken the corepressor activity because of attenuated recruitment of HDACs and increased nuclear export (16). Huq and Wei identified four acetylation sites in RD1 and three in RD2, and their experiments with an HDAC inhibitor suggested that hyperacetylation enhances the repressive activity of RIP140 (17). According to another study, acetylation of Lys446 within the N-terminal CtBP-binding motif reduces its interaction with CtBP, thereby attenuating transcription repression by RIP140 (13).

In contrast to covalent modification by small chemical groups, ubiquitination and SUMOylation represent bulky covalent modifications in which a protein, ubiquitin, or SUMO, is attached via an isopeptide linkage to lysine residues of target proteins (18, 19). The SUMO attachment lysines are typically found within a minimal consensus motif ΨKXE (where Ψ is a large hydrophobic residue and X is any residue). Mammalian SUMO-1, SUMO-2, and SUMO-3 are ~100-amino acid-long proteins. SUMO-2 and SUMO-3 are nearly identical, whereas SUMO-1 is only ~50% identical with SUMO-2/3. SUMOs are similar to ubiquitin in their three-dimensional structure, but their amino acid sequence and surface charge distribution differ considerably from those of ubiquitin. SUMO-2 and -3 con-

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1 To whom correspondence should be addressed: Institute of Biomedicine/Medical Biochemistry, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland. Tel.: 358-17-163109; Fax: 353-17-2811510; E-mail: jorma.palvimo@uku.fi.

2 The abbreviations used are: NR, nuclear receptor; SUMO, small ubiquitin-like modifier; CtBP, C-terminal binding protein; HDAC, histone deacetylase; ER, estrogen receptor; SENP, SUMO-specific protease; HA, hemagglutinin; RD, repression domain; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; DBD, DNA-binding domain; PBS, phosphate-buffered saline; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; PML, promyelocytic leukemia.

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tain an internal consensus site for SUMOylation, which allows SUMO chain formation (20). The role of SUMOylation in the regulation of protein stability and degradation is still elusive, but in some cases, attachment of SUMO-1 can protect a protein from being degraded (19, 21). The SUMO conjugation pathway is similar to that of ubiquitin, but cells contain distinct E1 (activating enzyme), E2 (conjugase), and E3 (ligase) activities for both processes. SUMOs are first activated by SAE1 and -2 dimer and subsequently conjugated by Ubc9. PIAS (protein inhibitor of activated STAT) proteins, nucleoporin RanBP2, and polycomb protein Pc2 can promote SUMOylation in a fashion that resembles the action of E3 ligases (19, 21, 22). Under cell-free conditions, the modification can, however, proceed efficiently without E3 activity. SUMOylation is a highly dynamic modification that is reversed by a family of SUMO-specific proteases (SENPs) (23). SUMOylation has emerged as an important regulatory mechanism, especially in transcription and signal transduction. The modification can affect, in a target-specific fashion, the subcellular and subnuclear localization of a protein, its ability to interact with other proteins, and/or its activity in transcription (19, 21).

Because SUMOylation has been shown to contribute to repression of transcription (reviewed in Refs. 21 and 24) and RIP140 possesses lysine residues conforming to the SUMOylation consensus, we investigated whether the protein is modified and regulated by SUMOs. Our results show that even though RIP140 contains four SUMOylation consensus sites, under cell-free conditions, the modification can, however, proceed efficiently without E3 activity. SUMOylation is a highly dynamic modification that is reversed by a family of SUMO-specific proteases (SENPs) (23). SUMOylation has emerged as an important regulatory mechanism, especially in transcription and signal transduction. The modification can affect, in a target-specific fashion, the subcellular and subnuclear localization of a protein, its ability to interact with other proteins, and/or its activity in transcription (19, 21).

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium, fetal bovine serum, and penicillin-streptomycin were purchased from Invitrogen, Hyclone, and EuroClone Ltd., respectively. TransIT-LT1 transfection reagent was from MIRUS Bio Corporation. Proteasome inhibitor Z-Leu-Leu-Leu-al (MG132), cycloheximide, 17β-estradiol, protease inhibitor mixture, and N-ethylmaleimide were from Sigma-Aldrich. Anti-HA mouse monoclonal HA.11 antibody, horseradish peroxidase-conjugated secondary antibody, and Rhodamine Red X-conjugated monoclonal HA.11 antibody were purchased from Santa Cruz Biotechnology Inc. Goat anti-mouse IgG (H&L) DyLight™ 680-conjugated secondary antibody, nitrocellulose membranes, and detection reagents for ECL were from Pierce.

