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Negative Modulation of RXRα Transcriptional Activity by Small Ubiquitin-related Modifier (SUMO) Modification and Its Reversal by SUMO-specific Protease SUSP1*

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Retinoid X receptor α (RXRα) belongs to a family of ligand-activated transcription factors that regulate many aspects of metazoan life. Here we demonstrate that RXRα is a target substrate of a small ubiquitin-related modifier (SUMO)-specific protease, SUSP1, which is capable of controlling the transcriptional activity of RXRα. RXRα was modified by SUMO-1 *in vivo* as well as *in vitro*, and the Lys-108 residue within the IKPP sequence of RXRα AF-1 domain was identified as the major SUMO-1 acceptor site. Prevention of SUMO modification by Lys-to-Arg mutation led to an increase not only in the transcriptional activity of RXRα but also in the activity of its heterodimeric complex with retinoic acid receptor-α or peroxisome proliferator-activated receptor-γ (PPARγ). SUSP1 co-localized with RXRα in the nucleus and removed SUMO-1 from RXRα but not from androgen receptor or PPARγ. Moreover, overexpression of SUSP1 caused an increase in the transcriptional activity of RXRα, whereas small hairpin RNA-mediated knockdown of endogenous SUSP1 led to a decrease in RXRα activity. These results suggest that SUSP1 plays an important role in the control of the transcriptional activity of RXRα and thus in the RXRα-mediated cellular processes.

Retinoic acids (RA),[4] natural and synthetic derivatives of vitamin A, modulate a wide variety of biological processes, including proliferation, homeostasis, and differentiation of many cell types (1, 2). RA exerts its effects through two classes of nuclear receptors acting as ligand-dependent transcriptional regulators: the retinoic acid receptors (RARs), which bind either all-*trans*-retinoic acid or 9-*cis*-RA (9*c*RA); and the retinoid X receptors (RXRs), which bind 9*c*RA only. There are three RAR isotypes (α, β, and γ) and three RXR isotypes (α, β, and γ) encoded by distinct genes, and for each isotype there are at least two main isoforms, which differ in their N-terminal region. Each receptor has an N-terminal A/B region that harbors the ligand-independent activation function-1 (AF-1), a central C region containing a DNA-binding domain, and a C-terminal E region containing a ligand-binding domain and a ligand-dependent AF-2.

RXRs play important roles in numerous nuclear receptor-dependent signaling pathways (3). Not only can an RXR function as a homodimer but also as an obligate heterodimeric partner for many other receptors, including those for retinoic acid, thyroid hormone, vitamin D, prostanoids, oxysterols, bile acids, and xenobiotics, as well as several orphan receptors. The homo- and heterodimeric complexes of RXR target specific DNA sequences known as hormone response elements. Correct receptor dimerization on target DNA is required for the recruitment of necessary co-activator or co-repressor proteins to the transcription complex (2, 4).

The small ubiquitin-related modifier (SUMO) is structurally related to ubiquitin and is also ligated to Lys residues within its target proteins (5–13). Mammalian cells contain at least three SUMO family members, SUMO-1/Smt3C, SUMO-2/Smt3A, and SUMO-3/Smt3B. Similar to ubiquitination, SUMO modification occurs through a three-step process involving SUMO activation by the E1 enzymes SAE1/SAE2, SUMO conjugation by the E2 enzyme UBC9, and SUMO ligation by E3-like ligases, including the nucleoporin RanBP2/Nup358 (14, 15), members of the PIAS (protein inhibitors of activated STAT) family of proteins (16–18), and Pc2 (19). The target Lys residues generally fall within a recognizable consensus sequence, namely ψ-Lys-*X*-Glu (where ψ is a large hydrophobic amino acid and *X* is any residue) (20).

SUMO modifies many proteins that participate in diverse cellular processes, including transcriptional regulation, nuclear transport, maintenance of genome integrity, and signal transduction (5–13, 21). Of these, many of the identified SUMO substrates are transcription factors or transcriptional co-regu-

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[4] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; 9*c*RA, 9-*cis*- retinoic acid; PPAR, peroxisome proliferator-activated receptor; AF, activation function; shRNA, small hairpin RNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SUMO, small ubiquitin-related modifier; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; HDAC, histone deacetylase; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; AR, androgen receptor; GFP, green fluorescent protein; GST, glutathione *S*-transferase; PML, promyelocytic leukemia.

lators, and in numerous cases, modification with SUMO leads to attenuation of transcriptional activation. Thus, elimination of SUMO acceptor site(s) enables transcription factors to become more potent activators; examples include Sp3 (22, 23), p300 (24), Elk-1 (25), c-Myb (26), C/EBP (27), and CtBP (28). A number of nuclear receptors have also been shown to be sumoylated, and their transcriptional activities are modulated by SUMO modification. The examples include androgen receptor (AR) (29), glucocorticoid receptor (30, 31), and progesterone receptor (32).

Sumoylation is a reversible process, and several SUMO-specific proteases have been described (6–13). Sequence analyses have suggested the presence of seven SUMO protease genes in mammals, which encode proteins with diverse N-terminal domain and conserved catalytic C-terminal domain. These enzymes include SENP3 (SMT3IP1), which localizes to the nucleolus (33); SUSP1 (SENP6), found primarily in the cytoplasm when GFP is fused to its N terminus (34); SENP1, which localizes to foci in the nucleus and the nuclear rim (35); and SENP2, found in at least in three different isoforms derived from alternatively spliced mRNAs (Axam, SMT3IP2/Axam2, SuPr-1). Axam localizes to the nucleoplasmic face of the nuclear pore complex (36, 37), and Axam2 and SuPr-1 have been detected in the cytosol and PML bodies, respectively (38, 39). Substrate specificity between different SUMO-specific proteases has been suggested based on the observed differences in subcellular localization (12).

