MCB
Journals.ASM.org

RSUME Enhances Glucocorticoid Receptor SUMOylation and Transcriptional Activity

Jimena Druker,[a] Ana C. Liberman,[a] María Antunica-Noguerol,[a] Juan Gerez,[a] Marcelo Paez-Pereda,[b] Theo Rein,[b] Jorge A. Iñiguez-Lluhí,[c] Florian Holsboer,[b] Eduardo Arzt[a]

Instituto de Investigación en Biomedicina de Buenos Aires (IBioBA), CONICET, Partner Institute of the Max Planck Society, and DFBMC, FCEN, Universidad de Buenos Aires, Buenos Aires, Argentina[a]; Max Planck Institute of Psychiatry, Munich, Germany[b]; Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan, USA[c]

Glucocorticoid receptor (GR) activity is modulated by posttranslational modifications, including phosphorylation, ubiquitination, and SUMOylation. The GR has three SUMOylation sites: lysine 297 (K297) and K313 in the N-terminal domain (NTD) and K721 within the ligand-binding domain. SUMOylation of the NTD sites mediates the negative effect of the synergy control motifs of GR on promoters with closely spaced GR binding sites. There is scarce evidence on the role of SUMO conjugation to K721 and its impact on GR transcriptional activity. We have previously shown that RSUME (RWD-containing SUMOylation enhancer) increases protein SUMOylation. We now demonstrate that RSUME interacts with the GR and increases its SUMOylation. RSUME regulates GR transcriptional activity and the expression of its endogenous target genes, FKBP51 and S100P. RSUME uncovers a positive role for the third SUMOylation site, K721, on GR-mediated transcription, demonstrating that GR SUMOylation acts positively in the presence of a SUMOylation enhancer. Both mutation of K721 and small interfering RNA-mediated RSUME knockdown diminish GRIP1 coactivator activity. RSUME, whose expression is induced under stress conditions, is a key factor in heat shock-induced GR SUMOylation. These results show that inhibitory and stimulatory SUMO sites are present in the GR and at higher SUMOylation levels the stimulatory one becomes dominant.

Glucocorticoid (GC) actions are mediated by the glucocorticoid receptor (GR), a ligand-activated transcription factor and member of the nuclear receptor (NR) superfamily. GR plays an important role in gene transcription (1), regulating processes such as inflammation, glucose and lipid metabolism, stress response, development, the cell cycle, and apoptosis (2–4). GR has a modular structure consisting of three major functional domains, the N-terminal domain (NTD), the central DNA-binding domain (DBD), and the C-terminal ligand-binding domain (LBD). Upon ligand binding, GR dissociates from multichaperone complexes, dimerizes, and translocates into the nucleus (5). In addition to its role in ligand recognition, the LBD contains a ligand-dependent activation function (AF-2) that is tightly regulated by hormone binding and several coactivators interacting with this domain (6). GR-mediated transcriptional activity is regulated not only by its binding to GC response elements (GREs) and the balance between coactivators and corepressors but also by posttranslational modifications, which include phosphorylation, ubiquitination, and SUMOylation (7–12).

The SUMO conjugation pathway has similarities to the ubiquitination process but uses a different set of enzymes involved in processing, attachment, and removal of SUMO (13, 14). First, SUMO is activated in an ATP-dependent manner by the heterodimeric E1-activating enzyme SAE1/2. Activated SUMO is then transferred to the E2-conjugating enzyme Ubc9 and is conjugated to specific lysine residues in substrate proteins. Efficient SUMO conjugation *in vivo* further requires the action of specific SUMO E3 ligases (15). Interestingly, SUMO2 and SUMO3 conjugation, but not SUMO1 conjugation, is stimulated by cellular stress (16). Furthermore, GR is present in the SUMO2 proteome upon heat stress (17, 18).

Our group has previously demonstrated that RSUME (RWD-containing SUMOylation enhancer), a small RWD domain-containing protein, enhances protein SUMOylation in an RWD-dependent manner. The RWD domain has been named after three major RWD-containing proteins—RING finger-containing proteins, WD repeat-containing proteins, and yeast DEAD (DEXD)-like helicases—and has been proposed to be a protein-protein interaction domain (19). RSUME promotes (i) the binding of E1 and E2 enzymes, (ii) the thioester linkage between SUMO and Ubc9, and (iii) the transfer of SUMO to specific target proteins (20). Interestingly, RSUME expression is induced under stress conditions, such as heat shock (20).

SUMO modification of GR has a strong influence on its transcriptional activity (8, 11, 21). GR transcriptional synergy is inhibited by SUMOylation in a context-dependent manner (8). Covalent attachment of SUMO to GR takes place in the absence of ligand, but GR agonists increase SUMOylation of the receptor (11). GR contains three consensus SUMOylation sites. The first two (K297 and K313 in rat GR, K277 and K293 in human GR) are located in the NTD, while the third one (K721 in rat GR, K703 in human GR) is located in the LBD (see Fig. 1A). The NTD SUMOylation sites (K297 and K313) are part of the synergy control (SC) motif sequence (11). SC motifs are short regulatory sequences that limit synergistic transactivation in a promoter-de-

Received 9 November 2012 **Returned for modification** 4 December 2013
Accepted 11 March 2013
Published ahead of print 18 March 2013
Address correspondence to Eduardo Arzt, earzt@fbmc.fcen.uba.ar.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/MCB.01470-12.

doi:10.1128/MCB.01470-12

pendent manner. They are present in several transcription factors (22, 23), including steroids and other nuclear receptors (24–27). It is well established that SUMO modification of K297 and K313 in the GR is responsible for the functional effect of the SC motifs, thereby exerting a negative effect on GR transcriptional activity at multiple GREs without altering GR-mediated transcription at promoters containing a single GRE (8). Indeed, disruption of these sites increases GR transcriptional activity, showing that SUMOylation exerts a negative effect on GR-mediated transcription. Importantly, this effect includes GR-mediated endogenous gene expression in the natural chromatin context (28).

Several components of the SUMO cascade are involved in GR-dependent transcription. It has been reported that Ubc9 binds to GR (29) and alters GR transactivation in a manner that is independent of its SUMOylation activity (30, 31). In addition, members of the PIAS (protein inhibitor of activated STAT) family of SUMO E3 ligases have also been described to regulate GR-directed transcription (32, 33).

It has been reported that the LBD SUMOylation site (K721) only marginally influences GR SUMOylation (11), and there is little evidence for the relevance of this site on GR-mediated transcription (11). Nevertheless, small changes in the GR LBD can alter the activity of bound cofactors without modifying cofactor binding, highlighting the impact of modifications in the LBD on GR transcriptional activity (34, 35). Considering that the third SUMOylation site is located within the LBD and knowing that RSUME is a SUMOylation enhancer, we decided to investigate the role of RSUME on GR transcriptional activity and to dissect the possible implication of the K721 site on this regulation.

In the present work, we demonstrate that RSUME increases GR SUMOylation and transcriptional activity and that K721 is critical for the RSUME effect, showing that this third site of the GR has a positive action on its activity. In addition, we show that both mutation of K721 and knocking down of RSUME compromise coactivator GRIP1-mediated GR activation. We further demonstrate that RSUME is a key regulator of heat shock-induced GR SUMOylation. Thus, RSUME plays an important role in GR-mediated transcription, modulating the cellular outcome to GC exposure and uncovering the positive action of the SUMO site on the GR over the negative ones.

MATERIALS AND METHODS

Cell culture and transfections. COS7, A549, HEK293T, and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM; pH 7.3) supplemented with 10% fetal bovine serum (FBS), 2.2 g/liter $NaHCO_3$, 10 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. For luciferase assays and small interfering RNA (siRNA), reverse transcription-quantitative PCR (RT-qPCR), protein half-life, and immunofluorescence experiments, cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For SUMOylation analysis, immunoprecipitation, and pulldown assays, the polyethylenimine (Polysience, Inc.) reagent was used.

Plasmids and siRNAs. The following plasmids used in this work were previously described, as indicated: the V5-tagged RSUME and V5-RSUME mutant (Y61A/P62A) expression vectors (20); the Rous sarcoma virus (RSV)-driven expression vectors for rat GR wild type (wt) (p6RGR) and SUMOylation mutants p6RGR K297/313R (2K/R), p6RGR K297/313/721R (3K/R), and p6RGR K721R (25); and the hemagglutinin (HA)-tagged version of wild type (HA-p6RGR), the SC mutant (HA-p6RGR K297R/K313R), and the reporter $p\Delta(TAT)_4$-Luc, which harbors four copies of a minimal GRE from the tyrosine aminotransferase gene (TAT) (8). The following plasmids were kindly provided: pEGFP-SUMO1(GG), pEGFP-SUMO1(GA) (26), and GRIP1 (36) were provided by Jorma Palvimo; pSG5-HA-GRIP1 was obtained from Michael Stallcup (37); pcDNA3.1-HA-SUMO2, V5-Ubc9, and 6× His-tagged SUMO2 (6× His-SUMO2) were provided by Ronald Hay (38–40); yellow fluorescent protein (YFP)-labeled SUMO2 (YFP-SUMO2) was provided by Mary Dasso (41); and the TK-$(GRE)_2$-Luc reporter was provided by Dietmar Spengler (42). To generate glutathione *S*-transferase (GST)-labeled RSUME (GST-RSUME), human RSUME cDNA was amplified by PCR and cloned into the BamHI-XhoI sites of pGEX-4T3 (GE Healthcare). The PGEX-4T3 vector was used to express GST. For knockdown experiments, siRNA duplexes were transfected at 20 nM. The siRNA sequences targeting RSUME or scramble are described elsewhere (20). siRNA targeting luciferase mRNA was used as a scramble in the experiments whose results are presented in Fig. 6 (43). The siRNA sequence targeting Ubc9 mRNA was 5′-GAGGCUUGUUUAAACUACGGAUGCU-3′.

Luciferase assays. COS7 cells (3×10^4/well) were seeded onto 24-well plates and transfected 24 h later with the indicated amount of expression plasmids or siRNA. The total amount of DNA was 0.3 μg/well. After 24 or 48 h (see Fig. 2B, 3A, 5B, and 6G), cells were incubated with medium containing 2% charcoal-stripped, steroid-free FBS with either 10 nM dexamethasone (Dex; Sigma Chemical Co., St. Louis, MO) or vehicle. Cells were lysed 8 h later (and 16 h later, for the experiments whose results are presented in Fig. 4D), and luciferase and β-galactosidase (β-Gal) activities were determined according to the manufacturer's instructions (Promega, Madison, WI). For the experiments whose results are presented in Fig. 5B and C, the total amount of protein in cell lysates was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL). To calculate transcriptional activity, each value was relativized to that for wt GR with Dex, and the mean ± standard error of the mean (SEM) of these values is shown.

