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## Sumoylation of the Progesterone Receptor and of the Steroid Receptor Coactivator SRC-1\*

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**SUMO-1 (small ubiquitin-like modifier) conjugation regulates the subcellular localization, stability, and activity of a variety of proteins. We show here that SUMO-1 overexpression markedly enhances progesterone receptor (PR)-mediated gene transcription. PR undergoes a sumoylation at lysine 388 located in its N-terminal domain. However, sumoylation of the receptor is not responsible for enhanced transcription because substitution of its target lysine did not abolish the effect of SUMO-1 and even converted the receptor into a slightly more active transactivator. Furthermore estrogen receptor  $\alpha$  (ER $\alpha$ )-driven transcription is also enhanced by SUMO-1 overexpression contrasting with the absence of sumoylation of this receptor. We thus analyzed SUMO-1 conjugation to the steroid receptor coactivator SRC-1. We showed that this protein contains two major sites of conjugation at Lys-732 and Lys-774. Sumoylation was shown to increase PR-SRC-1 interaction and to prolong SRC-1 retention in the nucleus. It did not prevent SRC-1 ubiquitinylation and did not exert a clear effect on the stability of the protein. Overexpression of SUMO-1 enhanced PR-mediated gene transcription even in the presence of non-sumoylated mutants of SRC-1. This observation suggests that among the many protein partners involved in steroid hormone-mediated gene regulation several are probably targets of SUMO-1 modification.**

The progesterone receptor (PR)<sup>1</sup> is a transcription factor belonging to the superfamily of nuclear receptors. It contains

two domains involved in transcription activation: the constitutive activation function 1 (AF-1) localized in the N-terminal region of the protein and the ligand-regulated activation function 2 (AF-2) corresponding to the ligand binding domain. X-ray diffraction analysis of receptor crystals have allowed a detailed study of the molecular mechanisms involved in ligand-induced conformational changes of the AF-2 region (1–9).

To activate transcription, steroid hormone receptors recruit coactivators among which the p160 family seems to be of special importance. This family comprises SRC-1/NCoA-1 (steroid receptor coactivator-1), SRC-2/TIF2/GRIP1 and SRC-3/TRAM-1/ACTRA/IB1/RAC3/pCIP (for reviews, see Refs. 10–11). CREB-binding protein (CBP) and p300 interact with both the nuclear receptors and the coactivators. Recruitment of histone acetylases allows local decondensation of chromatin thus facilitating transcription (for reviews, see Refs. 12–14).

Aside from their regulation by specific ligands, nuclear receptors and especially steroid hormone receptors are known to undergo covalent modifications. Phosphorylation (for review, see Ref. 15) and ubiquitinylation (15–20) have been shown to regulate their functions and stability. Recently a new covalent modification of proteins and especially of transcriptional regulators have been described: SUMO-1 has been found to be conjugated to a growing list of proteins: the Ran GTPase-activating protein RanGAP1 (21–24), the NF $\kappa$ B inhibitor I $\kappa$ B $\alpha$  (25), the promyelocytic leukemia protein PML (26–29), the nuclear dot protein Sp100 (27), the homeodomain-interacting protein kinase 2 HIPK2 (30), the topoisomerases I and II (31–32), the tumor suppressor protein p53, and the protooncogene c-Jun (33–35).

SUMO-1 is a protein of 101 amino acids that has a low (18%) but significant homology to ubiquitin. Modification by SUMO-1 (sumoylation) has a marked similarity with ubiquitin conjugation reactions (for reviews, see Refs. 36–37). It requires an E1-activating enzyme (SAE1/SAE2 in mammals, Aos1/Uba2 in yeast) and the E2-conjugating enzyme Ubc9. E3 enzymes have been recently identified in yeast (38–39) and in human cells (40–41) but do not appear to be required for SUMO conjugation *in vitro*.

Whereas mono-ubiquitinylation has been described as specifically involved in membrane receptor endocytosis (for review, see Refs. 42), polyubiquitinylation addresses proteins to proteasomes where they are degraded. In contrast, there is addition of a single SUMO-1 molecule to target lysine(s). Whereas ubiquitinylation usually targets proteins to degradation, sumoylation modifies their functional properties: subcellular

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<sup>1</sup> The abbreviations used are: PR, progesterone receptor; SRC-1, steroid receptor coactivator 1; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; SUMO, small ubiquitin-like modifier; ER $\alpha$ , estrogen receptor  $\alpha$ ; HA, hemagglutinin epitope; PRE, progesterone responsive element; ERE, estrogen responsive element; CAT, chloramphenicol acetyltransferase; AD, activation domain; DBD, DNA-binding domain; Ub, ubiquitin; WCE, whole cell extracts; Ni-NTA, nickel nitrilotriacetic acid-charged agarose beads; R5020, 17,21-di-methyl-19-norpregna-4,9-dien-3,20-dione; RU486, 17 $\beta$ -

hydroxy-11 $\beta$ -(4-dimethylaminophenyl)-17 $\alpha$ -(1-propynyl)-oestra-4,9-dien-3-one; ZK98299, 11 $\beta$ -(4-dimethylaminophenyl)-17 $\alpha$ -hydroxy-17 $\beta$ -(3-hydroxypropyl)-13 $\alpha$ -methyl-4,9-gonadien-3-one; DBD, DNA-binding domain; LBD, ligand-binding domain.

localization, stability, or ability to regulate gene transcription.

In this manuscript, we describe SUMO-1 conjugation to the progesterone receptor and to its coactivator SRC-1. We also analyze the effect of this modification on the subcellular localization and the transcription-activating properties of these proteins.

#### EXPERIMENTAL PROCEDURES

**Plasmids**—The plasmids encoding the full-length HA-tagged SRC-1 and the deletion mutants pSG5-HA-SRC-1  $\Delta$ 1–567, pSG5-HA-SRC-1  $\Delta$ 1–782, and pSG5-HA-SRC-1  $\Delta$ 1209–1440 have been previously described (43). PCR was used to generate the expression vector for the SRC-1 mutant pSG5-HA-SRC-1  $\Delta$ 785–1038, by inserting a *Bam*HI site in position 2343 corresponding to the amino acid 782. The deletion was generated by replacing the wild-type *Bam*HI/*Msc*I fragment with the PCR fragment digested by *Bam*HI/*Msc*I.

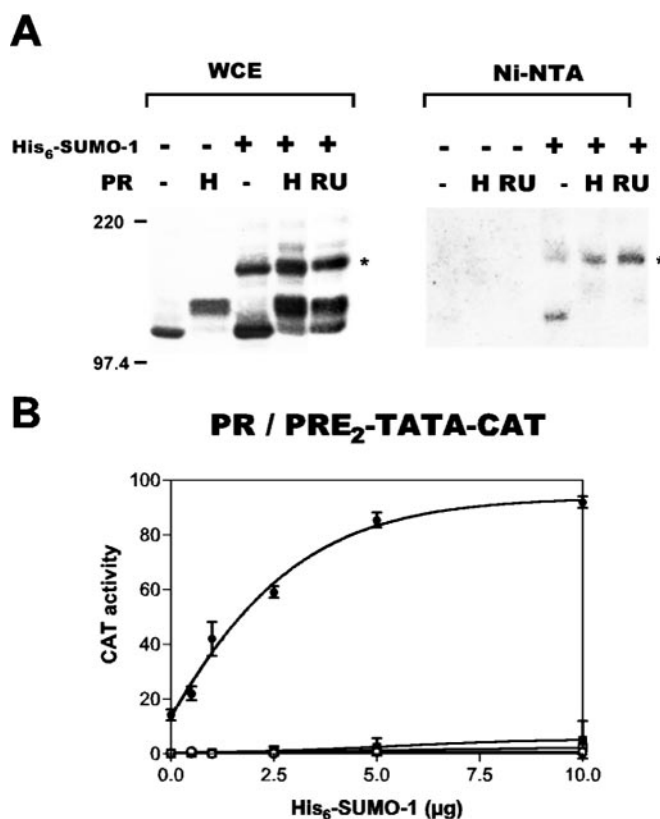
All point mutation mutants were generated by site-directed mutagenesis using PCR strategy. The generated fragments containing Lys/Arg substitution were cloned into the pSG5-HA-SRC-1 vector by restriction digestions. The multiple substituted mutants were obtained by consecutive fragment exchanges using the restriction sites *Eco*RV, *Bam*HI, *Bst*EII, *Msc*I, and *Bgl*II. All the constructs were verified by DNA sequencing on an automatic sequencer (ABI-Perkin 373A).

