SUMOylation of the Corepressor N-CoR Modulates Its Capacity to Repress Transcription

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In the absence of ligands the corepressor N-CoR mediates transcriptional repression by some nuclear hormone receptors. Several protein–protein interactions of N-CoR are known, of which mainly complex formation with histone deacetylases (HDACs) leads to the repression of target genes. On the other hand, the role of posttranslational modifications in corepressor function is not well established. Here, we show that N-CoR is modified by Sumo-1. We found SUMO-E2-conjugating enzyme Ubc9 and SUMO-E3 ligase Pias1 as novel N-CoR interaction partners. The SANT1 domain of N-CoR was found to mediate this interaction. We show that K152, K1117, and K1330 of N-CoR can be conjugated to SUMO and that mutation of all sites is necessary to fully block SUMOylation *in vitro*. Because these lysine residues are located within repression domains I and III, respectively, we investigated a possible correlation between the functions of the repression domains and SUMOylation. Coexpression of Ubc9 protein resulted in enhanced N-CoR-dependent transcriptional repression. Studies using SUMOylation-deficient N-CoR RDI mutants suggest that SUMO modification contributes to repression by N-CoR. Mutation of K152 to R in RD1, for example, not only significantly reduced repression of a reporter gene, but also abolished the effect of Ubc9 on transcriptional repression.

INTRODUCTION

The corepressor N-CoR and the related protein SMRT mediate transcriptional repression of unliganded nuclear hormone receptors (NRs; Chen and Evans, 1995; Hörlein *et al.*, 1995). These NRs are transcriptional regulators activating gene expression in the presence of agonistic ligands and play critical roles in development, growth, and homeostasis (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). N-CoR forms corepressor complexes with histone deacetylases (HDACs), which induce changes in local chromatin structure and thereby cause transcriptional repression (Heinzel *et al.*, 1997). The N-terminal half of N-CoR contains three repression domains and several protein–protein interaction domains that mediate the formation of a core complex containing WD-40 proteins TBL1/TBLR-1, GPS2, and HDAC 3 (Guenther *et al.*, 2000; Li *et al.*, 2000; Zhang *et al.*, 2002). The bipartite receptor interaction domain resides in the C-terminal half of the protein and recognizes and binds to unliganded NRs (Seol *et al.*, 1996; Zamir *et al.*, 1996). Between repression domains I and II there are two copies of a 50-amino acid motif, the SANT (SWI3, ADA2, N-CoR, and TFIIB) domain (Aasland *et al.*, 1996). The N-terminal SANT1

domain is part of a deacetylase activation domain that binds to and activates HDAC3, whereas the C-terminal SANT2 domain functions as a histone interaction domain (Guenther *et al.*, 2001; Guenther *et al.*, 2002; Zhang *et al.*, 2002; Yu *et al.*, 2003). Most of the more than 160 human SANT-domain proteins identified to date are involved in transcription or in chromatin remodeling. In addition to playing an important role as a histone-binding motif or as a deacetylase-activating domain the SANT domain may also serve as a protein–protein interaction interface (Boyer *et al.*, 2004).

Posttranslational modification of proteins by the small ubiquitin-like modifier (SUMO) is an important regulatory mechanism that impinges on many cellular processes (Müller *et al.*, 2001; Gill, 2003; Melchior *et al.*, 2003; Seeler and Dejean, 2003; Verger *et al.*, 2003; Johnson, 2004). SUMO conjugation regulates different protein functions including protein stability, subnuclear localization, DNA binding, and transcription (reviewed in Gill, 2003; Verger *et al.*, 2003; Johnson, 2004; Hay, 2005). Recent studies including many reports on SUMOylation of gene-specific transcription factors and coregulators have shifted the focus toward a nuclear function for SUMO modification (Kagey *et al.*, 2003; Lee *et al.*, 2003; Lin *et al.*, 2003; Yamamoto *et al.*, 2003; reviewed in Gill, 2003; Verger *et al.*, 2003). There are four mammalian SUMO proteins, SUMO-1, SUMO-2, SUMO-3, and SUMO-4 (Gill, 2004). SUMO is first activated for conjugation by the E1 enzymes Aos1/Uba2, subsequently transferred to the E2 conjugation enzyme Ubc9 and finally conjugated to target proteins by an E3 ligase (Hochstrasser, 2000; Yeh *et al.*, 2000; Müller *et al.*, 2001). Different classes of E3 ligase-proteins have been reported. Protein Inhibitors of Activated Stat (PIAS; Johnson and Gupta, 2001; Schmidt and Müller, 2003), RanBP2 (Pichler *et al.*, 2002), and Polycomb group protein Pc2 have been shown to act as SUMO E3 ligases (Kagey *et al.*,

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2003). SUMO modification of transcription factors is often associated with transcriptional repression (Gill, 2004; Hay, 2005) and SUMO negatively affects the transcriptional activating function of transcription factors, such as Elk-1 or nuclear hormone receptors (Abdel-Hafiz *et al.*, 2002; Sapschnig *et al.*, 2002; Girdwood *et al.*, 2003; Yang and Sharrocks, 2004). However, the molecular basis of how SUMO represses transcription factor function is not well understood.