Immunoprecipitation. COS7 cells (1×10^6) were seeded in 10-cm cell culture dishes and transfected 24 h later with the indicated plasmids. The total amount of DNA was 18 μg per dish. At 48 h posttransfection, cells were incubated with medium containing 2% charcoal-stripped, steroid-free FBS with 10 nM Dex (Sigma Chemical Co., St. Louis, MO) for 2 h. Cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1% NP-40, 150 mM NaCl, 2 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany]) and incubated with rotation for 30 min at 4°C. For the experiments whose results are presented in Fig. 6F, cells were lysed in buffer A (see below) containing 0.1% SDS. After centrifugation for 20 min at 4°C, supernatants were used immediately for immunoprecipitation. For the experiments whose results are presented in Fig. 6F, 1 mM dithiothreitol (DTT) was added before immunoprecipitation. After preclearing of the lysates with protein A-Sepharose beads (Sigma Chemical Co., St. Louis, MO), 1 μg of anti-V5 (Abcam, Cambridge, United Kingdom), anti-GR (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-HA, as indicated in each figure, plus 30 μl of protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) was used. After an overnight incubation at 4°C (1 h for the experiments whose results are presented in Fig. 6F), beads were washed three times with lysis buffer and once with phosphate-buffered saline (PBS). Immunoprecipitates were resuspended in 2× Laemmli sample buffer.

Recombinant proteins. GST and GST-Ubc9 (20) were expressed in *Escherichia coli* M15(pREP4) and GST-RSUME was expressed in *E. coli* BL21(DE3) Rosetta by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside and purified with glutathione-Sepharose 4B beads (GE Healthcare). Proteins were analyzed by SDS-PAGE with Coomassie R-250 staining for quantity and purity and Western blotting.

GST pulldown assays. HEK293T cells (3×10^6) were seeded in 10-cm cell culture dishes and transfected 24 h later with the indicated plasmids. The total amount of DNA was 12 μg/dish. At 48 h posttransfection, cells were treated as described for immunoprecipitation. Lysates were prepared in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 10%

glycerol, 1× Complete protease inhibitor [Roche Diagnostics, Mannheim, Germany]) and incubated with rotation for 30 min at 4°C. After centrifugation for 20 min at 4°C, supernatants were used immediately for pulldown assays. After a preclearance step with 2 μg GST plus glutathione-Sepharose beads, lysates were incubated with 2 μg of the corresponding GST-tagged recombinant protein (or GST alone as a control) for 1 h at 4°C. After extensive washing, elution was performed with 2× Laemmli sample buffer.

RT-qPCR analysis. A549 cells (3×10^5/well or 1.5×10^5/well) (see Fig. 2D) were plated in 6-well plates and transfected with the plasmids specified in each figure or siRNA. The total amount of DNA was 2 μg/well. Forty-eight hours (see Fig. 2C and F) or 72 h (see Fig. 2D) later, cells were treated with 100 nM Dex or vehicle, as indicated, in medium containing 2% charcoal-stripped, steroid-free FBS. Total RNA was isolated from samples by the guanidinium isothiocyanate phenol-chloroform extraction method, and 1 μg was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in the presence of RNasin RNase inhibitor (Promega, Madison, WI) for 1 h at 37°C. cDNAs were amplified using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) with SYBR green and an Eppendorf Mastercycler and analyzed with Realplex software. The primer sequences used to amplify FKBP51 variant 1 and S100P were described elsewhere (44). HSPCB was used as a housekeeping gene with the following primers: 5′-CCAAAAAGCACCTGGAG ATCA-3′ (forward) and 5′-TGTCGGCCTCAGCCTTCT-3′ (reverse). For the analysis of RSUME mRNA knockdown, semiquantitative PCR analysis was performed with the primers described previously (20). Aliquots were taken at cycles 22, 24, 26, 28, 30, and 35 to ensure that the samples were in the linear range of amplification. Amplified products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide.

Western blotting and antibodies. After treatments, cells were washed with ice-cold PBS and lysed in Laemmli sample buffer. Whole-cell lysates were sonicated, heated to 95°C for 5 min, and cooled on ice. Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline supplemented with 0.1% Tween 20 and probed overnight at 4°C with the primary antibodies listed below. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA). When V5-RSUME levels from anti-V5 immunoprecipitates were analyzed, membranes were developed using a TrueBlot kit (eBioscience, San Diego, CA). Membranes were developed using the enhanced chemiluminescence reagent Super Signal West Dura (Thermo Scientific, Rockford, IL). The primary antibodies used were anti-HA.11 (clone 16B12; Covance, Madison, WI), anti-V5 (ab9116; Abcam, Cambridge, United Kingdom), anti-GR (M20 sc-1004; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA). Anti-SUMO2 (45) and anti-Ubc9 antibodies were kindly provided by Ronald Hay. Western blots were quantified using the publicly available ImageJ 1.43u software (NIH, Bethesda, MD). The experiments shown are representative of at least three independent experiments with similar results.

SUMOylation experiments. COS7 cells (1.6×10^5/well) were seeded onto 6-well plates and transfected 24 h later with the indicated plasmids. The total amount of DNA was 3 μg/well. At 48 h posttransfection, cells were incubated with medium containing 2% charcoal-stripped, steroid-free FBS with 10 nM Dex (Sigma Chemical Co., St. Louis, MO) for 2 h. Cells were harvested in 2× Laemmli sample buffer containing 20 mM *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, MO) and analyzed by SDS-PAGE and Western blotting.

For heat shock treatment, COS7 cells (3×10^4/well) were seeded onto 24-well plates and transfected 24 h later with the indicated plasmids and siRNA. The total amount of DNA was 0.3 μg/well. At 48 h posttransfection, cells were incubated with medium containing 2% charcoal-stripped, steroid-free FBS with 10 nM Dex (Sigma Chemical Co., St. Louis, MO) for 2 h. For the last 30 min of stimulation, cells were transferred to a 42°C water bath or maintained at 37°C. Cells were harvested in 2× Laemmli sample buffer containing 20 mM *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, MO) and analyzed by SDS-PAGE and Western blotting.

For 6×His-SUMO2 conjugate purification, COS7 cells (1.10^6/well) were transfected in 10-cm dishes with the indicated plasmids. The total amount of DNA was 18 μg/dish. After 48 h, cells were treated with 10 nM Dex (Sigma Chemical Co., St. Louis, MO) for 2 h and 6×His-SUMO2 conjugates were purified under denaturing conditions using Ni-nitrilotriacetic acid (NTA) agarose beads (Qiagen, Valencia, CA). Briefly, cells were lysed in 6 M guanidine-HCl, 100 mM Na_2HPO_4-NaH_2PO_4, 10 mM Tris-HCl, 10 mM iodoacetamide, 5 mM imidazole, pH 8. Fifty microliters of Ni-NTA agarose beads was added, and after incubation for 4 h at room temperature, beads were sequentially washed with buffer A (8 M urea, 100 mM Na_2HPO_4-NaH_2PO_4, 10 mM Tris-HCl, 10 mM iodoacetamide, 5 mM imidazole, pH 8), buffer B (8 M urea, 100 mM Na_2HPO_4-NaH_2PO_4, 10 mM Tris-HCl, 0.2% Triton X-100, 10 mM iodoacetamide, 5 mM imidazole, pH 6.3), and buffer C (8 M urea, 100 mM Na_2HPO_4-NaH_2PO_4, 10 mM Tris-HCl, 0.1% Triton X-100, 10 mM iodoacetamide, 5 mM imidazole, pH 6.3). Elution was performed with 150 mM Tris-HCl, pH 6.7, 5% SDS, 200 mM imidazole, 30% glycerol, and 720 nM β-mercaptoethanol for 20 min at room temperature, and after centrifugation, the supernatant was directly analyzed by SDS-PAGE and Western blotting.

For protein half-life experiments, COS7 cells (3×10^4/well) were placed onto 24-well plates and transfected 24 h later with the indicated plasmids. At 48 h posttransfection, cells were incubated with medium containing 2% charcoal-stripped, steroid-free FBS containing (10 μg/ml) cycloheximide and 10 nM Dex. Cells were harvested at different times points, as indicated, in 2× Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting.

***In vitro* deSUMOylation assay.** COS7 cells were transfected as indicated above and lysed in 800 μl of lysis buffer A (20 mM Tris-HCl, pH 8, 400 mM NaCl, 1 mM EDTA, 1% NP-40, 20 mM *N*-ethylmaleimide [Sigma Chemical Co., St. Louis, MO], 1× protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany]). Cleared lysates were made to 1 mM DTT and immunoprecipitated with anti-HA antibody for 1 h. Complexes were recovered with protein A-Sepharose beads over 1 h. Beads were washed two times with 1 ml of buffer B (buffer A containing 150 mM NaCl) and once with the deSUMOylation reaction buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM DTT). GST-tagged human recombinant SENP2 (catalytic domain, amino acids 368 to 549; Enzo Life Sciences, Inc., NY) was added, and the mixture was incubated at 30°C for 1 h. Reactions were stopped by addition of 1 volume of Laemmli sample buffer, and each sample was examined for deSUMOylation by SDS-PAGE and Western blotting with an anti-HA antibody.

***In vitro* SUMOylation assay.** HA-tagged wt and K297/313R GR immunoprecipitates from transiently transfected COS7 cells were subjected to the *in vitro* SUMOylation assay. Briefly, HA-tagged GRs were incubated in a total volume of 20 μl with 150 ng of SUMO E1, 500 ng of SUMO E2, and 2 μg of SUMO2 (Enzo Life Sciences, Inc., NY) in a buffer containing 20 mM HEPES, 110 mM K acetate, 2 mM Mg acetate, 0.5 mM EGTA, 0.05% Tween 20, and 0.2 mg/ml ovalbumin. Reaction mixtures were incubated either with or without an ATP-regenerating system (50 mM Tris, pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase, 0.6 U/ml of inorganic pyrophosphatase) for 3 h at 30°C. After terminating the reactions with SDS sample buffer, the products were fractionated by SDS-PAGE and subject to Western blotting with an anti-HA antibody.

Immunofluorescence analysis. HeLa cells (2×10^5) were seeded on glass coverslips in 6-well plates and transfected with YFP-SUMO2 and V5-RSUME 24 h after plating. The total amount of DNA was 2 μg/well. Forty-six hours later, cells were stimulated with 10 nM Dex for 2 h, and in the last 30 min, cells were transferred to a 42°C water bath. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton X-100 in PBS. Cells were blocked with 1% FBS in PBS for 1 h. Afterward, cells were incubated with the primary antibody (anti-V5) for 1 h in blocking buffer.