The expression vectors encoding the full-length progesterone receptor, the human estrogen receptor  $\alpha$  (pKSV-ER $\alpha$ ), and their respective reporter plasmids PRE<sub>2</sub>-TATA-CAT and ERE<sub>2</sub>-TATA-CAT have been previously described (43). The point mutation K388R was generated by PCR. The mutated fragment was introduced into pSG5-hPR using *Mlu*I/*Msc*I sites. The construct was verified by sequencing. The expression vectors encoding the AD<sub>VP16</sub>-SRC-1, PM-PR fusion proteins have been described (43). For the AD<sub>VP16</sub>-SRC-1 2R and 5R mutants, the *Sma*I/*Bgl*II fragments of pSG5-HA-SRC-1 2R and pSG5-SRC-1 5R have been cloned, respectively, into the blunted *Eco*RI/*Bam*HI empty AD<sub>VP16</sub> vector. The His<sub>6</sub>-tagged SUMO-1 (pSG5-His<sub>6</sub>-SUMO-1) and the His<sub>6</sub>-tagged ubiquitin (pSG5-His<sub>6</sub>-Ub) expression vectors were kindly provided by Dr. S. Müller and Dr. D. Bohmann, respectively (35, 44).

**Cell Culture, Transfections, and CAT Assays**—CV-1 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (Seromed). For CAT assays and Western blotting analysis, transfections were performed by the standard calcium phosphate precipitation method on CV-1 cells as previously described in detail (43). Briefly, transfections were performed on 6-well dishes with 330  $\mu$ l of calcium phosphate precipitate per well containing the reporter gene and expression vectors as indicated for each experiment. Total DNA was normalized to 20  $\mu$ g/ml of precipitate using salmon sperm DNA. The CAT activity was measured with the CAT ELISA kit (Roche Molecular Biochemicals). Protein concentrations were determined by using the BCA protein assay (Pierce), and the CAT activity was corrected for protein content.

**Preparation of Cell Extracts, Ni-NTA Precipitation, and Western Blotting**—CV-1 cells seeded on 10-cm diameter Petri dishes were transfected with the indicated expression vectors. For the preparation of whole cell extracts (WCE), 40 h after transfection, cells were washed with ice-cold phosphate-buffered saline and harvested in 1 ml of lysis buffer supplemented with 50 mM sodium fluoride (34). Alternatively, for the nickel-affinity purification procedure (Ni-NTA), the transfected cells were lysed in parallel in 1 ml of buffer A (6 M guanidinium-HCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM imidazole, 10 mM Tris-HCl, pH 8). After sonication to reduce viscosity, the clarified lysates were incubated for 2 h at room temperature with 50  $\mu$ l of Ni-NTA-coupled agarose beads (Qiagen) prewashed in buffer A. The beads were washed twice with 1 ml of buffer B (8 M urea, pH 8, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM imidazole, 10 mM Tris-HCl, pH 8) and once with 1 ml of buffer C (8 M urea pH 8, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM imidazole, 10 mM Tris-HCl, pH 6.3). After a final wash with PBS, the bound proteins were eluted by boiling in Laemmli loading buffer and subjected to a 6.4% SDS-PAGE. The proteins were analyzed by Western blotting using primary anti-PR monoclonal antibody Let126 (45) or anti-HA 12CA5 monoclonal antibody (Roche Molecular Biochemicals) at a concentration of 2  $\mu$ g/ml. The ECL system (Amersham Biosciences) was used for band detection.

**Fluorescence Microscopy**—COS-7 cells were grown on 35-mm dishes and were transfected with the expression vectors using LipofectAMINE reagent (Invitrogen) according to the instructions of the manufacturer. After different times, cells were fixed as previously described (46). Incubations with the primary antibodies were performed overnight at 4 °C in the presence of 1% goat serum. In single labeling experiments, SRC-1 was detected with the rat monoclonal anti-HA 3F10 (Roche



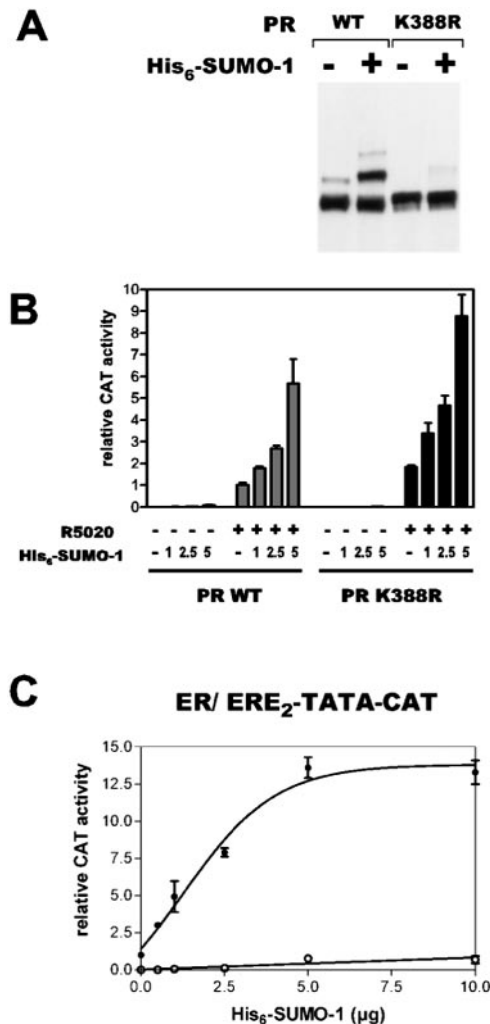
**FIG. 1. Sumoylation of the progesterone receptor; effect of SUMO-1 on PR-mediated gene transcription activation.** A, CV-1 cells were transfected with the expression vector encoding the progesterone receptor in the presence or absence of the His<sub>6</sub>-tagged SUMO-1. The cells were grown for 24 h in the presence or absence of 10 nM R5020 (H) or 10 nM RU486 (RU) as indicated. Total cell extracts (WCE) were analyzed by electrophoresis, and PR was detected by Western blotting using the PR-specific let126 monoclonal antibody. The co-transfected CV-1 cells were lysed in buffer containing guanidinium-HCl (Ni-NTA), and the SUMO-1-modified proteins were purified in parallel using Ni-NTA agarose beads as described under "Experimental Procedures." Eluted proteins were separated by electrophoresis and His<sub>6</sub>-SUMO-1 PR conjugates were detected by Western blotting using the PR-specific Let126 monoclonal antibody. WCE represent 15% of the input for Ni-NTA precipitation. Molecular size markers are shown on the right, and the SUMO-1-modified form of the PR is indicated by an asterisk. B, CV-1 cells were transfected with the PRE<sub>2</sub>-TATA-CAT reporter plasmid (5  $\mu$ g/ml) along with pSG5-rPR (0.5  $\mu$ g/ml) and increasing amount of pSG5-His<sub>6</sub>-SUMO-1 expression vector. The cells were treated 24 h before harvesting with 10 nM R5020 (●), 10 nM RU486 (■), 100 nM ZK98299 (□), or left untreated (○). The results are means  $\pm$  S.D. of three independent determinations.

Molecular Biochemicals) at 1  $\mu$ g/ml. The secondary antibody was the goat anti-rat Alexa 594 (Molecular Probes) (1/400). For double labeling, SRC-1 was detected with the mouse monoclonal anti-HA 12CA5 antibody (1  $\mu$ g/ml) and endogenous SUMO-1 was detected with the rabbit polyclonal anti-SUMO-1 FL-101 (Santa Cruz Biotechnology) (2  $\mu$ g/ml). The secondary antibodies sheep anti-mouse CY3-conjugated antibody (Sigma) and Alexa 594 IgG (1/400) were incubated with cells for 30 min at room temperature. Confocal images were acquired using the LSM410 system on an Axiocvert 135 M Zeiss microscope (Carl Zeiss, Thornwood, NY) using excitation wavelengths of 488 nm (for Alexa, green) and 543 nm (for CY3, red).