Studies with N-CoR knockout mice revealed the functional importance of N-CoR in early embryonic development with defects in neural cell differentiation and developmental progression of erythrocytes and thymocytes (Jepsen *et al.*, 2000; Hermanson *et al.*, 2002). Further identification and characterization of the constituents of N-CoR complexes is of great interest to understand the mechanistic basis of regulated events. Therefore, a yeast two-hybrid screen with the N-CoR SANT1 + 2 domains was used to identify putative interacting proteins. We identified the SUMO-E2 conjugation enzyme Ubc9 and the SUMO-E3 ligase Pias1 as interaction partners of the SANT1 domain. We demonstrate that the corepressor is SUMO-1 modified *in vivo* and characterized several consensus SUMOylation sites in N-CoR, all in repressor domains RDI and RDIII. Analysis of SUMOylation-deficient N-CoR mutants suggests that SUMO modification contributes to repression by N-CoR.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen

The Matchmaker Two-Hybrid System 3 (Clontech, Palo Alto, CA) was utilized to screen a mouse e17 cDNA library (Clontech) with an N-CoR region including the SANT domains (aa 429–683) as bait. Expression of the N-CoR bait protein was confirmed by Western blot. The library screen was performed using yeast strain AH109 under high stringency conditions (Ade⁻, His⁻, Leu⁻, Trp⁻ media) and ~1.1 × 10⁷ clones were screened. Colonies from the primary screen were restreaked and positives were confirmed with a β -galactosidase assay. All clones scoring positive for growth on selective media were confirmed using a β -galactosidase assay (unpublished data). Plasmid DNA from positive colonies was isolated, propagated in *Escherichia coli* and sequenced to identify clones.

Plasmids, Cell Lines, and Tissue Culture

PCR products of the murine N-CoR cDNA (Hörlein *et al.*, 1995) were cloned into the following plasmids: pGBKT7 (GAL4 DBD fusion for yeast expression, Clontech), pGEX (GST fusion, Amersham-Pharmacia, Piscataway, NJ), pcDNA3.1 (mammalian expression vector, Invitrogen, Carlsbad, CA). Full-length cDNAs for YFP-Ubc9 and YFP-SUMO were described previously or provided by F. Melchior (Pichler *et al.*, 2002). We made use of C93S Ubc9 (Ubc9m), a catalytically inactive mutant of the SUMO conjugating enzyme Ubc9, which can no longer conjugate SUMO-1 to target proteins (Girdwood *et al.*, 2003). Pias1-Flag, was a gift of M. Zörnig (originally provided by T. Mörry). pcDNA3-6xHis-SUMO-1 was kindly provided by R. Hay (University of St. Andrews, Fife, United Kingdom). The coding sequence of Gal DBD fused to N-CoR amino acids 1–549 or N-CoR mutant K₁₅₂R was cloned in frame into a pcDNA3-6 version with an N-terminal His-Flag tag. The plasmids for reporter gene assays ((DR5)₂-TK LUC, TRE Luc, 2X-GAL4 binding-site luciferase plasmid, pSV40 β -Gal) were described previously (Mangelsdorf *et al.*, 1991; Hörlein *et al.*, 1995; Heinzel *et al.*, 1997). The N-CoR K-to-R mutations were created using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). Cell lines utilized in this study include 293T, 293, and COS-7. All cell lines were grown in DMEM media supplemented with 10% fetal calf serum and 2 mM L-glutamine (Invitrogen). The Gam-1 expression vector has been described (Boggio *et al.*, 2004).

Whole Cell Extracts, Coimmunoprecipitation, and Western Blotting

Whole cell extract preparation and immunoprecipitation (IP) reactions were carried out as described (Desterro *et al.*, 1998). For coIPs cells were lysed in CoIP buffer (20 mM Tris [pH 8], 25 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM DTT) containing 25 mM N-ethylmaleimide (NEM) and protease inhibitors. To detect *in vivo* SUMOylation of N-CoR, cells were lysed in buffer as described in Gomez-del Arco *et al.* (2005). Lysates were prepared for affinity pulldown assays with Talon beads (Invitro-

gen) in denaturing conditions as described in Miller *et al.* (2005). Antibodies used were as follows: mouse α -GAL4-DBD RK5C1, goat α -HDAC3, rabbit α -mSin3A K20, goat α -Pias1/3 N-18 (Santa Cruz Biotechnology, Santa Cruz, CA), actin, mouse α -Flag M2 (Sigma, St. Louis, MO), mouse α -SUMO-1 (Zymed, South San Francisco, CA), rabbit anti-GFP antibody (Abcam, Cambridge, United Kingdom; 6556) and rabbit or guinea pig α -N-CoR.

Protein Expression and GST Pulldown

All cDNAs for protein production were cloned into the GST-fusion expression vector pGEX (Hörlein *et al.*, 1995). *E. coli* BL21 (DE3) Codon Plus-RP cells were utilized for protein expression according to the manufacturer's instructions (Stratagene). GST pulldown experiments were performed, as described (Hörlein *et al.*, 1995).

Luciferase Assays

293T cells were seeded in 12-well plates and transfected with calcium-phosphate. After 6 h, precipitates were replaced by fresh medium. Cells were grown for 48 h and then whole cell extracts were prepared and luciferase activity was measured. All reactions were performed in triplicate unless stated otherwise.