**Plasmid Constructions**—pSG5-HA-RIP140 and pCI-RIP140 were kindly provided by Drs. E. Treuter and M. G. Parker (2, 10). The lysine residues Lys111, Lys 170, Lys 756, and Lys1154 in RIP140 were converted to arginines using a site-directed mutagenesis kit (Stratagene) yielding vectors pSG5-HA-RIP140K111R, pSG5-HA-RIP140K170R, pSG5-HA-RIP140K756R, pSG5-HA-RIP140K1154R, pSG5-HA-RIP140K756R,K1154R, pCI-RIP140K756R, pCI-RIP140K1154R, and pCI-RIP140K756R,K1154R. To construct pM-RIP140(578–1158), pM-RIP140(578–1158)K756R, and pM-RIP140(578–1158)K756R,K1154R encoding N-terminal Gal4 DNA-binding fusions of RIP140 (repression domain 3–4) amino acids 578–1158, EcoRI-Sall fragments of RIP140 pSG4-HA-RIP140 or corresponding point-mutated versions were cloned into pM2 (from Dr. I. Sadowsky). pG2-Lex-Gal4-LUC containing binding sites for both LexA and Gal4 and pSG5-Lex-VP16 encoding LexA-VP16 fusion protein were a gift from Dr. M. G. Parker (10). pSG5-Hs-SUMO-1 was from Drs. J. Seeler and A. Dejean. pSG5-SUMO-2, pEGFP-SUMO-1, pEGFP-SUMO-1GA, pFLAG-mPIAS1, pFLAG-ARIP3 (PIASxα), pFLAG-Miz1 (PIASxβ), pFLAG-PiAS3, pEGFP-Eρα, pSG5-Eρα, and pERE-TATA-LUC have been described (25–27). pcDNA-SENP1 and pcDNA-SENP1C603S were provided by Dr. D. Bailey (28). pVP16-Eρα and pVP16-Eρα DEF (LBD) were a gift from Dr. E. Treuter (29). pMT107 coding for His6-ubiquitin was from Drs. D. Bohmann and M. Treier. pCMVβ encoding β-galactosidase was a gift from Clontech.

**Cell Lines, Transfections, and Reporter Gene Assays**—African green monkey kidney (COS-1) cells, human kidney (HEK 293) and HeLa cells were obtained from the ATCC and maintained as suggested by the ATCC. 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 hormone-activated nuclear receptors were studied, the cells received a fresh medium containing 2.5% charcoal-stripped fetal bovine serum 4 h before transfection, and vehicle/hormone (100 nm) was added 24 h after transfection. 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 (ThermoLabsystems) and for β-galactosidase assays as described previously (26). Total protein concentration was measured using protein assay dye (Bio-Rad). The relative LUC activities (i.e. luciferase light units divided by β-galactosidase values or protein concentration) are represented as the means ± the standard deviations. The experiments were done in triplicate and repeated at least three times.

**Immunoblotting and Protein Half-life Measurements**—Cell monolayers were washed with ice-cold PBS and harvested in PBS containing 20 mM N-ethylmaleimide. Cell pellets were suspended in SDS-PAGE sample buffer containing 10 mM N-ethylmaleimide with 1:200 protease inhibitor mixture 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 anti-mouse antibody using the ECL reagents according to the manufacturer’s instructions (Pierce). For protein half-life measurements, goat anti-mouse IgG DyLight™ 680 fluorescent dye-conjugated secondary antibody was used, and the membranes were analyzed with a Li-COR Odyssey infrared imaging system (Li-COR Inc.) according to the manufacturer’s instructions.
SUMOylation of Nuclear Receptor Corepressor RIP140