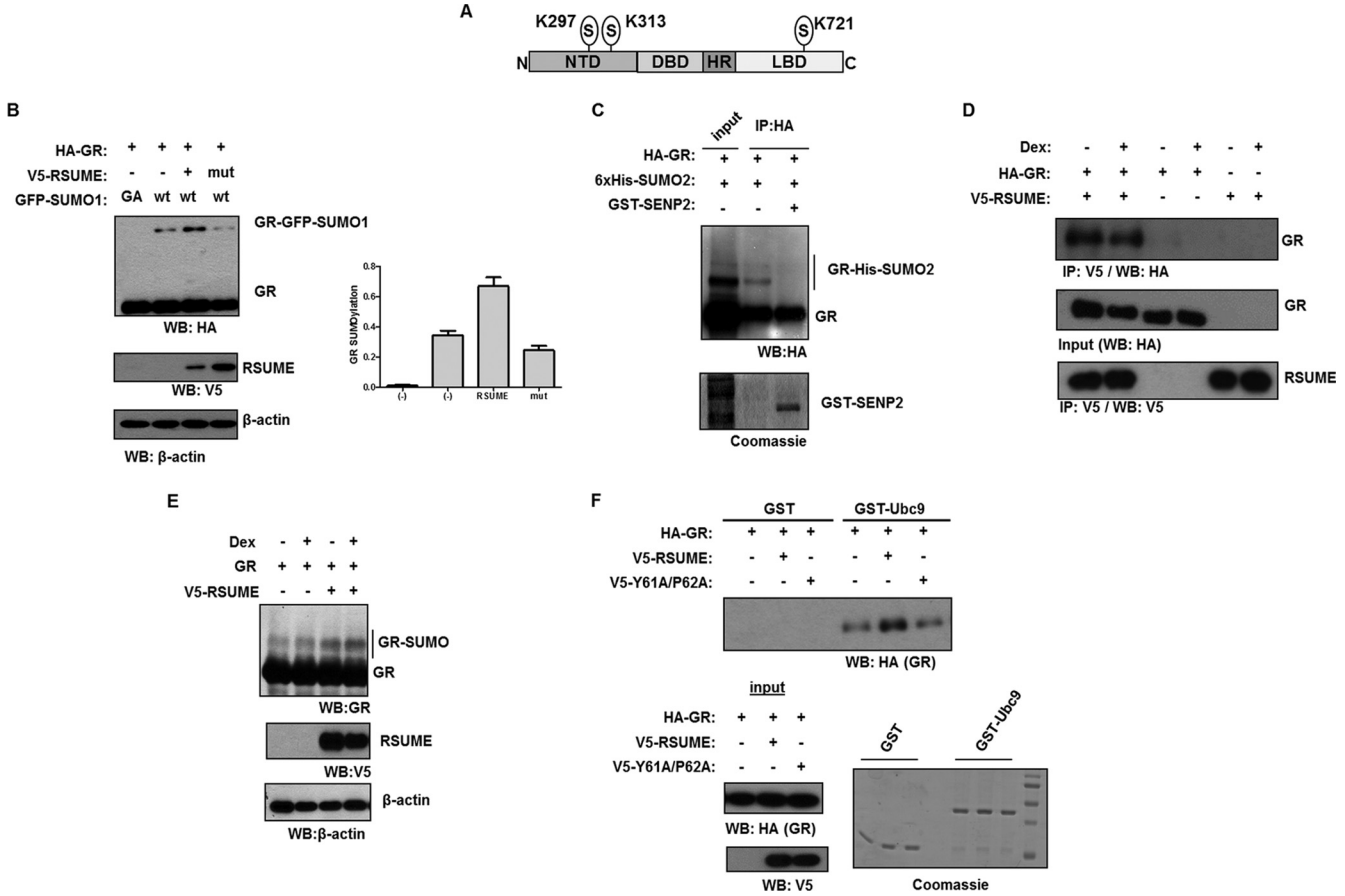

FIG 1 RSUME enhances GR SUMOylation. (A) Modular structure of the GR, N-terminal domain (NTD), DNA-binding domain (DBD), hinge region (HR), and ligand-binding domain (LBD). The three SUMO (S) attachment sites of rat GR are depicted. (B) COS7 cells were transfected with HA-p6RGR (1 μg) in the presence of wt or conjugation-deficient (GA) GFP-SUMO1 (1 μg) either with or without wt V5-RSUME or the RSUME Y61A/P62A mutant (mut) expression vector (1 μg). Cells were stimulated with 10 nM Dex 46 h after transfection and harvested 2 h later. Western blots (WB) were quantified using ImageJ software; GR SUMOylation, SUMOylation for GR-GFP-SUMO1 relative to that for unmodified GR. (C) COS7 cells were transfected with the wt HA-p6RGR (6 μg) and 6×His-SUMO2 (6 μg) expression vectors. Cells were stimulated with 10 nM Dex or vehicle 46 h after transfection and harvested 2 h later. Immunoprecipitation (IP) was performed with an anti-HA antibody, and the immunoprecipitate was incubated with recombinant GST-SENP2 (1 μg). (D) COS7 cells were transfected with V5-RSUME (6 μg) and HA-p6RGR (6 μg). Cells were stimulated with 10 nM Dex 46 h after transfection and harvested 2 h later for immunoprecipitation. (E) COS7 cells were transfected with the p6RGR (1 μg) and V5-RSUME (wt) expression vectors (1 μg). Cells were stimulated with 10 nM Dex or vehicle 46 h after transfection and harvested 2 h later for Western blotting. (F) HEK293T cells were transfected with HA-p6RGR (6 μg) either with or without V5-RSUME (6 μg) or the Y61A/P62A mutant (6 μg), and lysates were incubated with 2 μg GST or GST-Ubc9, followed by pulldown. HA-p6RGR binding to GST-Ubc9 was analyzed with an anti-HA antibody. The experiments shown are representative of four independent Western blots with similar results.

After washing with PBS, cells were incubated with Alexa Fluor 647-conjugated secondary antibody (Invitrogen, Carlsbad, CA) for another hour. Cells were extensively washed with PBS and mounted. The slides were examined using an FV300 BX61 laser-scanning confocal fluorescence microscope (Olympus, Melville, NY). Images were acquired with the Fluoview analyzer program.

Statistical analyses. Statistical analyses were performed by analysis of variance (ANOVA) in combination with Scheffé's test. Data are shown as the mean ± SEM. Experiments were performed at least in triplicate. For the experiments whose results are shown in Fig. 4D, statistics were performed by analysis of covariance (ANCOVA).

RESULTS

RSUME stimulates GR SUMOylation. To address the role of RSUME on GR SUMOylation, COS7 cells were cotransfected with GR and the green fluorescent protein (GFP)-SUMO1 expression vector either with or without RSUME. As shown in Fig. 1B, GR is SUMOylated by GFP-SUMO1 and is not by a mutated GFP-SUMO1 (GA) that is incapable of conjugating to proteins. More interestingly, RSUME overexpression increases GR SUMOylation, and mutation of the RWD domain (Y61A/P62A), known to abolish RSUME's SUMO conjugation enhancer activity (20), completely inhibits the stimulatory effect on GR SUMOylation (Fig. 1B). By performing an *in vitro* deSUMOylation assay using SENP2, we confirmed that the slower-migrating bands observed upon SUMO expression are indeed SUMO-GR conjugates (Fig. 1C). Moreover, coimmunoprecipitation assays showed that RSUME interacts with GR under constitutive or Dex stimulation conditions (Fig. 1D). Dex treatment also did not modify the RSUME stimulatory effect on GR SUMOylation (Fig. 1E). In order to start to address the mechanism by which RSUME increases GR SUMOylation, we tested the hypothesis that RSUME regulates GR-Ubc9 interaction. GST pulldown assays were performed using GST-Ubc9 and lysates from HEK293T cells that had been cotransfected with GR either with or without RSUME or RSUME mutant

