SUMO Modification of Repression Domains Modulates Function of Nuclear Receptor 5A1 (Steroidogenic Factor-1)*

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Steroidogenic factor 1 (SF-1 or NR5A1), is a Ftz-F1 member of the nuclear receptor superfamily that plays essential roles in endocrine development, steroidogenesis, and gonad differentiation. We investigated modifications that control SF-1 function and found that SF-1 could be conjugated by SUMO-1 both in vitro and in vivo. SF-1 was modified predominantly at Lys194 and much less at Lys119 when free SUMO-1 was supplied. Mutations of Lys194 and Lys119 enhanced transcriptional activity of SF-1, although the DNA binding activity of SF-1 was not affected. Sequences around Lys194 and Lys119 both repressed transcription intrinsically. The Lys194 motif repressed transcription more efficiently than the Lys119 domain, consistent with its ability to be a better substrate for SUMO conjugation. Thus, SUMO modification of SF-1 correlates with transcriptional repression. Wild-type but not conjugation-deficient SF-1 was localized at the nuclear speckles together with SUMO-1. Thus, SUMO-1 conjugation could also target SF-1 into nuclear speckles. Collectively, these results suggest that SUMO modification at the repression domains targets SF-1 to nuclear speckles; this could be an important mechanism by which SF-1 is regulated.

SF-1 contains an N-terminal DNA-binding domain, a proline-rich domain, and a C-terminal ligand-binding domain. The C-terminal region of its DNA-binding domain is a conserved 30-amino-acid basic Ftz-F1 (Fushi-tarazu factor 1) box shared by all members of the NR5 (FTZ-F1) subfamily (6, 7). An FP domain (aa 78–172), including the Ftz-F1 box and a proline-rich sequence, contains a nuclear localization signal and interacting regions for TFIIB and c-Jun and is important for the transactivation function of SF-1 (8). Unlike other nuclear receptors, SF-1 lacks the N-terminal A/B region, which usually contains the ligand-independent transactivation AF-1 domain. Amino acids 180–265 in the hinge region of SF-1 may function as AF-1. In this domain, the single serine residue (Ser203) is phosphorylated and required for activation by mitogen-activated protein kinase (9). However, phosphorylation of SF-1 at Ser203 does not respond to adrenocorticotropic and gonadotropin signals. The functional significance of this putative AF-1 domain remains unclear. Another activation domain, AF-2, is located at the C terminus of SF-1; this domain interacts with transcription co-activators such as steroid receptor co-activator 1 (10), glucocorticoid receptor-interacting protein 1 (9), and p300/CBP/co-integrator-associated protein (11).

Recently, SUMO-1 (also known as Pict1, Ubl1, hsSmt3, and sentrin) conjugation (sumoylation) has been reported to play an important role in many cellular processes (12). Sumoylation resembles ubiquitination, but the enzymes involved in these two processes are distinct. During sumoylation, four C-terminal amino acids of the precursor of SUMO-1 are removed to expose the conjugation site to the E1 activating enzyme. Subsequently, the activated SUMO-1 is transferred to the E2-conjugating enzyme UBC9. Although E1 and E2 enzymes are sufficient to trigger sumoylation in vitro, SUMO-1 is conjugated to target proteins by E3 ligase in vivo. Analysis of sumoylated proteins has revealed that SUMO-1 is conjugated to substrate at the consensus sequence KXE (ϕ is any hydrophobic amino acid, and X is any amino acid).

Protein sumoylation has been reported to participate in protein targeting (13, 14), protein stabilization (15), and protein-protein interaction (16, 17). A growing number of transcription factors have been found to be sumoylated, such as androgen receptor (18), ELK-1 (19), and p53 (20), but the outcome of transcription factor sumoylation is still not fully understood.