In Vitro SUMOylation

Expression and purification of recombinant SUMO-1, Uba2/Aos1, and Ubc9, and *in vitro* SUMOylation assays were performed as described (Pichler *et al.*, 2002). Three microliters of ³⁵S-labeled TNT-translated N-CoR was utilized as substrate for the *in vitro* SUMOylation reaction. Each reaction contained 150 ng of recombinant Aos1/Uba2, 10 ng of recombinant Ubc9, 1000 ng of recombinant SUMO-1 (1–97), 10 μ l of IP of Pias1-Flag, 1 mM ATP, Tween 20, 0.05% ovalbumin, 0.2 mg/ml, in a 25 μ l reaction volume. Reactions were performed for 1 h at 30°C. Sample buffer was then added and the products were separated on an SDS-PAGE gel, followed by autoradiography. SUMO modification reactions of GST-N-CoR RDI were carried out essentially as described (Tatham *et al.*, 2001).

RESULTS

N-CoR Interacts with Multiple Components of the SUMOylation Machinery In Vitro and In Vivo

The corepressor N-CoR consists of multiple modular domains (Figure 1A). Although interaction partners and functional properties have been identified for a subset of these domains, several other regions of N-CoR have not been characterized in detail. Thus, we decided to identify additional interactors, which may regulate the repressive functions of N-CoR. We performed a yeast two-hybrid screen with the N-CoR SANT 1 + 2 domains, fused to the GAL4-DNA binding domain as bait. Our screen of a mouse embryonic cDNA library led to the identification of multiple independent clones containing sequences of the SUMO-E2-conjugating enzyme Ubc9, and the SUMO-E3 ligase Pias1 (Figure 1B).

To verify yeast two-hybrid interactions, we performed GST pulldown assays with *in vitro* transcribed, ³⁵S-labeled, Ubc9 and Pias1, and different GST-N-CoR domains. We were able to map the binding of Pias1 and Ubc9 to the SANT1 domain of N-CoR (Figure 1C). Additionally, we found that Pias1 also interacts with N-CoR repression domain RDI (Figure 1D). Controls for equal loading of N-CoR GST fusion proteins are shown (Figure 1, C and D). To further confirm this interaction, we performed coimmunoprecipitations in both directions using cells transfected with Flag-tagged Pias1. Flag-tagged Pias1 coimmunoprecipitated with endogenous N-CoR and vice versa (Figure 1E). We were also able to verify this interaction with endogenous Pias1 and N-CoR (Figure 1F). The Western blot with anti-Pias1/3 antibody shows that only Pias1 coprecipitates with N-CoR; the lower migrating Pias3 protein is only detectable in the input lane. The interaction of Ubc9 and N-CoR could not be demonstrated in coimmunoprecipitations, probably because of the transient character of this interaction. In addition, interactions between N-CoR and Pias1 or Ubc9, were also confirmed by using immunofluorescence staining and

Figure 1. N-CoR interacts with Pias1 and Ubc9 in vitro. (A) Schematic diagram of N-CoR domains. N-CoR contains three separate repression domains (RDI, RDII, RDIII). Nuclear receptor interaction domains (NID) are located at the C-terminus. Three putative SUMOylation sites are located each in RDI and RDIII with target lysine residues (K) indicated. The SANT domains 1 and 2 are located in between RDI and RDII. (B) Interaction partners of the N-CoR SANT1 + 2 domain identified by yeast two-hybrid screen. (C) ^{35}S -labeled in vitro-translated Pias1 (top panel) or Ubc9 (middle panel) was incubated with glutathione agarose bound GST as negative control or GST-fusion's of the N-CoR SANT domains as indicated. After washing, bound proteins were analyzed by SDS-PAGE and phosphorimaging. Ten percent of the ^{35}S -labeled input was loaded for reference. Equal loading of N-CoR GST fusions was verified by Coomassie staining (bottom panel). (D) ^{35}S -labeled in vitro-translated Pias1 was incubated with GST or GST-N-CoR domains (excluding SANT) as indicated. The pull-down was performed as described in C. Equal loading of N-CoR GST fusions was verified by Coomassie staining (bottom panel). (E) 293T cells were transfected with a Pias1-Flag expression plasmid. Lysates were immunoprecipitated with α -N-CoR, α -Flag (for Pias1-Flag) antibodies or pre-immune serum (C). The immunoprecipitates were analyzed by Western blot using α -N-CoR (left panel) or α -Flag antibody (right panel). (F) Cos cells were lysed in CoIP buffer and lysates were immunoprecipitated with α -N-CoR antibody preimmune serum (C). The immunoprecipitates were separated by SDS-PAGE and Western blot was performed using α -Pias1/3.

confocal microscopy for each of the endogenous proteins (unpublished data). These results further support the notion that N-CoR interacts with components of the SUMOylation machinery *in vivo*.