In the present studies we show that RXRα is covalently modified by SUMO-1 *in vivo* as well as *in vitro* and that this SUMO-1 modification negatively regulates the transcriptional activity of the receptor. We further show that SUSP1 specifically removes SUMO-1 from RXRα and thereby enhances the transcriptional activity of the nuclear receptor. Thus, it appears that reversible SUMO modification serves as an important mechanism for regulation of RXRα-mediated transcriptional activation.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—pcDNA3-FLAG-RXRα was constructed by amplification of the RXRα coding sequence by PCR using a 5′ primer containing BglII site and a 3′ primer containing NotI site. The PCR fragment was ligated into BamHI and NotI sites of pcDNA3-FLAG. To eliminate the SUMO-1 acceptor site(s) in RXRα, Lys residues were replaced by Arg upon site-directed mutagenesis using pcDNA3-FLAG-RXRα as the template. To generate RXRα deletion mutants (amino acid sequences 1–100, 1–200, 1–300, 1–400, 101–462, 201–462, 301–462 and 401–462), the PCR fragments corresponding to the sequences were ligated into BamHI and NotI sites in pcDNA3-FLAG. To obtain vectors expressing SUMO-1, the coding region for the mature form of SUMO-1 was amplified by PCR using primers containing BamHI and XhoI sites. The PCR fragments were ligated into pGEX4T-2 (Amersham Biosciences) and pcDNA4-HisMax/C (Invitrogen), and the resulting vectors were referred to as pGEX-SUMO-1 and pcDNA4-HisMax-SUMO-1, respectively. Expression vectors for GAL4-fused RXRα deletion mutants were constructed by ligating the PCR fragments for the corresponding regions into pM vector (Clontech). pcDNA3.1-SUSP1-V5 expressing SUSP1 fused to the N terminus of V5 tag was prepared by TA cloning of its coding sequence into pcDNA3.1/V5/His-TOPO (Invitrogen) as specified by the manufacturer. pcDNA3.1-SUSP1/C1030S-V5 was generated by replacement of active site Cys-1030 by Ser upon site-directed mutagenesis. pEGFP-C1-SUSP1 was prepared as described previously (34).

Protein Purification—GST-SUMO-1, GST-SAE2/SAE1, and GST-RXRα were overproduced in BL21-CodonPlus bacteria (Stratagene). Each protein was then purified by using a glutathione-Sepharose 4B column. Purified GST-SUMO-1 was treated with thrombin and applied again onto the GST-affinity column. SUMO-1 was then recovered as the unbound protein. Partially purified SUSP1 and SUSP1/C1030S were prepared by transfection of HEK293T cells with pcDNA3.1-SUSP1-V5 or pcDNA3.1-SUSP1/C1030S-V5. Cell lysates were loaded on Ni^{2+}-nitrilotriacetic acid agarose columns, and bound proteins were obtained by following the standard procedure supplied by the manufacturer (Qiagen). His-UBC9 was also overproduced in BL21-CodonPlus bacteria and isolated by using nitrilotriacetic acid-agarose.

GST Pulldown Assay—GST (10 μg) or GST-RXRα (10 μg) was incubated with 10 μl of glutathione-Sepharose 4B beads for 1 h at 4 °C. The samples were further incubated with 1 μg of His-UBC9 in the presence or absence of 1 μM 9*c*RA for 2 h at 4 °C. Beads were washed five times with 1 ml of the pulldown buffer consisting of 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.1% Nonidet P-40, 0.1% Triton X-100, 1 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, and 1× protease inhibitor mixture (Roche Applied Science). Proteins bound to the beads were released by boiling in 2× SDS-PAGE sampling buffer and analyzed by immunoblot with anti-His monoclonal antibody (Qiagen).

Cell Culture and Transfections—All cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 25 units/ml penicillin, and 25 units/ml streptomycin. Cells were transfected with the appropriate vectors using Lipofectamine Plus reagent (Invitrogen). The total amounts of transfected vectors in each experiment were equalized by supplementing empty vector DNA.

In Vitro Sumoylation and Desumoylation Assays—For the *in vitro* sumoylation assay, RXRα or its mutant forms were radiolabeled using an *in vitro* transcription and translation system (Promega) in the presence of [^{35}S]Met. The labeled proteins were then subjected to SUMO modification by incubation at 37 °C for 2 h with 10 μg of SUMO-1, 10 μg of His-UBC9, 3 μg of GST-SAE2/SAE1, and an ATP-regenerating system (50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, and 0.6 unit/ml inorganic pyrophosphatase). The reaction was terminated by the addition of 2 × SDS-PAGE sampling buffer followed by boiling. The samples were resolved by SDS-PAGE and visualized by autoradiography.

For *in vitro* desumoylation assay, purified GST-RXRα was sumoylated as described above followed by incubation at 37 °C with partially purified SUSP1 or SUSP1/C1030S in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM dithiothreitol. The reaction was terminated by addition of 2× SDS-PAGE sampling

buffer and boiling. The samples were resolved by SDS-PAGE followed by immunoblot with anti-RXRα antibody (Santa Cruz Biotechnology).

Immunocytochemistry—HeLa cells were grown on coverslips and transfected with the appropriate vectors. After incubation for 36 h, cells were fixed by incubation with 2% formaldehyde in PBS for 30 min. They were washed three times with PBS containing 0.1% Triton X-100, permeabilized with PBS containing 0.5% Triton X-100 for 5 min, and incubated with PBS containing 0.1% Triton X-100, 10% normal goat serum, 1% bovine serum albumin, and 1% gelatin. Cells were incubated for 1 h with rabbit anti-FLAG antibody (Sigma), mouse anti-V5 antibody (Invitrogen), or anti-SUSP1 antibody (Abgent) in PBS containing 3% bovine serum albumin and 0.1% Triton X-100. After washing three times with PBS containing 0.1% Triton X-100, cells were incubated for 1 h with FITC-labeled goat anti-mouse IgG and TRITC-labeled goat anti-rabbit IgG or FITC-labeled goat anti-rabbit IgG in PBS containing 3% bovine serum albumin and 0.1% Triton X-100. After washing, cells were observed under a Carl Zeiss LSM510 confocal microscope or a Zeiss Axioplan II microscope.

Knockdown of SUSP1 mRNA—pSM2c-SUSP1 expressing SUSP1-specific short hairpin RNAs (nucleotides 2679–2698 from NM_015571), referred to as shRNA, was purchased from Open Biosystems. To knock down SUSP1 mRNA, HEK293T cells were transfected with shRNA or a negative control vector (shControl). Total RNAs were prepared from cells by extracting them with Trizol (Invitrogen) and were subjected to reverse transcription-PCR. Reverse transcription reactions were performed using Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primer by following the manufacturer's instruction. SUSP1 mRNA was amplified with primers (5′-GCT GTA ATT GAT TCC AAT CC-3′ and 5′-AGT CAA TCT GAG ATA CTA TTG ACA C-3′), and β-actin mRNA was amplified as an expression control.

RESULTS

SUMO Modification of RXRα—To identify novel proteins that interact with RXRα, we performed yeast two-hybrid screening by using RXRα as bait from a *Xenopus* embryo cDNA library. Among 5 independently identified clones, 2 clones encoded sequences identical to a SUMO-specific protease, SUSP1. Both clones contained the N-terminal regions of *Xenopus* SUSP1 spanning the amino acid sequence 5–826, which corresponds to that of the human SUSP1 sequence 5–813 containing a part of catalytic domain called the His box (34). Because SUSP1 interacted with RXRα (see below), we hypothesized that RXRα could be a target for SUMO modification. To test this hypothesis, we first examined whether RXRα could interact with UBC9, a SUMO-conjugating E2 enzyme. Purified GST-RXRα was incubated with His-UBC9 in the absence or presence of 9*c*RA. A pulldown assay using glutathione-conjugated Sepharose resin revealed that GST-RXRα could co-precipitate with UBC9 whether or not 9*c*RA was present (Fig. 1*A*), indicating that RXRα directly interacts with UBC9 and 9*c*RA does not influence their interaction. These results also suggest that RXRα is a target of SUMO modification.