In the present report, we show that SF-1 is sumoylated at Lys119 and Lys194. Mutations of sumoylation sites enhance the transcriptional activity of SF-1 but do not affect the DNA binding affinity of SF-1. The sumoylation sites of SF-1 function as intrinsic repression domains. Moreover, overexpression of SUMO-1 directs wild-type (WT) SF-1, but not sumoylation-deficient SF-1 mutant, to nuclear speckles. These results indicate that sumoylation at repression domains is an important posttranslational modification for the transcriptional activity of SF-1.
**EXPERIMENTAL PROCEDURES**

**Plasmids, Recombinant Proteins, and Antibody—**The following constructs have been described previously: pCDNA1-SF-1-1-HA (21), pCDX-TFHIB (8), and pS2.3H-Luc (22). Plasmids pCMV-tag2c-SUMO-1 and 5xGAL4-E1B-Luc were generously provided by Dr. H. M. Shih (National Health Research Institutes, Taiwan) and Dr. Y. S. Lin (Institute of Biomedical Sciences, Academia Sinica, Taiwan), respectively. SAE1/SAE2 (E1-activating enzyme), His-UBC9 (E2-conjugating enzyme), and His-SUMO-1 were kindly provided by Dr. J. Hwang (Institute of Molecular Biology, Academia Sinica, Taiwan). SF-1 mutants (K119R, K194R, and K119R/K194R) were generated by PCR-based site-directed mutagenesis in pCDNA1-SF-1-HA. SUMO-1 (aa 1–97) expression plasmids pEGFP-SUMO-1 WT, pEYFP-SUMO-1, and pDsRed2-SUMO-1 WT were generated after PCR amplification from pCMV-tag2c-SUMO-1. To generate conjugation-deficient SUMO-1, the last Gly (the 97th Gly residue) or the last two Gly residues (the 96th and 97th Gly residues) were mutated to Ala by PCR-based site-directed mutagenesis to construct pEGFP-SUMO-1 GA or pDsRed2-SUMO-2 AA, respectively. The recombinant proteins GST-UBC9, GST-TFIIb, and SF-1 were overexpressed in *Escherichia coli* strain BL21(DE3)pLysS and purified as described previously (23). Anti-SF-1 antiserum was raised against full-length SF-1 in rabbit; protein overexpression, purification, and immunization procedures were performed as described previously (4). Rat anti-HA antibody (clone 3F10) was obtained from Roche Applied Science.

**Transcriptional Activity Assay—**For transfection into NIH-3T3 or Y1 cells in 24-well culture plates, 100 ng of SF-1 expression plasmid was co-transfected with 100 ng of pS2.3H-Luc or 5xGAL4-E1B-Luc, and luciferase activities from phRLuc, used as an internal control. The results are the average of three independent transfection experiments, with error bars representing standard deviations.

**In Vitro Protein Binding Assay—**In *vitro* GST pull-down assays to measure protein binding were performed as described previously (8), with some modifications. Briefly, in *vitro* transcribed/translated SF-1-FH(S) fragment or full-length SF-1 was incubated with 1 μg of purified GST, GST-UBC9, or GST-TFIIb in NENT buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 150 mM NaCl). The protein complexes were then incubated with glutathione-coupled beads. After extensive washing, the samples were separated by 12% polyacrylamide gel and detected by autoradiography.

**In Vitro and in Vivo Sumoylation Assay—**The substrate proteins, luciferase and SF-1-HA, were in *vitro* transcribed/translated to generate radiolabeled proteins. Two microliters of *in vitro* translation products were added to sumoylation buffer (4 mM MgCl2, 1 mM dithiothreitol, and 2 mM ATP) containing 100 ng of SAE1/SAE2 and 1 μg of Hist-SUMO-1 in the presence or absence of 1 μg of His-UBC9 at 30 °C for 30 min. The reaction samples were separated by 10% polyacrylamide gel followed by autoradiography for 24 h. To examine SF-1 sumoylation in cells, SF-1-HA was co-expressed with SUMO-1 in 293T cells as indicated. Twenty-four hours after transfection, cells were washed twice with ice-cold phosphate-buffered saline containing 20 mM N-ethylmaleimide. Whole cell extracts were prepared in IPH buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 1× complete protease inhibitor mixture) containing 20 mM N-ethylmaleimide and subjected to immunoprecipitation with anti-HA antibody. The anti-HA immunoprecipitates were fractionated by 10% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was analyzed by anti-HA immunoblotting, stripped, and then re-blotted with anti-SUMO-1 antibody (Chemicon, Temecula, CA) and visualized by chemiluminescence detection.