Figure 2. Endogenous N-CoR is modified by SUMO-1 *in vivo*. (A) 293T cells untransfected or transfected with Ubc9-YFP and/or SUMO-1-YFP expression plasmids were lysed in 1% NP-40 buffer with or without NEM. Samples were analyzed by Western blot using α -N-CoR antibody. The positions of free and putative SUMO-1-conjugated N-CoR and marker are indicated. (B) α -N-CoR Western blot of 293T cells lysed in 1% SDS in the presence or absence of NEM. Cells were either untransfected or transfected with Ubc9-YFP and SUMO-1-YFP expression plasmids. The arrowheads show the position of N-CoR and two higher molecular weight N-CoR species. (C) 293T cells were cotransfected with N-CoR-Flag plasmid or in combination with His-SUMO-1. After direct lysis in 8 M urea, extracts were incubated with Talon-NTA beads and transferred to a column. After intensive washes bound proteins were pH eluted and transferred to SDS-PAGE. Western blot was performed using an α -Flag monoclonal antibody. I, input; C, control; NTA, His-SUMO-1 containing material eluted from NTA.

N-CoR Is Modified by SUMO In Vivo and In Vitro

Previous studies have shown that many proteins interacting with Ubc9 or Pias1 are also modified by SUMO-1. To determine whether N-CoR is SUMOylated *in vivo*, either untransfected or transfected (YFP-Ubc9, YFP-SUMO-1, or in combination) cells were lysed under nondenaturing conditions in the presence or absence of NEM, an inhibitor of SUMO hydrolyases and were subjected to Western blot analysis, using a rabbit α -N-CoR antibody. In extracts without NEM we observed a prominent α -N-CoR-cross-reactive band at 270 kDa, whereas in cells lysed in the same buffer in the presence of NEM, an additional more slowly migrating species appeared (Figure 2A). This modified N-CoR form was more abundant upon coexpression of YFP-Ubc9 and/or YFP-SUMO-1 protein. To further examine the possibility that these slower migrating species correspond to covalent adducts of SUMO-1, 293T cells were either lysed in a 1% NP-40 buffer or cotransfected with vectors expressing YFP-SUMO-1 and YFP-Ubc9 and lysed in a 1% SDS buffer in the presence of NEM. Western blots of extracts were performed and in the cotransfected cells, two additional bands with increments of ~ 40 kDa were detected that are likely to correspond to larger, SUMO-1-YFP modified forms of N-CoR (Figure 2B). Because of the high molecular weight of the full-length corepressor, separation between single and mul-

Figure 3. N-CoR is a substrate for SUMO-1 conjugation in vitro. (A) In vitro-translated ^{35}S -labeled N-CoR RDI (aa 1-549) or RDIII (aa 970-1502) were incubated in the presence (+) or absence (-) of the indicated components of the SUMOylation machinery and were tested for SUMO-1 conjugation in vitro. Marker positions are indicated. The schematic diagrams of repression domains I and III constructs are shown. SUMOylation sites are indicated as horizontal white bars and the SANT1 domain as a gray box. (B) As a negative control, in vitro-translated ^{35}S -labeled N-CoR SANT 1 + 2 domain was incubated in the presence (+) or absence (-) of the indicated components. (C) The influence of coregulator SUMOylation on the transcription of TR (left panel) and RAR (right panel) regulated genes was analyzed by reporter assay. 293T cells were transiently transfected with 1.5–2 μg total DNA in a 12-well format (0.5 μg RARE or 0.8 μg TRE, specific luciferase reporters with or without Gam-1 (0.5 μg) expression vectors and 0.1 μg of SV40 beta Gal reporter plasmid per well. Empty vector was added as required to transfect equal amounts of DNA. Cells were exposed to all-trans retinoic acid (ATRA; 100 nM), antagonist 193840 (100 nM), or triiodothyroacetic acid (Triac; 100 nM) 24 h before harvesting. The quantification shown represents the mean $\pm$ SD of triplicates from one typical out of a total of three experiments.

multiple SUMOylated forms of N-CoR is difficult to achieve even using a low percentage SDS Page. Furthermore, the exact size of SUMOylated N-CoR forms is difficult to determine, because of low gel resolution and limited availability of high-molecular-weight marker proteins. To demonstrate that these forms result specifically from SUMOylation, we adopted the approach of using His-tagged SUMO-1, which can be affinity-purified under denaturing conditions using metal-NTA resin. Using His-tagged SUMO-1 in cells transfected with Ubc9 and Flag-N-CoR, we were able to precipitate SUMOylated N-CoR (Figure 2C).

Inspection of the N-CoR sequence indicated that it contains several copies of a ψKxE motif often required for SUMO modification, all of which are located in repression domains I and III (Figure 1A). To analyze SUMO modification of N-CoR, we used an in vitro assay as described previously (Pichler *et al.*, 2002). In this assay, ^{35}S -labeled, in vitro-translated N-CoR domains were incubated with recombinant E1 (Aos1/Uba2), E2 (Ubc9), and E3 (Pias1-Flag) proteins, along with ATP in the presence or absence of recombinant SUMO-1 (Figure 3, A and B). N-CoR RDI as well as RDIII showed specific higher molecular weight bands when Pias1 was added to the SUMO mix (Figure 3A). A mobility shift of ~ 20 kDa was observed only when the E3 ligase Pias1 was added to the in vitro reaction but not with recombinant RanBP2 E3 ligase. Modification of N-CoR RDI was consistent with the addition of two SUMO molecules (Figure 3A, top panel) and for N-CoR RDIII the results suggest the addition of three monomeric SUMO molecules

or perhaps poly-SUMOylation (Figure 3A, bottom panel). As shown in Figure 3B the SANT 1 + 2 domain of N-CoR, which does not harbor any ψKxE motifs is not subject to SUMO modification.