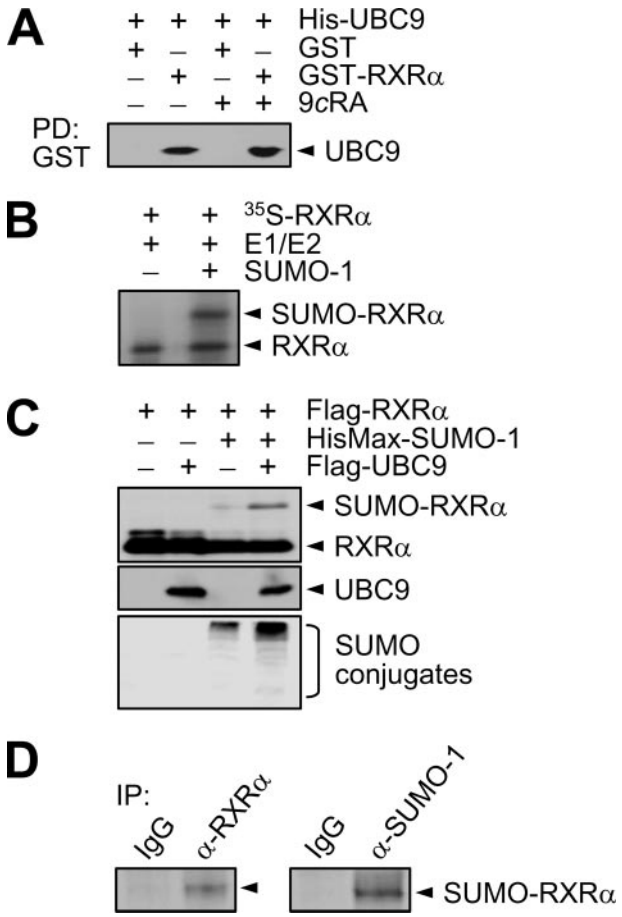

FIGURE 1. **Interaction of RXRα with UBC9 and sumoylation of RXRα.** *A*, purified His-UBC9 was incubated with GST or GST-RXRα at 4 °C for 2 h and then with glutathione-conjugated Sepharose 4B for the next hour. Proteins bound to the resin were pulled down (*PD*), and subjected to SDS-PAGE followed by immunoblot with anti-His antibody. *B*, *in vitro* translated ^{35}S-labeled RXRα was incubated with purified SUMO-1, His-UBC9, and GST-SAE2/SAE1 at 4 °C for 2 h. The samples were then subjected to SDS-PAGE followed by autoradiography. *C*, pcDNA3-FLAG-RXRα was transfected to HEK293T cells with pcDNA4-HisMax-SUMO-1, pcDNA3-FLAG-UBC9, or both. After incubation for 36 h, cells were collected and their lysates subjected to immunoblot with anti-FLAG antibody. The lysates were also probed with anti-Xpress antibody. *D*, lysates were obtained from HEK293T cells and subjected to immunoprecipitation (*IP*) with anti-RXRα antibody followed by immunoblot with anti-SUMO-1 antibody (*left panel*). The same cell lysates were subjected to immunoprecipitation with anti-SUMO-1 antibody followed by immunoblot with anti-RXRα antibody (*right panel*).

To determine whether RXRα can be sumoylated, ^{35}S-labeled RXRα was prepared and incubated with purified SUMO-1, E1 (SAE1/SAE2), and E2 (UBC9). Fig. 1*B* shows that RXRα can be modified by SUMO-1 *in vitro*. We also examined whether RXRα could be sumoylated *in vivo*. FLAG-RXRα was expressed in HEK293T cells with HisMax-SUMO-1, FLAG-UBC9, or both. Cell lysates were prepared and subjected to immunoblot with anti-RXRα antibody. A slow migrating band could be seen in the lysates from the cells expressing SUMO-1 (Fig. 1*C*). Moreover, co-expression of UBC9 led to an increase in the intensity of the slow migrating band as well as of SUMO-conjugated cellular proteins, suggesting that RXRα can be modified by SUMO-1. We then examined whether endogenous RXRα could be modified by SUMO-1. Lysates obtained from HEK293T cells were subjected to immunoprecipitation with anti-RXRα antibody followed by immunoblot with anti-

SUMO-1 antibody. A SUMO-conjugated band could be seen in the lysates precipitated by anti-RXRα antibody but not in those precipitated by control IgG (Fig. 1*D*, *left panel*), suggesting that endogenous RXRα can be sumoylated. As a confirmation, the same cell lysates were subjected to immunoprecipitation with anti-SUMO-1 antibody followed by immunoblot with anti-RXRα antibody. A SUMO-conjugated band again appeared in the lysates that had been precipitated by anti-SUMO-1 antibody but not in those precipitated by control IgG (Fig. 1*D*, *right panel*). These results indicate that RXRα is a natural substrate for SUMO modification.

Determination of SUMO Acceptor Site in RXRα—SUMO modification to target proteins occurs at specific Lys residues, which are commonly embedded in a consensus sequence, ψ-Lys-*X*-Glu (20, 40). RXRα contains two similar sequences, $MK^{201}RE$ and $PK^{245}TE$ in the hinge region and ligand-binding domain, respectively. Therefore, we first examined whether the Lys residues might serve as the SUMO acceptor sites upon substitution of each residue with Arg by site-directed mutagenesis. *In vitro* sumoylation assay revealed that neither the K201R nor the K245R mutation affected the SUMO modification of RXRα (data not shown). Neither did the double mutation (K210R/K245R) show any effect on RXRα sumoylation, indicating that Lys-210 and Lys-245 in the putative consensus sequences are not the SUMO acceptor sites in RXRα.

The Lys residues in non-consensus sequences have also been shown to serve as the SUMO acceptor sites (41, 42). To identify the SUMO acceptor site(s) in RXRα, we constructed serial deletion mutants (Fig. 2*A*) and subjected them to an *in vitro* sumoylation assay. As shown in Fig. 2*B*, slow migrating, sumoylated bands were not detected with the RXRα mutant proteins lacking the amino acid sequence from 101 to 200. These results suggest that the SUMO acceptor site is located in the RXRα sequence 101–200, which contains 10 Lys residues. To identify the SUMO acceptor Lys residue(s) in the 101–200 sequence, each of 10 Lys residues was replaced by Arg, and the resulting mutant proteins were subjected to *in vitro* sumoylation assay. Among the Lys-to-Arg mutations, only the K108R mutation blocked the sumoylation of RXRα (Fig. 2*C*), indicating that Lys-108 is the major acceptor site for SUMO under *in vitro* conditions. To determine whether the K108R mutation could prevent *in vivo* sumoylation of RXRα, HisMax-SUMO-1 was expressed in HEK293T cells with FLAG-RXRα or FLAG-RXRα/K108R. Immunoprecipitation analysis using anti-FLAG antibody demonstrated that wild-type RXRα (*wt*), but not RXRα/K108R (*mt*), can be sumoylated (Fig. 2*D*). These results indicate that Lys-108 serves as the major SUMO acceptor site in RXRα under both *in vivo* and *in vitro* conditions.