**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 (25). The reactions were stopped by addition of SDS-PAGE 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. Twenty-four hours after transfection, the cells were washed twice with ice-cold PBS, fixed with 3% (w/v) paraformaldehyde in PBS for 20 min at 22 °C, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 15 min at 22 °C. The cells were washed twice with PBS and unspecific binding was blocked using 1% (w/v) bovine serum albumin in PBS for 1 h at 22 °C. The primary antibody was incubated for 1 h in PBS containing 1% (w/v) bovine serum albumin, and the cells were washed four times for 5 min with PBS. The secondary antibody, Rhodamine Red X-labeled anti-mouse IgG, was incubated for 1 h at 22 °C and washed with PBS four times for 5 min. The coverslips were dipped in sterile water and put in mounting medium (Vector Laboratories, Inc.). The coverslips were sealed with nail polish and stored in the dark at 4 °C. The amount of transfected DNA was first titrated, and the lowest suitable dose was used. Any anomalous expression patterns and overexpressing cells were discarded. Each localization experiment was repeated at least three times. The micrographs were obtained with PerkinElmer UltraVIEW confocal imaging system connected to Nikon Eclipse TE300 microscope and 100× NA 1.3 oil immersion objective.

**RESULTS**

**Identification of SUMO Attachment Sites in RIP140**—The presence of potential SUMO attachment sites in RIP140 were analyzed by SUMOplot algorithm, a web-based tool for prediction of consensus SUMOylation sites. Four lysine residues, Lys$^{111}$, Lys$^{170}$, Lys$^{756}$, and Lys$^{1154}$, in the RIP140 sequence fulfill the consensus SUMO-1 attachment sequence ΨKXE (Fig. 1A). To examine whether these lysine residues serve as SUMO-1 or SUMO-2 acceptors in mammalian cells, HA epitope-tagged RIP140 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 SUMO-1 or SUMO-2. The cell lysates were resolved by SDS-PAGE and detected by fluorography.

The expression of wild type RIP140 with SUMO-1 yielded two to three additional, slower migrating forms that were detected with anti-HA antibody (Fig. 1B, asterisk, arrowhead, and arrow), suggesting that RIP140 is modified by SUMO-1 at more than one site. Formation of the additional RIP140 forms in the presence of SUMO-2 was weaker than with SUMO-1, yielding one to two slower migrating RIP140 species. To investigate which residues are involved in the modification, the four 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 Lys$^{111}$ and/or Lys$^{170}$ did not affect the pattern of RIP140 forms (data not shown). In contrast, conversion of Lys$^{756}$ to arginine (K756R) changed the pattern of RIP140 forms, abolishing the two slowest migrating RIP140 forms that were induced by SUMO-1 (arrowhead and arrow) and weakening the forms dependent on SUMO-2 (Fig. 1B). Mutation of Lys$^{1154}$ to arginine (K1154R) also influenced the pattern of RIP140 forms (data not shown). In contrast, conversion of Lys$^{756}$ to arginine (K756R) changed the pattern of RIP140 forms, abolishing the two slowest migrating RIP140 forms that were induced by SUMO-1 (arrowhead and arrow) and weakening the forms dependent on SUMO-2 (Fig. 1B). Mutation of Lys$^{1154}$ to arginine (K1154R) also influenced the pattern of RIP140 forms in the presence of SUMO-1; the higher molecular mass band (arrowhead) remained, but the faster migrating band (arrow) disappeared. The latter mutation did not clearly influence the main RIP140 form induced by SUMO-2. Importantly, concomitant mutation of the Lys$^{756}$ and Lys$^{1154}$ (K756R, K1154R) abolished all of the RIP140 forms that
were induced by SUMOs (Fig. 1B). Taken together, these results indicate that Lys\(^{756}\) and Lys\(^{1154}\) can serve as major SUMO acceptors in RIP140. Notably, the two SUMOylation consensus sites are conserved in vertebrates, from man to fish (Fig. 1D).