Next, we wanted to investigate whether SUMO influences the transcription of known N-CoR-dependent target genes. Therefore, 293T cells were transfected with a luciferase reporter containing binding sites for RAR or TR with or without coexpression of Gam1 in the presence of agonist or antagonist (Figure 3C). The adenoviral protein Gam1 is known to inhibit the SUMO pathway by interfering with the activity of E1 (SAE1/SAE2; Boggio *et al.*, 2004). The disappearance of both SAE1/SAE2 and UBC9 leads to an overall inhibition of protein SUMOylation (Boggio *et al.*, 2004; Degerny *et al.*, 2005). In the presence of agonist both RAR and TR reporter activity is strongly enhanced when Gam1 is coexpressed (Figure 3C). In the presence of a synthetic RAR antagonist maximal repression was observed, which was unchanged by coexpression of Gam1.

Repression of RDI Depends on SUMOylation of K152

To inactivate potential SUMOylation motifs, acceptor lysines 152, 194, and 260 in RDI and acceptor lysines 1117, 1330, and 1443 were mutated to arginine either alone or in combination. Corresponding ^{35}S -radiolabeled, in vitro-translated N-CoR mutants were subjected to in vitro SUMOylation as described. When K152 is substituted by arginine, the 100-kDa SUMO modification product of N-CoR RDI no longer appears, indicating that this amino acid is

Figure 4. Repression of N-CoR RDI depends on K152 SUMOylation. (A) In vitro-translated ³⁵S-labeled N-CoR RDI or RDIII wt, or K-to-R mutants (RDI: K152R, K194R, K260R, K152/194/260R and RDIII: K1117R, K1330R, K1117/1330R, K1117/1330/1443R) were tested for SUMO-1 conjugation in vitro as described in Figure 3. The bands corresponding to SUMO-conjugated N-CoR products are marked by asterisks. (B) 293 cells were transfected with either Gal4-N-CoR RDI wt or Gal4-N-CoR RDI mutant (K152R) and YFP-SUMO expression vectors. Extracts and Western blot with Flag antibody were performed as described in Figure 2C. The membrane was stripped with Restore Western Blot Stripping buffer (Pierce, Rockford, IL; 21059) for 15 min and incubated with rabbit α-GFP-antibody (Abcam; 6556). The positions of SUMO-1-conjugated N-CoR RDI are indicated. Markers are shown on the right. (C) Reporter activity (2UASx-TK-Luc) in 293T cells transfected with pCMXGAL4 or pCMXGAL4-N-CoR full-length, GAL4-N-CoR RDI

likely to serve as a SUMO acceptor (Figure 4A, top panel). Substitution of K194 and K260 showed the same triple modification as RDI wild type (wt), although at a reduced level for K260. To confirm the in vitro results in a cellular system, 293 cells were transfected with N-CoR wt or K₁₅₂R RDI and SUMO-YFP. We used His-Flag-tagged RDI wt or K₁₅₂R mutant for affinity purification under denaturing conditions using metal-NTA resin (Figure 4B). Both, wt and mutant N-CoR RDI were purified with high efficiency. Only for N-CoR RDI wt bands of reduced electrophoretic mobility appeared, which correspond to molecular sizes above 115 kDa. As in the in vitro SUMOylation assay (Figure 4A) the lower band of SUMOylated N-CoR RDI is more intense than higher migrating bands (Figure 4B). Consistent with these results, no bands of reduced electrophoretic mobility were detectable for the RDI K₁₅₂R mutant. The membrane was reprobed with a GFP antibody and a SUMOylated band could be detected only for N-CoR RDI wt (Figure 4B). This result confirms our in vitro data and supports the hypothesis that K152 in RDI is a SUMO acceptor site in vitro and in vivo.

K-to-R substitutions in RDIII are consistent with a role of amino acids 1117 and 1330 as acceptor sites for SUMO modification (Figure 4A, bottom panel). Furthermore, upon in vitro SUMOylation of N-CoR RDIII groups of modified bands appear that are separated by intervals corresponding to ~20 kDa higher molecular weight. Modification of the wt protein generates a closely spaced triplet of bands, each corresponding to the attachment of a single SUMO molecule (~20 kDa shift). Apparently, SUMOylation of different sites causes slight changes in electrophoretic mobility. The lower band of the triplet disappeared upon arginine substitution of K1117 of N-CoR, whereas the K₁₃₃₀R mutation eliminates the middle band of the triplet. Thus, these bands are likely to represent mono-SUMOylation of these lysine residues. Higher migrating bands could be indicative either of poly-SUMOylation of a single lysine or of mono-SUMOylation of multiple lysines.