Effect of K108R Mutation on Transcriptional Activity of RXRα—SUMO modification has been shown to influence the activity of many transcription factors. To determine whether the K108R mutation might affect the transcriptional activity of RXRα, the DNA-binding domain of GAL4 was fused to various deletions of RXRα or RXRα/K108R (Fig. 3*A*). HEK293T cells were transfected with vectors expressing the GAL4-fused RXRα mutants and a luciferase reporter vector. They were then cultured in the absence or presence of 9*c*RA. The transcriptional activity of GAL4-AF1/K108R was more than 1.5-fold

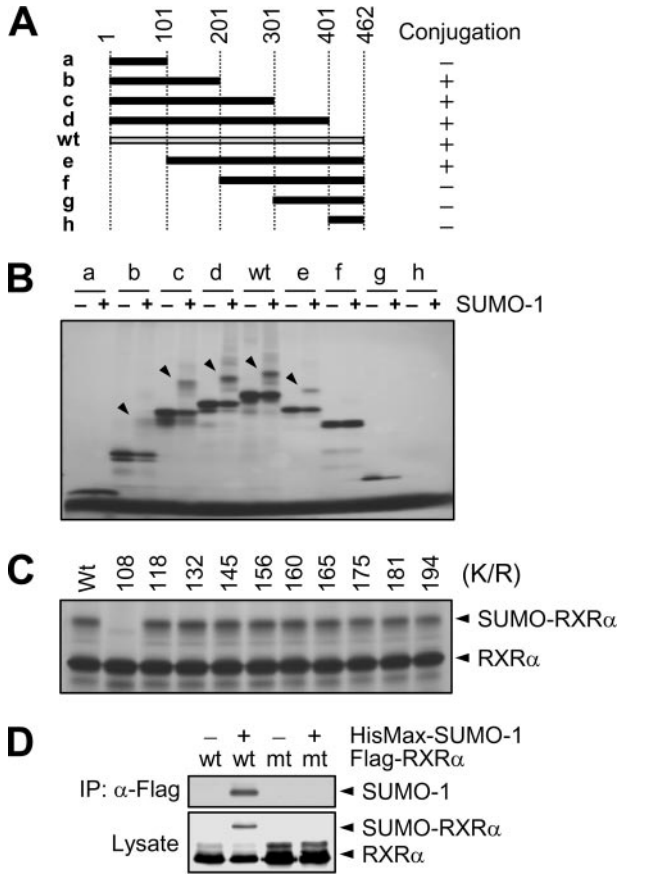

FIGURE 2. **Determination of SUMO-1 conjugation site in RXRα.** *A*, various deletion constructs of RXRα were generated as shown. A summary of the results from *B* is shown on the right: +, constructs that were SUMO-conjugated; −, those that were not SUMO-conjugated. *B*, ^{35}S-labeled RXRα and its deletion mutants were subjected to a sumoylation assay in the absence or presence of SUMO-1 as described under "Experimental Procedures." Proteins were resolved by SDS-PAGE and visualized by autoradiography. The *arrowheads* indicate the sumoylated proteins. *C*, each of 10 Lys residues in the RXRα sequence 101–200 was replaced by Arg, and the resulting mutant proteins were subjected to a sumoylation assay as described in *B*. The *numerals* shown *above* the gels indicate the position of each Lys residue in the sequence. *D*, pcDNA3-FLAG-RXRα (*wt*) or pcDNA3-FLAG-RXRα/K108R (*mt*) was transfected to HEK293T cells with or without pc-DNA4-HisMax-SUMO-1. After incubation for 36 h, cells were subjected to immunoprecipitation (*IP*) with anti-FLAG antibody followed by immunoblot with anti-Xpress antibody. Cell lysates were also directly probed with anti-FLAG antibody.

higher than that of GAL4-AF1; this effect of the K108R mutation was independent of 9*c*RA, as the constructs do not contain the ligand-binding domain (Fig. 3*B*, *lower panel*). The 9*c*RA-stimulated activities of GAL4-RXRα/K108R and GAL4-AF1/K108R-LBD lacking the DNA-binding domain were ~2-fold higher than those of their parental forms, respectively. The *upper panel* of Fig. 3*B* shows the expression levels of RXRα deletions as a control for transfection efficiencies. These results suggest that the transcriptional activity of RXRα is negatively regulated by SUMO modification.

RXRα is a combinational partner in the nuclear receptor family and can form a homodimer by itself and heterodimers with a variety of hormone and orphan receptors. The dimerization partners of RXRα include RAR, PPAR, TR (thyroid hormone receptor), VDR (vitamin D receptor), and so on (4). To determine whether the K108R mutation might also influence the transcriptional activity of heterodimeric RXRα,