#### RESULTS

**Sumoylation of the Progesterone Receptor**—To examine if the progesterone receptor undergoes sumoylation, CV-1 cells were transfected with a PR expression vector in the presence or absence of a vector encoding His-tagged SUMO-1. Western blotting with an anti-PR monoclonal antibody detected in cells expressing only PR and not incubated with a ligand, a single protein band of  $\sim$ 115 kDa (Fig. 1A, WCE). Treatment

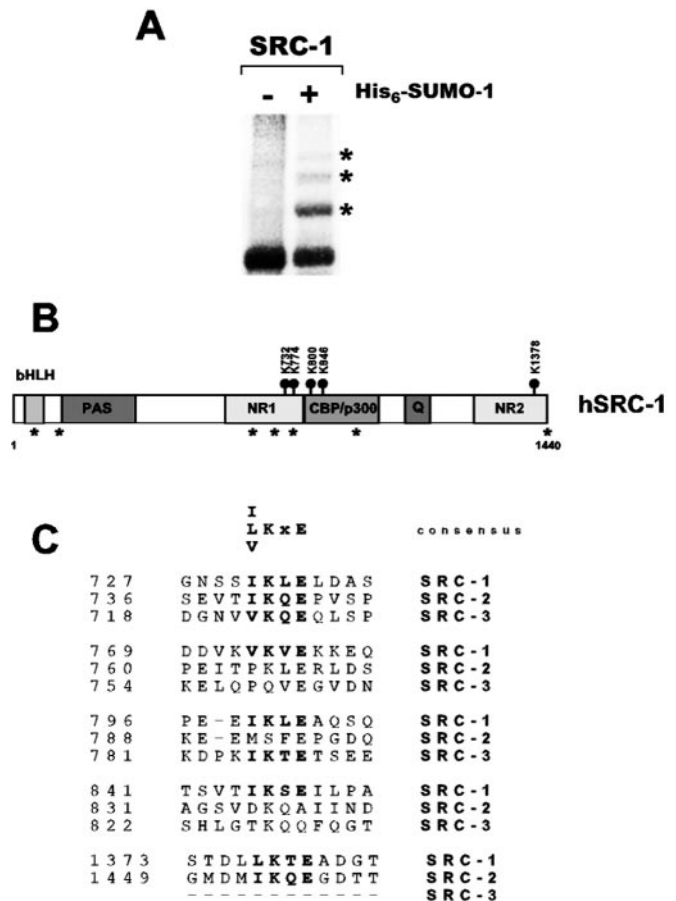




**FIG. 2. Receptor sumoylation and SUMO-1 enhancement of transcription.** A, lysine 388 is the major sumoylation site of PR. CV-1 cells were transfected with the expression vector encoding the wild-type progesterone receptor (pSG5-hPR) or the K388R mutant (pSG5-hPR K388R) in the presence or absence the His<sub>6</sub>-tagged SUMO-1. The cells were grown in the presence of 10 nM R5020 for 24 h before harvesting. Total cell extracts (WCE) were analyzed by electrophoresis and PR was detected by Western blotting using the PR-specific let126 monoclonal antibody. B, SUMO-1 enhances PR K388R-mediated gene transcription activation. CV-1 cells were transfected with the PRE<sub>2</sub>-TATA-CAT reporter plasmid (5 μg/ml) along with pSG5-hPR (1 μg/ml) or pSG5-hPR K388R (1 μg/ml) as indicated and increasing amount (1; 2.5, and 5 μg/ml) of pSG5-His<sub>6</sub>-SUMO-1 expression vector. The cells were treated with 10 nM R5020 (+) or untreated (-) 24 h before harvesting. The results are means ± S.D. of three independent determinations. C, SUMO-1 enhances ERα-mediated gene transcription activation. CV-1 cells were cotransfected with the plasmid encoding the estrogen receptor ERα (0.5 μg/ml) with the ERE<sub>2</sub>-TATA-CAT reporter plasmid (5 μg/ml) and increasing amounts of pSG5-His<sub>6</sub>-SUMO-1 expression vector. The cells were treated with 10 nM estradiol (●) or untreated (○) 24 h before harvesting. The results are means ± S.D. of three independent determinations.

by the progesterone agonist R5020 provoked a decrease in receptor migration previously shown to be due to its phosphorylation (47). In contrast, in cells expressing both PR and SUMO-1, an additional band of ~155 kDa was detected in both hormone- or antiprogestin (RU486)-treated and untreated cells.

To confirm that this band corresponded indeed to the sumoylated receptor the His-tagged proteins were purified by chromatography on nickel-charged agarose beads (Ni-NTA). The eluted proteins were analyzed by Western blotting with an anti-PR antibody. The same band of ~155 kDa was observed in

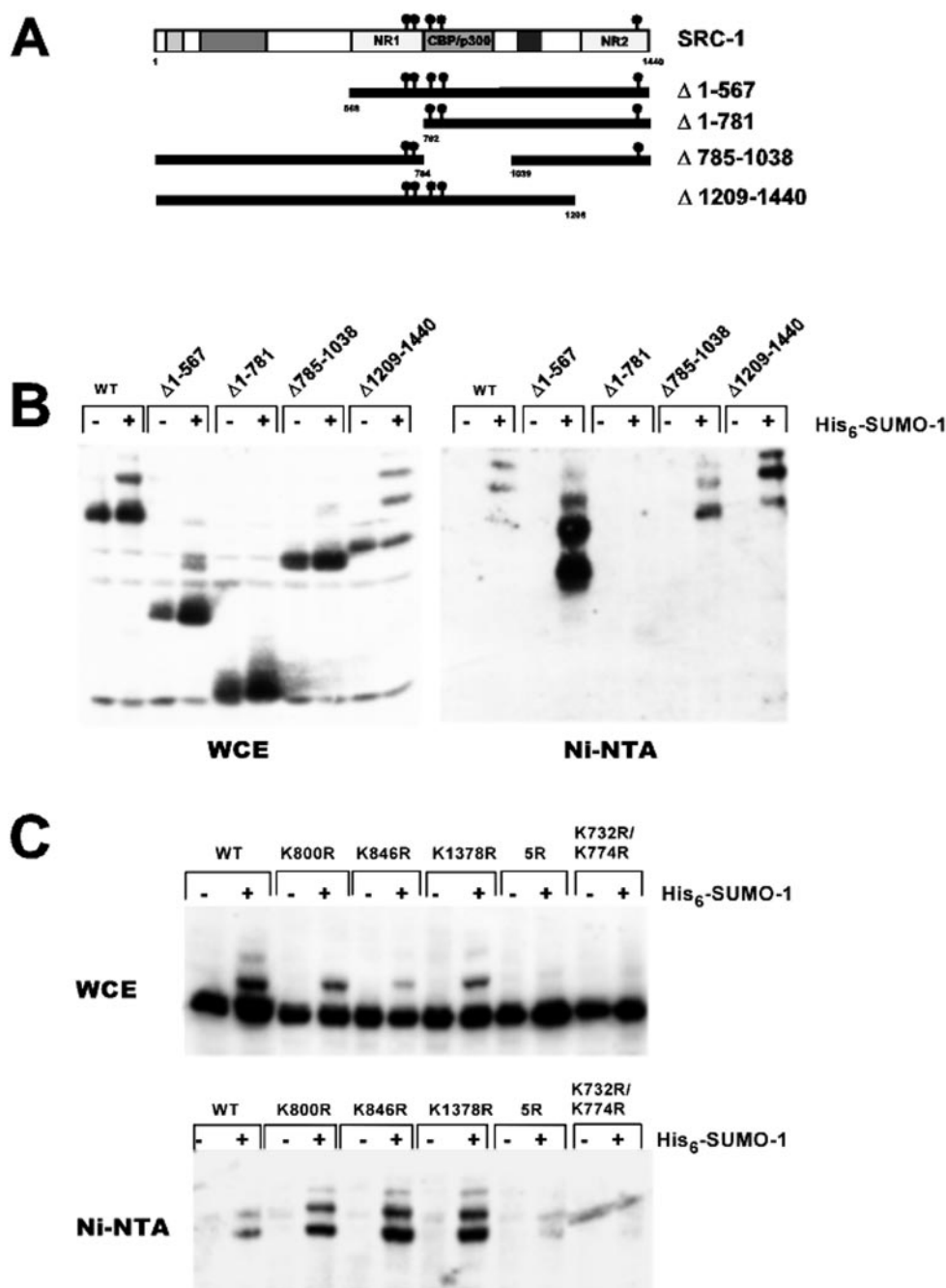


**FIG. 3. Covalent modification of SRC-1 by SUMO-1. Putative sumoylation sites.** A, CV-1 cells were transfected with the expression vector encoding the HA-tagged SRC-1 in the presence or absence the His<sub>6</sub>-tagged SUMO-1. Total cell extracts were separated by electrophoresis on a 6.4% SDS-PAGE and SRC-1 was immunoblotted with an anti-HA monoclonal antibody. The SUMO-1-modified forms of SRC-1 are indicated by an asterisk. B, a schematic representation shows the sites corresponding to consensus sequences for SUMO-1 conjugation (indicated by ●). The functional domains identified in SRC-1 are also represented: bHLH, helix-loop-helix motif; PAS, Per Arnt-Sim motif; CBP/p300, CBP/p300 interaction domain; Q, glutamine rich domain; NR, nuclear receptors interacting domains 1 and 2. The amino acids corresponding to the putative target lysines are indicated above. The localization of LXXLL motifs is indicated with an asterisk. C, potential sumoylation consensus motifs of the human SRC-1 based on the consensus ((I/L/V)KXE) (52–53) and conservation of this motif among the members of the p160 coactivator family. SRC-2 designates the GRIP1 and TIF2 coactivators and SRC-3 designates AIB1/RAC3/ACTR/TRAM-1 proteins. The amino acids matching the consensus are indicated in bold.

this experiment (Fig. 1A, Ni-NTA). Besides the sumoylated receptor minor bands corresponding to the non-modified receptor species were also observed. They were probably retained on the beads because of a low affinity of receptor zinc fingers toward Ni-NTA.