**Electrophoretic Mobility Shift Assay—**Electrophoretic mobility shift assay was performed as described previously (24), with some modifications. SF-1-containing samples were prepared from *in vitro* synthesis/sumoylated assay without [35S]methionine and incubated with [3P]labeled oligonucleotide containing the SF-1 binding sequence on ice for 30 min. Subsequently, the samples were separated by 5% native acrylamide gel followed by autoradiography. The intensity of each band was quantified using Image Gauge Version 3.2 software with a FujiFilm LAS-1000plus image reader.

**Immunostaining and Confocal Microscopy—**Y1 cells were cultured on coverslips at a density of 1 × 10^5 cells/well in 6-well plates. Expression plasmids were transfected into cells as indicated. Twenty-four hours after transfection, cells were fixed in 3.7% formaldehyde for 10 min, permeabilized with 0.2% Triton X-100/phosphate-buffered saline (PBST), and blocked in 2% blocking reagent (Roche Applied Science).

**RESULTS**

*SF-1 Is Sumoylated in Vitro and in Vivo—*To understand the components that regulate SF-1 action, we searched for SF-1-interacting proteins by yeast two-hybrid screening using the FH(S) fragment (aa 78–213) of SF-1 as bait. During this screening, UBC9 was identified as one of the many proteins that interacted with SF-1 (data not shown). An *in vitro* protein binding assay further confirmed this interaction (Fig. 1). Both the SF-1-FH(S) fragment and full-length SF-1 interacted with UBC9, SF-1-FH(S) and SF-1 also interacted with TFIIb (Fig. 1, lanes 3 and 5), as demonstrated previously (8). They did not interact with GST (Fig. 1, lane 2), which was used as a negative control. The addition of 25-hydroxycholesterol, a proposed ligand for SF-1, did not affect the interaction between SF-1 and UBC9 (Fig. 1, lane 4). Because UBC9 is the single E2-conjugating enzyme for SUMO-1, and many sumoylation substrates interact with UBC9 (25), this result led us to investigate SF-1 conjugation by SUMO-1.

To determine whether SF-1 can be conjugated by SUMO-1, *in vitro* translated, [35S]labeled SF-1-HA was incubated with SUMO-1 and the E1-activating enzyme in the presence or absence of UBC9. Luciferase (Fig. 2A, Luc), a negative control, was not modified, regardless of whether UBC9 was present or absent (lanes 1 and 2). Whereas SF-1 remained unmodified in the absence of UBC9 (Fig. 2A, lane 3), in the presence of UBC9 two slow-migrating bands of 73 and 90 kDa appeared in addition to SF-1-HA (lane 4). The slow-migrating bands indicated the presence of SUMO-1-conjugated SF-1. This result indicated that SF-1 was modified by SUMO-1 in *vitro*.

To examine SUMO-1 conjugation of SF-1 in cells, SF-1-HA was co-expressed with GFP- or YFP-tagged SUMO-1 in 293T cells. After immunoprecipitation with anti-HA antibody, the presence of SF-1-HA was detected by immunoblotting with an anti-HA antibody (Fig. 2B, top panel). In the absence of SF-1-HA transfection, no band was detected, indicating the specificity of the immunoprecipitation and immunoblotting (Fig. 2B, lanes 1 and 2). The SF-1-HA band appeared when it was co-transfected with GFP alone (Fig. 2B, lane 3), whereas an additional slow-migrating band besides SF-1-HA was detected.
in the presence of GFP- or YFP-tagged wild-type SUMO-1 (lanes 4 and 6). These slow-migrating bands were further confirmed as SUMO-SF-1 by anti-SUMO-1 immunoblotting (Fig. 2B, bottom panel). To verify the involvement of SUMO-1, Gly to Ala mutation was performed at the 97th Gly residue of SUMO-1, which is essential for the ability of SUMO-1 to conjugate to substrates (25). When the resulting GFP-SUMO-1 GA mutant was co-transfected instead of wild-type GFP-SUMO-1, no additional band was detected (Fig. 2B, lane 5). This indicated the specificity of SUMO-1 conjugation. Unlike the in vitro assay, only one slow-migrating band of SF-1 was detected in the in vivo analysis. These results indicated that although SF-1 can be conjugated by two SUMO-1 molecules in vitro, it is probably sumoylated at only one site in vivo.