We next investigated the K-to-R substitutions within the SUMO consensus motifs of RDI in reporter assays. N-CoR is ubiquitously expressed and N-CoR^{-/-} cell lines could not be used due to low viability. Therefore, we decided to co-transfect 293T cells with vectors expressing Gal4 fusions of N-CoR and an artificial luciferase reporter containing 2 × Gal4 DNA binding sites. The 2 × Gal4-TK-luciferase reporter construct has a high level of basal promoter activity, which is repressed upon cotransfection of Gal4-N-CoR (Figure 4C). Repression was observed when N-CoR was tethered to the promoter by fusion to the Gal4-DBD. N-CoR RDI alone is a much more efficient repressor in comparison to full-length N-CoR. RDI constructs harboring the K₁₅₂R mutation exhibit significantly reduced repression (Figure 4C). These results suggest that SUMO-1 modification of K152 in RDI substantially contributes to N-CoR repression although repression of N-CoR RDI clearly does not exclusively depend on SUMO-modification. This is to be expected as RDI had been shown to recruit HDAC activity.

(aa 1-549) or GAL4-N-CoR RDI mutant or (K-to-R) constructs is shown. Fold repression was determined relative to the basal reporter activity in the presence of GAL4 DBD. K → R substitutions within the SUMO consensus sequences of N-CoR RDI alter their ability to repress basal transcriptional repression. Each transfection was performed in duplicates and repeated at least twice. A Western blot with α-Gal DBD antibody indicates equal expression of N-CoR RDI wt and mutant proteins.

Ubc9 Enhances the Ability of N-CoR to Repress Transcription

Next, we tested whether the ability of N-CoR to repress transcription would be affected by increased SUMOylation. As shown in Figure 2A we found that coexpression of Ubc9 increases the posttranslational modification of N-CoR. To test the effect of SUMO-1 modification on the ability of full-length N-CoR to repress transcription, Gal4-N-CoR, causing significant repression of a luciferase reporter containing 2 × Gal4 DNA binding sites, was cotransfected with Ubc9 or with Ubc9m and/or Pias1 expression vectors into 293T cells. The C93S active site mutation (Ubc9m) renders Ubc9 catalytically inactive so that it can no longer conjugate SUMO-1 to target proteins. Ubc9 overexpression significantly enhanced Gal4-N-CoR-mediated transcriptional repression, whereas this effect was not observed when Ubc9m was transfected (Figure 5A). Overexpression of both Ubc9 and Pias1 had an even more pronounced effect on reporter gene repression, whereas this effect was abolished by using Ubc9m (Figure 5A).

Importantly, the effect of Ubc9 on N-CoR repression was completely abolished when the RDI triple mutant was used, indicating that Ubc9 functions via N-CoR SUMOylation rather than via an effect on other SUMO target proteins (Figure 5B). Cotransfection of Ubc9m did not alter the repression ability of N-CoR RDI or RDI mutant. This clearly demonstrates that SUMOylation of acceptor lysines within RDI (most likely K152) is important for N-CoR repression. Furthermore, we detected a significant reduction in the repression of full-length N-CoR when RDI was no longer accessible for SUMOylation on K152 (Figure 6A). Thus, we confirmed our results that K-to-R substitutions in RDI interferes with repression in the context of full-length N-CoR (Figure 6A). We conclude that SUMOylation of N-CoR RDI is important for the ability of N-CoR to repress basal transcription of a reporter. Using His-tagged SUMO-1 and Flag-tagged N-CoR wt or N-CoR mutants, we were able to purify SUMOylated N-CoR wt and N-CoR K_{1117, 1330, 1443}R (Figure 6B). The N-CoR RDI mutant K_{152, 260}R could not be purified on the metal-NTA column, indicating that these sites are critical for His-SUMO-1 modification of N-CoR in vivo.

In principle, lysine mutations in N-CoR RDI could change protein–protein interaction motifs or the overall conformation of N-CoR and thus reduce repression by affecting interactions with known components of corepressor complexes such as HDAC3 and Sin3A. To verify that this is not the case and the N-CoR RDI mutant is still conformationally intact, we compared interactions of wt and mutated full-length N-CoR with HDAC3 and Sin3A in coimmunoprecipitations (Figure 6C). This experiment clearly demonstrates that the interaction of HDAC3 and Sin3A with N-CoR is not impaired by mutating K152 and K260 in repression domain I. Furthermore, wt and mutant N-CoR constructs were equally expressed and by using immunofluorescence staining and confocal microscopy (unpublished data) found to be predominantly distributed in the nucleus.

DISCUSSION

In this article, we provide the first evidence that the corepressor N-CoR belongs to a subfamily of transcriptional regulators that are subject to covalent modification by SUMO-1. We identified several components of the SUMO pathway as novel N-CoR interactors and have presented evidence that SUMOylation of N-CoR occurs in vivo and that RDI (K152) and RDIII (K1117 and K1330) are modified

Figure 5. Ubc9 enhances N-CoR transcriptional repression activity. (A) Luciferase assays were performed after transfection of 293T cells with a 2xUAS TK-Luc reporter plasmid together with pCMXGal4DBD or pCMX Gal4 DBD-N-CoR (full-length, f.l.) either with or without Ubc9/Ubc9m or Pias1 as indicated. pCMXSV40-βGal plasmid was cotransfected to monitor the efficiency of transfection. Fold repression was determined relative to the basal reporter activity in the presence of GAL4 DBD. Transfections were performed in triplicate and repeated at least three times. A Western blot with α-Flag antibody indicates equal expression of the Gal-DBD N-CoR protein in the reporter assay. (B) pCMX Gal4-N-CoR (aa 1–549) or Gal4-N-CoR RDI mutant (K152/194/260R) constructs were either transfected alone or with YFP-Ubc9/Ubc9m, and reporter activity (2UASx-TK-Luc) in 293T cells was measured as described in A.

by SUMO-1. We demonstrate that although SUMOylation of N-CoR does not appear to affect its interaction with HDAC3 or HDAC-dependent corepressors like Sin3A, K152 within the SUMO consensus motif in RDI plays an important role in N-CoR repression.