**SUMO-1 Enhances Progesterone Receptor-mediated Transcriptional Activation**—To examine the effect of SUMO-1 on receptor-driven transcription activation, CV-1 cells were co-transfected with an expression vector encoding PR, the reporter gene PRE<sub>2</sub>-TATA-CAT and increasing amounts of an expression vector for SUMO-1.

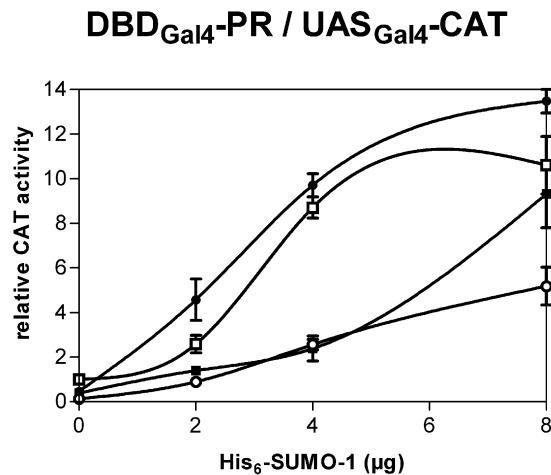
As shown in Fig 1B, SUMO-1 expression markedly increased hormone-induced transcription. There was no significant effect of SUMO-1 on transcription in the absence of hormone or in the presence of the antagonists RU486 and ZK98299. The effect of SUMO-1 was thus strictly hormone-dependent.



**FIG. 4. Identification of SUMO-1 conjugation sites in SRC-1.** A, schematic representation of the deletion mutants of SRC-1 used for the determination of the SUMO-1-conjugated sites. The residues conserved are indicated below the *bold lines*. The putative (consensus-derived) SUMO-1 conjugated sites are designated as in Fig. 3. B, CV-1 cells were transfected with expression vector encoding the HA-tagged SRC-1 deletion mutants respectively, in the presence or absence the His<sub>6</sub>-tagged SUMO-1. Total cell extracts (WCE) were analyzed by 6.4% SDS-PAGE and immunoblotted with anti-HA monoclonal antibody. Alternatively, the same co-transfected CV-1 cells were lysed in buffer containing guanidinium-HCl (Ni-NTA), and the SUMO-1-modified proteins were purified using Ni-NTA agarose beads as described under "Experimental Procedures." Eluted proteins were separated by electrophoresis and His<sub>6</sub>-SUMO-1 SRC-1 conjugates were detected by Western blotting using the anti-HA monoclonal antibody. WCE represent 15% of the input for Ni-NTA precipitation. C, CV-1 cells were transfected with the expression vector encoding the substitution Lys/Arg mutants of SRC-1, in the presence or absence the His<sub>6</sub>-tagged SUMO-1. The total cell extracts (WCE) or Ni-purified precipitates (Ni-NTA) were analyzed as in B. The mutant name indicated the position of the substituted lysine with arginine residue (5R corresponds to the substitution of the five lysines).

**Receptor Sumoylation and SUMO-1 Enhancement of Transcription**—We examined the possibility that receptor sumoylation was responsible for the enhancement by SUMO-1 of hormone-induced transactivation. Examination of the sequence of the progesterone receptor showed a single sumoylation consensus at lysine 388 in the N-terminal domain of the protein. Indeed, compared with the wild-type receptor, the mutant K388R was not sumoylated (Fig. 2A). (It should however be noted that a very faint sumoylated band was still present in

mutant K388R. It may correspond to a second site of very weak affinity, and which does not fit the target consensus). However the enhancement of transcription of the reporter gene in the presence of hormone and of SUMO-1 was also observed with the K388R mutant (Fig. 2B). Furthermore, the non-sumoylated mutant was more active than the wild-type receptor, in agreement with the recent data of Abdel-Hafiz *et al.* (48). These observations suggested that sumoylation of PR could not explain the overall effect of SUMO-1 on receptor-mediated gene



**FIG. 5. SUMO-1 expression enhances PR-SRC-1 interaction *in vivo*.** The effect of SUMO-1 on the formation of PR-SRC-1 complexes was analyzed by the two-hybrid system in CV-1 cells. The cells were co-transfected with the pG5-CAT reporter plasmid (5 μg/ml) (Clontech), the vector encoding AD<sub>VP16</sub>-SRC-1 (●) or AD<sub>VP16</sub>-SRC-1 2R (□) or AD<sub>VP16</sub>-SRC-1 5R (■) (2 μg/ml), the vector encoding the full-length PR fused to the DBD<sub>Gal4</sub> (PM-PR) (0.2 μg/ml), along with increasing amount of pSG5-His<sub>6</sub>-SUMO-1 expression vector. The cells were treated with 10 nM R5020. In control experiments, AD<sub>VP16</sub>-SRC-1 construct was replaced by AD<sub>VP16</sub> (○). The results are means ± S.D. of three independent determinations.

transcription. It should also be noted that antiprogesterin-complexed receptor, which does not provoke enhanced transcription of target genes, is sumoylated in a manner similar to that of hormone-complexed PR.

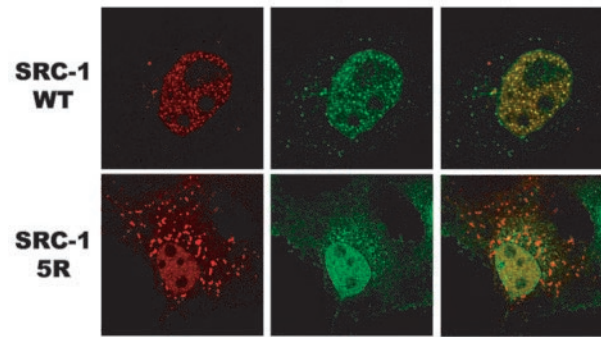
To further investigate the role of receptor sumoylation, we analyzed SUMO-1 effect on ERα-stimulated gene transcription. Although ERα has previously been shown to be devoid of SUMO-1 conjugation consensus site and to not undergo sumoylation *in vitro* (49), cotransfection with SUMO-1 did markedly enhance estradiol-driven reporter gene transcription (Fig. 2C). Enhancement of transcription by SUMO-1 thus involves modification of proteins other than the receptor.

**Sumoylation of SRC-1 *in Vivo***—The hormone-induced transcriptional activation mediated by nuclear receptors involves a multistep recruitment of several coactivators leading to the final chromatin remodeling necessary for gene transcription. SRC-1 was the first coactivator identified by its ligand-induced binding to the AF-2 region of the progesterone receptor (50). It has also been shown to be a closely related partner of PR *in vivo* (51). We thus examined the possibility that SRC-1 could be a target for SUMO-1 conjugation.