Interestingly, the location of the SUMO attachment site influenced the migration of RIP140 on SDS-PAGE. When SUMO-1 was conjugated at Lys\(^{1154}\), the protein migrated clearly faster than when it was modified at Lys\(^{756}\). The phenomenon may be due to the fact that the modification at Lys\(^{756}\) near the center of the protein creates a branched structure that migrates more slowly than the Lys\(^{1154}\)-modified RIP140 form that is basically linear, because the modification site is essentially at the C terminus of the protein. A similar situation has been recognized in the SUMOylation of BKLF (30). It is also of note that the SUMOylation pattern of RIP140 differed slightly between experiments; the slowest migrating SUMO-1-induced RIP140 form (depicted by asterisk in Fig. 1B) that very likely corresponds to a RIP140 form SUMOylated concomitantly at Lys\(^{756}\) and Lys\(^{1154}\) was not always readily detectable. The reason for this is currently not known. It is possible that other covalent modifications and/or changes in cellular SUMO E3 activity contribute to its formation. To investigate RIP140 SUMOylation under cell-free conditions, \(^{35}S\)-labeled RIP140 proteins produced by \textit{in vitro} translation were incubated with bacterially produced SAE1/SAE2 and Ubc9 in the presence and absence of SUMO-1. When (mature form of) SUMO-1 was present in the reactions, the \textit{in vitro} SUMOylation machinery produced three additional higher molecular mass RIP140 bands (Fig. 1C) that, because of the fusion of GST with SUMO-1, migrated considerably more slowly than the SUMOylated forms in Fig. 1B. The effects of mutations of Lys\(^{756}\) and Lys\(^{1154}\) on SUMOylation \textit{in vitro} are comparable with the assays in intact cells. These \textit{in vitro} assays thus confirmed Lys\(^{756}\) and Lys\(^{1154}\) as main acceptor lysines of SUMO-1 in RIP140.

**Effect of PIAS Proteins and SUMO-specific Proteases on the SUMOylation of RIP140**—We and others have shown that PIAS proteins can promote SUMOylation in a fashion that resembles the action of RING finger ubiquitin E3 ligases (25, 31). To study whether PIAS proteins are capable of enhancing SUMO-1 or SUMO-2 modification of RIP140, PIAS proteins were coexpressed with RIP140 and SUMO-1 or -2 in COS-1 cells. PIAS proteins had clearly different effects on the SUMOylation of RIP140, in that overexpression of PIAS1 and PIAS3, but not PIAS\(\alpha\) or PIAS\(\beta\), markedly enhanced the modification of RIP140 by SUMO-1 (Fig. 2A), whereas in the presence of SUMO-2, only PIAS1 had a moderate stimulatory effect on the modification (Fig. 2B). In addition to enhancing the SUMOylated species present in the absence of ectopic PIAS expression, PIAS1 and PIAS3 also promoted formation of additional, even slower migrating RIP140 forms. Because SUMO-1 is not thought to mediate chain formation \textit{in vivo} (20), and the appearance of the latter PIAS-promoted RIP140 species was less pronounced with SUMO-2 that can form SUMO polymers, PIAS-promoted SUMOylation of otherwise cryptic sites may be at least in part responsible for this phenomenon. Ectopic expression of PIAS1 or PIAS3 in fact induced weak SUMOylation of the RIP140K756R,K1154R mutant (Fig. 2). However, it is of note that, even in the presence of PIAS proteins, the modification of the compound mutant was dramatically weaker than that of the wild type protein.

SUMOylation is thought to be a highly reversible modification, and the reverse reaction is catalyzed by a family of SENPs (23). As shown in Fig. 2C, ectopic expression of SENP1 decreased the amount of SUMO-1-modified RIP140 forms in COS-1 cells, whereas expression of a catalytically inactive SENP1 mutant contrastingly increased their amount (Fig. 2C). Also SENP2, but not SENP5, was capable of deconjugating SUMOs from RIP140.\(^3\) These results indicate the reversible nature of the modifications.

**Effect of SUMOylation Sites on the RIP140 Protein Stability**—Small ubiquitin-like modifiers have been in some cases shown to influence the longevity of proteins and to subsequently protect them from proteasomal degradation (19, 21). To study whether the main SUMOylation sites of RIP140 influence its degradation by proteasomes, RIP140 was expressed in COS-1 cells in the presence and absence of proteasome inhibitor MG132. Inhibition of proteasome activity resulted in the appearance of higher molecular mass RIP140 species that were considerably strengthened with an ectopic expression of ubiquitin (Fig. 3A), implying that RIP140 is degraded via the ubiquitin-proteasome pathway. The high molecular mass smear formation, a characteristic of ubiquitinated proteins, by the SUMOylation-deficient RIP140K756R,K1154R mutant was only slightly stronger than that by the wild type protein. To investigate the effect of SUMOylation on the stability of the corepressor protein further, wild type and SUMOylation site-
SUMOylation of Nuclear Receptor Corepressor RIP140