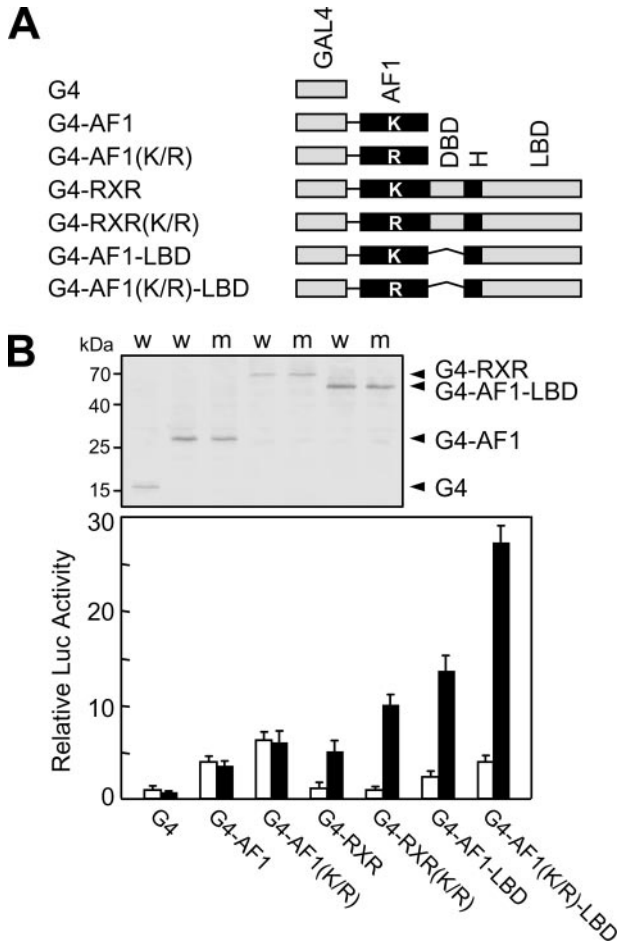

FIGURE 3. **Effect of K108R mutation on transcriptional activity of RXRα.** *A*, the DNA-binding domain of GAL4 was fused to various deletions of RXRα or RXRα/K108R. *G4*, GAL4 DNA-binding domain; *K/R*, K108R mutation; *DBD*, DNA-binding domain; *LBD*, ligand-binding domain; *H*, hinge region. *B*, HEK293T cells were transfected with pGAL4-UAS-Luc, pCMV-β-galactosidase, and pM vectors expressing the GAL4-fused RXRα mutants. After incubation for 24 h, cells were further cultured in DMEM containing 0.2% charcoal-stripped FBS in the absence (*open bars*) or presence of 1 μM 9cRA (*closed bars*). Cell lysates were then assayed for luciferase (*Luc*) activity (*lower panel*). Total amounts of transfected vector DNAs were kept constant by supplementing the appropriate amount of empty vector. Luciferase activities were then normalized relative to β-galactosidase activity. Data represent mean ± S.D. of triplicates. The lysates from cells cultured in the absence of the ligand were subjected to SDS-PAGE followed by immunoblot with anti-Gal4-DBD antibody (*upper panel*). *w*, wild type; *m*, mutant.

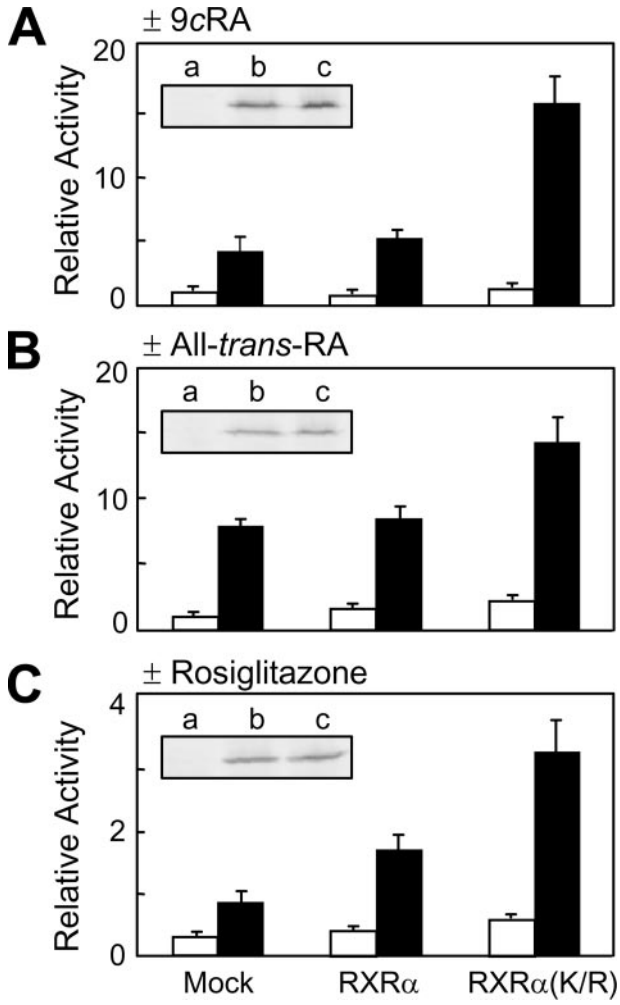

FIGURE 4. **Effect of K108R mutation on transcriptional activity of RXRα homo- or heterodimers.** pcDNA3 (*lane a*), pcDNA3-FLAG-RXRα (*lane b*), or pcDNA3-FLAG-RXRα/K108R (*lane c*) was transfected to HEK293 cells with pCMV-β-galactosidase. They were also co-transfected with p3XPPRE-Luc (*A*), p3XRARE-Luc and pcDNA-RARα (*B*), or p3XPPRE-Luc and pcDNA-PPARγ (*C*). After incubation for 24 h, cells were further cultured in DMEM containing charcoal-stripped FBS in the absence (*open bars*) or presence (*closed bars*) of 1 μM 9cRA (*A*), 1 μM all-*trans*-RA (*B*), or 5 μM rosiglitazone (*C*). They were then subjected to luciferase assay. Data represent mean ± S.D. of triplicates. The lysates from cells cultured in the absence of ligand were subjected to SDS-PAGE followed by immunoblot with anti-FLAG antibody (*insets*).

HEK293T cells were transfected with luciferase reporter vectors containing various response elements and vectors expressing the RXRα binding partners. They were then cultured in the absence or presence of corresponding ligands (*i.e.* 9*c*RA for RXRα homodimer; all-*trans*-RA for RARα/RXRα heterodimer, and rosiglitazone for PPARγ/RXRα heterodimer). As a control, we first examined the effect of K108R mutation on the transcriptional activity of RXRα homodimer using the cells transfected with p3xPPRE-Luc and a RXRα expression vector. RXRα/K108R had about 3-fold higher activity than wild-type RXRα (Fig. 4*A*), again indicating that SUMO modification negatively regulates the transcriptional activity of RXRα homodimer. In the cells transfected with pRARE-tk-Luc and expression vectors for both RXRα and RARα, the K108R mutation caused about a 1.5-fold increase in the transcriptional activity (Fig. 4*B*). Similarly, the same mutation caused about a 2-fold increase in the transcriptional activity in cells transfected with p3xPPRE-Luc and expression vectors for both RXRα and PPARγ (Fig. 4*C*). The *insets* in Fig. 4 show the expression levels of RXRα and RXRα/K108R as a control for transfection efficiencies. Taken together, these results suggest that SUMO modification negatively regulates the transcriptional activity of RXRα heterodimers as well as of RXRα homodimer.