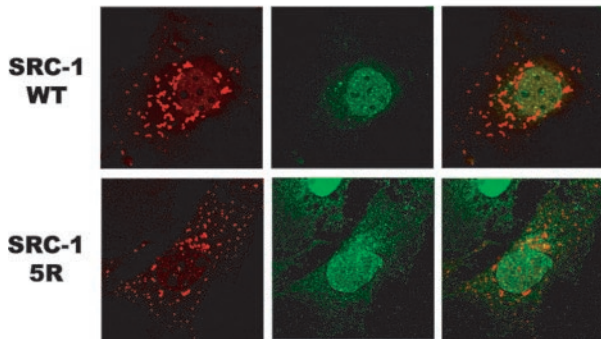
CV-1 cells were transfected with expression vectors encoding the full-length HA-tagged SRC-1 in the presence or absence of an expression vector encoding His<sub>6</sub>-SUMO-1. Total cell extracts were prepared and analyzed by Western blotting (Fig. 3A). In the absence of SUMO-1, the anti-HA antibody detected a major band of ~175 kDa. In the cells cotransfected with SUMO-1 expression vector three other bands were observed (apparent molecular sizes of ~220, 245, and 260 kDa). These bands probably corresponded to conjugation of SUMO-1 on one, two, or three lysines of SRC-1, because SUMO-1 unlike ubiquitin is not able to self-conjugate. The intensity of the bands decreased from the mono- to the tri-conjugated forms. The latter was barely visible in several experiments.

**Mapping of SUMO-1 Modification Sites in SRC-1**—Examination of the sequence of SRC-1 showed the presence of five consensus motifs ((IL/V)KXE) for SUMO-1 conjugation (52–53). Interestingly, all the potential target lysines were localized

## A 24h expression



## B 48h expression



**FIG. 6. Substitution of SUMO-1 target lysines modifies the kinetics of the trafficking of SRC-1 between the nucleus and the cytoplasm.** Subcellular colocalization of wild-type (WT) or mutated non-sumoylated (5R) SRC-1 with SUMO-1. Wild-type or mutated HA-tagged SRC-1 expression vector was transfected into COS-7 cells. The cells were fixed 24 h (A) or 48 h after transfection (B), and SRC-1 proteins were detected using the monoclonal anti-HA antibody (red channel). The endogenous SUMO-1 was detected with the anti-SUMO-1 FL-101 antibody (green channel). A merge of both signals is shown in the right panels with overlapping staining appearing in yellow.

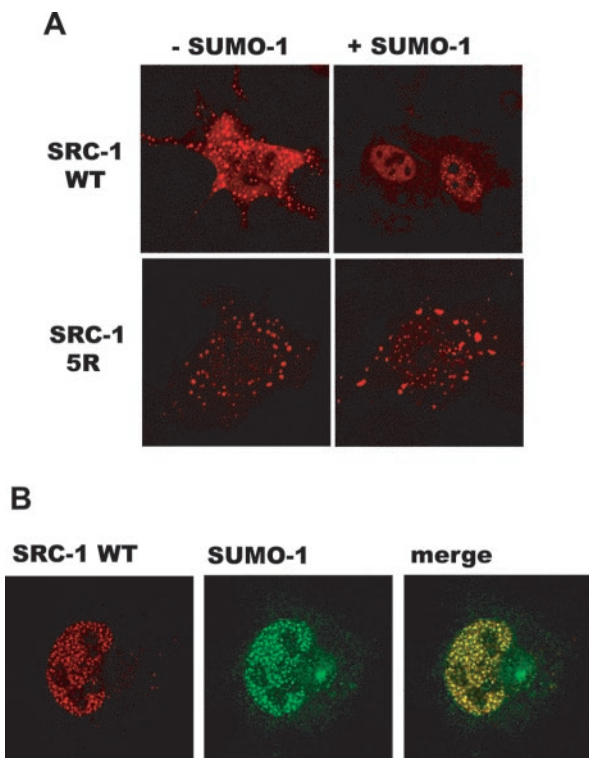
in protein-protein interaction domains close to LXXLL motifs (Fig. 3B).

The lysines 732 and 774 were localized in the first nuclear receptor interaction domain (NR1). The lysines 800 and 846 resided in the CBP/p300 interaction domain. Lysine 1378 was located in the C-terminal extremity of the protein corresponding to the second receptor-interacting domain (NR2). Interestingly only one of the consensus sequences (Lys-732) is conserved in all the three members of the p160 family including SRC2/TIF2/GRIPI and SRC-3/TRAM1/ACTR/AIB1/RAC3. Two other motifs (Lys-800 and Lys-1378) are conserved in two of these proteins; the remaining two motifs are present only in SRC-1 (Fig. 3C).

Mutagenesis studies were undertaken to verify these predictions. CV-1 cells were cotransfected with expression vectors encoding various deletion mutants of SRC-1 and His<sub>6</sub>-SUMO-1 (Fig. 4A). Total cell extracts and Ni-NTA precipitates were analyzed by Western blotting using the anti-HA antibody.

Removal of the nuclear interaction domain 1 (NR1) in mutant Δ1–781 completely abolished SUMO-1 conjugation (Fig. 4B). The mutants Δ785–1038 and Δ1209–1440, which retain this domain, were sumoylated. This result suggested that lysines 732 and 774 could be the major sumoylation sites. Point mutations were used to verify this hypothesis. When all five putative target lysines (Lys-732, Lys-774, Lys-800, Lys-846, and Lys-1378) were substituted by arginines (5R SRC-1), the sumoylation reaction was suppressed. Individual substitutions of Lys-800, Lys-846, and Lys-1378 did not affect SRC-1 overall





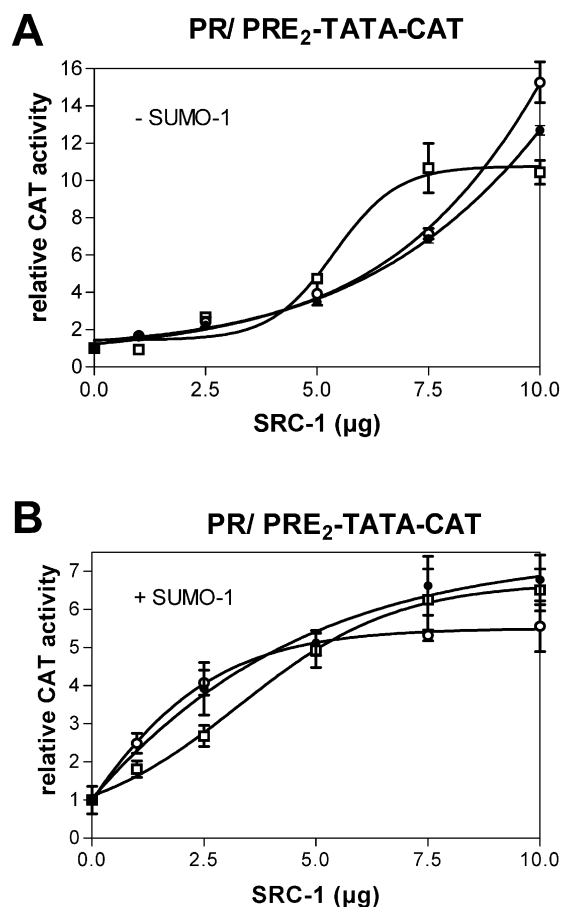
**FIG. 7. Overexpression of SUMO-1 retains SRC-1 in the nucleus.** A, subcellular localization of p160 SRC-1 in the presence of overexpressed SUMO-1. COS-7 cells were either transfected with the HA-tagged SRC-1 expression vector alone (SRC-1 wt) or co-transfected with a 4-fold excess of the His<sub>6</sub>-SUMO-1 expression vector (SRC-1 wt + SUMO-1). After 48 h, SRC-1 was detected with the rat monoclonal anti-HA 3F10 antibody using confocal fluorescence. The same experiment was repeated with non-sumoylated 5R-SRC-1 mutant (lower part of A). B, subcellular colocalization of ectopically expressed HA-tagged SRC-1 wt with endogenous SUMO-1 in the presence of the proteasome inhibitor clasto-lactacystin. COS-7 cells were transfected with the wild-type SRC-1 and treated with 50  $\mu$ M clasto-lactacystin for the last 24 h. Forty-eight hours after transfection, SRC-1 and SUMO-1 proteins were revealed as in Fig. 6. Bar, 5  $\mu$ m. A merge of both signals is shown in the right panels with overlapping staining appearing in yellow.

modification by SUMO-1. In contrast, the double substitution on Lys-732R and Lys-774R abolished sumoylation (Fig. 4C).

We thus concluded that lysines 732 and 774 were the main sites of SUMO-1 conjugation in SRC-1 whereas the other lysine(s) may serve as weak affinity target sites.