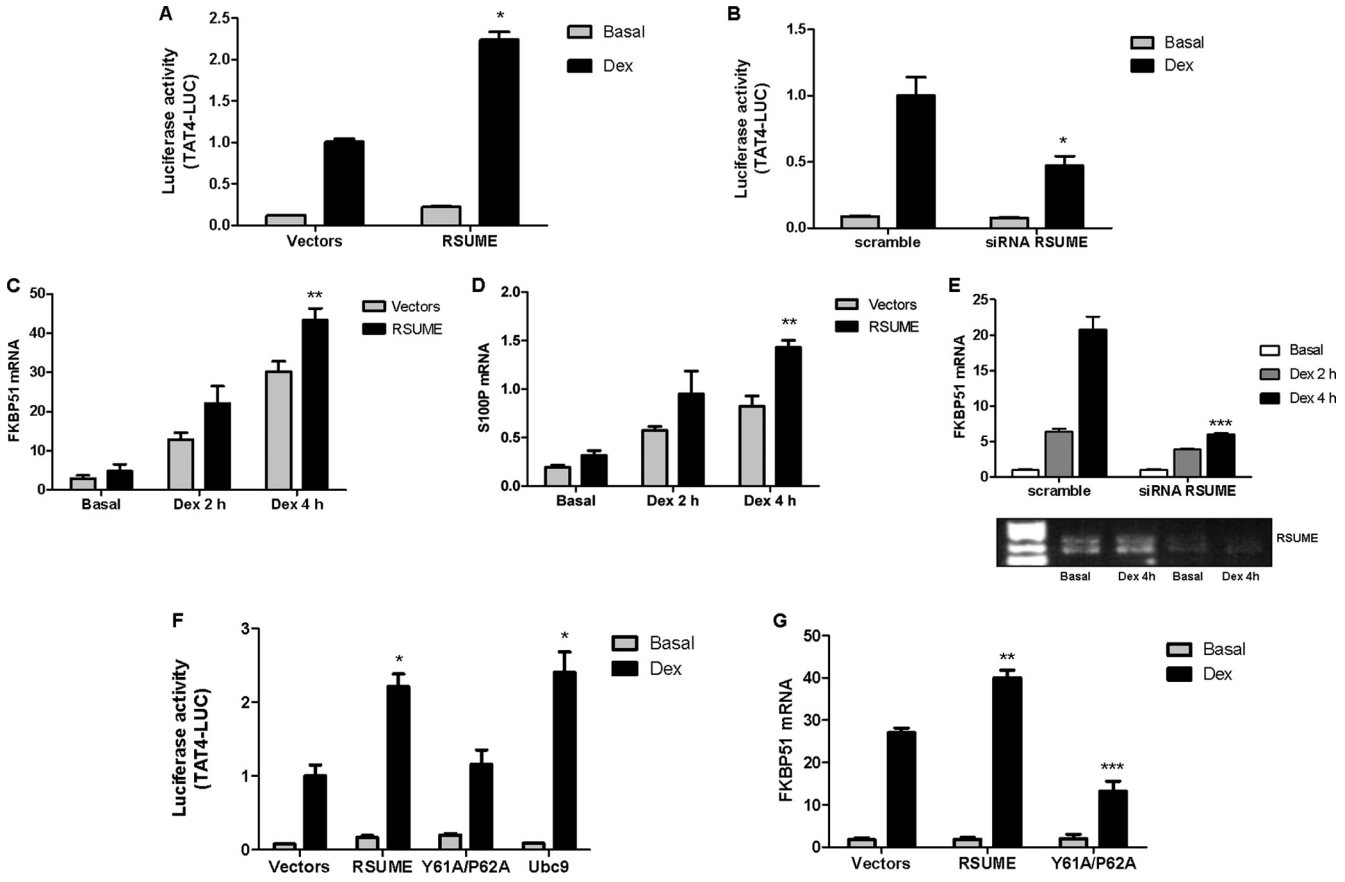

FIG 2 RSUME modulates GR transcriptional activity. (A) COS7 cells were transfected with a reporter plasmid bearing four tandem GREs, pΔ(TAT)$_4$-Luc (60 ng), together with p6RGR (10 ng) either with (RSUME) or without (vectors) RSUME (100 ng) expression vectors, as indicated. A plasmid carrying RSV–β-Gal (60 ng) was used as a control. At 24 h after transfection, cells were stimulated with 10 nM Dex or vehicle (basal) for 8 h, and luciferase activity in the cell extract was measured. (B) COS7 cells were transfected as described in the legend to panel A with 20 nM siRNA directed against RSUME or scramble siRNA. At 48 h posttransfection, cells were stimulated and analyzed as described in the legend to panel A. (C and D) A549 cells were transfected with or without the RSUME (1 μg) expression vector. At 46 h posttransfection, cells were stimulated with 100 nM Dex or vehicle for 2 h or 4 h and FKBP51 (C) or S100P (D) mRNA was analyzed by RT-qPCR as described in Materials and Methods. (E) (Top) A549 cells were transfected with 20 nM siRNA directed against RSUME or scramble siRNA. At 72 h posttransfection, cells were treated as described in the legend to panel C and FKBP51 mRNA was analyzed by RT-qPCR. (Bottom) Knockdown of endogenous RSUME mRNA was analyzed by RT–semi-qPCR. (F) COS7 cells were transfected as described in the legend to panel A, together with wild-type RSUME or the RSUME Y61A/P62A mutant (100 ng) or Ubc9 (10 ng) expression vector. A plasmid carrying RSV–β-Gal (60 ng) was used as a control and treated as described in the legend to panel A. (G) A549 cells were transfected with RSUME or with the RSUME Y61A/P62A expression vector and treated as described in the legend to panel C. For luciferase assays, each value was normalized to β-galactosidase activity, and results are expressed as the mean ± SEM of triplicates of one representative experiment from three independent experiments with similar results. The activity of GR with Dex was set equal to 1.*, $P < 0.05$ compared with cells transfected with RSUME empty vector (A and F) or scramble siRNA (B) stimulated with Dex (ANOVA with Scheffé's test). For RT-qPCR, the relative mRNA levels represent the mean ± SEM of triplicates of one representative experiment from three independent experiments with similar results. **, $P < 0.05$ compared with cells transfected with RSUME empty vector (C and D [at the same corresponding time] and G); ***, $P < 0.01$ compared with cells transfected with scramble siRNA at the same corresponding time (E) or the RSUME empty vector (G) and in all cases stimulated with Dex (ANOVA with Scheffé's test).

Y61A/P62A. RSUME increased the GR-Ubc9 interaction and Y61A/P62A did not (Fig. 1F), while increasing the RSUME concentration enhanced the interaction (see Fig. S1 in the supplemental material). Taken together, these results show that RSUME increases GR SUMOylation and this effect depends on the integrity of RSUME's RWD domain.

RSUME increases GR transcriptional activity. To examine the role of RSUME on GR transcriptional activity, COS7 cells (lacking endogenous GR) were transfected with a GR expression vector, the pΔ(TAT)$_4$-Luc construct bearing four tandem GREs, either with or without RSUME. As shown in Fig. 2A, RSUME stimulated GR-dependent transcription. The effect of RSUME was not cell line dependent, as similar results were obtained in EL-4, CV-1, and HeLa cells (data not shown). In order to evaluate the effect of endogenous RSUME on GR-directed transcription, COS7 cells were transfected as described above, in combination with a specific siRNA targeting RSUME mRNA. Interestingly, GR-dependent transcription was attenuated in RSUME-depleted cells (Fig. 2B). We further analyzed the role of RSUME on endogenous GR-responsive genes. We performed RT-qPCR in A549 cells to examine the Dex-induced expression of FKBP51 and S100P, known GR target genes (44). As shown in Fig. 2C and D, RSUME overexpression led to enhanced FKBP51 and S100P expression. On the contrary, knocking down endogenous RSUME by siRNA

led to a significant inhibition of Dex-induced FKBP51 expression (Fig. 2E). Basal activities of GR did not drop following RSUME knockdown, probably because of the remaining activity of endogenous RSUME (as shown in the semiquantitative PCR). These experiments indicate that endogenous RSUME plays a regulatory role in the endogenous GR transcriptional program.

Having shown that RSUME increases both the SUMOylation and transcriptional activity of GR, we further evaluated whether the SUMOylation-enhancing activity of RSUME is responsible for stimulating GR-mediated transcription. As shown in Fig. 2F, RSUME increased GR transcriptional activity to the same extent as Ubc9, while the RSUME RWD mutant (Y61A/P62A) had no effect. Overexpression of Y61A/P62A inhibited Dex-induced FKBP51 mRNA expression (Fig. 2G). Altogether, these results demonstrate that RSUME modulates GR responses in the natural context and that the RSUME effect on GR-directed transcription is dependent on its SUMO enhancer activity.

The RSUME effect on GR transcriptional activity is independent of the NTD SUMOylation sites. Since the RSUME effect on GR-mediated transcription depends on its SUMOylation enhancer activity, we decided to test the relevance of the different GR sumoylation sites in this regulation. In agreement with previous results, mutation of the two NTD SUMOylation sites within GR (K297/313R, designated 2K/R) led to enhanced transcriptional activity (Fig. 3A). Regardless of this increased transcriptional activation, RSUME overexpression was still able to increase 2K/R GR activity (Fig. 3A). Knocking down endogenous Ubc9 by siRNA inhibited both RSUME-mediated GR and 2K/R transcriptional stimulation (Fig. 3A), further confirming that this action of RSUME is due to SUMOylation. The remaining action of RSUME might have been related to the fact that there were still residual levels of Ubc9 (Fig. 3A, Western blot). Moreover, as shown in Fig. 3B, GST pulldown experiments demonstrated that RSUME interacts both with wild-type GR and also with 2K/R GR. Since SUMO conjugation occurred not only in the two NTD sites within the GR, we tested whether RSUME enhances 2K/R GR mutant SUMOylation. Figure 3C shows that RSUME enhanced not only SUMOylation of wild-type GR but also that of the 2K/R mutant. By performing an *in vitro* de-SUMOylation assay using recombinant SENP2, we confirmed that the slower-migrating bands observed in the 2K/R mutant upon SUMO and Ubc9 expression were indeed SUMO-GR conjugates (Fig. 3D). The decreased remaining band after SENP2 treatment was not a product of the immunoprecipitation because it was similarly present in the input. In addition, 2K/R SUMOylation was demonstrated by means of an *in vitro* SUMOylation assay (see Fig. S2 in the supplemental material). Altogether, these results indicate that the RSUME transcriptional effect is actually due to SUMOylation and that RSUME acts on GR independently of the two NTD SUMOylation sites, pointing to the LBD SUMO attachment site K721 as the relevant one.

The RSUME effect on GR transcriptional activity depends on K721. We then analyzed the effect of RSUME on GR SUMOylation when K721 was mutated to arginine to maintain the basic charge but inhibit SUMO attachment. COS7 cells were cotransfected with either the wild-type GR or the 2K/R, K297/313/721R (3K/R), or K721R GR mutant together with HA-SUMO2 in the presence or absence of RSUME. As shown in Fig. 4A, while RSUME enhanced wt, 2K/R, and K721R GR SUMOylation, it was not able to increase SUMO conjugation to 3K/R GR. These results indicate that RSUME is able to stimulate SUMO conjugation to all three GR SUMOylation sites. Since SUMO attachment to NTD or LBD sites had opposite effects, RSUME could function as a molecular switch of the negative to positive action of SUMO conjugation to GR.

Since the NTD sites are not acting in positive RSUME-mediated GR transcriptional regulation, the effect of RSUME on GR transcriptional activity most likely involves SUMO conjugation to this LBD site. Indeed, while RSUME increased wt and 2K/R GR transcription, it was not able to activate transcription driven by either the K721R or the 3K/R GR mutant, where the K721 SUMOylation site is absent (Fig. 4B). The effect of RSUME on GR transcriptional activity of the wild type and all GR SUMOylation mutants (2K/R, K721R, and 3K/R) was not due to differences in the amounts of expressed receptor protein levels, as assessed by Western blotting (Fig. 4C). We then assessed if SUMOylation at the K721 residue alters the stability of the receptor. COS7 cells were transfected with either wt GR or the K721R mutant and treated with Dex in the presence of the protein synthesis inhibitor cycloheximide. As shown in Fig. S3A in the supplemental material, the wild type and the K721R GR SUMOylation mutant have similar half-lives. We also tested if SUMOylation affects the Dex 50% effective concentration (EC_{50}) of GR in COS7 cells transiently transfected with the wild-type GR expression vector or the 2K/R, 3/KR, or K721R SUMOylation mutant. Figure S3B in the supplemental material shows that the Dex dose-response curves for the wt and the 2K/R, 3K/R, and K721R GR SUMOylation mutants have similar EC_{50}s without statistically significant differences between them (EC_{50}s, 1.7e−9 M, 9.76e−10 M, 1.25e−9 M, and 1.49 e−9 M, respectively). These results show that although 2K/R and 3K/R GRs have higher transactivation activities than the wt GR, SUMOylation of GR does not affect the EC_{50} of the receptor. Together, these results demonstrate that the SUMO attachment site within the GR LBD is critical for the positive effect of RSUME on GR transcriptional activity.

The coactivator GRIP1 is involved in the regulation of GR transcriptional activity by RSUME. Small changes in the GR LBD can alter the activity of the bound cofactor without modifying cofactor binding (34). We tested the relationship of RSUME and the K721 site of the GR and GRIP1's coactivator activity. As shown in Fig. 5A, RSUME interacts with GRIP1. To explore the functional consequences of the RSUME-GRIP1 interaction on GR's transcriptional activity, we performed transactivation assays. Notably, downregulation of RSUME expression by siRNA reduced GRIP1's effect on GR-dependent transcription (Fig. 5B). Also, since the third SUMOylation site of the GR, K721, is located in the LBD, we speculated that K721 could be involved in the activity of the GR coactivator. To address this, we analyzed GRIP1's coactivator activity on the K721R mutant. Most strikingly, as shown in Fig. 5C, mutation of the K721 SUMOylation site in the LBD inhibited GRIP1 coactivator activity, even though mutation in K721 did not disrupt the GRIP1-GR interaction (Fig. 5D). The fact that knocking down RSUME or mutating K721 affected the stimulatory GRIP1 activity suggests that the action of RSUME on the positive K721 site involves the modulation of GRIP1 coactivator activity.