Different lines of evidence demonstrate that N-CoR directly interacts with components of the SUMO pathway. The SUMO-E2 conjugation enzyme Ubc9 and the SUMO-E3 ligase Pias1 were found in yeast two-hybrid interactions with the N-CoR SANT1 + 2 domains. Pulldown experiments using different N-CoR domains have shown that both proteins interact with the SANT1 domain of N-CoR. Furthermore, an additional interaction between N-CoR RDI and Pias1 was found, which was not further investigated, because this region of N-CoR appears to be involved in non-specific interactions. In Figure 3A we clearly show that in the

Figure 6. K-to-R substitutions within the SUMO consensus sequences of N-CoR RDI reduces the ability to repress transcription. (A) Reporter activity (2xUAS-TK-Luc) in 293T cells transfected with pCMXGAL4 or pCMXGAL4-N-CoR full-length constructs is shown. Luciferase assays were performed as in Figure 5A. Equal expression of N-CoR wt and mutant proteins was verified by Western blot (right panel). (B) 293T cells were transfected with pCMX-N-CoR-Flag constructs and 6xHis-SUMO-1 and lysed in 8 M urea. His-tagged proteins were purified under denaturing conditions. The middle panel shows lysates for the input, whereas the top panel shows Cobalt-NTA affinity-purified proteins. Western blot for both panels was performed with Flag antibody. The bottom panel shows actin protein to verify equal loading of inputs. (C) 293T cells were transfected with a Flag-tagged vector containing the DNA of N-CoR wt or K-to-R substitution mutants. Whole cell extracts were immunoprecipitated with Flag antibody, subjected to Western blot analysis and probed with α -N-CoR, α -Sin3A and α -HDAC3 antibody. Input is shown in the left lane.

presence of Pias1 N-CoR RDIII is specifically SUMOylated, although no interaction between this N-CoR domain and the E3 ligase could be detected in pulldown experiments (Figure 1 D). This could be due to low expression levels of this GST-N-CoR fusion and substantial degradation (Figure 1D). SUMOylation of N-CoR RDIII suggests at least some transient interaction between this domain and Pias1. In addition, immunofluorescence studies with endogenous or recombinant N-CoR, Ubc9, and Pias1 have demonstrated that these proteins colocalize in mammalian cells (unpublished data). Finally, N-CoR immunoprecipitations from mammalian cells revealed an association with recombinant or endogenous SUMO E3 ligase Pias1.

Using His-tagged SUMO-1 or His-tagged N-CoR RDI protein, we found that full-length N-CoR (Figure 6B) as well as N-CoR RDI (Figure 4B) is modified by SUMO-1 in vivo. N-CoR consists of multiple modular domains, and interestingly only repression domains RDI and RDIII contain the SUMO consensus motif ψ KxE. There may however exist additional SUMO sites in N-CoR that do not conform to this consensus. Using a highly specific in vitro SUMOylation

assay, we provide evidence that RDI (K152) and RDIII (K1117 and K1330) are modified by SUMO-1 in vitro. The SANT1 domain, which mediates the interaction to the components of the SUMO pathway, is not SUMO modified. Thus, N-CoR provides an interesting example for the spatial separation of the recruitment of E3 ligase activity and SUMO modification of acceptor sites.

The SUMO modification sequences in N-CoR RDI are also present in the equivalent repression domain of SMRT. These motifs are highly conserved in human and mouse homologues and are also found in the SNOR (SMRTER, SMRT, and N-CoR) domain of SMRTR, which is a *Drosophila* homologue of SMRT and N-CoR (Tsai *et al.*, 1999). This high degree of conservation suggests a similar role of SUMO modification of the corepressor N-termini. Here we show that N-CoR K152 in RDI is a target for SUMO-1 modification in vitro and in vivo. Interestingly, mutagenesis reveals a correlation between SUMO modification and N-CoR repression activity because repression by RDI is impaired in the absence of the functional SUMO acceptor site K152. Substitution of this lysine by arginine drastically reduces repression of both full-length N-CoR and RDI alone (Figures 4C and 6A). Consistent with this finding, N-CoRs repression is enhanced by coexpression of SUMO-E2 or SUMO-E3 protein.