A

FIGURE 3. SUMOylation sites do not affect the stability of RIP140. A, COS-1 cells were transfected with pSGS-HA-RIP140 (0.8 μg) or its SUMOylation mutants in the presence and absence of His6-tagged ubiquitin encoding vector (pMT107, 0.25 μg). After 24 h, the cells were treated with and without MG132 (10 μm) as depicted by + and − signs for 16 h before cell harvesting. The cell extracts were analyzed as described in Fig. 2. B and C, COS-1 cells were transfected with 1 μg of pSGS-HA-RIP140 or pSGS-HA-RIP140K756R,K1154R, and after 24 h, 10 μg/ml of cycloheximide (CHX) was added, and the cells were harvested at indicated times. Proteins were detected by immunoblotting using anti-HA and anti-tubulin antibodies. Duplicate immunoblots were performed and detected either conventionally by ECL (B) or with a Li-COR Odyssey infrared imaging system for precise quantification of RIP140 band intensities (C). The graph illustrates the amounts of RIP140 and RIP140K756R,K1154R normalized by the amount of tubulin in the sample. The points in the graph represent the mean ± S.D. values of three samples, wt, wild type.

mutated RIP140 were expressed in COS-1 cells, and the cells were treated with cycloheximide. The amount of RIP140 at various times after the addition of cycloheximide was quantified by immunoblotting with the Li-COR Odyssey infrared imaging system. As shown in Fig. 3 (B and C), there was no significant difference between the stability of the wild type and that of the mutant RIP140, with the half-life of both proteins being about 2 h. Together these results indicate that the SUMOylation sites do not play a major role in the degradation of RIP140 protein and that the weaker repressive activity of RIP140K756R,K1154R is not due to the instability of the mutant.

Substitutions of the SUMOylation Sites in RIP140 Compromise Its Transcription Repression Function—We next assessed the potential effects of SUMOylation on the function of RIP140 as a transcriptional repressor. To that end, we used a system based on a reporter gene under the control of LexA- and Gal4-binding sites (pGL2-Lex-Gal-LUC) and activation by a chimeric LexA-VP16 fusion protein (10, 11). As shown in Fig. 4A, expression of LexA-VP16 increased transcription by ≥1000-fold, and coexpression of increasing amounts of Gal4 DBD-fused full-length wild type RIP140 resulted in a dose-dependent repression of transcription in HEK 293 cells. Interestingly, the repression potential of both SUMOylation-deficient mutants RIP140K756R and RIP140K1154R was significantly decreased at the smallest doses (2 and 10 ng) in comparison with the wild type protein (Fig. 4A). Also the compound mutant RIP140K756R,K1154R displayed compromised repressor function, although the double mutation did not have an additive effect in the context of the full-length RIP140 in this assay. Similar results were obtained in COS-1 and HeLa cells. Because the RIP140 SUMOylation sites are located in RD3 and RD4 at the C-terminal region of the molecule, we also investigated how these sites influence the repression function of RIP140 in the absence of RD1, whose function has been shown to be dependent on the HDAC activity (11). To that end, the C-terminal half (amino acids 578–1158) of the wild type RIP140 and the corresponding regions of the SUMOylation mutants were fused to Gal4 DBD and analyzed in the transcription repression assay. In keeping with the notion that RIP140 harbors separate and independent repression domains (10), also the RIP140(578–1158) fragment efficiently and dose-dependently repressed transcription. Also in this context, mutation of the SUMOylation sites compromised the repression function, with the mutant K756R,K1154R fragment being ~50% less repressive than the corresponding wild type RIP140 region (Fig. 4B). The expression levels of the Gal4-DBD fused RIP140, and mutants were verified to be comparable by immunoblotting with anti-Gal4 antibody (Fig. 4C). Taken together, these results strongly suggest that SUMOylation modulates the repressive activity of RIP140, and the modifications are needed for the full repressive function of the protein.