To map the site of SUMO conjugation, we analyzed SF-1 for the consensus sumoylation KXE sequence and found Lys119 in the sequence of FKLE and Lys194 in the sequence of IKSE of SF-1 to be potential sumoylation sites (Fig. 2C). To determine whether these two sites are indeed SUMO-conjugated, Lys to Arg mutagenesis was carried out at both sites to generate the K119R, K194R, and K119R/K194R mutants. The mutants were analyzed using both in vitro and in vivo sumoylation assays. In vitro sumoylation reactions revealed that whereas two slow-migrating SUMO-SF-1 bands were detected in the presence of wild-type SF-1 (Fig. 2D, lane 2), only one additional band was detected with K119R (lane 3), and no extra band besides the

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SF-1-HA band was visible in the presence of K194R and K119R/K194R (lanes 4 and 5). The absence of modification in the K194R mutant indicated that Lys 194 of SF-1 is a major conjugation site. In the absence of SUMO-conjugated Lys 194, Lys119 can not be conjugated. Therefore, Lys 119 is a minor conjugation site, which may depend on prior conjugation at Lys194 in vitro. Analysis of these mutants in 293T cells also revealed similar results, except that Lys 119 was not used for SUMO conjugation in vivo (Fig. 2E). Collectively, these results showed that SF-1 is sumoylated predominantly at Lys194 in vitro and in vivo.

Sumoylation Sites of SF-1 Are Intrinsic Repression Domains—To determine whether sumoylation affects SF-1 DNA binding activity, WT and mutated SF-1-HA (K119R, K194R, and K119R/K194R) were synthesized and SUMO-conjugated in vitro before being subjected to DNA binding and electrophoretic mobility shift assays. As calculated from the anti-HA immunoblot, about 70% of wild-type SF-1-HA and the K119R protein were modified in vitro (Fig. 3, top panel, lanes 4 and 6). However, the intensities of the DNA-protein complexes of sumoylated and non-sumoylated SF-1 were similar (Fig. 3, bottom panel). Therefore, sumoylation does not appear to affect the DNA binding activity of SF-1.

To examine the effect of SUMO conjugation on SF-1 transcriptional activity, wild-type or sumoylation-deficient SF-1 mutants were co-transfected with a SF-1-dependent reporter gene in NIH-3T3 cells. As shown in Fig. 4A, wild-type SF-1 activated the expression of the reporter; however, all of the sumoylation-deficient mutants (K119R, K194R, and K119R/K194R) were even more active. Because SF-1 is not endogenous to NIH-3T3 cells, we examined the activity of mutant SF-1 in a physiologically relevant cell line, Y1. Because Y1 expresses abundant SF-1, to avoid interference of endogenous SF-1, we examined the ability of exogenous GAL4-SF-1 fusion proteins (GAL4-DBD fused to aa 110–462 from wild-type or sumoylation-deficient SF-1) to activate a GAL4-dependent reporter gene (Fig. 4B). The activity of wild-type GAL4-SF-1 (Fig. 4B, WT) was very low in Y1 cells. The activities of all the GAL4-SF-1 mutants were enhanced, and the K119R, K194R, and K119R/K194R mutants had activities 2-, 39-, and 12-fold greater than the WT activity, respectively. These results indi-
cated that the regions surrounding Lys\(^{119}\) and Lys\(^{194}\) function as repression domains for SF-1.