In addition, we investigated whether inhibition of the SUMO pathway by Gam1 (Boggio *et al.*, 2004; Degerny *et al.*, 2005) influences the transcription of known N-CoR-dependent target genes. Our data show that in the presence of agonistic ligand the transcription of a RAR and TR reporter is significantly increased when Gam1 is coexpressed. This result suggests that either the nuclear receptor or coactivators may be influenced in their activity by SUMO. Several publications showed a connection of this posttranslational modification to nuclear receptors and coactivators (Abdel-Hafiz *et al.*, 2002; Girdwood *et al.*, 2003). More importantly, we investigated the active repressive function of RAR using the synthetic RAR antagonist 193840. An increased level of repression was observed, which was unchanged by coexpression of Gam1. Binding of antagonist to the receptor leads to a conformational change of the ligand-binding domain, which then enables binding of corepressor to the receptor. Even when Gam1 may inhibit N-CoR repression activity on a RAR reporter this would not necessarily lead to a recruitment of coactivator activity. Differences in N-CoR repression activity might be difficult to study under those conditions. Coexpression of SUMO-deficient N-CoR mutants on RAR or TR reporters would furthermore compete with high levels of endogenous N-CoR present in the cell. Therefore, we decided to use a Gal-N-CoR reporter assay to study N-CoR repressive events.

To date, several roles for SUMO modification have been proposed. SUMO alters interactions of substrates with other proteins or with DNA, changes the subcellular localization of proteins, or acts by blocking ubiquitin attachment sites (reviewed in Verger *et al.*, 2003; Johnson, 2004). Because SUMOylation can affect the subcellular distribution of proteins, we investigated the localization of N-CoR. The corepressor is predominantly localized in the nucleus and we found that this pattern is not affected by substitution of SUMO-acceptor lysines in N-CoR RDI or RDIII (unpublished data).

Instead, the ability of N-CoR to repress transcription is strongly affected by increased SUMOylation. Our results indicate that Ubc9 coexpression significantly enhances Gal4-N-CoR-mediated transcriptional repression of a reporter (Figure 5A). Overexpression of both Ubc9 and Pias1 had an

even more pronounced effect (Figure 5A). This effect was not found by using a mutant of Ubc9, which is unable to transfer SUMO to target proteins. This result demonstrates that SUMOylation of K152 is an important modulator of N-CoR repressor function. It is conceivable that SUMOylation of N-CoR might create new surfaces for the interaction of other proteins, which enhance repression. Alternatively, the addition of relatively bulky SUMO moieties might affect the conformation of N-CoR, e.g., by altering intramolecular interactions.

SUMO modification has been described to influence the assembly of transcription factors on promoters and the recruitment of chromatin-modifying enzymes. SUMOylation of Elk-1, for example, results in the recruitment of histone deacetylase activity, in particular HDAC-2, to promoters (Yang and Sharrocks, 2004). The N-terminal portion of N-CoR interacts with HDAC3 and Sin3A (Heinzel *et al.*, 1997; Guenther *et al.*, 2000; Wen *et al.*, 2000). We could not observe any SUMOylation-induced changes in the binding of these proteins to N-CoR (Figure 6C and unpublished data). The association of HDACs with the N-CoR K₁₅₂R mutant may also explain its residual repression activity (Figure 4C). We conclude that K152 of N-CoR RDI plays a major role for the ability of N-CoR to repress transcription and that the effects of SUMOylation are more complex than a change in HDAC binding.

We speculate that recruitment of the SUMO machinery by N-CoR may lead to more efficient SUMOylation of N-CoR-associated factors. Thus, N-CoR could serve as a platform for the recruitment of Ubc9 and Pias1, which could then lead to more efficient SUMO-modification of N-CoR-associated proteins such as HDACs. SUMOylation of N-CoR may also affect its interaction with nuclear receptors. We found a higher activation of TR and RAR reporters by cotransfection of Gam1 (Figure 3C). This could be due to more efficient dissociation of nonSUMOylated N-CoR from the ligand-binding domain of nuclear receptors. Interestingly, a molecular pathway by which peroxisome proliferator-activated receptor- γ (PPAR- γ) represses the transcriptional activation of inflammatory response genes was recently described (Pascual *et al.*, 2005). Ligand-dependent SUMOylation of the ligand-binding domain targets PPAR- γ to NCoR-HDAC3 complexes. The nuclear receptor interaction domain of N-CoR was not involved in this interaction. Furthermore, N-CoR binding to PPAR- γ was Ubc9-dependent and HDAC3 binding was not altered, in response to Ubc9 and Pias1 siRNA treatment, similar to our findings (Figure 6C). SUMOylation of N-CoR RDI or RDIH may thus very well play important roles in regulating the interaction with nuclear receptors. Several reports showed that class I (HDAC1, HDAC2) and class II HDACs (HDAC4, HDAC6) are targets for SUMO modification (David *et al.*, 2002; Kirsh *et al.*, 2002; Girdwood *et al.*, 2003; Yang and Sharrocks, 2004). Interestingly, histones are also subject to SUMOylation and post-translational modifications play important roles in the regulation of chromatin structure and function. In particular, histone H4 is modified by SUMO, which mediates gene silencing through the recruitment of HDACs and HP-1 (Shiio and Eisenman, 2003). Furthermore, N-CoR can interact with histones through the SANT domain, and the SANT1 domain itself can also interact with SUMO E2 and E3 proteins, which could induce a more repressive state.

In conclusion, our finding that N-CoR undergoes SUMO modification adds a new aspect to our understanding of transcriptional repression and further strengthens the evidence implicating this type of posttranslational modification in regulatory events occurring at the transcriptional and

chromatin level. It is likely that the coordinated recruitment of proteins of the SUMOylation machinery and of SUMO substrates may be an important property of N-CoR and related corepressors.

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