Disruption of SUMO-1 Acceptor Sites Blunts the Corepressor Activity of RIP140 on ERα-dependent Transcription—To study the importance of RIP140 SUMOylation sites in the context of nuclear receptor signaling, COS-1 or HeLa cells were transfected with ERα,TATA-LUC containing three estrogen response elements in front of a TATA box together with ERα and increasing doses of wild type RIP140 or RIP140 mutants. Wild type RIP140 repressed ERα-dependent transcription in a dose-dependent manner, with the highest doses abolishing the transcription (Fig. 5A). In agreement with the above data with the chimeric repression assay, the RIP140K756R,K1154R mutant was unable to repress ERα-dependent transcription to the same extent as wild type RIP140, the repressor activity of the mutant being ~50–60% of that of the wild type protein at all doses tested. The effects of the substitutions of SUMO acceptor sites in RIP140 on the function of ERα were not specific for COS-1 cells, because RIP140K756R,K1154R displayed significantly attenuated ability to repress the receptor also in HeLa cells (Fig. 5B). The weaker repressor activity of the RIP140K756R,K1154R mutant cannot be explained by its protein level, because, if anything, the expression level of the
mutant was somewhat stronger than that of the wild type protein (Fig. 3). Moreover, the effects of SUMOylation mutations were not selective for ERα/H9251, because these mutations also weakened the corepressor activity of RIP140 on vitamin D receptor/retinoid X receptor/H9251-dependent transcription.3

To study whether SUMOylation of RIP140 influences its interaction with ERα, we employed mammalian two-hybrid assays by expressing RIP140 forms as Gal4 DBD fusions and ERα proteins as VP16 activation domain fusions with a reporter gene driven by Gal4-binding sites. As shown in Fig. 6, the estradiol-induced interaction of RIP140 with full-length ERα/H9251 was relatively weak, but there was no difference between the wild type and the RIP140 mutant. In agreement with the previous yeast two-hybrid data (32), the hormone-induced interaction of the ERα LBD with RIP140 was stronger than that with the full-length receptor (Fig. 6). Interestingly, the SUMOylation-defi-
SUMOylation of Nuclear Receptor Corepressor RIP140

FIGURE 6. Effect of SUMOylation site mutations on the interaction of RIP140 with ERα. The interaction of RIP140 with ERα was studied by mammalian two-hybrid assay. COS-1 cells were transfected with 200 ng of pG5-LUC, 200 ng of pVP16-ERα or pVP16-ERα-LBD, and 200 ng of Gal4 DBD fusion-encoding pCl-RIP140, pCl-RIP140K756R,K1154R, or empty Gal4 DBD vector as indicated. After 24 h, the cells were treated with 100 nm of 17β-estradiol or vehicle. LUC activities were measured after 16 h of estradiol treatment. The graph illustrates mean values of a representative experiment with samples in triplicate. wt, wild type.

DISCUSSION

In this work, we have characterized SUMO modification of the NR corepressor RIP140 (NRIPI) and shown that lysine residues Lys756 and Lys1154 of RIP140 act as SUMO acceptors, showing preference for SUMO-1 isoform. These lysines are located in repression domains 3 and 4, respectively, and their concomitant mutation abolished RIP140 SUMOylation. Both of these lysines conform to the SUMO attachment consensus, and importantly, they are evolutionary conserved across vertebrates. The repression domain 1 of RIP140 also harbors two lysines, Lys111 and Lys1154, fulfilling the consensus sites. The latter sites are conserved only in mammals, and they were not modified by SUMOs. These findings add to the notion that all lysine residues showing perfect fit with the SUMOylation consensus are not necessarily modified. Moreover, because certain modification sites clearly deviate from the consensus, other factors, such as subcellular environment, local concentration of E3 SUMO ligase activity and/or other covalent modifications, must influence the modification and affect the target site specificity (22). Interestingly, the Lys111 of RIP140 appears to be acetylated in vivo (17), which may explain its lack of SUMOylation. Overexpression of SUMO E3 ligases PIAS1 and PIAS3 that promoted SUMO-1 modification of wild type RIP140 also brought about weak SUMO-1 modification of the RIP140K756R,K1154R mutant, suggesting that secondary sites, such as Lys170, can become SUMOylated under conditions promoting the modification.