**SUMO-1 Modification of SRC-1 Enhances PR/SRC-1 Interaction *in Vivo***—The two major sites of SUMO-1 conjugation (Lys-732 and Lys-774) are located in the first nuclear receptor interaction domain of SRC-1. This domain contains three LXXLL motifs (named NR boxes), which are involved in receptor-SRC-1 interaction (54–57). Residues flanking NR boxes on both sides make significant contacts with the ligand binding domain of receptors (58). These residues have also been shown to be important for the preferential binding of various NR boxes to different nuclear receptors and thus to determine a preference of the various receptors for specific p160 coactivators (58). The sumoylation sites perfectly flank NR box 3. This suggested that covalent binding of SUMO-1 molecule(s) could alter SRC-1-receptor interaction.

Few methods are available to study interactions between sumoylated proteins since cell extracts must contain protein denaturing and SH-blocking agents to prevent desumoylation (34). We thus used a mammalian two-hybrid system to analyze the effect of sumoylation *in vivo*. CV-1 cells were cotransfected with a Gal<sub>4</sub> UAS-driven reporter gene, a vector encoding the full-length PR fused to the Gal<sub>4</sub> DBD (DBD<sub>Gal4</sub>-PR) and a



**FIG. 8. Non-sumoylated mutants of SRC-1 enhance PR-mediated transcription.** CV-1 cells were transfected with the PRE<sub>2</sub>-TATA-CAT reporter plasmid (2.5  $\mu$ g/ml) along with pSG5-rPR (0.2  $\mu$ g/ml) and increasing amount of pSG5-HA vector encoding respectively wild type (●), K732R/K774R substitution mutant (○), or 5R mutant version (□) of SRC-1 (A) in the absence or in the presence (B) of co-expressed pSG5-His<sub>6</sub>-SUMO-1 vector (2.5  $\mu$ g/ml). The cells were treated with 10 nM R5020 24 h before harvesting. The results are means  $\pm$  S.D. of three independent determinations.

vector encoding the SRC-1 sequence fused to the VP16 activation domain (AD<sub>VP16</sub>-SRC-1). The transfected cells were incubated with hormone (Fig. 5). The hormone-driven transcription observed in the presence of the DBD<sub>Gal4</sub>-PR construct was increased by SUMO-1 coexpression. However when AD<sub>VP16</sub>-SRC-1 was added, SUMO-1 determined a markedly stronger enhancement of transcription, showing that it increased DBD<sub>Gal4</sub>-PR interaction with AD<sub>VP16</sub>-SRC-1. Mutation of lysines 732 and 734 (AD<sub>VP16</sub>-SRC-1 2R) hardly modified the interaction between PR and SRC-1 but this interaction was markedly affected in the non-sumoylated SRC-1 (AD<sub>VP16</sub>-SRC-1 5R), suggesting that the extent of sumoylation of SRC-1 plays a role in receptor-coactivator interaction.

**Effect of SUMO-1 on the Nucleocytoplasmic Trafficking of SRC-1**—In previous studies, we have observed that the intracellular localization of SRC-1 corresponds to a dynamic process, the protein being initially addressed to the nucleus and thereafter exported into the cytoplasm.<sup>2</sup> In both nuclear and cytoplasmic compartments, SRC-1 is present in dot-like structures. To analyze the effect of sumoylation, we transfected cells with expression vectors encoding the wild-type SRC-1 or the mutant

<sup>2</sup> L. Amazit, Y. Alj, R. K. Tyagi, A. Chauchereau, H. Loosfelt, C. Pichon, J. Pantel, E. Foulon-Guinard, P. Leclerc, E. Milgrom, and A. Guiochon-Mantel, manuscript in preparation.

with five substituted arginines (5R-SRC-1), which cannot be sumoylated. 24 h after transfection the wild-type SRC-1 was predominantly present in the nucleus (Fig. 6A) where it colocalized with endogenous SUMO-1. Forty-eight hours after transfection, SRC-1 was mainly present in the cytoplasm, and only the few remaining nuclear dots colocalized with endogenous SUMO-1 (Fig. 6B). In contrast, the non-sumoylated 5R-SRC-1 mutant was mainly localized in the cytoplasm already 24 h after transfection (only the few remaining intranuclear SRC-1 stained dots colocalized with endogenous SUMO-1) (Fig. 6A). The pattern observed with this mutant 24 h after transfection was very similar to that observed 48 h after transfection with wild-type receptor (Fig. 6B).

If sumoylation retards SRC-1 export from the nucleus, overexpression of SUMO-1 should increase the nuclear residency time of SRC-1. This was indeed observed in cells cotransfected with SUMO-1 where SRC-1 remained in the nucleus even 48 h after transfection (Fig. 7A). No such nuclear retention could be observed with the non-sumoylated 5R-SRC-1.

Finally, as previously observed by us in the case of SRC-1<sup>2</sup> and others, proteasome inhibitors have been reported to provoke the nuclear sequestration of various transcription factors (59–62). Indeed in the presence of proteasome inhibitor clasto-lactacystin, and 48 h after transfection, SRC-1 was still present mainly in the nucleus presenting a hyperspeckled pattern. In this case, there was a perfect colocalization between these speckles and endogenous SUMO-1 (Fig. 7B).

**Mutation of SUMO-1 Acceptor Sites in SRC-1 Does Not Alter Its Effect on PR-mediated Transcription**—To obtain further insight into the transcriptional role of SUMO-1 conjugation to SRC-1, we studied the impact of mutations of SUMO-1 acceptor sites.

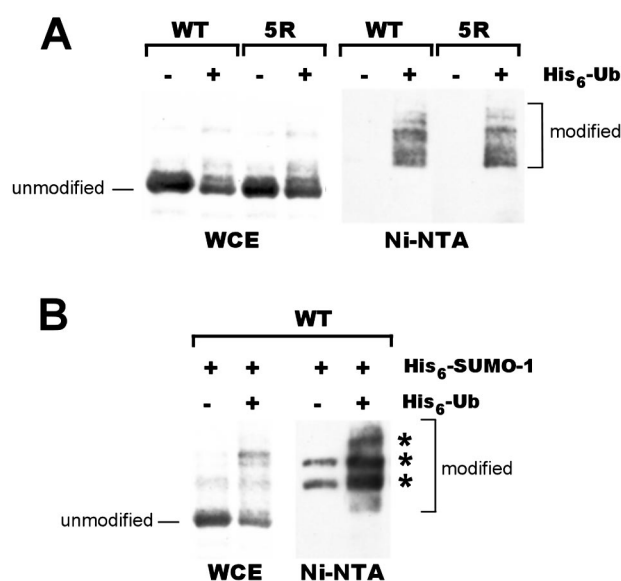
Expression vectors encoding PR and a reporter gene (PRE<sub>2</sub>-TATA-CAT) were transfected into cells. Various amounts of an expression vector encoding wild-type SRC-1 were co-transfected and the cells treated by hormone. As previously described (63–65), cotransfection of increasing amounts of SRC-1 enhanced the hormone-induced transcription.

If wild-type SRC-1 was replaced by non-sumoylated mutants (double substituted K732R/K774R SRC-1 or 5×-substituted 5R-SRC-1), the effect on the reporter gene transcription was very similar (Fig. 8A).

Since endogenous SUMO-1 protein may be limited in the cells, we repeated the experiment in the presence of overexpressed SUMO-1 (Fig. 8B). Again, disruption of SUMO-1 target lysines in SRC-1 did not modify its co-transcriptional activity.

**SRC-1 Sumoylation, Ubiquitinylation, and Stability**—SUMO-1 conjugation has been involved in the regulation of the stability of several proteins (25, 66). Competition between ubiquitinylation and sumoylation for the same lysines has been shown to prevent proteasome targeting and degradation. We have previously shown that SRC-1 is subjected to a proteasome-mediated proteolysis.<sup>3</sup> We thus examined the effect of sumoylation on ubiquitinylation. Ubiquitinylation and sumoylated sites were clearly different since the non-sumoylated 5R-SRC-1 mutant underwent normal ubiquitinylation (Fig. 9A).