RSUME is required for heat shock-induced SUMO conjugation to GR. In the quest for a physiological scenario in which RSUME could regulate GR SUMOylation, we realized that heat shock leads to the induction of RSUME (20) and also GR SUMOylation (17, 18). It

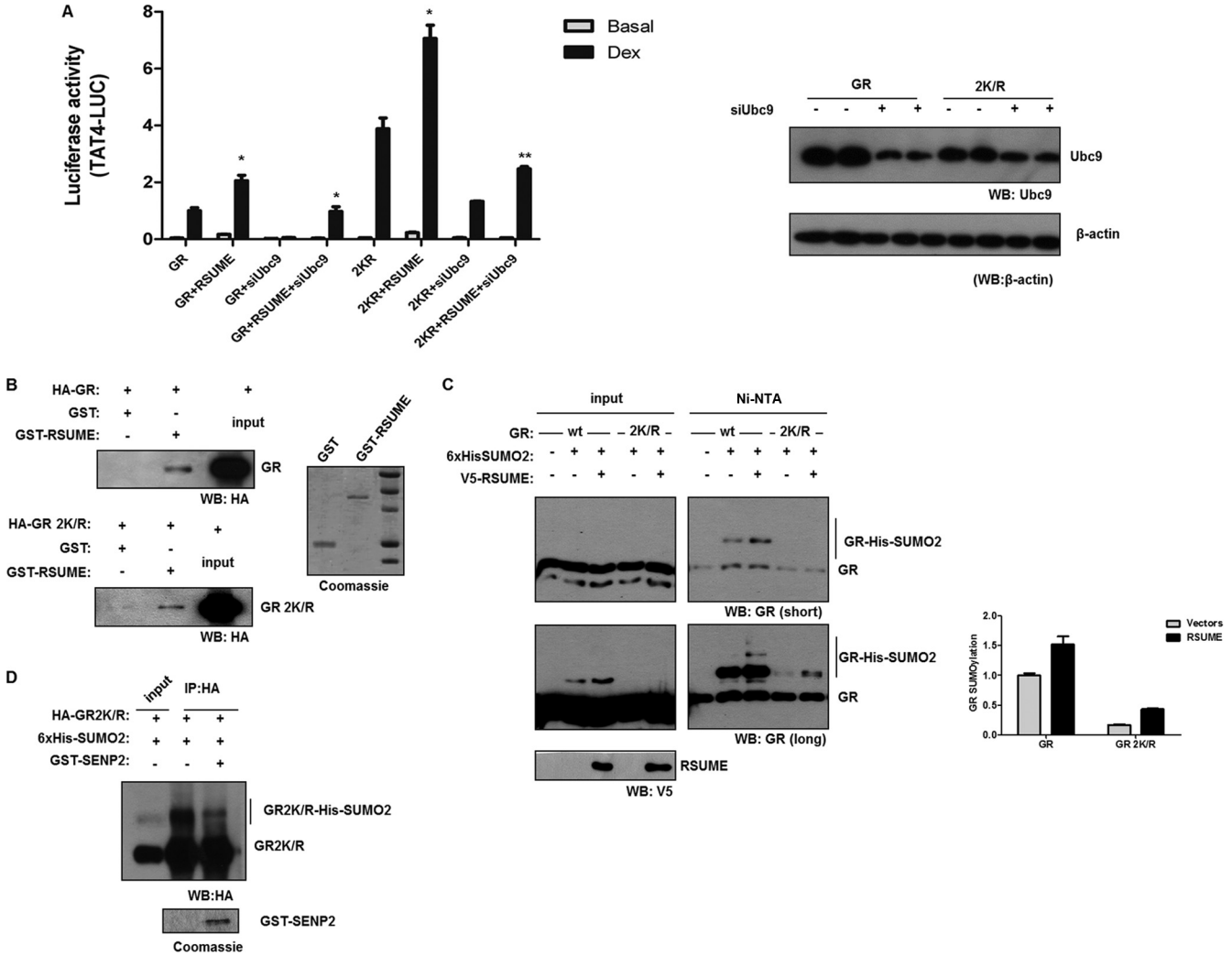

FIG 3 RSUME regulates GR transcriptional activity independently of the two N-terminal SUMO conjugation sites. (A) COS7 cells were transfected with $p\Delta(TAT)_4$-Luc (60 ng) together with the wt or the two site SUMOylation-deficient p6RGR K297/313R mutants (10 ng) with either RSUME or the empty (100 ng) expression vector and with 20 nM siRNA directed against Ubc9 or scramble siRNA. A plasmid carrying RSV–β-Gal (60 ng) was used as a control. At 48 h after transfection, cells were stimulated with 10 nM Dex or vehicle (basal) for 8 h and luciferase activity was measured in cell extracts. Each value was normalized to β-galactosidase activity, and results are expressed as the mean ± SEM of triplicates of one representative experiment from three independent experiments with similar results. The activity of GR with Dex was set equal to 1. *, $P < 0.05$ compared with the corresponding cells transfected with the p6RGR wt plus the RSUME empty vector, RSUME, or the p6RGR 2K/R mutant plus the RSUME empty vector stimulated with Dex; **, $P < 0.01$ compared with cells transfected with the p6RGR 2K/R mutant plus RSUME stimulated with Dex (ANOVA with Scheffé's test). (Right) Cell extracts stimulated with Dex were subjected to Western blotting with anti-Ubc9 antibody or anti-β-actin. (B) HEK293T cells were transfected with wild-type HA-p6RGR (6 μg) or the HA-p6RGR K297/313R mutant (6 μg). Cells were harvested at 48 h after transfection and at 2 h after stimulation with 10 nM Dex for pulldown. (C) COS7 cells were transfected with wt p6RGR (6 μg) or the p6RGR 2K/R mutant (6 μg) and 6×His-SUMO2 (6 μg) either with or without V5-RSUME (6 μg). Cells were harvested at 48 h after transfection and at 2 h after stimulation with 10 nM Dex. An aliquot of the lysates was directly analyzed by Western blotting (input), and the remainder was used for Ni^{2+} affinity chromatography to purify 6×His-SUMO2 conjugates (Ni-NTA). Short and long exposures of the Western blots are shown and were quantified using ImageJ software; GR SUMOylation, results for wt or the 2K/R GR-6×His-SUMO2 mutant relative to those for unmodified wt or the GR 2K/R mutant are shown. (D) COS7 cells were transfected with the HA-p6RGR 2K/R (6 μg), 6×His SUMO2 (6 μg), and V5-Ubc9 (6 μg) expression vectors. Cells were stimulated with 10 nM Dex at 46 h after transfection and harvested 2 h later. Immunoprecipitated proteins were incubated with recombinant GST-SENP2 (1 μg). The experiments shown are representative of three independent Western blots with similar results.

has been reported that during heat stress SUMO2 accumulates in discrete intranuclear granules. We examined the subcellular localization of RSUME by immunofluorescence analysis during heat stress. As shown in Fig. S4 in the supplemental material, RSUME is present in SUMO2 granules. In order to analyze whether endogenous RSUME is involved in the heat shock-induced GR SUMOylation, COS7 cells were transfected with HA-tagged GR and 6× His-tagged SUMO2 expression vectors in combination with a RSUME targeting siRNA (20). As shown in Fig. 6A, heat shock-triggered GR SUMOylation was attenuated when RSUME was knocked down. Interestingly, heat shock treatment increased not only wild-type GR SUMOylation but also 2K/R GR SUMOylation. Most importantly, siRNA against RSUME diminished heat shock-triggered SUMO conjugation to the 2K/R GR mutant (Fig. 6B). Next, we investigated if endogenous SUMO conjugation to GR is affected by heat stress. As shown in Fig. 6C,