Interaction of RXRα with SUSP1 and Their Subcellular Localization—Interaction of RXRα with SUSP1 was initially identified by yeast two-hybrid screening. To confirm the interaction of RXRα with SUSP1, FLAG-RXRα, SUSP1-V5, or both were expressed in HEK293T cells. Immunoprecipitation of cell lysates with anti-FLAG antibody shows that SUSP1 can be co-precipitated with RXRα and RXRα/K108R (Fig. 5*A*), indicating that these proteins interact with each other in *vivo* and that

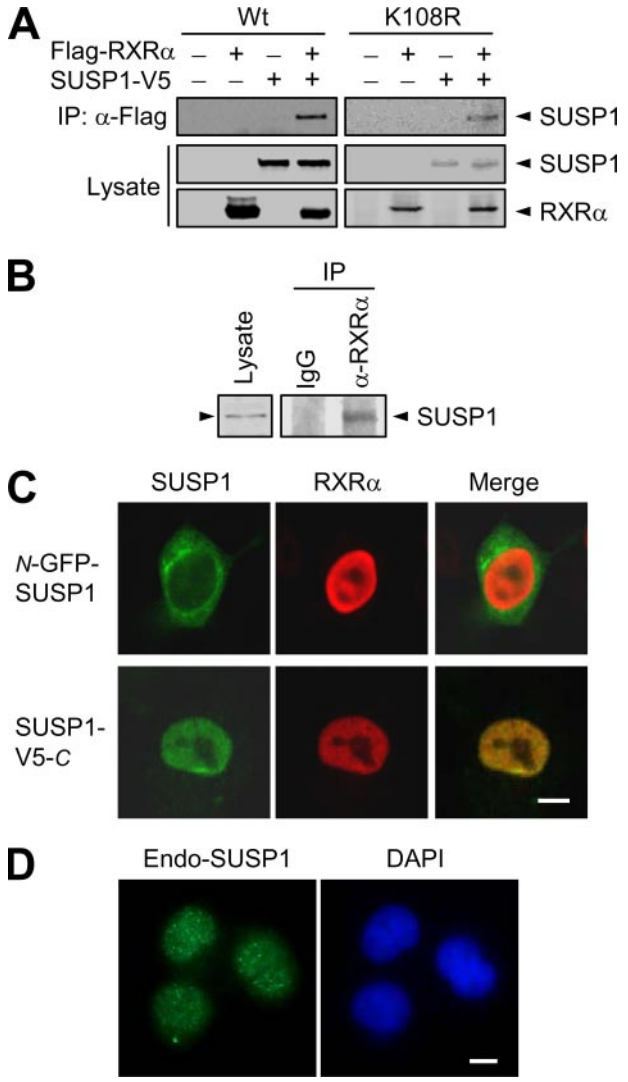

FIGURE 5. **Interaction of RXRα with SUSP1 and their subcellular localization.** *A*, pcDNA3-FLAG-RXRα or pcDNA3-FLAG-RXRα/K108R was transfected to HEK293T cells with or without pcDNA3.1-SUSP1-V5. Cell lysates were prepared and subjected to immunoprecipitation (*IP*) by anti-FLAG antibody followed by immunoblot with anti-V5 antibody. The lysates were also directly probed with anti-FLAG or anti-V5 antibody. *Wt*, wild type. *B*, lysates were prepared from HEK293T cells and subjected to immunoprecipitation with anti-RXRα antibody followed by immunoblot with anti-SUSP1-antibody. *C*, pcDNA3-FLAG-RXRα was transfected to HeLa cells with pEGFP-C1-SUSP1 (*upper panels*) or pcDNA3.1-SUSP1-V5 (*lower panels*). Cells were then fixed and stained with anti-FLAG or anti-V5 antibody. *D*, HeLa cells were stained with anti-SUSP1 antibody. The nuclei were stained with 4,6-diamidino-2-phenylindole (*DAPI*). The *bars* indicate 10 μm.

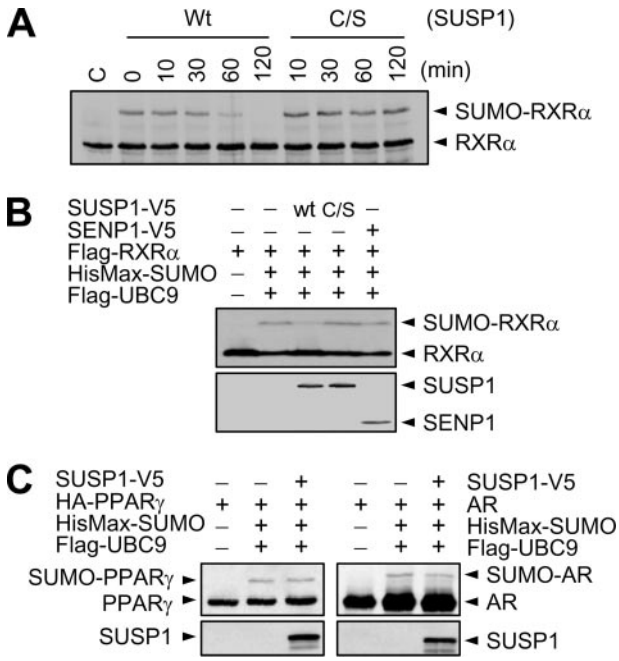

FIGURE 6. **Desumoylation of RXRα by SUSP1.** *A*, GST-RXRα that had been sumoylated were incubated with wild-type SUSP1 (*Wt*) or SUSP1/C1030S (*C/S*) for increasing periods of time. The samples were subjected to immunoblot with anti-RXRα antibody. *B*, pcDNA3-FLAG-RXRα, pcDNA4-HisMax-SUMO-1, and pcDNA3-FLAG-UBC9 were transfected to HEK293T cells with pcDNA3.1-SUSP1-V5 (*wt*), pcDNA3.1-SUSP1/C1030S-V5 (*C/S*), or pcDNA3.1-SENP1-V5. Cell lysates were then subjected to immunoblot with anti-FLAG or anti-V5 antibody. *C*, experiments were performed as in *B*, except that cells were transfected with pcDNA3-HA-PPARγ (*left panel*) or pcDNA3-AR (*right panel*) in place of pcDNA3-FLAG-RXRα.

SUMO modification of RXRα is not required for its binding to SUSP1. We then examined whether endogenous RXRα and SUSP1 could interact with each other. Lysates were prepared from HEK293T cells and subjected to immunoprecipitation with anti-RXRα antibody followed by immunoblot with anti-SUSP1-antibody. Fig. 5*B* shows that RXRα co-precipitates with SUSP1, indicating that the endogenous proteins interact with each other in cells.

RXRα localizes in the nucleus. However, we have previously reported that SUSP1 fused to the C terminus of GFP (*i.e.* GFP-SUSP1) resides in the cytoplasm (34). These findings provoke a discrepancy in the interaction of two proteins that are localized in different cellular compartments. To clarify this discrepancy, we first expressed GFP-SUSP1 in HeLa cells with FLAG-RXRα. Cells were then fixed and stained with anti-FLAG antibody. FLAG-RXRα appeared exclusively in the nucleus as expected, whereas GFP-SUSP1 localized predominantly in the cytoplasm (Fig. 5*C*, *upper panels*) in accord with our previous report (34). Therefore, we suspected that the presence of GFP at the N terminus of SUSP1 might interfere with the translocation of SUSP1 to the nucleus. To test this possibility, FLAG-RXRα was expressed in cells with SUSP1 fused to the N terminus of V5 (*i.e.* SUSP1-V5). Immunostaining of the cells shows that FLAG-RXRα and SUSP1-V5 co-localize in the nucleus (Fig. 5*C*, *lower panels*). SUSP1-GFP, unlike GFP-SUSP1, also co-localized with FLAG-RXRα in the nucleus (data not shown), indicating that the position of tagged proteins alters the subcellular localization of SUSP1. We then examined the subcellular localization of endogenous SUSP1 by staining cells with anti-SUSP1 antibody. SUSP1 was stained almost exclusively in the nucleus (Fig. 5*D*). Collectively, these results indicate that SUSP1 is a nuclear protein.