To investigate the SF-1 repression activity around its SUMO conjugation sequences, we inserted peptides with either wild-type or mutant sequences centered around Lys\(^{119}\) or Lys\(^{194}\) sites of SF-1 into a transcriptional activator composed of a DNA-binding domain from GAL4 and an activation domain from VP16 (Fig. 4C, left panel). The resulting P-119WT and P-194WT plasmids encoded proteins with the wild-type Lys\(^{119}\) and Lys\(^{194}\) motifs, respectively, whereas P-119R and P-194R contained the mutated sequences. After transfection, P-119WT and P-194WT repressed reporter gene expression by 15% and 48%, respectively (Fig. 4C, right panel). This repression was statistically significant (\(p < 0.05\) and \(p < 0.01\), respectively). In contrast, P-119R and P-194R had the same activity as the control. Thus, the mutants lost the ability to repress transcription. This result indicated that both Lys\(^{194}\) and Lys\(^{119}\) lie in intrinsic repression domains, and the repression domain at Lys\(^{194}\) is stronger than that at Lys\(^{119}\).

**SF-1 Is Co-localized with SUMO-1 in Nuclear Speckles**—SUMO-1 conjugation affects the localization of some substrate proteins (25). To study whether sumoylation regulates the localization of SF-1, GFP-tagged SF-1 and RFP-tagged SUMO-1 were co-expressed in Y1 cells. Consistent with other reports, wild-type RFP-SUMO-1 formed many nuclear speckles in transfected cells (Fig. 5A). In about 60% of transfected cells, wild-type SF-1-GFP was located in nuclear speckles in the transfected cells (Fig. 5A). In contrast to wild-type SUMO-1, the conjugation-deficient SUMO-1 mutant, RFP-SUMO-1 AA, did not form nuclear speckles, and the co-expressed SF-1 was found homogenously in the nucleus (Fig. 5A, middle panels). To ascertain that localization of SF-1 to the nuclear speckles is due to SUMO-1 conjugation, the SF-1 mutant K194R, which cannot be conjugated, was co-expressed with wild-type RFP-SUMO-1. This SF-1-GFP K194R was homogenously distributed in the nucleus of all examined transfected cells, whereas the co-transfected SUMO-1 still formed nuclear speckles (Fig. 5A, bottom panels). Thus, the lack of SUMO-1 conjugation renders SF-1-GFP unable to go into nuclear speckles. These data indicate that targeting of SF-1-GFP into nuclear speckles by SUMO-1 requires intact conjugation sites in both SUMO-1 and SF-1-GFP.

Because the localization of exogenous SF-1-GFP is affected by SUMO-1 conjugation, we next examined whether the localization of endogenous SF-1 was also affected by SUMO-1. We transfected YFP-SUMO-1 into Y1 cells and then examined the location of SF-1 by indirect immunofluorescence (Fig. 5B). In some cells that expressed YFP-SUMO-1, nuclear speckles formed, and endogenous SF-1 was localized in these speckles (Fig. 5B, arrows). When YFP-SUMO-1 was absent, SF-1 was evenly distributed in the nucleus (Fig. 5B). These data suggest that overexpression of SUMO-1 can direct SF-1 to nuclear speckles formed by SUMO-1.

**DISCUSSION**

In this report, we demonstrate that SF-1 is modified by SUMO-1 in vitro and in vivo. We detect two SUMO conjugation sites, Lys\(^{119}\) and Lys\(^{194}\), in SF-1. SUMO conjugation does not affect the DNA binding activity of SF-1 but is associated with transcriptional repression. In addition, SUMO-1 conjugation modulates the localization of SF-1 in the nucleus. These results suggest that SUMO modification at the repression domains is an important posttranslational regulation of SF-1 activity.