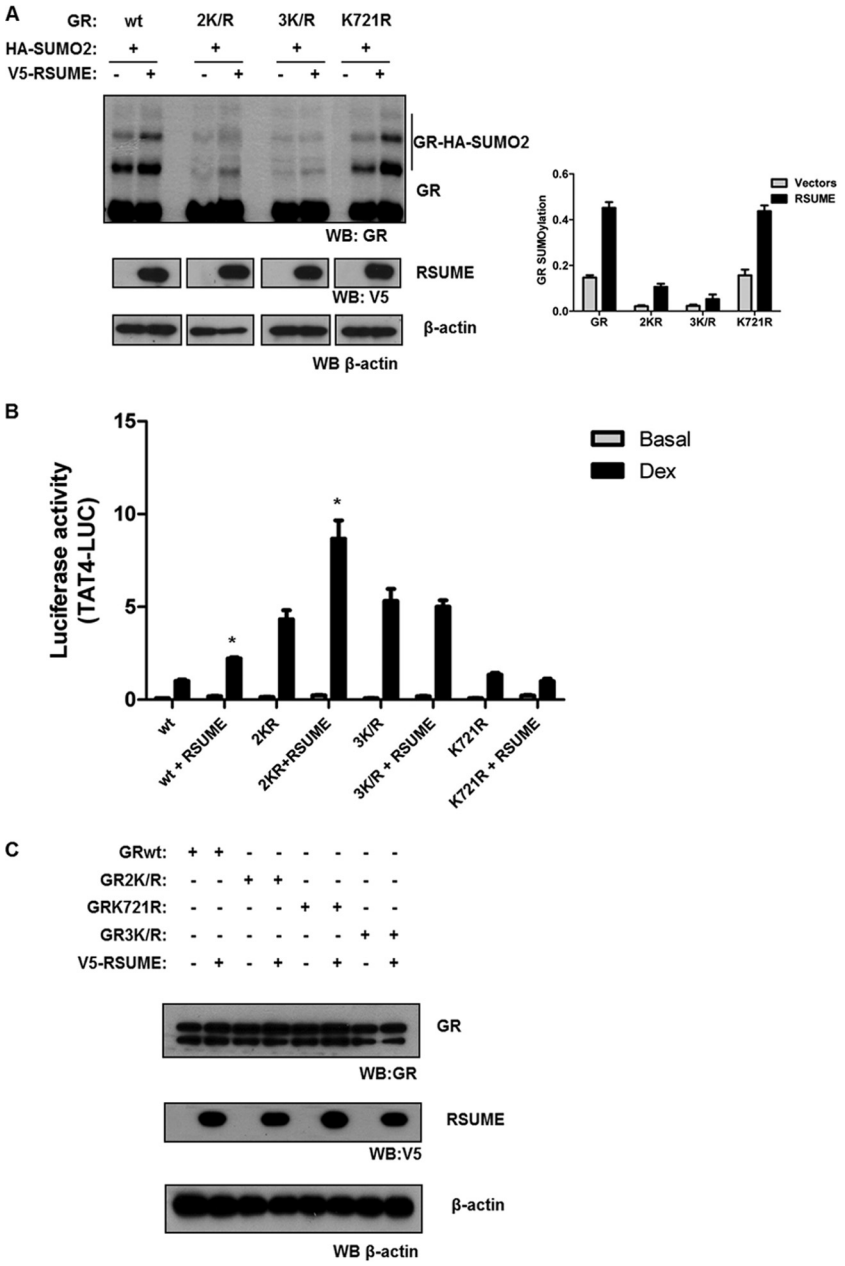

FIG 4 RSUME effect on GR transcriptional activity depends of K721. (A) COS7 cells were transfected with wt or 2K/R, 3K/R, or K721R mutant p6RGR (1 μg) and HA-tagged SUMO2 (1 μg) either with or without V5-RSUME (1 μg). Cells were harvested at 48 h after transfection and 2 h after stimulation with 10 nM Dex. Western blots were quantified using ImageJ software; GR SUMOylation, results for wt or 2K/R, 3K/R, or K721R mutant GR-HA-SUMO2 relative to those for the unmodified wt or 2K/R, 3K/R, or K721R mutant GR are shown. (B) COS7 cells were transfected with the $p\Delta(TAT)_4$-Luc reporter plasmid (60 ng) together with wt or 2K/R, 3K/R, or K721R mutant p6RGR (10 ng) with either RSUME or empty vector (100 ng). A plasmid carrying RSV–β-Gal (60 ng) was used as a control. At 24 h after transfection, cells were stimulated with 10 nM Dex or vehicle (basal) for 8 h and luciferase activity in the cell extract was measured. Results are expressed as the mean ± SEM of triplicates of one representative experiment from three independent experiments with similar results. *, $P < 0.05$ compared with cells transfected with p6RGR wt or the p6RGR 2K/R mutant plus the RSUME empty vector stimulated with Dex (ANOVA with Scheffé's test). (C) COS7 cells were transfected under the same conditions described in the legend to panel B. At 24 h after transfection, cells were stimulated with 10 nM Dex for 8 h and cell extracts were subjected to Western blotting. The experiments shown are representative of three independent Western blots with similar results.

hyperthermic stress stimulated endogenous SUMO conjugation to GR. Notably, downregulation of RSUME expression by siRNA reduced this effect, and, on the contrary, RSUME overexpression potentiated heat shock-mediated endogenous SUMO conjugation to the GR (Fig. 6C). Moreover, immunoprecipitation of GR followed by anti-SUMO2 blotting confirmed that the bands lost upon RSUME knockdown correspond to endogenous GR SUMOylation (Fig. 6E). Increased GR transcriptional activity during the heat shock is shown in Fig. 6F. This effect was abolished by siRNA directed against RSUME, indicating that RSUME is involved in GR-mediated transcription upon heat stress (Fig. 6F).

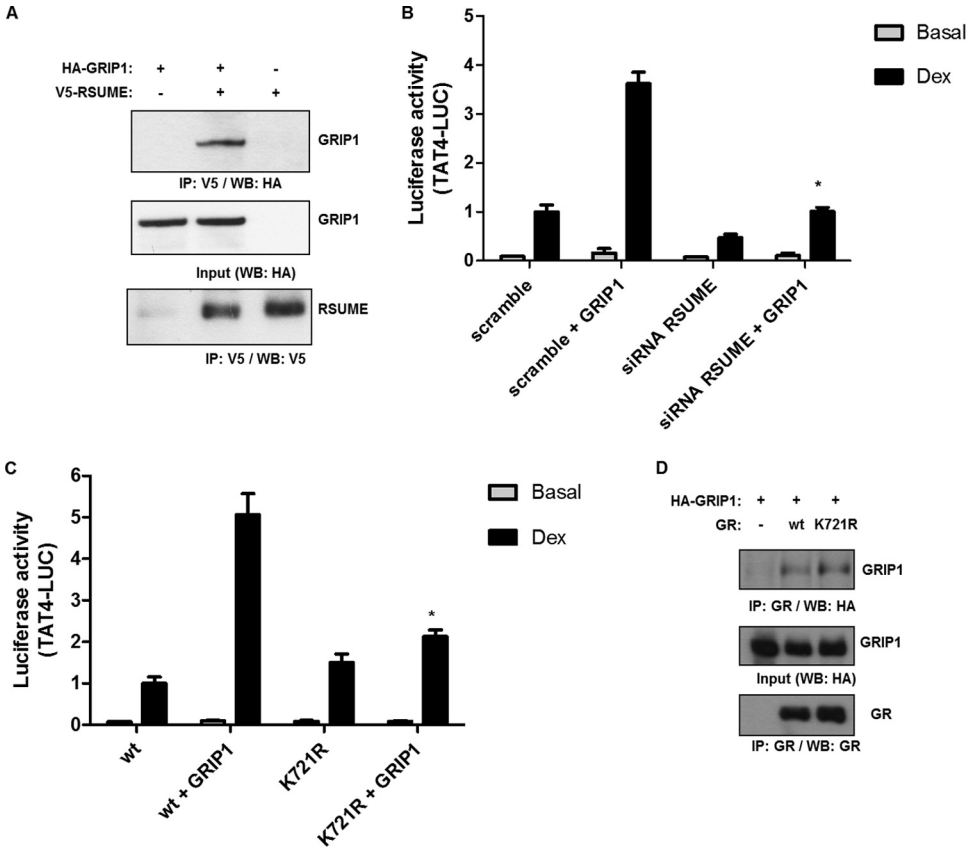

FIG 5 The coactivator GRIP1 is involved in the regulation of GR transcriptional activity by RSUME. (A) RSUME interacts with GRIP1 in whole-cell lysates, as assessed by coimmunoprecipitation. COS7 cells were transfected with V5-RSUME (6 μg) and HA-GRIP1 (6 μg). Cells were harvested at 48 h after transfection. Immunoprecipitation was with an anti-V5 antibody. (B) COS7 cells were transfected with $p\Delta(TAT)_4$-Luc (60 ng), p6RGR (10 ng), or GRIP1 (20 ng), together with 20 nM siRNA against RSUME or scramble siRNA. At 48 h after transfection, cells were stimulated with 10 nM Dex or vehicle for 8 h and luciferase activity in the cell extract was measured. (C) COS7 cells were transfected with wt or K721R mutant pS6GR with GRIP1 or empty expression vector. At 24 h after transfection, cells were stimulated with 10 nM Dex or vehicle (basal) for 8 h and luciferase activity in cell extracts was measured. (D) COS7 cells were transfected with GRIP1-HA (6 μg) together with p6RGR (6 μg) or the K721R mutant (6 μg). Cells were stimulated with 10 nM Dex at 46 h after transfection and harvested 2 h later. Immunoprecipitation was with an anti-GR antibody. For luciferase assays, each value was normalized to the protein content per well, and results are expressed as the mean ± SEM of triplicates of one representative experiment from four (B) or three (C) independent experiments with similar results. The activity of pS6GR with Dex was set equal to 1.*, $P < 0.05$ compared with cells transfected with GRIP1 plus scramble siRNA (B) or GRIP1 plus pS6GR wt (C) stimulated with Dex (ANOVA with Scheffé's test). The experiments shown are representative of five independent Western blots with similar results.

DISCUSSION

In the present work, we demonstrate that the SUMOylation enhancer RSUME is a novel regulator of GR transcriptional activity. RSUME interacts with GR and increases its SUMOylation and transcriptional activity in an RWD domain-dependent manner, pointing to SUMO conjugation as the cause for transcriptional stimulation. This functional link was demonstrated in reporter gene assays and on endogenous targets. Knocking down RSUME by siRNA leads to inhibition of FKBP51 expression, proving that endogenous RSUME plays an important role in GR transcriptional activity. Interestingly, RSUME action on GR-dependent transcription is independent of SUMO attachment to the N-terminal sites K297 and K313. On the contrary, RSUME-mediated GR SUMOylation at K721 is responsible for the consequent transcriptional regulation. In the same line, we show that mutation of K721 or siRNA against RSUME impairs GRIP1-mediated activation of GR transcription. Also, we demonstrate that RSUME, whose expression is induced under stress conditions, is a key factor in heat shock-induced GR SUMOylation. Altogether, we show that the presence of a SUMOylation enhancer, such as RSUME, uncovers a positive role for the LBD SUMOylation site on GR-mediated transcription. This third site could become relevant in a scenario of increased RSUME expression, such as under cellular stress conditions, in which RSUME can function as a molecular switch of the negative to positive action of SUMO conjugation to the GR.

Several reports have addressed the relevance of SUMO conjugation to nuclear receptors (NRs) in regulating their transcriptional output. The most-studied NRs are the androgen receptor (AR) and GR, which were among the first transcription factors shown to be SUMOylated (11, 26). In both cases, the two main sites of conjugation reside in the NTD (11, 26). One of the prominent functional roles of SUMOylation in both AR and GR is to mediate the regulatory effects of SC motifs. These motifs exert an inhibitory effect and restrain the transcriptional activation of factors when stably bound to multiple, closely spaced response elements. It was demonstrated that SC motifs in GR and AR exert their effects by serving as sites for SUMO modification (8, 11, 26,