When both ubiquitin and SUMO-1 were co-expressed with SRC-1, the two modified forms of SRC-1 were purified on nickel-charged agarose beads (Fig. 9B) showing that SRC-1 can simultaneously be the target of both modifications. Furthermore, SRC-1 sumoylation did not constitute a mechanism for ubiquitin antagonism because even in the presence of



**FIG. 9. SRC-1 can be both sumoylated and ubiquitinated.** A, CV-1 cells were transfected with the expression vector encoding the HA-tagged wild-type SRC-1 or the 5R-SRC-1 mutant in the presence of the His<sub>6</sub>-tagged ubiquitin expression vector (His<sub>6</sub>-Ub) (10  $\mu$ g/ml). Total cell extracts (WCE) were analyzed by electrophoresis on 6.4% SDS-PAGE and immunoblotted with anti-HA monoclonal antibody. Alternatively, the same co-transfected CV-1 cells were lysed in buffer containing guanidium-HCl (Ni-NTA). The ubiquitin-modified proteins were purified using Ni-NTA agarose beads as described under "Experimental Procedures." Eluted proteins were separated by electrophoresis, and His<sub>6</sub>-SRC-1 conjugates were detected by Western blotting using the anti-HA monoclonal antibody. WCE represent 15% of the input for Ni-NTA precipitation. The ubiquitin conjugates of SRC-1 are indicated with brackets. B, CV-1 cells were transfected with the HA-tagged SRC-1, and the His<sub>6</sub>-tagged SUMO-1 (10  $\mu$ g/ml) expression vectors in the presence or absence of the His<sub>6</sub>-tagged ubiquitin expression vector (5  $\mu$ g/ml). Molecular species of SRC-1 were detected in WCE or in Ni-NTA as described in A. The SUMO-1-modified forms of SRC-1 are indicated by an asterisk, the ubiquitin-conjugates are indicated with brackets.

SUMO-1, the overall SRC-1 form was decreased in the presence of ubiquitin.

## DISCUSSION

Steroid hormone receptors undergo a variety of covalent modifications which can modify their functional properties (phosphorylation, ubiquitinylation, acetylation). In this manuscript, we describe SUMO-1 conjugation to the progesterone receptor at lysine 388. The non-sumoylated PR mutant was still transcriptionally active and the gain of activity observed in this mutant suggested that the sumoylation of the receptor may have a repression effect. Indeed, it has recently been shown that the sumoylation of the PR is implicated in the regulation of its autoinhibition and transrepression activities (48). Sumoylation has different effects on different steroid receptors: the estrogen receptor  $\alpha$  is not sumoylated (49) whereas the androgen receptor (AR) is sumoylated in its N-terminal domain in an androgen-enhanced fashion (49). SUMO-1 conjugation of AR apparently decreases its transcriptional activity as observed after substitution of the sumoylated lysines. On the contrary this substitution has no effect on AR-mediated transrepression (49). Ubc9, the conjugating enzyme for SUMO-1, interacts with both AR and GR (49, 67–68). In transiently transfected cells, coexpression of Ubc9 enhances AR-dependent transcription. However, mutated Ubc9 having lost its SUMO-1-ligating activity retains its effect on AR-mediated transactivation (68). Thus, the role of Ubc9 in sumoylation reactions does not explain its binding to AR and its effect on transactivation.

<sup>3</sup> L. Amazit, A. Chauchereau, Y. Alj, R. K. Tyagi, P. Leclerc, E. Milgrom, and A. Guiochon-Mantel, unpublished data.



We have observed that overexpression of SUMO-1 very markedly enhanced PR- and ER $\alpha$ -mediated gene transcription. This effect was not related to receptor sumoylation. We thus analyzed SUMO-1 modification of the coactivator SRC-1. Two major sites of conjugation were localized. They flanked at a distance of 20 amino acids a NR box situated in the nuclear receptor-interacting region 1 (NR1). Since the function of the nuclear receptor binding LXXLL motifs is known to be modulated by adjacent amino acids (58), we examined the effect of sumoylation on PR-SRC-1 interaction. Using a mammalian two-hybrid system, we observed an increased interaction of PR with sumoylated SRC-1. Substitution of the two lysines 732 and 734 showed little effect on PR-SRC-1 interaction. In contrast, we observed a markedly decreased interaction of PR with the non-sumoylated SRC-1 (5R mutant). The significant difference between the two mutants underlines the functional relevance of the NR box located at the C terminus of SRC-1, close to the sumoylation target lysine 1378. These results suggest that sumoylation may be implicated in the formation or stability of receptor-coactivator complexes.

SUMO-1 modification of various proteins seems to play a major role in their subcellular and especially intranuclear targeting. The sumoylation of PML directs this protein into nuclear bodies (27–28, 69). Actually it has been shown that sumoylated PML is necessary for the assembly and/or the stability of these intranuclear structures (69). SUMO-1 conjugation to HIPK2 directs it into nuclear speckles (30). The non-conjugated form of RanGAP1 is found in the cytoplasm. Once modified by SUMO-1, it becomes localized in the nuclear pore complex (22–23). Sumoylation of RanGAP1 is necessary for its interaction with Ran GTP-binding protein RanBP2 (70). It must be emphasized that the SAE (SUMO-1 activating) and Ubc9 (SUMO-1 conjugating) enzymes are mainly localized in the nucleus (52). We have shown SRC-1 to localize initially in the nucleus and to be thereafter exported into the cytoplasm.<sup>2</sup> In both cellular compartments, it is associated with dot-like structures. In the non-sumoylated SRC-1 mutant, the time of residency in the nucleus is markedly decreased. SUMO-1 conjugation thus seems to play a role in the nuclear retention of SRC-1. These results suggest that sumoylation may regulate the kinetics of SRC-1 subcellular localization and/or of the assembly of transcriptional complexes. This may explain why we did not observe any differences between the transcriptional activity of wild-type and of non-sumoylated SRC-1. Indeed, at the time necessary for the transcriptional assay, the different SRC-1 constructs have recovered the same subcellular localization.

Several proteins have been shown to be stabilized by sumoylation. In I $\kappa$ B $\alpha$ , SUMO-1 and ubiquitin compete for conjugation to the same lysines. Thus I $\kappa$ B $\alpha$  sumoylation renders it resistant to signal-induced degradation. NF $\kappa$ B transcriptional activation is then blocked (25). A similar mechanism has been observed for Mdm2, the E3 ubiquitin ligase for p53. Sumoylation stabilizes this protein (66). On the contrary, sumoylation of SRC-1 does not compete with its ubiquitinylation and has no clear effect on its stability.

A variety of transcriptional regulators undergo SUMO modification and in several cases, their activity is modulated by this reaction. This is the case of the tumor suppressor p53, which regulates the transcription of a number of genes involved in cell cycle arrest and apoptosis (33–35, 71–72). The transcriptional activator c-Jun (35), as well as the transcriptional repressors or corepressors TEL (73), HIPK<sub>2</sub> (30), and *Drosophila* Tramtrack-69 (74) also undergo sumoylation.

We have shown here that SUMO-1 overexpression very strongly enhances PR-mediated gene transcription. However a

similar effect was observed when using non-sumoylated mutants of PR and SRC-1. This suggests that besides its action on SRC-1 and PR, SUMO-1 exerts effects on other proteins involved in gene transcription regulation by nuclear receptors. Further studies will be necessary to define these unknown and functionally important SUMO-1 targets.

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**Note Added in Proof**—While preparing this manuscript, SUMO-1 modification of the coactivator GRIP-1 has been described by Kotaja *et al.* (75). They have shown that the non-sumoylated mutant of the coactivator GRIP1 displayed half of the transcription enhancing activity of the wild-type protein. However, these authors have studied androgen receptor/GRIP1 interaction by using the isolated LBD domain of the receptor. As seen in the case of PR, N-terminal sumoylation also plays a role in these interactions.