Desumoylation of RXRα by SUSP1—Because SUSP1 interacts with RXRα, we examined whether SUSP1 shows desumoylating activity toward RXRα *in vitro*. GST-RXRα that had been modified by SUMO-1 was incubated with partially purified SUSP1 or SUSP1/C1030S, of which the active site Cys-1030 was replaced by Ser. Fig. 6*A* shows that wild-type SUSP1 (*Wt*), but not SUSP1/C1030S (*C/S*), is capable of cleaving SUMO-1-conjugated RXRα in a time-dependent fashion, suggesting that RXRα is a target substrate of SUSP1. To determine whether

SUSP1 could also cleave off SUMO-1 from RXRα under *in vivo* conditions, FLAG-RXRα, HisMax-SUMO-1, and FLAG-UBC9 were expressed in HEK293T cells with SUSP1 or SUSP1/C1030S. Immunoblot analysis of cell lysates with anti-FLAG antibody shows that SUSP1, but not SUSP1/C1030S, could cleave SUMO-1-conjugated RXRα (Fig. 6*B*). On the other hand, SENP1 showed relatively little activity toward SUMO-1-conjugated RXRα, suggesting that RXRα is a specific target of SUSP1. We also examined whether SUSP1 could remove SUMO from other nuclear receptors. However, neither AR nor PPARγ was desumoylated by SUSP1 (Fig. 6*C*), again suggesting that SUSP1 acts specifically on RXRα.

Effect of SUSP1 on Transcriptional Activity of RXRα—To determine whether SUSP1 is involved in the control of RXRα transcriptional activity, SUSP1 was expressed in HEK293T cells with GAL4-RXRα or GAL4-RXRα/K108R. Increased expression of SUSP1 led to a gradual increase in the transcriptional activity of GAL4-RXRα (Fig. 7*A*). On the other hand, SUSP1 at all of the concentrations tested showed little or no effect on the transcriptional activity of GAL4-RXRα/K108R. These results again show that the transcriptional activity of RXRα is negatively regulated by SUMO modification. These results also suggest that SUSP1 specifically acts on RXRα but not on other target proteins, in which SUMO modification might influence the transcriptional activity of RXRα.

We then examined whether the desumoylating activity of SUSP1 is required for the increase in transcriptional activity of RXRα. Expression of catalytically inactive SUSP1/C1030, unlike that of wild-type enzyme, showed little or no effect on the transcriptional activity of RXRα (Fig. 7*B*). On the other hand, neither the wild-type nor mutant form of SUSP1 showed any effect on the activity of RXRα/K108R, which lacks the SUMO acceptor site. In addition, SENP1 did not affect the transcriptional activity of either RXRα or RXRα/K108R, again demonstrating that RXRα is a specific target substrate of SUSP1. These results indicate that the desumoylating activity of SUSP1 is required for the positive control of RXRα activity.

To determine whether endogenous SUSP1 is indeed involved in the control of transcriptional activity of RXRα, HEK293T cells were transfected with a SUSP1-specific small hairpin RNA. Transfection of shRNA, but not a control RNA vector (shControl), led to a marked reduction in the level of SUSP1 protein as well as in its transcript level (Fig. 7*C*, *left panel*). Consistently, the level of sumoylated RXRα in shRNA-transfected cells was significantly higher than that in cells transfected with shControl. Furthermore, SUSP1 knockdown caused a significant decrease in the transcriptional activity of RXRα (Fig. 7*C*, *right panel*). These results demonstrate that reversible SUMO modification could be a mechanism that regulates the transcriptional activity of RXRα.