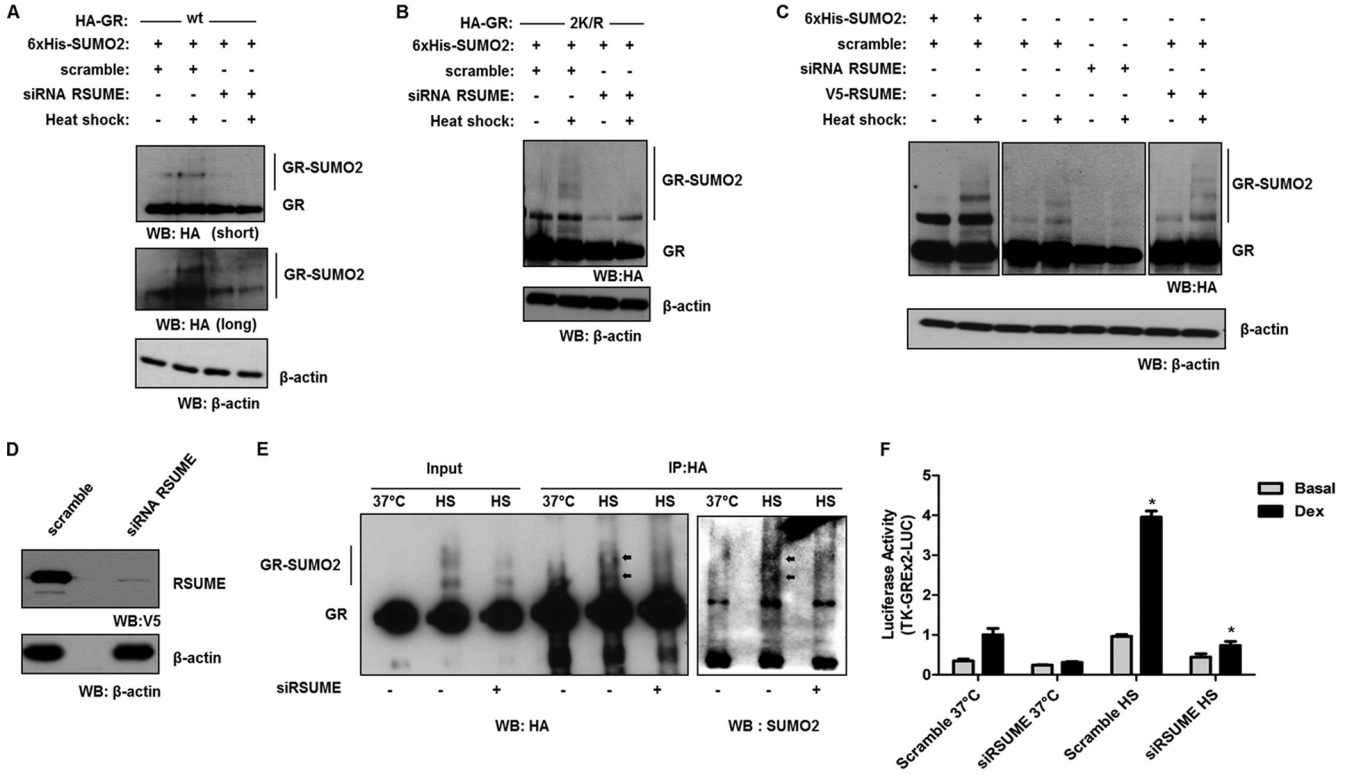

FIG 6 RSUME is required for heat shock-induced SUMO conjugation to GR. (A) COS-7 cells were transfected with 20 nM siRNA directed against RSUME or scramble siRNA together with wt HA-p6RGR or the HA-p6RGR 2K/R (100 ng) (B) and 6×His-SUMO2 (100 ng) expression vectors. At 48 h posttransfection, cells were stimulated with 10 nM Dex for 2 h, and in the last 30 min, cells were subjected to heat conditions (42°C for 30 min) or left untreated (37°C). Extracts were subjected to Western blotting. Short and long exposures of Western blots are shown in panel A. (C) COS-7 cells were transfected with 20 nM siRNA directed against RSUME or scramble siRNA, together with wt HA-p6RGR (100 ng) and the V5-RSUME (100 ng) or 6×His-SUMO2 (100 ng) expression vector, as indicated. At 48 h posttransfection, cells were treated as described in the legends to panels A and B, and cells extracts were subjected to Western blotting. (D) COS7 cells were transiently transfected with the V5-RSUME (100 ng) expression vector in combination with 20 nM RSUME or scramble siRNA. After 48 h, cell extracts were subjected to Western blotting with an anti-V5 antibody to confirm RSUME depletion. Membranes were probed with an anti-β-actin antibody as a loading control. (E) COS7 cells were transfected with 20 nM siRNA directed against RSUME or scramble siRNA together with wt HA-p6RGR. At 48 h posttransfection, cells were stimulated with 10 nM Dex for 2 h, and in the last 30 min, cells were subjected to heat conditions (42°C for 30 min, heat shock [HS]) or untreated (37°C) and harvested for immunoprecipitation. Samples were analyzed by Western blotting with anti-HA or anti-SUMO2 antibodies. Arrows, SUMO-modified GR bands. The experiments shown are representative of three independent Western blots with similar results. (F) COS7 cells were transfected with a reporter plasmid bearing two tandem GREs, TK-GREx2-Luc (100 ng) together with p6RGR (10 ng) and 20 nM siRNA directed against RSUME or scramble siRNA. At 48 h posttransfection, cells were stimulated with 10 nM Dex or vehicle (basal) and subjected to 42°C for 1 h or untreated (37°C). Cells were harvested 7 h after recovery at 37°C, and luciferase activity was measured. Each value was normalized to the protein content per well. The activity of GR with Dex was set equal to 1. *, $P < 0.05$ compared with the corresponding cells transfected with p6RGR wt plus scramble at 37°C or scramble heat shock stimulated with Dex (ANOVA with Scheffé's test). Results are expressed as the mean ± SEM of triplicates of one representative experiment from three independent experiments with similar results.

28). As recently reported, not only is AR SUMOylation linked to transcriptional repression, but also it affects the dynamics of AR-chromatin interactions and activity in a target gene-specific manner (46).

Interestingly, the functional effect of RSUME does not involve the SC-motif SUMOylation sites within GR but instead requires a third site located within the LBD, which has not been studied in detail until now. Indeed, mutation of K721 abolishes the effect of RSUME on GR transcriptional activity independently of SUMOylation at the NTD sites, demonstrating the importance of K721 as a positive element of GR activity. Thus, it is expected that other factors that favor SUMO conjugation will underscore a positive role of the third SUMOylation site on GR-dependent transcription. The GR mutant with the mutation in humans that is equivalent to K721R in the rat, K703R, consistently shows lower activity than wt GR, as already observed (11). Moreover, mutation of K703R also inhibits the RSUME effect on GR-mediated transcription (data not shown). In spite of the differences that might account for the interspecies differences, the C-terminal Lys seems to have an important positive role under certain cellular conditions (e.g., stress) in which factors such as RSUME act. Our study also proposes that NR SUMOylation cannot be regarded as a general inhibitory mechanism, but a more specified view is needed.

It has been reported that small changes in the LBD lead to altered transactivation by the GR (35). Those changes can alter the activity of bound cofactors, such as GRIP1 or Ubc9, without modifying their binding capabilities (34, 35). Therefore, we speculate that SUMOylation of K721 influences GR activity by modifying coactivator function due to its localization in the LBD. In fact, we demonstrate here that GRIP1-mediated GR activation is compromised when K721 is mutated and this effect is not due to the lack of GRIP1-GR interaction. These results suggest that SUMOylation of K721 might not be necessary for GRIP1 binding to GR but might be critical for this coactivator's action.

Interestingly, RSUME interacts with GRIP1, and knocking down RSUME by siRNA inhibits GRIP1-dependent GR transcriptional activation, showing a positive correlation between RSUME, K721, and GRIP1. Further studies are needed in order to shed light onto the mechanistic role of SUMO modification of K721 in GR coactivator recruitment. Nevertheless, it is tempting to speculate that RSUME influences GRIP1 coactivator activity on GR-mediated transcription indirectly by modifying GR K721 SUMOylation. Since RSUME is a SUMOylation enhancer, we do not discard the possibility that RSUME affects the SUMOylation pattern of other proteins involved in the GR transcriptional complex. RSUME might be acting at different levels to stimulate GR transcriptional activity, either directly by stimulation of K721 SUMOylation, by GRIP1 SUMOylation, or by the SUMOylation of other proteins involved in GR activity.

Both GR and RSUME have been reported to interact with Ubc9 (20, 29). Our present results show that RSUME increases the GR-Ubc9 interaction and the RSUME SUMOylation mutant does not, providing a plausible mechanistic explanation for RSUME-mediated GR SUMOylation enhancement. Regarding the role for different SUMO paralogues, we have shown that RSUME increases SUMO2 conjugation to all three GR sites. A possible differential role for different SUMO isoforms on the RSUME effect on GR SUMOylation cannot be ruled out until a systematic quantitative comparison of all of them is performed.

In addition, RSUME's effect on GR SUMOylation and its transcriptional activity depend on the integrity of RSUME's RWD domain. However, several components of the SUMOylation pathway regulate NR transcriptional activity through mechanisms that can be either dependent on or independent of their SUMOylation activity. PIAS1 stimulates AR transcriptional activity, and this effect is dependent on its SUMO E3 activity (47). In contrast, Ubc9 stimulates GR SUMOylation (data not shown) and transcriptional activity (30, 31), but this transcriptional stimulation does not involve its SUMO E2 activity (31), which occurs with the mineralocorticoid receptor (MR) (48). This additional activity of Ubc9 as a GR modulator could explain the fact that silencing its expression yields a weaker transcriptional activity compared to that of the 3K/R mutant. In the case of the steroidogenic factor-1, the stimulatory effect of both Ubc9 and PIAS1 is independent of their SUMOylation activity (49). Thus, it has become apparent that the functional interplay between SUMO conjugation pathway components and NR's transcriptional activity has no easily predictable outcome, as this will depend on the specific NR, the site of modification, and the specific regulator.

Cellular stressors have been shown to increase global SUMO conjugation (16). In particular, heat shock treatment was shown to rapidly stimulate SUMOylation of GR and AR (17, 18, 46). On the other hand, it has been reported that GR-mediated transcription is potentiated in response to stressors such as heat shock (50, 51) and hypoxia (52). Furthermore, here we show that heat stress stimulates GR transcriptional activity and that RSUME is critically involved in this physiological process. A recent study on the natural chromatin context of AR target genes shows that heat stress results in a marked release of AR from chromatin and a consequent decrease in the mRNA levels of these genes (46).

Moreover, heat and chemical stresses induce translocation of the unliganded GR to the nucleus (51, 53). Interestingly, RSUME colocalizes with SUMO2 granules in heat-treated cells. Most strikingly, heat shock enhances GR SUMOylation, and RSUME is involved in this effect. Hence, a positive correlation between GR SUMOylation and transcriptional activity seems to occur during the heat shock response. However, taking into account the fact that heat shock induces a rapid SUMO2 conjugation of proteins which is reversed during the recovery phase, further studies will be required to determine if heat-induced GR SUMOylation and transcription are indeed functionally coupled.

The fact that RSUME regulates GR SUMOylation in a specific pattern leads us to speculate that RSUME could fine-tune the cellular response to GCs through the regulation of GR SUMOylation status. RSUME and other regulators of GR SUMOylation determine the outcome of the complex interaction between the negative and positive SUMO sites on the GR. Thus, understanding these mechanisms contributes to the establishment of potential targets to modulate physiological and therapeutic responses to GCs.

ACKNOWLEDGMENTS

This work was supported by grants from the Max Planck Society, Germany; the University of Buenos Aires; CONICET; the Agencia Nacional de Promoción Científica y Tecnológica, Argentina; the National Institutes of Health (R56DK061656 to J.A.I.-L.); and FOCEM-Mercosur (COF 03/11). Jimena Druker is a postdoctoral fellow of the Bunge & Born Foundation, Argentina.

We thank Ronald Hay, Jorma Palvimo, Michael Stallcup, Mary Dasso, and Dietmar Spengler for providing the plasmids and antibodies used in this work and Federico Pelisch for technical comments and discussion of the manuscript.