## REFERENCES

- Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fletterick, R. J. (1995) *Nature* **378**, 690–697
- Renaud, J. P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **378**, 681–689
- Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **375**, 377–382
- Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389**, 753–758
- Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998) *Nature* **395**, 137–143
- Williams, S. P., and Sigler, P. B. (1998) *Nature* **393**, 392–396
- Tanenbaum, D. M., Wang, Y., Williams, S. P., and Sigler, P. B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5998–6003
- Rochel, N., Wurtz, J. M., Mitschler, A., Klaholz, B., and Moras, D. (2000) *Mol. Cell* **5**, 173–179
- Matias, P. M., Donner, P., Coelho, R., Thomaz, M., Peixoto, C., Macedo, S., Otto, N., Joschko, S., Scholz, P., Wegg, A., Basler, S., Schafer, M., Egner, U., and Carrondo, M. A. (2000) *J. Biol. Chem.* **275**, 26164–26171
- McKenna, N. J., Xu, J., Nawaz, Z., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1999) *J. Steroid Biochem. Mol. Biol.* **69**, 3–12
- Leo, C., and Chen, J. D. (2000) *Gene (Amst.)* **245**, 1–11
- Jenster, G. (1998) *Mol. Cell. Biol.* **18**, 1–7
- Glass, C. K., and Rosenfeld, M. G. (2000) *Genes Dev.* **14**, 121–141
- Lee, K. C., and Kraus, W. L. (2001) *Trends Endocrinol. Metab.* **12**, 191–197
- Weigel, N. L. (1996) *Biochem. J.* **319**, 657–667
- Nirmala, P. B., and Thampan, R. V. (1995) *Biochem. Biophys. Res. Commun.* **213**, 24–31
- Syvala, H., Vienonen, A., Zhuang, Y. H., Kivineva, M., Ylikomi, T., and Tuohimaa, P. (1998) *Life Sci.* **63**, 1505–1512
- Lange, C. A., Shen, T., and Horwitz, K. B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1032–1037
- Li, X. Y., Boudjelal, M., Xiao, J. H., Peng, Z. H., Asuru, A., Kang, S., Fisher, G. J., and Voorhees, J. J. (1999) *Mol. Endocrinol.* **13**, 1686–1694
- Boudjelal, M., Wang, Z., Voorhees, J. J., and Fisher, G. J. (2000) *Cancer Res.* **60**, 2247–2252
- Matunis, M. J., Coutavas, E., and Blobel, G. (1996) *J. Cell Biol.* **135**, 1457–1470
- Matunis, M. J., Wu, J., and Blobel, G. (1998) *J. Cell Biol.* **140**, 499–509
- Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997) *Cell* **88**, 97–107
- Mahajan, R., Gerace, L., and Melchior, F. (1998) *J. Cell Biol.* **140**, 259–270
- Desterro, J. M., Rodriguez, M. S., and Hay, R. T. (1998) *Mol. Cell* **2**, 233–239
- Boddy, M. N., Howe, K., Etkin, L. D., Solomon, E., and Freemont, P. S. (1996) *Oncogene* **13**, 971–982
- Sternsdorf, T., Jensen, K., and Will, H. (1997) *J. Cell Biol.* **139**, 1621–1634
- Müller, S., Matunis, M. J., and Dejean, A. (1998) *EMBO J.* **17**, 61–70
- Kamitani, T., Nguyen, H. P., Kito, K., Fukuda-Kamitani, T., and Yeh, E. T. (1998) *J. Biol. Chem.* **273**, 3117–3120
- Kim, Y. H., Choi, C. Y., and Kim, Y. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12350–12355
- Mao, Y., Sun, M., Desai, S. D., and Liu, L. F. (2000a) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4046–4051
- Mao, Y., Desai, S. D., and Liu, L. F. (2000b) *J. Biol. Chem.* **275**, 26066–26073
- Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. (1999) *EMBO J.* **18**, 6462–6471
- Rodriguez, M. S., Desterro, J. M. P., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999) *EMBO J.* **18**, 6455–6461
- Müller, S., Berger, M., Lehenbre, F., Seeler, J.-S., Haupt, Y., and Dejean, A. (2000) *J. Biol. Chem.* **275**, 13321–13329
- Melchior, F. (2000) *Annu. Rev. Cell Dev. Biol.* **16**, 591–626
- Schwiehorst, I., Johnson, E. S., and Dohmen, R. J. (2000) *Mol. Gen. Genet.* **263**, 771–786
- Johnson, E. S., and Gupta, A. A. (2001) *Cell* **106**, 735–744
- Takahashi, Y., Toh-e, A., and Kikuchi, Y. (2001) *Gene (Amst.)* **275**, 223–231

40. Kahyo, T., Nishida, T., and Yasuda, H. (2001) *Mol. Cell* **8**, 713–718
41. Pichler, A., Gast, A., Seeler, J.S., Dejean, A., and Melchior, F. (2002) *Cell* **108**, 109–120
42. Hicke, L. (1999) *Trends Cell Biol.* **9**, 107–112
43. Chauchereau, A., Georgiakaki, M., Perrin-Wolff, M., Milgrom, E., and Loosfelt, H. (2000) *J. Biol. Chem.* **275**, 8540–8548
44. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) *Cell* **78**, 787–798
45. Lorenzo, F., Jolivet, A., Loosfelt, H., Vu Hai, M. T., Brailly, S., Perrot-Applanat, M., and Milgrom, E. (1988) *Eur. J. Biochem.* **176**, 53–60
46. Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., and Milgrom, E. (1989) *Cell* **57**, 1147–1154
47. Chauchereau, A., Loosfelt, H., and Milgrom, E. (1991) *J. Biol. Chem.* **266**, 18280–18286
48. Abdel-Hafiz, H., Takimoto, G. S., Tung, L., and Horwitz, K. B. (2002) *J. Biol. Chem.* **277**, 33950–33956
49. Poukka, H., Karvonen, O. A., and Palvimo, J. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 14145–14150
50. Onate, S. A., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1995) *Science* **270**, 1354–1357
51. McKenna, N. J., Nawaz, Z., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11697–11702
52. Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001) *J. Biol. Chem.* **276**, 12654–12659
53. Sampson, D. A., Wang, M., and Matunis, M. J. (2001) *J. Biol. Chem.* **276**, 21664–21669
54. McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T.-M., Krones, A., Inostroza, J., Torchia, J., Nolte, R. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998) *Genes Dev.* **12**, 3357–3368
55. Bramlett, K. S., Wu, Y., and Burris, T. P. (2001) *Mol. Endocrinol.* **15**, 909–922
56. Heery, D. M., Hoares, S., Hussain, S., Parker, M. G., and Sheppard, H. (2001) *J. Biol. Chem.* **276**, 6695–6702
57. Sheppard, H. M., Harries, J. C., Hussain, S., Bevan, C., and Heery, D. M. (2001) *Mol. Cell. Biol.* **21**, 39–50
58. Needham, M., Raines, S., McPheat, J., Stacey, C., Ellston, J., Hoare, S., and Parker, M. (2000) *J. Steroid Biochem. Mol. Biol.* **72**, 35–46
59. Chilov, D., Camenisch, G., Kvietikova, I., Ziegler, U., Gassmann, M., and Wenger, R. H. (1999) *J. Cell Sci.* **112**, 1203–1212
60. Chen, F., Chang, D., Goh, M., Klibanov, S. A., and Ljungman, M. (2000) *Cell Growth Diff.* **11**, 239–246
61. Santiago-Josefat, B., Pozo-Guizado, E., Mulero-Nvarro, S., and Fernandez-Salguero, P. M. (2001) *Mol. Cell. Biol.* **21**, 1700–1709
62. Xirodimas, D. P., Stephen, C. W., and Lane, P. (2001) *Exp. Cell Res.* **270**, 66–77
63. Smith, C. L., Onate, S. A., Tsai, M.-J., and O'Malley, B. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8884–8888
64. Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7879–7884
65. Onate, S. A., Boonyaratankornkit, V., Spencer, T. E., Tsai, S. Y., Tsai, M.-J., Edwards, D. P., and O'Malley, B. W. (1998) *J. Biol. Chem.* **273**, 12101–12108
66. Buschmann, T., Fuchs, S. Y., Lee, C.-G., Pan, Z.-Q., and Ronai, Z. (2000) *Cell* **101**, 753–762
67. Göttlicher, M., Heck, S., Doucas, V., Wade, E., Kullmann, M., Cato, A. C. B., Evans, R. M., and Herrlich, P. (1996) *Steroids* **61**, 257–262
68. Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J. J., and Jänne, O. A. (1999) *J. Biol. Chem.* **274**, 19441–19446
69. Zhong, S., Müller, S., Ronchetti, S., Freemont, P. S., Dejean, A., and Pandolfi, P. P. (2000) *Blood* **95**, 2748–2753
70. Saitoh, H., Sparrow, D. B., Shiomi, T., Pu, R. T., Nishimoto, T., Mohun, T. J., and Dasso, M. (1998) *Curr. Biol.* **8**, 121–124
71. Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Sternsdorf, T., Jensen, K., Pandolfi, P. P., Will, H., Schneider, C., and Del Sal, G. (2000) *EMBO J.* **19**, 6185–6195
72. Kwek, S. S. S., Derry, J., Tyner, A. L., Shen, Z., and Gudkov, A. V. (2001) *Oncogene* **20**, 2587–2599
73. Chakrabarti, S. R., Sood, R., Nandi, S., and Nucifora, G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 13281–13285
74. Lehenbre, F., Badenhorst, P., Müller, S., Travers, A., Schweisguth, F., and Dejean, A. (2000) *Mol. Cell. Biol.* **20**, 1072–1082
75. Kotaja, N., Karvonen, U., Jänne, O. A., and Palvimo, J. J. (2002) *J. Biol. Chem.* **277**, 30283–30288