Our results strongly suggest that SUMOylation of RIP140 regulates its corepressor activity in vivo. RIP140K756R,K1154R mutant that carries substitutions of the main SUMOylation site did not display compromised, but stronger, interaction with the ERα LBD. Our mammalian two-hybrid data thus rule out the possibility that the compromised repressive activity of the SUMOylation-deficient RIP140 on ERα-dependent transcription is due to its weaker interaction with the receptor.

The Subnuclear Localization of RIP140 Is Influenced by SUMO-1—Because SUMOylation has often been shown to play an important role in protein targeting and subnuclear architecture in the nucleus (19, 33); we studied the role of SUMOylation in the subcellular localization of RIP140 by immunocytochemistry and confocal microscopy. Imaging of RIP140 in COS-1 cells showed that a large portion of RIP140 is localized in small, discrete nuclear foci (Fig. 7A), as recently shown by Tazawa and colleagues (34). The nuclear distribution of SUMOylation-deficient RIP140 mutants did not differ from that of the wild type protein in the absence of coexpressed SUMO-1. Coexpression of wild type RIP140 with SUMO-1 fused to enhanced green fluorescent protein (EGFP-SUMO-1) showed partial colocalization of the two proteins, whereas the colocalization was less evident with the K756R mutant and undetectable with the K1154R and the K756R,K1154R mutant (Fig. 7B). Notably, coexpressed SUMO-1 had a marked dispersing effect on the distribution pattern of wild type RIP140 in nuclei, in that the percentage of the cells with discrete RIP140 foci was reduced to one-third of that seen in the absence of ectopic SUMO-1 expression (Fig. 7C). Interestingly, SUMO-1 had much smaller effects on the distribution pattern of K756R mutant as well as that of K756R,K1154R, and in the case of K1154R, it actually increased the percent of the cells with the mutant in small nuclear foci. To study whether the effect of SUMO-1 was due to its covalent attachment to proteins, we used a EGFP-SUMO-1GA mutant (26) that is unable to conjugate to proteins. In contrast to the wild type SUMO-1, coexpression of the conjugation-deficient SUMO-1 with RIP140 did not alter the nuclear distribution of the corepressor (Fig. 7B). Taken together, these results suggest that covalent conjugation by SUMO-1 contributes to the intranuclear compartmentalization of RIP140.

Substitutions of the SUMOylation Sites in RIP140 Do Not Alter Its Colocalization with ERα in Nuclei—To elucidate whether substitutions of SUMO-1 acceptor sites in RIP140 can also influence localization of RIP140 in relation to ERα, full-length ERα fused to enhanced green fluorescent protein (EGFP-ERα)) and wild type RIP140 or RIP140K756R,K1154R mutant were coexpressed in COS-1 cells. Coexpression of ERα with wild type RIP140 in the presence of estradiol lead to redistribution of the corepressor from the small foci to considerably larger nuclear domains in which it colocalized with the receptor (Figs. 7 and 8). Interestingly, when SUMO-1 was coexpressed with the corepressor and the receptor, the RIP140-ERα colocalization pattern was altered, showing finer and more uniform-sized nuclear cogranules. However, there was no apparent difference between the wild type and the SUMOylation negative corepressor with respect to their colocalization with the ERα neither in the presence nor absence of ectopically expressed SUMO-1 (Fig. 8). These results imply that SUMOylation sites in RD3 and RD4 of RIP140 do not play a major role in recruitment of the corepressor to ERα-containing subnuclear sites.
sites exhibited significantly blunted repressor domain function as well as compromised capacity to repress holo-ERα-dependent transcription. Moreover, the effects of these mutations were not restricted to ERα, because they also weakened the corepressor activity of RIP140 on vitamin D-dependent transcription. The RIP140 harbors four distinct repressor domains, all contributing to repression. The N-terminal RD1 has been shown to interact with both class I and II HDACs and RD2 bind CtBP (11–13). Castet et al. (11) showed that recruitment of CtBPs only partially explains the transrepression by RIP140. The roles of individual RDs and HDAC recruitment appears to be complex, because the HDAC activity has been reported to be dispensable for active repression in the context of full-length RIP140 molecule. Moreover, interaction partners, mechanism(s) of repression, and regulatory modifications of the RIP140 RD3 and RD4 have thus far remained unknown. Our results demonstrating that these protein domains are subjected to bulky modifications that can create new binding sites for SUMO-interacting motif-containing repressive proteins can at least to some extent explain their repressive capacity (35, 36).