REFERENCES

1. **Beato M, Chavez S, Truss M.** 1996. Transcriptional regulation by steroid hormones. Steroids **61**:240–251.
2. **Barnes PJ.** 1998. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin. Sci. (Lond.) **94**:557–572.
3. **Gross KL, Cidlowski JA.** 2008. Tissue-specific glucocorticoid action: a family affair. Trends Endocrinol. Metab. **19**:331–339.
4. **Sapolsky RM, Romero LM, Munck AU.** 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrinol. Rev. **21**:55–89.
5. **Ricketson D, Hostick U, Fang L, Yamamoto KR, Darimont BD.** 2007. A conformational switch in the ligand-binding domain regulates the dependence of the glucocorticoid receptor on Hsp90. J. Mol. Biol. **368**:729–741.
6. **Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE.** 2002. Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell **110**:93–105.
7. **Davies L, Karthikeyan N, Lynch JT, Sial EA, Gkourtsa A, Demonacos C, Krstic-Demonacos M.** 2008. Cross talk of signaling pathways in the regulation of the glucocorticoid receptor function. Mol. Endocrinol. **22**:1331–1344.
8. **Holmstrom S, Van Antwerp ME, Iniguez-Lluhi JA.** 2003. Direct and distinguishable inhibitory roles for SUMO isoforms in the control of transcriptional synergy. Proc. Natl. Acad. Sci. U. S. A. **100**:15758–15763.
9. **Ismaili N, Garabedian MJ.** 2004. Modulation of glucocorticoid receptor function via phosphorylation. Ann. N. Y. Acad. Sci. **1024**:86–101.
10. **Mason SA, Housley PR.** 1993. Site-directed mutagenesis of the phosphorylation sites in the mouse glucocorticoid receptor. J. Biol. Chem. **268**:21501–21504.
11. **Tian S, Poukka H, Palvimo JJ, Janne OA.** 2002. Small ubiquitin-related modifier-1 (SUMO-1) modification of the glucocorticoid receptor. Biochem. J. **367**:907–911.
12. **Wallace AD, Cidlowski JA.** 2001. Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J. Biol. Chem. **276**:42714–42721.
13. **Hay RT.** 2005. SUMO: a history of modification. Mol. Cell **18**:1–12.
14. **Melchior F.** 2000. SUMO—nonclassical ubiquitin. Annu. Rev. Cell Dev. Biol. **16**:591–626.

15. **Gareau JR, Lima CD.** 2010. The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. Nat. Rev. Mol. Cell Biol. **11**:861–871.
16. **Saitoh H, Hinchey J.** 2000. Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J. Biol. Chem. **275**: 6252–6258.
17. **Golebiowski F, Tatham MH, Nakamura A, Hay RT.** 2010. High-stringency tandem affinity purification of proteins conjugated to ubiquitin-like moieties. Nat. Protoc. **5**:873–882.
18. **Tatham MH, Matic I, Mann M, Hay RT.** 2011. Comparative proteomic analysis identifies a role for SUMO in protein quality control. Sci. Signal. **4**:rs4. doi:10.1126/scisignal.2001484.
19. **Doerks T, Copley RR, Schultz J, Ponting CP, Bork P.** 2002. Systematic identification of novel protein domain families associated with nuclear functions. Genome Res. **12**:47–56.
20. **Carbia-Nagashima A, Gerez J, Perez-Castro C, Paez-Pereda M, Silberstein S, Stalla GK, Holsboer F, Arzt E.** 2007. RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1alpha during hypoxia. Cell **131**:309–323.
21. **Le Drean Y, Mincheneau N, Le Goff P, Michel D.** 2002. Potentiation of glucocorticoid receptor transcriptional activity by sumoylation. Endocrinology **143**:3482–3489.
22. **Chupreta S, Brevig H, Bai L, Merchant JL, Iniguez-Lluhi JA.** 2007. Sumoylation-dependent control of homotypic and heterotypic synergy by the Kruppel-type zinc finger protein ZBP-89. J. Biol. Chem. **282**:36155–36166.
23. **Subramanian L, Benson MD, Iniguez-Lluhi JA.** 2003. A synergy control motif within the attenuator domain of CCAAT/enhancer-binding protein alpha inhibits transcriptional synergy through its PIASy-enhanced modification by SUMO-1 or SUMO-3. J. Biol. Chem. **278**:9134–9141.
24. **Abdel-Hafiz H, Takimoto GS, Tung L, Horwitz KB.** 2002. The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. J. Biol. Chem. **277**: 33950–33956.
25. **Iniguez-Lluhi JA, Pearce D.** 2000. A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. Mol. Cell. Biol. **20**:6040–6050.
26. **Poukka H, Karvonen U, Janne OA, Palvimo JJ.** 2000. Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc. Natl. Acad. Sci. U. S. A. **97**:14145–14150.
27. **Vu EH, Kraus RJ, Mertz JE.** 2007. Phosphorylation-dependent sumoylation of estrogen-related receptor alpha1. Biochemistry **46**:9795–9804.
28. **Holmstrom SR, Chupreta S, So AY, Iniguez-Lluhi JA.** 2008. SUMO-mediated inhibition of glucocorticoid receptor synergistic activity depends on stable assembly at the promoter but not on DAXX. Mol. Endocrinol. **22**:2061–2075.
29. **Gottlicher M, Heck S, Doucas V, Wade E, Kullmann M, Cato AC, Evans RM, Herrlich P.** 1996. Interaction of the Ubc9 human homologue with c-Jun and with the glucocorticoid receptor. Steroids **61**:257–262.
30. **Cho S, Kagan BL, Blackford JA, Jr, Szapary D, Simons SS, Jr.** 2005. Glucocorticoid receptor ligand binding domain is sufficient for the modulation of glucocorticoid induction properties by homologous receptors, coactivator transcription intermediary factor 2, and Ubc9. Mol. Endocrinol. **19**:290–311.
31. **Kaul S, Blackford JA, Jr, Cho S, Simons SS, Jr.** 2002. Ubc9 is a novel modulator of the induction properties of glucocorticoid receptors. J. Biol. Chem. **277**:12541–12549.
32. **Kotaja N, Aittomaki S, Silvennoinen O, Palvimo JJ, Janne OA.** 2000. ARIP3 (androgen receptor-interacting protein 3) and other PIAS (protein inhibitor of activated STAT) proteins differ in their ability to modulate steroid receptor-dependent transcriptional activation. Mol. Endocrinol. **14**:1986–2000.
33. **Tirard M, Jasbinsek J, Almeida OF, Michaelidis TM.** 2004. The manifold actions of the protein inhibitor of activated STAT proteins on the transcriptional activity of mineralocorticoid and glucocorticoid receptors in neural cells. J. Mol. Endocrinol. **32**:825–841.
34. **Lee GS, Simons SS, Jr.** 2011. Ligand binding domain mutations of the glucocorticoid receptor selectively modify the effects with, but not binding of, cofactors. Biochemistry **50**:356–366.
35. **Tao YG, Xu Y, Xu HE, Simons SS, Jr.** 2008. Mutations of glucocorticoid receptor differentially affect AF2 domain activity in a steroid-selective manner to alter the potency and efficacy of gene induction and repression. Biochemistry **47**:7648–7662.
36. **Kotaja N, Karvonen U, Janne OA, Palvimo JJ.** 2002. The nuclear receptor interaction domain of GRIP1 is modulated by covalent attachment of SUMO-1. J. Biol. Chem. **277**:30283–30288.
37. **Teyssier C, Ou CY, Khetchoumian K, Losson R, Stallcup MR.** 2006. Transcriptional intermediary factor 1alpha mediates physical interaction and functional synergy between the coactivator-associated arginine methyltransferase 1 and glucocorticoid receptor-interacting protein 1 nuclear receptor coactivators. Mol. Endocrinol. **20**:1276–1286.
38. **Desterro JM, Rodriguez MS, Hay RT.** 1998. SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. Mol. Cell **2**:233–239.
39. **Jaffray EG, Hay RT.** 2006. Detection of modification by ubiquitin-like proteins. Methods **38**:35–38.
40. **Tatham MH, Jaffray E, Vaughan OA, Desterro JM, Botting CH, Naismith JH, Hay RT.** 2001. Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. J. Biol. Chem. **276**:35368–35374.
41. **Ayaydin F, Dasso M.** 2004. Distinct in vivo dynamics of vertebrate SUMO paralogues. Mol. Biol. Cell **15**:5208–5218.
42. **Rupprecht R, Arriza JL, Spengler D, Reul JM, Evans RM, Holsboer F, Damm K.** 1993. Transactivation and synergistic properties of the mineralocorticoid receptor: relationship to the glucocorticoid receptor. Mol. Endocrinol. **7**:597–603.
43. **Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T.** 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411**:494–498.
44. **Paakinaho V, Makkonen H, Jaaskelainen T, Palvimo JJ.** 2010. Glucocorticoid receptor activates poised FKBP51 locus through long-distance interactions. Mol. Endocrinol. **24**:511–525.
45. **Tatham MH, Geoffroy MC, Shen L, Plechanovova A, Hattersley N, Jaffray EG, Palvimo JJ, Hay RT.** 2008. RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. Nat. Cell Biol. **10**:538–546.
46. **Rytinki M, Kaikkonen S, Sutinen P, Paakinaho V, Rahkama V, Palvimo JJ.** 2012. Dynamic SUMOylation is linked to the activity cycles of androgen receptor in the cell nucleus. Mol. Cell. Biol. **32**:4195–4205.
47. **Kotaja N, Karvonen U, Janne OA, Palvimo JJ.** 2002. PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. Mol. Cell. Biol. **22**:5222–5234.
48. **Yokota K, Shibata H, Kurihara I, Kobayashi S, Suda N, Murai-Takeda A, Saito I, Kitagawa H, Kato S, Saruta T, Itoh H.** 2007. Coactivation of the N-terminal transactivation of mineralocorticoid receptor by Ubc9. J. Biol. Chem. **282**:1998–2010.
49. **Suda N, Shibata H, Kurihara I, Ikeda Y, Kobayashi S, Yokota K, Murai-Takeda A, Nakagawa K, Oya M, Murai M, Rainey WE, Saruta T, Itoh H.** 2011. Coactivation of SF-1-mediated transcription of steroidogenic enzymes by Ubc9 and PIAS1. Endocrinology **152**:2266–2277.
50. **Li DP, Periyasamy S, Jones TJ, Sanchez ER.** 2000. Heat and chemical shock potentiation of glucocorticoid receptor transactivation requires heat shock factor (HSF) activity. Modulation of HSF by vanadate and wortmannin. J. Biol. Chem. **275**:26058–26065.
51. **Sanchez ER, Hu JL, Zhong S, Shen P, Greene MJ, Housley PR.** 1994. Potentiation of glucocorticoid receptor-mediated gene expression by heat and chemical shock. Mol. Endocrinol. **8**:408–421.
52. **Leonard MO, Godson C, Brady HR, Taylor CT.** 2005. Potentiation of glucocorticoid activity in hypoxia through induction of the glucocorticoid receptor. J. Immunol. **174**:2250–2257.
53. **Sanchez ER.** 1992. Heat shock induces translocation to the nucleus of the unliganded glucocorticoid receptor. J. Biol. Chem. **267**:17–20.