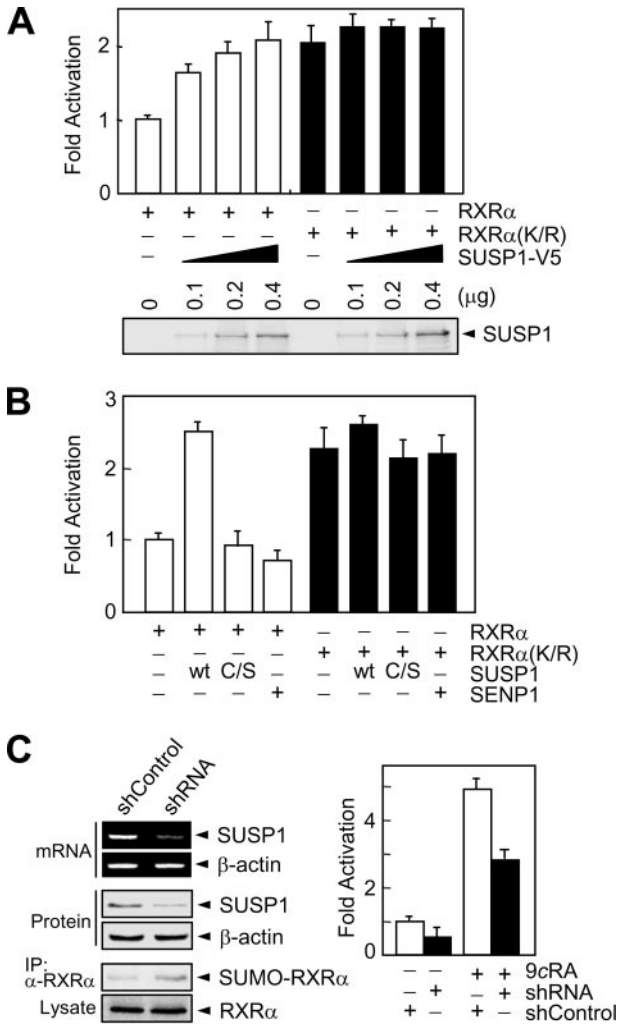

FIGURE 7. **Effect of overexpression or knockdown of SUSP1 on transcriptional activity of RXRα.** *A*, increasing amounts of pDNA3.1-SUSP1-V5 were transfected to HEK293T cells with pGAL4-RXRα or pGAL4-RXRα/K108R. Cells were also transfected with pGAL4-UAS-Luc and pCMV-β-galactosidase. After incubation for 24 h, cells were further cultured in DMEM containing 0.2% charcoal-stripped FBS and 1 μM 9cRA. Cell lysates were then subjected to a luciferase assay (*upper panel*). To confirm the expression of SUSP1, the same lysates were subjected to immunoblot with anti-V5 antibody (*lower panel*). The numerals on the top of the gel indicate the amounts of pDNA3.1-SUSP1-V5 DNA transfected to cells. *B*, pGAL4-RXRα or pGAL4-RXRα/K108R was transfected to cells with pcDNA3.1-SUSP1-V5 (*wt*), pcDNA3.1-SUSP1/C1030S-V5 (*C/S*), or pcDNA3.1-SENP1-V5. Cells were then cultured and subjected to luciferase assay as described in *A*. *C*, pGAL4-RXRα was transfected to cells with pSM2c-SUSP1 (*shRNA*) or pSM2c (*shControl*). Total RNAs levels were obtained and subjected to reverse transcription-PCR to determine the mRNA levels for SUSP1 and β-actin. Lysates were prepared from cells and subjected to immunoblot with anti-SUSP1 or anti-β-actin antibody. Lysates were also subjected to immunoprecipitation (*IP*) with anti-RXRα antibody followed by immunoblot with anti-SUMO-1 antibody (*left panels*). After transfection, duplicated cells were further cultured in the absence or presence of 1 μM 9cRA and subjected to a transcription assay as in *A* (*right panel*). Data represent mean ± S.D. of triplicates.

DISCUSSION

In the present studies we have demonstrated that RXRα is covalently modified by SUMO-1 and that this SUMO modification negatively regulates the transcriptional activity of RXRα. We further demonstrated that a SUMO-specific protease, SUSP1, removed SUMO-1 from RXRα, thereby reversing the sumoylation-mediated repression of RXRα activity. Therefore, we suggest that reversible SUMO modification could serve as an important mechanism for the control of RXRα-dependent transcription. Of note was the finding that the SUMO acceptor site of RXRα resides within the IKPP sequence of the AF-1 domain, which appears distinct from the common ΨK*X*E sequence. Interestingly, RXRβ and RXRγ contain VKPP and IKPL, respectively, in their AF-1 domains, suggesting that the atypical sequences may also serve as the SUMO acceptor sites of the RXRα isoforms. Unlike the retinoid receptors, other

nuclear receptors, including PPARγ, AR, and the glucocorticoid and progesterone receptors, have the consensus ΨK*X*E sequence as their SUMO acceptor sites (29–32, 43). However, the sumoylation sites of the latter receptors also are located in their AF-1 domain. Therefore, it appears that sumoylation-mediated transcriptional repression occurs mainly (if not exclusively) through the modification of Lys residue(s) in the AF-1 domains of the nuclear receptors.

We have previously reported that GFP-SUSP1 localizes predominantly in the cytoplasm (34). However, lines of evidence provided in this study indicate that SUSP1 is a nuclear protein. First, upon yeast two-hybrid screening, SUSP1 was found to interact with RXRα, which is known to reside exclusively in the nucleus. Second, endogenous SUSP1 could be co-immunoprecipitated with RXRα. Third, SUSP-V5 could also be co-immunoprecipitated with FLAG-RXRα. Fourth, ectopically expressed SUSP-V5 or SUSP1-GFP, but not GFP-SUSP1, co-localized with FLAG-RXRα in the nucleus. Finally, endogenous SUSP1 was found to locate exclusively in the nucleus upon staining with anti-SUSP1 antibody. These results indicate that GFP tagged to the N terminus of SUSP1, but not to its C terminus, somehow interferes with the translocation of the protease into the nucleus. Therefore, we wondered whether any nuclear localization signal sequence might be located immediately adjacent to the N terminus of SUSP1. However, three putative nuclear localization signal sequences were found in the sequences 188–205 (KKTEESESQVEPEIKRK), 419–422 (KRRK), and 1086–1090 (KRKHK), all of which are quite remote from the N terminus of SUSP1. Although it remains unclear why GFP-SUSP1 cannot be translocated into the nucleus, unlike SUSP1-V5 or SUSP1-GFP, we should have more carefully analyzed the localization of SUSP1 in our previous report by alternating the position of the tags.

An increasing number of sumoylated transcription factors and co-regulators have been identified. In most cases, SUMO modification appears to repress the activity of targeted transcription factors through altering their subcellular localization and/or their interaction with co-repressors. For examples, sumoylation reduces the transcriptional activity of Sp3 by translocating it to PML nuclear bodies (23). Sumoylation of Elk-1 not only regulates the nucleocytoplasmic shuttling of the transcription factor but also recruits histone deacetylase-2 (HDAC-2) to Elk-1-regulated promoters, thereby repressing their transcription (44, 45). Sumoylation of transcriptional co-activator also recruits HADC-6, leading to repression of p300-mediated transcriptional activity (24). Replacement of the Smad4 sumoylation site Lys-159 by Arg blocks Smad4-Daxx interaction and relieves Daxx-mediated repression of Smad4 transcriptional activity (46). However, SUMO modification of RXRα showed little or no effect on its subcellular localization or interaction with co-regulators tested thus far, such as HDACs, Daxx, and SRC-1 (data not shown). Thus, the mechanism for sumoylation-mediated control of RXRα activity remains to be investigated.

In addition to SUSP1, several SUMO-specific proteases have been shown to reverse the sumoylation-mediated transcriptional repression of nuclear factors (5). SuPr-1, a spliced form of SENP2, was shown to induce c-Jun-dependent transcription independently of c-Jun phosphorylation (39). The mechanism underlying SuPr-1 action on c-Jun activity is through SuPr-1 binding of SUMO-modified PML, thereby altering the distribution of PML in nuclear bodies and nuclear body-associated proteins. SENP1 was also shown to enhance c-Jun-dependent transcription, independently, however, of the sumoylation and phosphorylation status of c-Jun (47). SENP1 action on c-Jun activity is through desumoylation of the CRD1 domain of p300, thereby releasing the *cis*-repression of CRD1 of p300. It has been demonstrated that SENP1 also enhances AR-dependent transcription (48). This stimulatory effect of SENP1 is not mediated by desumoylation of AR but rather through its ability to remove SUMO from HDAC-1, thereby reversing the repressive function of the deacetylase on AR-dependent transcription. Thus, it appears that SuPr-1 and SENP1 act on co-regulators, but not directly on nuclear factors, for the control of transcriptional activity.

Unlike these SUMO-specific proteases, SUSP1 appears to directly act on RXRα for the control of transcriptional activity. Increased expression of SUSP1 resulted in the enhancement of RXRα activity up to the extent seen with the mutant protease (*i.e.* SUSP1/C1030S), which lacks desumoylating activity (Fig. 7*A*). Thus, it is likely that SUSP1-dependent control of RXRα transcriptional activity is mediated by direct desumoylation of the nuclear receptor other than its co-regulators, although the possibility that SUSP1 might also desumoylate the transcriptional co-regulators of RXRα cannot be excluded. Taken together, we suggest that reversible SUMO modification RXRα is a potential mechanism for the control of RXRα transcriptional activity and that SUSP1 is involved in the positive control of the nuclear receptor function.

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