SUMO modifications of transcription factors appear to have a general tendency to inhibit transcription factor activity and repress transcription (21, 24). Still, the molecular mechanisms by which SUMOylation represses transcription are largely elusive, but they may involve recruitment of HDACs and changes in histone acetylation as well as SUMOylation of histones themselves (37–39). However, the RIP140 RD3 and/or RD4 have not been found to bind HDACs, and our experiments with trichostatin A, an HDAC inhibitor, strongly suggest that HDAC recruitment is not involved in the SUMOylation-dependent regulation of RIP140 activity. Recently, Tiefenbach et al. (40) reported that nuclear receptor corepressor, in contrast to RIP140 typically interacts with apo-NRs, is also modified by SUMO-1 at its RDs. Similar to our results, SUMOylation-deficient nuclear receptor corepressor mutants showed attenuated ability to repress transcription, but the modification did not appear to affect nuclear receptor corepressor interaction with HDAC3 or HDAC-dependent corepressor Sin3A (40).

The effect of SUMO-1 modification on the transcriptional activity of Sp3 is particularly dramatic; it acts as a major switch that converts Sp3 from an activator to a repressor (41).
the case of Sp3, SUMOylation regulates its activity in a trichostatin A-insensitive fashion (42). Instead of being involved in recruitment of HDACs, the modification was shown to be needed for targeting of Sp3 to discrete subnuclear domains, some of which colocalized with promyelocytic leukemia (PML) protein-containing bodies, suggesting that SUMOylation represses Sp3 transcriptional activity at least in part by regulating subnuclear localization of the protein (41). SUMO modifications have previously been shown to be important to the regulation of subnuclear compartmentalization, with the importance of SUMOylation in the targeting of PML protein and the assembly of PML nuclear bodies being particularly well established (33). We analyzed the subcellular localization of RIP140 and the potential effect of SUMOylation on its subnuclear localization. In agreement with the recent work by Tazawa et al. (34), our confocal analyses showed RIP140 to mainly localize in small nuclear domains. However, as noted by Tazawa et al. (34), RIP140-containing nuclear domains do not colocalize with PML bodies.3 In the absence of ectopically expressed SUMO-1, the distribution patterns of SUMOylation-deficient RIP140 forms did not differ from that of the wild type protein. Interestingly, however, SUMO-1 influenced the nuclear distribution of RIP140 in a fashion that was dependent on the SUMOylation sites in RIP140. Overexpression of SUMO-1, but not that of a SUMO-1 mutant deficient in protein conjugation, had a marked effect on the nuclear localization of wild type RIP140, resulting in redistribution of RIP140 into finer nuclear foci, whereas the SUMOylation-deficient double mutant K756R,K1154R remained in small foci despite the overexpressed SUMO-1. We have previously shown that SUMOylation of the nuclear receptor coactivator GREP1 (SRC-2) regulates its ability to colocalize with androgen receptor in the nucleus (43). In the case of RIP140, the reduced corepressor activity of its SUMOylation-deficient mutant on ERα-dependent transcription was not accompanied by its impaired interaction or colocalization with ERα in nuclei, suggesting that SUMOylation of RIP140 is not directly involved in targeting of the corepressor to ERα-containing nuclear domains. Instead, it is plausible that the modification is needed for the recruitment of other proteins to the RIP140 holo-corepressor complex that exerts the repressive function on ERα target genes.

In conclusion, our findings that RIP140 repression domains 3 and 4 are subject to reversible SUMO modifications and that these SUMOylations regulate the subnuclear localization and the capacity of RIP140 to repress transcription add a novel level to the regulation of the RIP140 activity. Thus, signals influencing the activity of cellular SUMO modification machinery may have ramifications in the control of gene networks exerted by RIP140.

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SUMOylation of Nuclear Receptor Corepressor RIP140

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