**SF-1 Is Modified by SUMO-1**—We investigated SF-1 sumoylation because SF-1 interacts with UBC9, and many UBC9-interacting proteins are sumoylated (12). In the present study, we demonstrate that SF-1 interacts with E2-conjugating enzyme UBC9 in vitro and is a substrate for sumoylation in vitro and in vivo.

The potential sumoylation sites in SF-1 are located at Lys\(^{119}\) and Lys\(^{194}\). Our data showed that Lys\(^{194}\) was efficiently sumoylated by SUMO-1 in vitro and that Lys\(^{119}\) was poorly conjugated by SUMO-1 in vitro (Fig. 2D). In vivo, only Lys\(^{194}\) was sumoylated (Fig. 2E). This indicates that Lys\(^{119}\) is inefficiently sumoylated, although its sequence matches sumoylation consensus well. This suggests that regulation of SUMO conjugation depends on many factors in addition to pure substrate recognition. The large difference in the sumoylation efficiency of Lys\(^{194}\) and Lys\(^{119}\) could be of relevant importance to physiological condition. Similar results were also observed with c-Myb, which is also unequally sumoylated at two different sites (26).

**Sumoylation Sites Are Intrinsic Repression Domains**—The single SF-1 sumoylation site of Lys\(^{194}\) can repress transcription more efficiently than Lys\(^{119}\) (Fig. 4C). In our experiments,
mutation of Lys\textsuperscript{194} enhanced the transcriptional activity of full-length SF-1 by 2-fold and of GALA-SF-1 by 39-fold. Mutation of Lys\textsuperscript{119} also enhanced the transcriptional activity of SF-1, although at a much reduced level. One would expect that the K119R/K194R mutant would have an even higher activity than that of the single mutants, K119R and K194R, but our data showed that the activities of the double mutant were between those of the single mutants (Fig. 4 A and B). We do not know how to explain this because the mechanism of this repression is still unclear. The Lys\textsuperscript{194} sequence directly interacts with a DEAD box-containing protein, DP103, which represses the activity of SF-1 (27). It will be interesting to find out whether sumoylation of Lys\textsuperscript{194} is related to DP103 interaction or to recruitment of other co-repressor proteins. Furthermore, this SUMO conjugation motif also overlaps with the synergistic control motif, which has been suggested to modulate higher-order interactions among transcriptional regulators (28). These observations reveal that Lys\textsuperscript{194} could be an important and highly regulated domain involved in interactions among transcriptional regulators.

**SF-1 Is Localized to SUMO-1-formed Nuclear Speckles**—It has been proposed that SUMO-1 conjugation targets proteins to different cellular localizations. For example, sumoylation directs RanGAP1 from the cytoplasm to the nuclear membrane (14). SUMO-1 modification of promyelocytic leukemia and p53 also directs them into promyelocytic leukemia nuclear bodies (20, 29). Besides this, SUMO-1-conjugated homeodomain-interacting protein kinase 2 is concentrated in nuclear speckles, which are distinct from promyelocytic leukemia bodies (30). Like most transcription factors, SF-1 is evenly distributed in the nucleus. Both endogenous and exogenous SF-1 can be detected into nuclear speckles in the presence of SUMO-1, but not in the presence of conjugation-deficient SUMO-1. The SF-1 SUMO conjugation-deficient mutant also cannot be directed into nuclear speckles (Fig. 5A). This re-localization of SF-1 may be associated with transcriptional repression. Therefore, we propose that sequestration of SF-1 from the nucleoplasm might be a mechanism regulating the transcriptional activity of SF-1.

Based on our data, SUMO conjugation sites of SF-1 are transcriptional repression domains. Repression could be achieved by two separate means: (a) directly, probably through recruitment of co-repressor proteins to the transcriptional activation complex and/or disruption of the transcriptional activation complex; and (b) through the sequestration of SF-1 to nuclear speckles, thus removing it from transcriptionally active sites. Both possibilities are not mutually exclusive, and more experiments are needed to test these possibilities.

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**Note Added in Proof**—After this manuscript was accepted, there was one paper that appeared online that also showed SUMO conjugation of SF-1 (31).

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